Rho Family Binding Proteins In Human Neutrophils.

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To Mum and Dad.
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Abstract

Rho family proteins mediate diverse cellular functions including regulation of the actin cytoskeleton, transcriptional activation, and the NADPH oxidase of phagocytic leukocytes. To understand the molecular mechanisms by which Rho family proteins act, Cdc42Hs and Rac1 interacting proteins were identified in human neutrophils. Purification of the major Cdc42Hs binding activity from neutrophil cytosol demonstrated the presence of distinct binding proteins at approximately 62, 65, and 68 kDa, which interacted with Cdc42Hs and Rac1 in a GTP-dependent manner. Purified protein fractions contained three Ste20 immunoreactive proteins, and included a p21-activated kinase (PAK) with similarity to brain enriched α-PAK, suggesting the existence of multiple isoforms of PAK in neutrophils. Cdc42Hs and Rac1 interacting proteins were also identified in membrane-cytoskeletal fractions and with the Arp2/3 complex. In the latter case these proteins included a β-PAK-like protein and p57 the human coronin-like protein. Rac1 interacted directly with the NADPH oxidase component p67phox in a GTP-dependent manner, but not with the other components, p47phox, p40phox or the cytochrome b. Rac1 and Rac2 bound amino acid residues 170-199 of p67phox. Recombinant β-PAK was found to phosphorylate p67phox in vitro. Potential PAK phosphorylation sites were located close to the Rac binding and polyproline domains. Deletion of the C-terminal (amino acids 239-526), the C-terminal SH3 domain (amino acids 461-526) or the polyproline domain (amino acids 226-236) of p67phox stimulated Rac1 binding ~8-fold and also β-PAK phosphorylation. These observations suggest that an intramolecular SH3-polyproline domain interaction may exist within p67phox and that the Rac1 and β-PAK-interacting sites in p67phox are cryptic. This study identifies three p21 binding partners in human neutrophils, PAK, p67phox and p57. Characterisation of these target proteins will be important for understanding the signal transduction pathways through which Rho family proteins carry out their cellular functions.
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### Abbreviations

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<tbody>
<tr>
<td>β-ARK</td>
<td>β-adrenergic receptor kinase</td>
</tr>
<tr>
<td>aa</td>
<td>amino acid(s)</td>
</tr>
<tr>
<td>AA</td>
<td>arachidonic acid</td>
</tr>
<tr>
<td>ACK</td>
<td>activated Cdc42-associated kinase</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
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<td>Arp</td>
<td>actin related protein</td>
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<td>adenosine triphosphate</td>
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<td>bis-(o-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid</td>
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<td>break point cluster region gene product</td>
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</tr>
<tr>
<td>CGD</td>
<td>chronic granulomatous disease</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>CRIB</td>
<td>Cdc42Hs/Rac1 interactive binding</td>
</tr>
<tr>
<td>CSF</td>
<td>colony-stimulating factor</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>ddH_{2}O</td>
<td>double distilled water</td>
</tr>
<tr>
<td>DH</td>
<td>Dbl homology</td>
</tr>
<tr>
<td>DIFP</td>
<td>diisopropyl flurophosphate</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleotide 5'-triophosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein Barr Virus</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced Chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol-0,0'-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular-signal-regulated kinase</td>
</tr>
<tr>
<td>F-actin</td>
<td>filamentous actin</td>
</tr>
<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
</tr>
<tr>
<td>fMLP</td>
<td>N-formyl-methionyl-leucyl-phenylalanine</td>
</tr>
<tr>
<td>G-actin</td>
<td>monomeric actin</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GDI</td>
<td>guanine-nucleotide dissociation inhibitor</td>
</tr>
<tr>
<td>GEF</td>
<td>guanine-nucleotide exchange factor</td>
</tr>
<tr>
<td>GMPPNP</td>
<td>guanosine-5'-(β-imino)triphosphate</td>
</tr>
<tr>
<td>G-protein</td>
<td>guanosine-nucleotide-binding protein</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione S-transferase</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IP&lt;sub&gt;3&lt;/sub&gt;</td>
<td>inositol 1,4,5-trisphosphate</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropylthio-β-D-galactoside</td>
</tr>
<tr>
<td>JNK</td>
<td>c-jun N-terminal kinase</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LB</td>
<td>L-Broth (Lauria-Bertani medium)</td>
</tr>
<tr>
<td>LPA</td>
<td>lysophosphatidic acid</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MAPKK</td>
<td>MAPK kinase</td>
</tr>
<tr>
<td>MAPKKK</td>
<td>MAPKK kinase</td>
</tr>
<tr>
<td>MBP</td>
<td>myelin basic protein</td>
</tr>
<tr>
<td>MES</td>
<td>4-morpholineethanesulfonic acid</td>
</tr>
<tr>
<td>NADPH</td>
<td>nitotinamide adenine dinucleotide phosphate (reduced)</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear transcription factor κB</td>
</tr>
<tr>
<td>NP40</td>
<td>Nonidet P-40; ethylphenyl-polyethylene glycol</td>
</tr>
<tr>
<td>PA</td>
<td>phosphatidic acid</td>
</tr>
<tr>
<td>PAB</td>
<td>p-aminobenzamidine</td>
</tr>
<tr>
<td>PAF</td>
<td>platelet activating factor</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAK</td>
<td>p21 (Cdc42/Rac)-activated kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin homology</td>
</tr>
<tr>
<td>phox</td>
<td>phagocytic oxidase</td>
</tr>
<tr>
<td>PI</td>
<td>phosphatidylinositol</td>
</tr>
<tr>
<td>PI 3-Kinase</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PIP</td>
<td>phosphatidylinositol 4-phosphate</td>
</tr>
<tr>
<td>PIP 5-kinase</td>
<td>phosphatidylinositol 4-phosphate 5-kinase</td>
</tr>
<tr>
<td>PIP₂</td>
<td>phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PIP₃</td>
<td>phosphatidylinositol 3,4,5-trisphosphate</td>
</tr>
<tr>
<td>PIPES</td>
<td>1,4-piperazinediethanesulphonic acid</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLA₂</td>
<td>phospholipase A₂</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PLD</td>
<td>phospholipase D</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulphonyl fluoride</td>
</tr>
<tr>
<td>PP5</td>
<td>protein phosphatase 5</td>
</tr>
<tr>
<td>ROK</td>
<td>RhoA binding kinase</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SAPK</td>
<td>stress activated protein kinase</td>
</tr>
<tr>
<td>SDS</td>
<td>lauryl sulphate</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2 domain</td>
</tr>
<tr>
<td>SH3</td>
<td>Src homology 3 domain</td>
</tr>
<tr>
<td>SOP2</td>
<td>suppressor of profilin 2</td>
</tr>
<tr>
<td>Sos</td>
<td>Son of sevenless</td>
</tr>
<tr>
<td>SRF</td>
<td>serum response factor</td>
</tr>
<tr>
<td>Ste20</td>
<td>Sterile20</td>
</tr>
<tr>
<td>Temed</td>
<td>N,N,N',N'-tetramethylethylene diamine</td>
</tr>
<tr>
<td>TLCK</td>
<td>N²-p-tosyl-l-lysine chloromethyl ketone</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>TPR</td>
<td>tetraicopeptide repeat</td>
</tr>
<tr>
<td>Tris</td>
<td>2-amino-2(hydroxymethyl)-1,3-propandiol</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>octylphenoxypolyethoxyethanol</td>
</tr>
<tr>
<td>Tween20</td>
<td>polyoxethylene sorbitan monolaurate</td>
</tr>
<tr>
<td>VASP</td>
<td>vasodilator-stimulated phosphoprotein</td>
</tr>
<tr>
<td>WASP</td>
<td>Wiskott-Aldrich Syndrome protein</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction
1.1. Neutrophils.

Neutrophils play a central role within the immune system in host defence against invading pathogens. They respond quickly to a number of different inflammatory signals and are the first cells to be recruited to a site of infection. Neutrophil cells are highly motile enabling migration from blood into tissues in response to chemical signals or chemoattractants, and possess an impressive array of cytotoxic mechanisms that are capable of killing a wide range of microbial pathogens.

Neutrophils are the most abundant white blood cells (leukocytes) found in the blood representing 40-65% of leukocytes which also include eosinophils, basophils, monocytes, and macrophages. As with all blood cells neutrophils develop in the bone marrow from multipotent stem cells by the process of haematopoiesis. This process is regulated by the activities of different colony-stimulating factors (CSFs). In addition to interleukin (IL) 3 (IL-3 or multi-CSF), which affects the replication of multipotent stem cells and precursor cells of all haematopoietic cell lineages, neutrophils are stimulated to differentiate by granulocyte-CSF (G-CSF), and granulocyte macrophage-CSF (GM-CSF). The bone marrow generates ~2 x 10^10 neutrophil cells/day and neutrophils are found at a concentration of 3-5 x 10^6 cells/ml of blood, although this number can drastically increase in cases of infection, up to 10-fold. Neutrophils have a half-life of 8-20 h in the circulation but this may be extended to up to several days if the cells leave the circulation and enter tissues.

Neutrophil cells have a multilobed nucleus and belong to a group of cells referred to as polymorphonuclear leukocytes, which in addition includes eosinophils, and basophils. The cytoplasm of neutrophils and other polymorphonuclear leukocytes is granular in appearance (hence these cells are also termed as granulocytes) due to the presence of a large number of granules which are membrane bound organelles that act to store proteins required for the process of killing invading pathogens, including membrane receptors. The molecular content of the granules defines the properties of these cells in immunity and distinguishes neutrophils from other polymorphonuclear leukocytes.

Neutrophils function in collaboration with the other components of the immune system which includes other leukocytes, lymphocytes and molecular components such as complement factors, antibodies, acute phase proteins and cytokines. The cellular and
molecular components of the immune system constitute a coordinated and sophisticated network that has evolved in order to maximise the survival of the host against the range of pathogens it encounters daily. Microbial infectious agents which include bacteria, yeast, and fungi, viruses and protozoa, can infect bodily fluids, penetrate tissues and even survive and multiply within an individual cell (e.g. pathogenic bacteria including *Listeria* and *Legionella*). Upon infection the immune system is responsible for destroying microbes before they can multiply and disperse throughout the body.

A variety of molecules activate the defence systems of neutrophils including cytokines (e.g. IL-1 and IL-8), antibody-antigen complexes and bacterial products (e.g. the formylated oligopeptide N-formyl-methionyl-leucyl-phenylalanine; fMLP). These factors generally elicit their effects by binding to specific cell surface receptors, which results in the activation of numerous second messengers and signalling pathways (Morel *et al.*, 1991).

The process by which neutrophil cells destroy microbial pathogens is regulated at several stages, hence the signalling systems that transduce chemical signals from the outside of a neutrophil cell to the inside are complex. These stages require neutrophil cells to: - detect pathogens or signals generated during infection (which may be generated by host tissues, immune cells or the pathogens themselves); attach to the cells of the capillary walls prior to leaving the circulation (margination); squeeze through the gaps between adjacent endothelial cells (diapedesis); migrate into tissues (chemotaxis); recognise pathogens as foreign; initiate phagocytosis and activation of microbicidal mechanisms; and release cytotoxic products and pro-inflammatory molecules (e.g. chemoattractants) or other immune stimulants (cytokines) if further recruitment of immune cells including neutrophils to the site of infection is required (Edwards, 1994).

Circulating neutrophils are ~10 μm in diameter and are generally spherical, and are said to be in a resting or non-activated state. Once activated by either a chemical stimuli or by attachment to the surfaces of the cells of the vascular system their morphology changes. They become polarised as required for directional movement and then flatten to assume a classical amoeboid shape with protruding pseudopodia. At this point they are said to be primed and thus are ready for subsequent functions. Priming may happen in response to pathological or physiological stimuli and involves preparing the neutrophil for a state of
readiness prior to its full activation status. The process of priming also involves the fusion of some of the cytoplasmic granules with the plasma membrane and during this process there is an increase in the number of receptor molecules present on the surface of the cell.

Neutrophils kill their targets by the process of phagocytosis. A rapid increase in the consumption of oxygen of 50-100-fold, unrelated to mitochondrial respiration accompanies phagocytosis and results from the activation of the NADPH oxidase enzyme (also known as the respiratory burst enzyme). Once enclosed in the phagocytic vacuole cytotoxic agents must be delivered to the pathogen. The NADPH oxidase, which has a complex structure and a complex mechanism of activation, resides in the membranes of neutrophils and is capable of generating a series of reactive oxygen species (ROS) with broad antimicrobial properties. These ROS are delivered into the phagocytic vacuole and with the contents of the cytoplasmic granules (released into the phagocytic vacuole during the process of degranulation) are involved in the degradation of the engulfed particles. The contents of the granules includes a range of proteases, hydrolytic enzymes, a peroxidase (myeloperoxidase) and also a number of highly specialised proteins that affect the permeability of microbial targets. The board range of biochemically distinct antimicrobials available enables neutrophils to attack a wide variety of targets.

Multiple signalling systems coordinate to specifically activate neutrophils, between which exists an element of overlap and redundancy to safe guard against defects that might abolish cell function. The importance of the NADPH oxidase of phagocytes, for host defence against invading pathogens is illustrated in chronic granulomatous disease (CGD), an immunodeficiency syndrome in which ROS production is lost or severely reduced, which renders patients highly susceptible to recurrent infections. In addition the two stage activation process noted above, may help prevent non-specific activation of neutrophils. The toxic nature of ROS utilised for pathogen killing requires that their production must be under tight control, as uncontrolled release from phagocytes may be involved in the pathogenesis of rheumatoid arthritis, in tissue damage following ischaemia, in stroke and myocardial infarction and respiratory distress syndrome (DeLeo and Quinn, 1996). Dissection of the signalling pathways involved during neutrophil activation may add to the knowledge of the mechanisms by which neutrophils function.
and facilitate the design of therapies to mediate control of this branch of the immune system.

1.2. Cellular signalling pathways.

Cell-cell communication and the process by which a cell assesses its environment is of particular importance in multicellular organisms. The transmission of signals between cells is required during the process of development, for cell growth, differentiation, and migration, and for assembly of cells into specific tissues within an embryo to occur in an organised fashion. Cell signalling continues to be of importance throughout life for normal cellular behaviour and to coordinate responses to tissue damage or infections. The process by which extracellular signals are relayed from the plasma membrane to specific intracellular sites is an essential aspect of cell regulation. Defects in signalling pathways form the basis of cancer, and disorders of the immune system.

Many factors interact with the outside of cells to direct changes in cell growth and differentiation, cell morphology, cell adhesion, cell migration, and the activation of specific enzyme systems, for example the NADPH oxidase of neutrophils. Extracellular factors include growth factors (e.g. platelet derived growth factor; PDGF), polypeptide hormones (e.g. insulin), cytokines (e.g. IL-8), chemoattractants (e.g. fMLP), antigen-antibody complexes, proteins of the extracellular matrix (e.g. fibronectin), and adhesion molecules displayed on the surfaces of cells (e.g. members of the selectin family). These extracellular molecules interact with specific integral membrane protein receptors, which as a result become activated and transmit signals to the inside of the cell. Transmembrane receptors may possess catalytic activity, for example tyrosine kinase receptors, including the PDGF receptor family; or may be linked via their cytoplasmic domain to proteins or protein complexes with catalytic activity, for example heterotrimeric G-protein coupled receptors, which include the fMLP and IL-8 receptors. Ligand binding to receptors without endogenous catalytic activity can lead to conformational changes that promote tyrosine phosphorylation of residues in the intracellular domain by cytoplasmic kinases. This is the case for members the Fc receptor family, which recognise the Fc portion of immunoglobulin G. Integral membrane integrins transmit signals upon clustering and activation induced by interaction with an extracellular ligand, for example the extracellular matrix protein fibronectin.
Signals can be transmitted from an activated receptor via a number of different mechanisms. For example by the generation of second messenger molecules, including cyclic AMP (cAMP), calcium (Ca^{2+}), and phospholipids, or via the activation of more specific pathways involving protein tyrosine kinases, serine/threonine kinases and kinase cascades, and pathways that signal via the members of the Ras superfamily of GTPases. Transmission of signalling pathways involves the formation of multiprotein complexes, containing proteins with and without catalytic activity. Those proteins without catalytic activity may act to form a scaffold or as adapters protein, for example to hold numerous proteins within a signalling pathway in complex together, or to direct the subcellular localisation of enzymes to within proximity of a specific substrate. Protein-protein interactions required for transduction of signalling pathways can occur via specific protein modules. For example, the prototypic protein module Src homology 2 (SH2) domain, which was identified in Src family tyrosine kinases, directs interactions with phosphotyrosine containing peptide targets, whereas Src homology 3 (SH3) domains interact with polyproline motifs (Pawson, 1995; see below).

1.2.1. Tyrosine kinase receptors.

Tyrosine kinase receptors typically possess an extracellular domain, a single transmembrane domain and an intracellular kinase domain. Autophosphorylation of the cytosolic domains on tyrosine residues results from receptor clustering induced by the binding of ligands such as PDGF or insulin to the extracellular domain, with the receptor molecules within a dimer phosphorylating each other (Heldin et al., 1995). The phosphorylated tyrosine residues provide sites for interaction with intracellular signalling molecules that contain SH2 domains. The cytosolic domains of tyrosine kinase receptors can become phosphorylated on multiple tyrosine residues. However, protein binding to these phosphorylated sites is highly specific and is determined by the amino acid sequence surrounding the phosphorylated residue.

1.2.2. Heterotrimeric G-protein coupled receptors.

Receptors coupled to heterotrimeric G-proteins include the fMLP receptor. Heterotrimeric G-proteins are comprised of α, β, and γ-subunits. At least 20 distinct α-subunits, ~5 β-subunits and ~12 γ-subunits have been identified. The β and γ-subunits are tightly associated and exist as a hydrophobic dimer (Gβγ), as the γ-subunit is isoprenylated. Gβγ acts to anchor the α-subunit, which is more freely disassociated, to

27
the membrane. The α-subunit contains the guanine nucleotide binding site and possess intrinsic GTPase activity. Dissociation of the Gαβγ complex occurs upon ligand binding to the receptor, resulting in the formation of active Gα-GTP and a Gβγ dimer, the former being free to interact with downstream effector molecules. The sensitivity of particular G-protein coupled receptors to pertussis toxin has enabled the investigation of signalling pathways transduced via these receptors in vitro. Pertussis toxin disrupts the association between receptors and certain Gα-subunits and has been used in experiments that demonstrated that both pertussis toxin-sensitive and insensitive G-proteins transduce signals between receptors and the stimulation of cellular events, such as activation of phospholipase Cβ (PLCβ), (for reviews Neer, 1994; Hamm and Gilchrist, 1996).

1.2.3. Second messenger molecules.
The activity of adenylate cyclase is mediated by the activation of G-protein coupled receptors. Active adenylate cyclase catalyses the conversion of ATP to produce the second messenger cAMP. Raised levels of cytosolic cAMP are observed upon cellular stimulation with factors such as fMLP. One effect of cAMP is to mediate the activation of the cAMP-dependent protein kinase, protein kinase A (PKA). By interacting with the regulatory subunit of PKA cAMP induces activation of the catalytic subunit. Disruption of the actin cytoskeletal changes required for phagocytosis by neutrophils in response to fMLP, caused by raised levels of cAMP were abrogated by PKA inhibitors suggesting that cAMP can elicit downstream actions via activation of PKA (Zalavary and Bengtsson, 1998).

An increase in the intracellular concentration of Ca^{2+} results from activation of cells with a number of different stimuli, for example fMLP or PDGF, and is important for numerous cellular functions including cell-cycle progression, gene transcription, and activation of the neutrophil NADPH oxidase. A rise in intracellular Ca^{2+} can result from release of Ca^{2+} from intracellular stores by inositol 1,4,5-trisphosphate (IP_3) (see below), or from Ca^{2+} ions crossing the plasma membrane (Berridge, 1993). Ca^{2+} binding proteins may act to buffer or lower intracellular Ca^{2+} and control localisation of raised Ca^{2+} levels or to trigger second messenger pathways, for example activation of protein kinases including members of the Ca^{2+}-dependent PKC family (Clapham, 1995).
Lipid second messengers are derived from the cleavage of membrane phospholipids by the action of phospholipases or are generated by the activity of phosphoinositol kinases. Phosphatidylinositol specific phospholipase C (PLC) enzymes catalyse the hydrolysis of membrane phosphatidylinositols, for example phosphatidylinositol 4,5-bisphosphate (PIP₂) generating hydrophobic and hydrophilic components; 1,2-diacylglycerol (DAG) and IP₃. DAG which remains in the membrane activates members of the protein kinase C (PKC) family, whereas IP₃ is released into the cytoplasm where it interacts with intracellular receptors to mobilise calcium from intracellular stores (Berridge, 1993). PLC enzymes have been grouped (α-ε). PLCβ isoforms are activated by G-protein coupled receptors (Sternweiss and Smrcka, 1992), whereas PLCγ isoforms, which contain SH2 and SH3 domains, are activated by SH2 domain directed interactions with activated tyrosine kinase receptors (Yeo et al., 1994). The phospholipase A₂ (PLA₂) family can be divided into two groups based upon whether they remain in the cytoplasm or are secreted (Dennis, 1997). The cytosolic members catalyse the hydrolysis of membrane phospholipids to produce lysophospholipids and free fatty acids including arachidonic acid (AA) and lysophosphatidylcholine. Phospholipase D (PLD) enzymes catalyse the hydrolysis of phospholipids, mainly phosphatidylcholine, to generate phosphatidic acid (PA) and a free polar head group. PA can be further metabolised into DAG. Roles of PA produced by PLD include control of the NADPH oxidase in neutrophils (Exton, 1997).

Phosphatidylinositol 3-kinase (PI 3-kinase) phosphorylates the D-3 position of membrane phospholipids for example PIP₂ to generate PIP₃. Several different isoforms of PI 3-kinase have been identified. The isoform of PI 3-kinase known to be activated by tyrosine kinase receptors is a heterodimer composed of an 85 kDa regulatory subunit (p85) containing two SH2 domains and a 110 kDa (p110) catalytic subunit (Kapeller and Cantely, 1994). Binding of the p85 subunit to phosphotyrosine residues within specific peptide sequences, via its SH2 domains leads to activation of the kinase activity. PIP₃ which was first identified in activated neutrophils (Traynor-Kaplan et al., 1988) has been suggested to play roles in activation of the NADPH oxidase and also in regulation of the actin cytoskeleton (Toker and Cantley, 1997).

Phosphatidylinositol 4-phosphate 5-kinase (PIP 5-kinase) phosphorylates PIP generating PIP₂. PIP 5-kinases can be divided into two subtypes (type-I and type-II) on the basis of
their structural and biochemical features. Type-I PIP 5-kinases of different molecular weights (68 kDa, 80 kDa, 90 kDa and 110 kDa) and type-II PIP 5-kinases (53 kDa), have been identified in a number of different tissues. The existence of multiple PIP 5-kinase isoforms suggests that their regulation and function may be complex (Loijens et al., 1996). PIP_2 is a key precursor in phosphoinositide signalling that can also regulate some proteins and cellular processes directly. A role for PIP_2 in the organisation of the actin cytoskeleton by regulating the activities of a number of different actin binding proteins has been suggested, for example PIP_2 may inhibit the actin filament capping and severing properties of gelsolin (Ayscough, 1998), see section 1.3.

1.2.4. Protein kinases and kinase cascades.
Many signalling pathways are transmitted by altering the phosphorylation states of serine, threonine and tyrosine residues of target proteins, which is mediated by balancing the action of proteins kinases and phosphatases. Cytosolic protein tyrosine kinases include members of the Src family; serine/threonine kinases include PKA, PKC, and members of the mitogen-activated protein kinase (MAPK) and the p21 (Cdc42/Rac)-activated kinase (PAK) families, see below.

1.2.4.1. Cytosolic tyrosine kinases.
The first cytosolic tyrosine kinase to be identified was the v-Src oncoprotein. Numerous enzymes related to Src have been identified from their transforming properties (e.g. Yes, Fgr and Lck) or by sequence homology (e.g. Fyn, Yrk, Hck, Lyn and Blk). Although Src, Fyn, Yes and Yrk are widely expressed, most members of this family of proteins show a restricted tissue distribution, with expression in specific haematopoietic cells. Structurally Src family members are highly conserved, each containing a site that directs membrane localisation, a unique domain that defines the distinct family members, an SH3, and an SH2 domain, a catalytic domain and a C-terminal regulatory sequence. Src kinases associate with the activated PDGF receptor via an SH2 domain directed interaction with a specific phosphotyrosine residue (Mori et al., 1993). Src family proteins are thought to be involved in the signalling pathways that mediate cell growth and morphology, the stress activated response and mitosis and are activated in response to many different stimuli, including PDGF, IL-2 and lysophosphatidic acid (LPA), (reviewed by Erpel and Courtneidge, 1995).
1.2.4.2. Protein kinase C (PKC).
The PKC family, which includes at least 11 isoenzymes encoded by different genes, is well conserved between species. PKC family members have been divided into three groups according to their cofactor requirements and structure: - classical PKCs (α, βI, βII, γ); novel PKCs (δ, ε, η, θ) and atypical PKCs (ξ, λ, μ). These serine/threonine protein kinases are activated by DAG, and are dependent upon calcium. The interaction with DAG stabilises the association of PKC with the membrane. PKC activation can occur downstream of G-protein coupled receptors for example following cellular stimulation with fMLP and can also be activated in vitro by phorbol esters such as the DAG analogue phorbol 12-myristate 13-acetate (PMA), (reviewed in Dekker and Parker, 1994; Dekker et al., 1995). A role for PKC in NADPH oxidase activation has been described (Morel et al., 1991; see section 1.5).

1.2.4.3. Mitogen-activated protein kinase (MAPK) cascades.
MAPK cascade is a major signalling system by which cells transduce extracellular signals from stimuli including growth factors, cytokines and also UV irradiation, to intracellular responses culminating in the activation of MAPKs which translocate into the nucleus to regulate the activity of transcription factors. The proteins involved in these kinase cascades are conserved between species and homologues have been identified in organisms ranging from yeast to humans. MAPKs are proline direct kinases that phosphorylate sites containing the consensus S/T-P, and are activated by dual specificity MAPK kinases (MAPKKs), which are activated by MAPKK kinases (MAPKKKs). The MAPKKK → MAPKK → MAPK cascade may function in a complex. For example the members of the yeast S. cerevisiae pathway activated in response to mating pheromone including Ste11 ⇒ Ste7 ⇒ KSS1/FUS3 all interact with the scaffolding protein Ste5 which holds the components in a functional unit. Activation of this cascade requires the activity of Sterile20 (Ste20) protein kinase (Treisman, 1996).

The mammalian family of MAPKs includes; extracellular-signal-regulated kinases (ERKs) ERK1 and ERK2; the c-jun N-terminal kinase/stress activated protein kinase (JNK/SAPK) subfamily, which includes JNK1 and JNK2 (Hibi et al., 1993; Derijard et al., 1994) and SAPKα, SAPKβ and SAPKγ (Kyriakis et al., 1994); and p38. Transcription factor substrates for activated ERK1 and ERK2 may include the ternary complex factor (TCF) Elk-1 which is involved in the induction of the potential oncogene
c-fos. JNK1 and JNK2 bind the N-terminus and phosphorylate serines 63 and 73 of c-jun, and were identified during investigation of the kinases activated by UV irradiation of human cells (Hibi et al., 1993; Derijard et al., 1994). SAPKα, SAPKβ and SAPKγ were cloned from human liver and rat brain cDNA libraries (Kyriakis et al., 1994). The mammalian MAPK p38 is similar to the yeast osmosensing MAPK, high-osmolarity-glycerol response 1 (HOG1), and also shares 46-49% identity with ERKs (Han et al., 1994).

1.2.5. Protein modules and protein motifs.

A number of conserved protein modules have been identified as important in signalling pathways by their ability to direct specific protein-protein interactions, and mediate activation induced changes in subcellular localisation of proteins. The prototype protein modules were first characterised from the Src family of tyrosine kinases namely the SH2 and SH3 domains, however a number of different domains have now been identified (Pawson, 1995; Cohen et al., 1995). SH2 and protein tyrosine binding (PTB) domains interact with phosphotyrosine containing peptide targets, SH3 and WW domains direct interactions with polyproline motifs, and pleckstrin homology (PH) domains influence protein-phospholipid binding (Pawson, 1995).

Proteins with catalytic activity or those solely involved in generating multiprotein signalling complexes can contain multiple protein modules in tandem, indicating the complexity of signalling pathways. Protein modules that may provide a specific function in directing protein-protein interactions or securing protein structure and folding are found in different types of proteins including enzymes, adapter proteins, transcription factors and cytoskeletal proteins. For example the adapter protein Grb2 contains two SH3 domains and an SH2 domain and has been shown to link receptor tyrosine kinases to Ras signalling (Lowenstein et al., 1992). The SH2 domain of Grb2 interacts with phosphorylated tyrosine residues on the cytosolic domain of an activated receptor (e.g. the β-PDGF receptor). The SH3 domain of Grb2 interacts with a polyproline region in the Ras exchange factor Sos. These protein-protein interactions form a multiprotein complex which brings Sos to the membrane where it is able to interact with its substrate Ras, mediating exchange of the bound nucleotide on Ras for GTP and activation of downstream signalling events.
1.2.5.1. **Src homology 3 (SH3) domains.**

SH3 domains are highly conserved non-catalytic regions of 50-60 residues, which can mediate the direction of proteins to the membrane cytoskeleton (Shpetner et al., 1996; Bar-Sagi et al., 1993), and appear to be required for the intermolecular interactions between the neutrophil NADPH oxidase components that mediate the assembly of the active NADPH enzyme complex (DeLeo and Quinn, 1996; see section 1.5).

The basic structure of an SH3 domain contains five anti-parallel β-sheets packed to form two perpendicular β-sheets. The ligand-binding site consists of a hydrophobic patch containing a cluster of conserved aromatic residues and is surrounded by two charged and variable loops. SH3 domains bind to proline-rich peptides, with the minimal consensus P-X-X-P, which adopt a left-handed polyproline type II helix. An aliphatic residue often precedes each proline residue and these pairs (aliphatic-proline) bind to a hydrophobic pocket on the SH3 domain. In principal peptide targets can bind in either orientation. An additional non-proline residue, frequently arginine, can form part of the binding core and contact the SH3 domain. Usually the affinity of such an interaction lies within the micromolar range. However, affinity and specificity can be markedly increased by tertiary interactions involving the loops within the SH3 domain (Lin et al., 1997a).

1.2.5.2. **Tetratricopeptide repeat (TPR) motifs.**

The tetratricopeptide repeat (TPR) motif is a degenerate 34 amino acid residue sequence identified in a wide variety of proteins. These motifs are found in tandem arrays of 3-16 motifs, and fold to form scaffolds that mediate protein-protein interactions and often the assembly of multiprotein complexes. The N-terminal of the NADPH oxidase component p67phox contains four TPR motifs (Ponting, 1996). Alignment of TPR motif containing proteins has demonstrated that within the 34 amino acid sequence 8 residues are conserved in terms of their size, hydrophobicity and spacing, with the consensus as shown below.
Alignment of the TPR motifs (1-4) of p67\textsuperscript{phox} with the consensus (Lamb et al., 1995).

<table>
<thead>
<tr>
<th>Consensus 1</th>
<th>XXXW XXLGXX</th>
<th>XXXXXXA</th>
<th>XXXX</th>
<th>XXXA</th>
<th>XPXX</th>
</tr>
</thead>
<tbody>
<tr>
<td>p67\textsuperscript{phox} 1</td>
<td>3-36 AISLWNEGVL AADKKDWKGA LDAESA QDP H</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p67\textsuperscript{phox} 2</td>
<td>37-70 SRICFNIGCM VTLKNMTEA EKAIRESINR DKHL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p67\textsuperscript{phox} 3</td>
<td>71-104 AVAYFOQGRML YQTEKYDLA IKDIKEALIQ LRGN</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p67\textsuperscript{phox} 4</td>
<td>121-154 CEVLYNFAFM YAKKEEWKA EEQALATSM KSEP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Each TPR motif folds into two alpha-helices of the same length. Residues at positions 4, 7, 8 and 11 (shown in green) locate on one side of the first alpha-helix and form a hydrophobic pocket, or ‘hole’, whereas positions 20, 24 and 27 (shown in pink) which locate to one surface of the second alpha-helix form a bulky ‘knob’ structure. It has been predicted that the two alpha-helical domains fit together with the bulky structure of the second fitting into the hydrophobic pocket on the surface of the first, by the so called ‘knob-hole’ model (Lamb et al., 1995). The lysine residue at position 58 and the glycine residue at position 78 of p67\textsuperscript{phox} deleted in forms of autosomal CGD (Leusen et al., 1996; de Boer et al., 1994) are shown above in lower case and bold type face. In protein phosphatase 5 (PP5) three TPR motifs are found in tandem array and adjacent TPR motifs pack together in a regular series of anti-parallel alpha-helices (Das et al., 1998). A linker sequence may also exist between TPR motifs within a polypeptide, for example between TPR 3 and 4 of p67\textsuperscript{phox} (Ponting, 1996).

1.2.5.3. WD repeat motifs.

WD repeat containing proteins are made up of highly conserved repeating units which usually end with tryptophan-aspartic acid (WD) residues. These proteins, which are only found in eukaryotes are generally thought to be involved in regulatory but not catalytic processes, and possess the potential to interact with other proteins through their repeats. WD repeats were first found in the β-subunits of heterotrimeric G-proteins in which there a seven repeats. WD repeats have been identified in a number of different proteins in which the conserved unit can recur 4 to 10 times, including the human coronin-like protein p57 which contains 5 repeats (Suzuki et al., 1995; see appendix A.7, for amino acid sequence and domain structure of p57).

The WD repeat has a region of variable length (X\textsubscript{6-94}), followed by a core of more or less constant length (X\textsubscript{23-41}), which is bracketed by two characteristic elements; GH (glycine-
histidine) and WD; \{X_{6.94-23.41}-\text{WD}\}^{n=10}. Analysis of the primary sequence of the WD repeat indicates that this motif may fold with a variable loop defined by the variable region which proceeds the GH, followed by \(\beta\)-strand-turn-\(\beta\)-strand-turn-\(\beta\)-strand ending with the WD element. That the predicted \(\beta\)-structure is small suggests that stabilisation by interaction with a ligand (such as a metal ion) or contact with other WD repeats may be required to maintain a stable folded structure. Determination of the structure of \(\beta\)-subunits of heterotrimeric G-proteins has shown that tandem WD repeat motifs fold together to form a propeller structure, with each of the seven propeller blades corresponding to a WD repeat motif (Neer et al., 1996). WD repeats function to direct the formation of multiprotein complexes; for example G\(\beta\)-subunits assist in the formation of the ternary complex of heterotrimeric G-protein components with receptor molecules. WD repeat containing proteins also play roles in assembling protein complexes required for mRNA splicing, mRNA modification, and transcription (reviewed by Neer et al., 1994). WD repeats have also been shown to interact with PH domain ligands; for example the G\(\beta\gamma\) dimer brings the \(\beta\)-adrenergic receptor kinase (\(\beta\)-ARK) to the receptor by binding \(\beta\)-ARK through its PH domain (Neer et al., 1994). PH domains direct protein-protein interactions and can simultaneously bind to phospholipids and hence may direct target proteins to the membrane (Lemmon et al., 1996; Shaw, 1996).

1.3. The cytoskeleton.

The cytoskeleton of eukaryotic cells consists of a filamentous protein network that provides the basis for the underlying structure determining cell shape. Three major filament systems coexist in most cells. Actin microfilaments and microtubules consist of globular protein subunits; actin monomers and tubulin dimers respectively. The third type, intermediate filaments are made up of \(\alpha\)-helical subunits (intermediate filament proteins) twisted together. At least five types of intermediate filaments have been identified each being made up of different proteins, for example vimentin. The structure of the cytoskeleton is mediated by the actions of proteins which associate with these filamentous structures.

In addition to determining cell shape and morphology the organisation of the cytoskeleton provides support for cell extensions such as villi, axons in nerve cells, and is involved in cell movement. The cytoskeleton is also involved in mediating interactions between the cell and the substratum and intracellular transport. The cytoskeleton does
not exist as a permanent structure, the different filament types continually assemble and
disassemble and the coordinated control of these processes is required for cell
morphology changes. Dynamic cell shape changes are associated with a number of
cellular processes for example cytokinesis, phagocytosis and cell motility (Stossel, 1993).

1.3.1. The actin cytoskeleton.

Actin is an abundant protein in all cells; cellular concentrations for example in neutrophils
may be as high as 25 mg/ml. Actin exists in two forms. Actin monomers (G-actin)
polymerise to form filamentous actin (F-actin) microfilaments which are polar structures.
Actin monomers have four binding sites capable of interacting with other actin
monomers, two of which interact within actin microfilaments whereas two direct
interactions between actin monomers in adjacent actin microfilaments. The two ends of
an actin microfilament appear structurally distinct as actin monomers polymerise in a
particular orientation and are referred to as either the pointed or the barbed ends.

The actin cytoskeleton provides the basis for a number of distinct cellular structures, the
formation of which is directed by actin filament polymerisation. In fibroblasts
polymerised actin is assembled at the cell periphery to form filopodia, retraction fibres,
membrane ruffles and lamellipodia and is assembled into thick actin cables that traverse
the cell known as stress fibres. In neuronal cells actin polymerisation in the growth cone
drives axonal extension. The growth cone is a highly dynamic structure consisting of
filopodial and lamellipodial protrusions. Filopodia are thin finger-like cytoplasmic
extensions thought to play a sensory function in Swiss 3T3 cells and neuronal growth
cones. Lamellipodia are thin veil-like structures free of organelles that contain a network
of branched actin filaments (Svitkina et al., 1997), which can form the leading edge
lamellae of motile cells if contacts are made with the substratum (Stossel, 1993).
Membrane ruffles, which are observed as dark areas at the cell periphery using phase
contrast microscopy, also contain a branched network of actin filaments. These are
dynamic structures that appear to result from areas of membrane that protrude from
the cell periphery and then fold up away from the substrate and back onto the cell (Stossel,
1993). Stress fibres are thick bundles of actin filaments that meet the plasma membrane
at adhesion complexes, for example focal adhesions. Stress fibres contain actin binding
proteins including myosin, which appears to attach to stress fibres with a polar
orientation.
Addition of actin monomers during elongation of F-actin can occur at both ends of the microfilament. However, polymerisation occurs more readily at the barbed end, also known as the ‘fast-growing’ end. Generation of an actin microfilament may occur via the formation of a nucleation complex that requires the formation of a stable actin dimer and so on or from small fragments of F-actin severed from preexisting microfilament structures. Disassembly of F-actin may result from removal of actin monomers from the pointed end or by the severing of actin filaments. In highly motile areas of the cell such as lamellipodia continual cycling of assembly and disassembly of filaments appears to occur, whereas in areas in which actin filaments are bundled into thicker F-actin structures (stress fibres) exchange of actin monomers is slow.

1.3.2. Actin dynamics.

G-actin is an ATP-binding protein and association with this nucleotide is required for polymerisation into F-actin microfilaments. Upon association with the ends of an actin filament ATP hydrolysis occurs, the phosphate group remaining associated with the actin subunit until released later which may depend upon the addition of further actin monomers. Exchange of the bound ADP for ATP occurs on actin monomers following dissociation from the actin filament, generating free ATP-G-actin available for polymerisation.

In the physiological cell medium F-actin and G-actin are maintained in a dynamic steady state whereby the association of ATP-G-actin onto the barbed ends of actin filaments is balanced by the disassociation of actin monomers from the pointed end of a filament. This phenomenon results in a process known as treadmilling. However, in the steady state the growth rate at the barbed end of an actin filament would generate a maximum rate approximately 2 to 3-fold lower than the velocity required for actin-based motile processes such as lamellipodial protrusion. That the protrusion of lamellipodia is powered by the rapid turnover of actin filaments by a treadmilling mechanism has been indicated in a number of cell types (Small, 1995; Small et al., 1995; Mogilner and Oster, 1996). The control of actin polymerisation, the development of an organised network of actin filaments and links to the plasma membrane required for the formation of structures such as the leading edge lamella of a motile cell, or extending pseudopodia of a
phagocytic cell, require the coordinated action of a number of different actin binding proteins.

### 1.3.3. Actin binding proteins.

A number of actin binding proteins have been identified that may affect the stability and dynamics of the actin cytoskeleton in vivo by interacting with either G-actin or F-actin. Actin associated proteins fall into a number of different categories depending upon their properties regarding control of the actin cytoskeleton. Many actin binding proteins are regulated by second messenger signalling molecules including Ca\(^{2+}\) and inositol phospholipids, which act to control their activities in the regulation of actin filament polymerisation. Actin binding proteins also provide the link between the actin cytoskeleton and other cellular structures for example the plasma membrane at focal complexes.

The cytosolic pool of ATP-G-actin is maintained by actin binding proteins that interact with ATP-G-actin and act as actin monomer sequestering proteins (e.g. profilin) which control actin polymerisation through mediating monomer availability. A role for profilin in promoting actin filament formation has been suggested in a number of cell types. Over expression of human profilin in clonal cells was found to increase the level and stabilise F-actin containing structures, and raised levels of profilin expression altered the distribution of actin filaments, whereby a loss of actin bundles that traversed the cell was accompanied by an increase in the density of the submembranous F-actin network (Finkel et al., 1994). The actin binding properties of profilin are regulated by binding PIP\(_2\), which acts to reduce the affinity of profilin for actin and hence releases actin monomers increasing the availability of G-actin for polymerisation onto actin filaments (Ayscough, 1998).

Barbed end capping proteins include gelsolin and capping protein \(\beta_2\) which is the non-muscle cell homologue of Cap Z, which act by preventing actin polymerisation at the barbed end. Gelsolin, which also acts to sever actin filaments is regulated by Ca\(^{2+}\) and inositol phospholipids, and has been implicated in the signalling systems that control actin polymerisation in neutrophils in response to fMLP (Stossel, 1993). Roles for gelsolin in mediating cell motility have been suggested as overexpression of gelsolin in NIH-3T3 fibroblasts increased cell motility and electroinjection of anti-gelsolin antibody into
human fibroblasts was found to inhibit cell migration. Both PIP and PIP$_2$ act to uncap gelsolin-blocked barbed ends and inhibit the actin severing properties of gelsolin (Ayscough, 1998).

The organisation and stabilisation of actin microfilaments into higher order structures including stress fibres and filament networks is controlled by a number of different proteins with various activities, for example tropomyosin, and the actin-bundling and crosslinking protein $\alpha$-actinin. Tropomyosins are elongated proteins that bind along actin filaments, spanning several actin monomers. Deletion of one of the two tropomyosin genes of $S.~cerevisiae$ results in a loss of cytoplasmic actin cables, suggesting a role in stabilising actin filaments (Ayscough, 1998). Actin binding proteins that bundle or cross-link actin filaments can act by their ability to self associate (e.g. $\alpha$-actinin), or by interacting with actin through multiple sites (e.g. fimbrin, which possesses two actin binding sites). The importance of actin bundling proteins in the control of the actin cytoskeleton has been demonstrated in $S.~cerevisiae$, and $Dictyostelium$. $S.~cerevisiae$ mutants that lack yeast fimbrin do not form normal actin structures and are defective in morphogenesis and endocytosis. Mutation of $\alpha$-actinin and actin-bundling proteins-120 (ABP-120) in $Dictyostelium$ resulted in enlarged cells with reduced rates of cell movement and phagocytosis (Ayscough, 1998).

1.3.4. Adhesion complexes.

Adhesive structures are found at sites where cells form contacts with the substratum, for example focal adhesions and focal contacts, or with other cells for example adherens junctions and tight junctions. Focal adhesions and focal complexes are discreet regions of the plasma membrane which mediate the attachment of cultured cells to the substratum, and transduce signals that relate to cell-growth control. Focal adhesions provide the structural link between the actin cytoskeleton and the extracellular matrix, and are found at sites where stress fibres meet the plasma membrane. Focal adhesions contain aggregated extracellular matrix receptors (integrins) that span the plasma membrane, interacting on the outside with extracellular matrix components (e.g. fibronectin). In addition to actin many proteins have been identified in focal adhesions, which may either provide a structural role, for example vinculin, profilin and vasodilator-stimulated phosphoprotein (VASP), or be involved in signal transduction, for example focal adhesion kinase (FAK), PKC, and the adapter protein Grb2. Signalling through focal
adhesions results from cell-adhesion induced integrin clustering, followed by tyrosine phosphorylation of components associated with the cytoplasmic face of the focal adhesion complex. As a result signalling complexes are generated that transmit downstream signals required for cell-cycle progression, cell survival and prevention of apoptosis (Yamada and Geiger, 1997).

1.3.5. The Arp2/3 complex.
The Arp2/3 complex, which has been implicated in the control of actin polymerisation in cells, consists of seven polypeptides, including the actin related proteins (Arp) 2 and 3, giving the complex its name. The seven components are highly conserved between yeast, amoebas and mammals and exist in the Arp2/3 complex with approximately equal stoichiometry (Welch et al., 1997b). The Arp2/3 complex was first identified in Acanthamoeba where it was found associated with the actin-rich cortex (Machesky et al., 1994). In addition to Arp2 and Arp3 the complex contains polypeptides of 40, 35, 19, 18 and 14 kDa in Acanthamoeba and proteins of similar sizes in humans (Welch et al., 1997b; Machesky et al., 1997). Apart from Arp 2 and 3 little is known about these proteins although peptide sequence information has been obtained for each of the component proteins. The 40 kDa protein shares homology with suppressor of profilin 2 (SOP2), which has been shown in fission yeast, using profilin affinity columns and genetic interactions, to interact with Arp3 protein and to modulate the function of the actin binding protein profilin (Welch et al., 1997b; Balasubramanian et al., 1996). Like SOP2 the 40 kDa Arp2/3 complex component contains WD repeat motifs and may be involved in directing protein-protein interactions perhaps acting as an adapter protein that links the Arp2/3 complex to the cell membrane by interacting with PH domain containing proteins.

In yeast cells Arp2 and Arp3 are involved in regulating the distribution of cortical actin patches which are involved in chitin distribution, budding, osmotic sensitivity and endocytosis in S. cerevisiae and for actin cytoskeletal organisation during the cell-cycle in S. pombe. (Balasubramanian et al., 1996; Moreau et al., 1996; Winter et al., 1997; Moreau et al., 1997; Schwob, et al., 1992). A role for the Arp2/3 complex in actin polymerisation required for the propulsion of Listeria monocytogenes through infected cells has been indicated from immunocytochemical localisation of the complex and that the Arp2/3 complex purified from human platelets can direct actin polymerisation on the
surface of *L. monocytogenes* in vitro (Welch et al., 1997a). In addition the Arp2/3 complex is dynamically associated with the peripheral regions of the actin cytoskeleton of Swiss 3T3 fibroblasts, to lamellipodia of stationary fibroblasts and to the leading edge of motile fibroblasts. The complex becomes redistributed to cellular regions of dynamic actin filament assembly, for example peripheral lamella upon stimulation of cells with agents such as PDGF. This association with actin appears to be spatially regulated as it is not found with larger actin bundles (Welch et al., 1997b; Machesky et al., 1997). These observations suggest an involvement for the Arp2/3 complex in actin polymerisation and organisation of the actin cytoskeleton in fibroblasts.

1.3.6. Motility of *Listeria monocytogenes*.

The mechanism by which *L. monocytogenes* employs host cell proteins to polymerise actin at its cell surface serves as a model for the control of actin dynamics at the cell periphery. Upon infection *L. monocytogenes* recruits a number of host cell proteins to its surface, which are required for movement of bacteria cells through the cytoplasm of an infected cell. These include the enabled/VASP family of proteins and the Arp2/3 complex proteins. *L. monocytogenes* propels itself through the cytoplasm of an infected cell followed by an actin comet tail composed of actin filaments and actin binding proteins. The addition of actin monomers at the barbed ends of actin filaments which abut the surface of *L. monocytogenes* appears to generate the force required to propel this pathogen through the cytoplasm, as actin polymerisation is tightly coupled to propulsion of the bacteria (Sanger et al., 1992; Theriot et al., 1992). This process resembles the generation of actin containing filopodial and lamellipodial extensions at the cell periphery which are coupled to actin polymerisation at the cell membrane and may result from the extension of actin filaments. The polymerisation process may provide the force to push the membrane out (Small, 1995; Mogilner and Oster, 1996).

ActA is the only bacterial protein required for an association *L. monocytogenes* with the actin cytoskeleton of the host cell. The microfilament and focal adhesion associated protein VASP has been reported to interact with the bacterial surface protein ActA in vitro. Enabled/VASP family members can interact with focal adhesion components zyxin and vinculin and also profilin via proline-rich motifs (Reinhard et al., 1995; Brindle et al., 1996) and have been implicated in the control of actin polymerisation. Over expression of one enabled/VASP family member (Mena) led to the development of abnormal
structures rich in F-actin in fibroblasts (Gertler et al., 1996). The interactions between ActA-VASP-profilin are thought to form a complex which provides the basis for actin filament elongation at the surface of *L. monocytogenes* as profilin binds actin in a 1:1 complex and as a complex can stimulate the addition of actin to the barbed ends of actin filaments. Other host actin binding proteins have also been associated with the comet tail. These include α-actinin (Dabiri et al., 1990), filamin (Dold et al., 1994), fimbrin (Kocks and Cossart, 1993) and ezrin/radixin (Temmgrove et al., 1994).

A number of the Arp2/3 complex components possess actin binding sites and collectively the complex components can interact with more than one actin microfilament (Machesky et al., 1994; Kelleher et al., 1996; Mullins et al., 1997). The Arp2/3 complex components can cross-link actin filaments *in vitro*, are seen to decorate the entire length of the comet tail following *L. monocytogenes* in cells and may act to bundle actin filaments (Mullins et al., 1997). Arp2 and Arp3 do not polymerise like conventional actin, but do form heterodimers which appear similar to but more stable than an actin dimer. Chemical cross-linking and electron microscopy studies have predicted that the components are arranged in complex in a horseshoe shape (Kelleher et al., 1996; Mullins et al., 1997). The topology of this structure suggests that the Arp2/3 complex may provide a nucleus for actin polymerisation at the barbed ends of actin filaments. This may indicate that the Arp2/3 complex mediates actin polymerisation by nucleating new actin filaments *in vivo*, the step which has been defined as rate limiting for actin filament assembly *in vitro*. Taken together these observations suggest that the Arp2/3 complex plays a role in directing actin filament assembly and organisation in cells and may provide the link between actin filaments and focal adhesion/focal complexes at the cell membrane.

1.4. The Rho family of small molecular weight GTPases.

1.4.1. The Ras superfamily of GTPases.

Small Ras-related GTPases constitute a superfamily of proteins that play central roles in regulating many aspects of cell biology. The prototypical member of this superfamily is the 21 kDa mammalian H-Ras protein. Ras proteins were first discovered as H-Ras and K-Ras were found to be encoded by the oncogenes in Harvey and Kirsten rat sarcoma viruses respectively (Barbacid, 1987). The members of the Ras superfamily which includes at least 50 distinct proteins can be divided into a number of different subgroups.
based upon their primary sequences, namely the Ras, Rho, Rab, Arf, Sar, Ran and Rad subfamilies (Bourne et al., 1991; Boguski and McCormick, 1993; Hall, 1994).

The Ras subfamily includes four different Ras proteins, H-Ras, K-RasA, K-RasB and N-Ras which are found in mammalian cells, with H-Ras for example being ubiquitously expressed (Hall, 1994). These proteins control cell-growth and neuronal cell differentiation and can elicit oncogenic properties when they are constitutively bound to GTP (Boguski and McCormick, 1993). The Ras subfamily also includes the Rap proteins, Rap 1A, Rap 1B and Rap2 (Downward, 1990). A role for Rap 1A (also known as Krev-1) in regulating NADPH oxidase activity in phagocytic leukocytes has been suggested (DeLeo and Quinn, 1996). The members of the Rab or Arf and Sar families have been suggested to have related roles; Rab proteins in vesicle trafficking and Arf and Sar families in formation and budding of vesicles. Ran proteins are involved in mRNA and protein transport across the nuclear membrane, and potential roles for members of the Rad subfamily have been suggested in growth related signalling (Boguski and McCormick, 1993; Hall, 1994). The members of the Rho subfamily have been implicated in a wide variety of cellular processes see below.

1.4.2. The molecular switch.

All members of the Ras superfamily are GTP-binding proteins which possess an intrinsic GTPase activity, and act as molecular switches cycling between GTP-bound and GDP-bound states, also known as active/"on" and inactive/"off" states respectively (Bourne et al., 1991). Numerous groups of regulatory proteins act to affect this cycle in vivo, thereby controlling the level of active p21 protein present (Boguski and McCormick, 1993). These regulatory proteins include GTPase activating proteins (GAPs), which bind to GTP-bound p21s and catalyse the hydrolysis of GTP, thereby enhancing the intrinsic GTPase activity, stimulating conversion to the GDP-bound state, guanine nucleotide exchange factors (GEFs), which direct exchange of bound GDP for GTP catalyse the conversion of the nucleotide bound state to GTP, and guanine nucleotide dissociation stimulators (GDIs), which bind p21s in either nucleotide state and prevent the exchange of the bound nucleotide. That members of the Ras superfamily are active, and transmit downstream signals when bound to GTP has been indicated by the finding that most Ras proteins that possess oncogenic activity are constitutively active as a result of point mutations that lock them in the GTP-bound state (Downward, 1990). In addition genetic
loss of Ras-GEF activity, in a number of different organisms, leads to a phenotype that resembles loss of Ras proteins themselves, thus suggesting that Ras activation requires conversion from the GDP to GTP-bound states, catalysed by GEFs (McCormick, 1994).

1.4.3. The Rho family of GTPases.

Rho family proteins are ubiquitously expressed in organisms from yeast to humans. Mammalian Rho family members includes at least 10 distinct proteins; RhoA, B, C, D, and E, Rac1, Rac2 and RacE, Cdc42 and TC10. Divergence in distribution of this family of proteins is observed in lower eukaryotes, for example the *S. cerevisiae* and *S. pombe* yeast cells do not contain Rac protein whereas numerous distinct Rac proteins have been reported in the slime mould *Dictyostelium*. Rho family proteins have been implicated in mediating a wide variety of cellular functions including organisation of the actin cytoskeleton, transcriptional activation, cell growth control, Ras cell transformation and membrane trafficking (Van Aelst and D’Souza-Schorey, 1997). A requirement for Rac1/2 in the production of superoxide via the NADPH oxidase of phagocytic leukocytes has been identified (Abo *et al.*, 1991; Knaus *et al.*, 1991) and more recently evidence has suggested an involvement of Rho family proteins in the generation of ROS in non-phagocytic cells (Finkel, 1998).

Rho family proteins share approximately 50% primary sequence homology between species. Mammalian Rac1 and Rac2 share approximately 92% sequence identity diverging at their C-terminal ends where Rac1 but not Rac2 contains a polybasic motif (Didsbury *et al.*, 1989). However, tissue distribution of these two Rac proteins is markedly different; Rac1 is ubiquitously expressed whereas Rac2 is found primarily in cells of haematopoietic lineage (Didsbury *et al.*, 1989).

1.4.4. Structure.

Information obtained from the crystal structure, biochemical analysis and mutational analysis of Ras protein has enabled assigning discreet functions to specific structural elements and even particular amino acids. Whilst Ras superfamily proteins share approximately 30% primary sequence identity, marked conservation is observed within regions of sequence involved in GDP/GTP exchange, GTP-binding and GTP-hydrolysis, and also within structural regions that undergo GTP-induced conformational change.
Comparison of the crystal structures of Rac1 and H-Ras, bound to the non-hydrolysable GTP analogue guanosine-5'-(βγ-imino)triphosphate (GMPPNP), demonstrates that these proteins share a similar folded structure. Both H-Ras and Rac1 are comprised of a central β-sheet made up of six β-strands, which are linked by hydrophilic loops and α-helices (Hirshberg et al., 1997). Of the ten loop regions defined in H-Ras some have been ascribed particular properties. Loop 1 contains the sequence motif GXXXXGK(S/T), corresponding to residues 10-17 in Ras. Amino acids in this motif, including the highly conserved lysine 16, form bonds with the α and β-phosphate groups of GTP or GDP. The loop1 nucleotide binding motif also forms part of the guanine nucleotide binding pocket in Rac1 (Hirshberg et al., 1997). The hydroxyl group of serine 17 in H-Ras (which corresponds to threonine 17 in Rac1 and other members of the Rho family) participates in coordinating the Mg²⁺ ion, which is linked to the oxygens of the β and γ-phosphates of GTP, and is essential for GTP hydrolysis (Valencia et al., 1991). The Mg²⁺ ion is also coordinated by the hydroxyl in the side-chain of threonine 35 in loop 2.

Loop 2 and the N-terminal part of the adjacent β-strand (β-strand 2) of Ras, constitute a region of structure that was found to undergo marked conformational change during the conversion between the two nucleotide states, and is called the Switch I region (residues 30-38). Mutational analysis of amino acids within and around the switch I region identified particular residues which were required for Ras signalling, but did not affect the GTP binding capacity. These residues were defined as being required for the Ras-effector interaction and include asparagine 38 (reviewed by Marshall, 1993). Thus the region of sequence containing amino acid residues 32-44 in Ras is described as the ‘effector domain’. The corresponding sequence in Rho family proteins is highly conserved with that of H-Ras. However, certain residues are substituted with hydrophobic amino acids, and analysis of the crystal structure predicts that the corresponding loop in Rac1 is not as exposed or as flexible as in H-Ras (Hirshberg et al., 1997).

A second region of Ras, known as the Switch II region (residues 60-76), was also found to undergo marked conformational change during conversion between the two nucleotide states. The Switch II region, which contains part of the third β-strand, loop 4 and residues in the second α-helix, can adopt many different conformations and is
thought to be involved in determining the specificity of Ras-effector interactions. This region contains the DXXG sequence motif (residues 57-62 in Ras and Rho family proteins) which is conserved in all GTPases. The invariant aspartate (D57) and glycine (G60) residues bind the Mg\(^{2+}\) ion and the \(\gamma\)-phosphate of GTP respectively.

Comparison of the amino acid sequences of Rho family proteins with Ras reveals a 14 amino acid insertion, which lies within loop 8 and is not present in any other members of the Ras superfamily. The so called ‘insert region’ corresponds to residues 120-137 in Rho family proteins (Valencia et al., 1991). Whereas loop 8 in Ras forms a short structure, the additional amino acids in Rac1 appear to form two \(\alpha\)-helices followed by an extended loop, thought to make up a highly mobile and exposed surface structure (Hirshberg et al., 1997).

Members of the Ras superfamily also contain a C-terminal sequence motif that is modified by the post-translational attachment of an isoprenyl group. Rho family proteins all contain a C-terminal CAAX box motif, where A is an aliphatic amino acid; in Rac and Rho X=L and in Cdc42 X=F. These proteins are modified by the addition of a 20 carbon geranylgeranyl group to the cysteine residue of the CAAX box motif by prenyltransferases. This modification acts to direct Rho family proteins to the membrane (Boguski and McCormick, 1993).

1.4.5. Rho family regulatory proteins.

1.4.5.1. Guanine nucleotide exchange factors (GEFs).

GEFs catalyse removal of bound GDP and due to the relatively high ratio of GTP:GDP existing in the cell, the nucleotide is replaced with GTP. This group of proteins stimulates activation of p21s. Rho p21 proteins are activated by a group of GEFs related in their catalytic domains to the protooncogene Dbl (Hart et al., 1991), which include Ost, Lbc and Tiam-1 (Cerione and Zheng, 1996). Each member of this group possesses a Dbl homology (DH) domain in tandem with a PH domain. The DH domain appears to be responsible for the catalytic activity, whereas the PH domain is thought to be essential for directing cellular localisation. Members of this group of proteins such as Cdc24 and Vav have been implicated in cell growth regulation and others, for example Ost, Lbc and Ect-2 were identified by virtue of their oncogenic capacity. Characterisation of the biochemical activities of Dbl related GEFs \textit{in vitro} suggests promiscuous activities
towards Rho family proteins. For example, Ost exhibits GEF activity towards RhoA and Cdc42Hs and is also capable of binding Rac1. Whether the \textit{in vitro} activities extend to the \textit{in vivo} situation is unclear, as only a few examples have been described. Biochemical and genetic analyses suggest that Cdc24 acts as a physiological activator of Cdc42 in \textit{S. cerevisiae} and that the protooncogene Vav has been shown to activate Rac1 \textit{in vivo}. In addition Trio, which possesses two DH domains, appears to have dual specificity \textit{in vivo} activating both Rac and Rho (Zheng \textit{et al.}, 1994a; Crespo \textit{et al.}, 1996; Bellanger \textit{et al.}, 1998).

\textbf{1.4.5.2. GTPase activating proteins (GAPs).}

GAPs enhance the intrinsic GTPase activity by catalysing hydrolysis of the bound GTP nucleotide, thereby increasing the rate of conversion from GTP to GDP-bound forms, down regulating the activity of the p21. There is a large family of sequence related proteins that possess GAP activity towards Rho family p21s, which have homology to the break point cluster region (Bcr) gene product (Lamarche and Hall, 1994). The first to be identified was p50RhoGAP, which led to the identification of Bcr and the \textit{n}-chimaerin family of GAPs by sequence similarity. Biochemical analysis has suggested a certain amount of overlap between the activities of Rho family-GAPs. p50RhoGAP is active towards Rho, whereas Bcr and \textit{n}-chimaerin were found to be active towards Rac \textit{in vitro} (Diekman \textit{et al.}, 1991). \textit{n}-chimaerin has also been described as a Cdc42Hs-GAP (Ahmed \textit{et al.}, 1994). The RasGAP-binding protein p190 has Cdc42Hs-GAP activity (Settleman \textit{et al.}, 1992). Other Rho family GAPs include 3BP-1 (Cicchetti \textit{et al.}, 1992 and 1995), \textit{\beta}-chimaerin (Leung \textit{et al.}, 1993), Abr (Tan \textit{et al.}, 1993), p122, Myr5, RalBP1 and Graf (Van Aelst and D'Souza-Schorey, 1997). Interestingly, \textit{n}-chimaerin, which was found to bind preferentially to GTP-bound Rac1 (Ahmed \textit{et al.}, 1994), has been implicated as a potential downstream effector for Rac1 in mediating morphological changes in fibroblasts (Kozma \textit{et al.}, 1996).

\textbf{1.4.5.3. Guanine nucleotide dissociation inhibitors (GDIs).}

GDIs bind tightly to GDP-p21 proteins preventing the action of exchange factors thereby inhibiting activation and are also found to associate with GTP-p21s inhibiting nucleotide hydrolysis. These proteins act to solubilise p21 proteins and appear to play a role in regulating the translocation of Rho proteins from the cytosol to the membrane (Boguski and McCormick, 1993). For example, Rac is found in association with RhoGDI in the
cytosol of resting neutrophils and dissociation of this complex is required for Rac translocation to the membrane which correlates with the conversion of Rac to the GTP-bound state and activation of the NADPH oxidase (Abo et al., 1994). Without a factor such as RhoGDI, Rho family proteins would remain firmly attached to the membrane by their C-terminal isoprenyl groups.

1.4.6. Mutational analysis of Rho family proteins.

Much of the mutational analysis applied to investigate the molecular mechanisms by which the members of the Rho family act has been based upon structural and biochemical analysis of Ras. The introduction of single amino acid substitutions can be used to generate proteins that are (i) GTPase negative and so constitutively active, (ii) invariably bound in the GDP nucleotide state due to a reduced affinity for GTP and so act as ‘dominant negative’ proteins or (iii) defective in directing interactions with target proteins, so called ‘effector domain’ mutants.

1.4.6.1. GTPase negative proteins.

Ras oncogenes, which have been found in ~30% of human tumours, encode forms of Ras which are activated by point mutations which occur at, or near sites that interact with the β and γ-phosphates of GTP. For example, single amino acid substitutions at positions 12 or 61 have the effect of reducing the GTPase activity which results in accumulation of GTP-bound Ras protein. The Ras-Q61L GTPase negative mutant has increased effector function in terms of transformation ability and Ras-GAP interaction (Brownbridge et al., 1993). Introduction of the Q61L point mutation in Rac2 leads to an increased activity in promoting superoxide production via the neutrophil NADPH oxidase (Xu et al., 1994) and in Rac1 membrane ruffling in fibroblasts. G12V and Q61L mutations in Rac, and analogous mutations in other Rho family proteins (for example G14V in RhoA), also cause loss of sensitivity to GAPs.

1.4.6.2. Dominant negative proteins.

Mutation of residue 17 in Ras, Rac1 and Cdc42, or 19 in RhoA, to an asparagine has been widely used to generate dominant negative proteins, which have been used to investigate actions of p21s by specifically blocking their activation in whole cell situations. Proteins harbouring this mutation are thought to interact with GEFs, but to have reduced affinity for GTP. Therefore such proteins remain bound to the GEF and act
to inhibit the activation of equivalent endogenous p21 by sequestering GEFs, as was
described for Ras protein (Feig and Cooper, 1988a and 1988b). Dominant negative
proteins have provided useful tools in defining p21 functions (for example Ridley et al.,
1992). However, the actions of these proteins are not fully understood and care must be
taken when interpreting results obtained from such experiments.

1.4.6.3. ‘Effector domain’ mutants.
Point mutations in the ‘effector domain’ of Ras, for example D38A in which residue 38 is
mutated from asparagine to alanine, have been used to determine the specificity of
interactions with potential target proteins. The Ras-D38A mutant does not interact with
effectors such as Raf, and is deficient of transforming ability (Warne et al., 1993).
Mutational analysis has been applied to the effector domain region of Rho family proteins
to dissect the pathways by which these proteins elicit their diverse functions. Single
amino acid substitutions at different positions in this domain have been used in order to
discern which of the potential Rho family target proteins participate as downstream
effectors (for example Joneson et al., 1996).

In addition to the introduction of point mutations, the use of bacterial cytotoxins has
provided useful information regarding the functions of Rho family proteins. C3 toxin
from Clostridium botulinum specifically inactivates Rho protein by catalysing ADP-
ribosylation of sites within the ‘effector domain’. C3 toxin has been widely used to
investigate the actions of Rho in mediating the control of the actin cytoskeleton (as
reviewed by Machesky and Hall, 1996).

1.4.7. Signalling pathways and functions of Rho GTPases.
1.4.7.1. Organisation of the actin cytoskeleton.
Members of the Rho family have been implicated in regulating the organisation of the
actin cytoskeleton in response to extracellular stimuli. RhoA mediates the formation of
stress fibres in quiescent fibroblasts in response to serum, the active component of which
was found to be LPA (Ridley and Hall, 1992). Furthermore, injection of active RhoA-
G14V induces formation of stress fibres and focal adhesion complexes in quiescent
fibroblasts (Nobes and Hall, 1995).
Growth factors such as PDGF, bombesin and insulin stimulate the formation of filamentous actin at the plasma membrane that extends into structures such as lamellipodia and membrane ruffles. The use of dominant negative proteins demonstrated a requirement for Rac in PDGF induced membrane ruffling. Microinjection of Rac1-G12V into fibroblasts induced the formation of membrane ruffles and lamellipodia, resembling those structures seen in response to PDGF, and also subsequent stress fibre formation (Ridley et al., 1992). Dominant negative Cdc42Hs-T17N specifically blocked the formation of filopodia in fibroblasts in response to Bradykinin stimulation. Filopodia formation was also observed following microinjection of fibroblasts with Cdc42Hs, and was accompanied by cell retraction and subsequent membrane ruffling and lamellipodia formation (Kozma et al., 1995). Cdc42 and Rac have also been shown to stimulate the formation of focal complex structures containing vinculin, paxillin and FAK, that were distinct from the ‘classical’ Rho-type focal adhesions in their morphology (Nobes and Hall, 1995).

It has been suggested that Cdc42, Rac and Rho operate in a linear pathway, in terms of their morphological effects. In fibroblasts, protrusion of filopodia induced by activation of Cdc42 was followed by the formation of lamellipodia. Also in fibroblasts, Rac1-G12V induced the formation of membrane ruffles, lamellipodia and subsequently stress fibres (Ridley and Hall, 1992). However, the proposed cascade is not conserved between a number of different cell types and evidence now indicates that Rac1 and Cdc42 can act in competition with RhoA. For example, the signalling pathways that transmit LPA or acetylcholine-induced morphological effects observed in the neuroblastoma cell line NIE-115 cells (mediated by RhoA and Cdc42 and/or Rac1 respectively), appear to act in competition (Kozma et al., 1997).

Rho family GTPases have been linked to the control of the actin cytoskeleton in leukocytes. Leukocytes are highly motile cells and dramatic cytoskeletal changes accompany the processes of chemotaxis and phagocytosis stimulated by agonists such as fMLP. Involvement of Cdc42 in the phagocytic uptake of, and nuclear responses induced by, *Salmonella* into COS-1 cells, and for Rho in invasion of epithelial cells by *Shigella* has been noted (Chen et al., 1996; Adam et al., 1996; Watarai et al., 1997). This information suggests a role for Rho proteins in the specific reorganisation of the actin cytoskeleton required for phagocytosis, which includes the formation of the F-actin-rich
phagocytic cup at the point of particle attachment. Evidence has also suggested a role for Rho family proteins in the control of the actin cytoskeleton in the murine macrophage cell lines RAW 264.7 and Bac1.2F5. RhoA, Rac1 and Cdc42 induced the formation of distinct F-actin containing structures when injected into Bac1 cells, resembling those seen in fibroblasts. Furthermore the use of C3 toxin or dominant negative forms of Rac1 and Cdc42 suggested a requirement for these GTPases in regulating CSF-1 induced actin cytoskeletal changes and also in assembly of focal contacts (Allen et al., 1997). Dominant negative Rac1 and Cdc42 proteins and the GAP-domain of n-chimaerin inhibited the cytoskeletal responses (e.g. membrane ruffling) stimulated by fMLP or CSF-1, and phagocytosis of IgG-coated red blood cells by RAW 264.7 cells (Allen et al., 1997; Cox et al., 1997).

1.4.7.2. Transcriptional activation.

It has been noted that the Rho family proteins RhoA, Cdc42 and Rac mediate signalling pathways upstream of transcriptional activation. Expression of constitutively active forms of Rac1 and Cdc42Hs in HeLa, NIH-3T3 and COS cells resulted in the stimulation of JNK and p38 (Coso et al., 1995; Minden et al., 1995), and similar effects were obtained with oncogenic GEFs for these p21s (Minden et al., 1995). However, a lack of conservation of these activities between different cell types has been suggested. An involvement for RhoA and Cdc42, but not Rac1, in JNK activation in human kidney 293 T-cells was described (Teramoto et al., 1996). RhoA, Rac1 and Cdc42 can also stimulate the activity of serum response factor (SRF) (Hill et al., 1995). SRF initiates transcription of genes with serum response elements in their promoter enhancer regions, for example the Fos promoter. RhoA has been found to be required for LPA, serum and AIF4 -induced transcriptional activation by SRF (Hill et al., 1995).

Rho GTPases have been implicated in the activation of nuclear transcription factor κB (NF-κB), perhaps by directing phosphorylation of the inhibitory factor IκB, causing its dissociation from the Rel-related protein hetero/homodimer, enabling transport of the latter complex to the nucleus. NF-κB results from the action of a number of stimuli including cytokines, phorbol esters, UV light and TNFα (Perona et al., 1997). Evidence has suggested that Rac1 mediation of NF-κB activation by cytokines involves a redox-dependent pathway. A number of NF-κB stimuli induce an increase in the intracellular level of ROS. In HeLa cells, Rac1-G12V expression increased the level of ROS whereas
dominant negative Rac1-T17N appeared to block ROS increase and the activation of NF-κB in response to cytokines (Sulciner et al., 1996). Ras and Rac1 have also been shown to be required for ROS generation in response to cytokines and growth factors (Sundaresan et al., 1996). The enzymes by which Rac mediates production of ROS in non-phagocytic cells have not been identified.

1.4.7.3. Cell growth control.
Important roles for Rho GTPases have been suggested in mitogenesis, proliferation and invasiveness. Injection of Rho, Rac or Cdc42 proteins into Swiss 3T3 fibroblasts stimulates progression through the G1 stage of the cell-cycle and subsequent DNA synthesis. The dominant negative forms of these proteins blocked serum induced DNA synthesis (Olson et al., 1995).

Oncogenic and transforming potential for Rac, RhoA and Cdc42 in different cell lines has been suggested. Expression of constitutively active forms of RhoA and Rac1 led to enhanced growth in low serum, anchorage independent growth and tumorigenic ability (Khosravi et al., 1995). A requirement of Rac and RhoA for transformation by Ras has also been demonstrated; that these proteins appear to be activated by pathways distinct from the Raf/MAPK pathway is consistent with the idea that a number of pathways contribute to Ras transformation (Khosravi et al., 1995; Qiu et al., 1995a and 1995b). RhoA and Rac appear to have independent roles downstream of Ras as a synergy between these two proteins in the formation of foci was observed (Qiu et al., 1997). A role for Cdc42 in cell transformation has also been established. Expression of active Cdc42-G12V in Rat-1 fibroblasts demonstrated anchorage independent growth and proliferation in nude mice. Unlike Rac and RhoA, Cdc42 was required to cycle between the nucleotide states to induce enhanced growth in low serum. Use of a mutant Cdc42-F28L that can undergo GTP exchange in the absence of GEF demonstrated enhanced growth in low serum (Lin et al., 1997b).

1.4.8. Rho family interacting proteins.
In order to understand the mechanisms by which Rho family proteins function in controlling diverse cellular processes, it is important to identify and characterise the biochemical and cellular properties of proteins with which they interact. A large number of proteins that preferentially interact with GTP-bound Rho proteins, and hence
represent potential downstream targets have now been identified (Van Aelst and D'Souza-Schorey, 1997).

The use of p21 binding assays for screening for proteins that specifically interact with GTP-bound p21s, led to the identification of activated Cdc42-binding kinase p120\(^{\text{ACK}}\), a tyrosine kinase (Manser et al., 1993), and identified the presence of a number of Cdc42Hs, Rac1 and RhoA binding partners in different rat tissue extracts. p21 binding assays are carried out by incubating proteins immobilised on nitrocellulose filter with \([\gamma^{32}\text{P}]\text{GTP-p21}\) probes. The filters are then exposed to X-Omat film and dark signals on a clear background indicate the position of interacting proteins.

Purification of Cdc42Hs binding activity from rat brain cytosol using the p21 binding assay led to the identification of a 65 kDa Cdc42Hs and Rac1 binding partner. This protein was found to be a p21(Cdc42Hs/Rac)-activated kinase (PAK) with serine/threonine kinase activity, and to be highly related to the \emph{S. cerevisiae} protein Ste20 (Manser et al., 1994). Ste20 was also found to interact with Cdc42-GTP via a motif with sequence similarity to the p21 binding motifs of p120\(^{\text{ACK}}\) and PAK (Manser et al., 1994). Ste20 is required for pheromone-mating in \emph{S. cerevisiae} (Ramer et al., 1993; Leberer et al., 1992) and acts as the first kinase in the pheromone response kinase cascade, which results in the activation of the transcription factor Ste12, leading to the expression of pheromone response elements (reviewed by Herskowitz, 1995; see section 1.2).

1.4.8.1. The PAK family of serine/threonine kinases.

The presence of at least three different mammalian PAK isoforms has been reported; \(\alpha\)-PAK (PAK1), a 68 kDa protein highly expressed in brain, muscle and spleen (Manser et al., 1994; Knaus et al., 1995; Brown et al., 1996); \(\beta\)-PAK (mPAK3), a 65 kDa protein highly enriched in brain (Manser et al., 1995a; Bagrodia 1995a); and \(\gamma\)-PAK (hPAK65, PAK2, PAK-1), a ubiquitously expressed 62 kDa protein (Teo et al., 1995; Martin et al., 1995; Jakobi et al., 1996).
1.5. The neutrophil NADPH oxidase.

The NADPH oxidase is specifically activated in the wall of the phagocytic vacuole in response to a number of different stimuli, for example the immature peptide secreted from bacteria at sites of infection fMLP, opsonised particles and the PKC agonist PMA. Once activated the NADPH oxidase catalyses electron transfer from the NADPH donor via a membrane associated cytochrome \( b \) to molecular oxygen, resulting in the one electron reduction of oxygen producing superoxide anion (\( O_2^- \)). Superoxide is specifically discharged into the lumen of the phagocytic vacuole, where it is rapidly converted into secondary species such as hydrogen peroxide (\( H_2O_2 \)), hydroxyl radical (\( OH \)) and hypochlorous acid (HOCl). These highly reactive oxygen metabolites are capable of killing microorganisms and in collaboration with the contents of the granules are the principal factors of the host defence mechanism of neutrophils. Maturation of the phagocytic vacuole occurs from the fusion with intracellular granules during the process of degranulation, which accompanies the respiratory burst. The contents of the granules are released into the phagocytic vacuole and the resulting phagolysosome is highly acidic and rich in hydrolytic enzymes (Segal and Abo, 1993).

Prevention of uncontrolled release of superoxide metabolites from phagocytes may be brought about by the complex nature of this enzyme system. The neutrophil NADPH oxidase which lies dormant in resting cells is a multicomponent enzyme that consists of five essential components; four phagocytic oxidase (\( phox \)) proteins, gp91\(^{phox} \) and p22\(^{phox} \) (which comprise the membrane associated cytochrome \( b \)), p47\(^{phox} \) and p67\(^{phox} \) (which reside in the cytosol of resting cells) and the Ras-related small molecular weight GTP-binding protein Rac1/2 (also found in the cytosol of resting cells). Each of the \( phox \) proteins was identified by an absence or loss of function in different forms of chronic granulomatous disease (CGD). The involvement of Rac protein was established by investigation of NADPH oxidase activity \textit{in vitro}.

1.5.1. Chronic granulomatous disease (CGD).

CGD which occurs in about 1 in 250 000 individuals results from a heterogeneous group of inherited molecular disorders. Biochemically CGD is characterised by the inability of phagocytic cells including neutrophils, eosinophils, monocytes, and macrophages, to produce superoxide, leaving patients highly susceptible to infections. Histologically CGD is recognised by the appearance of granulomata, which result from the fusion of
phagocytic leukocytes that have engulfed but are unable to destroy invading pathogens. In the various forms of CGD described, mutations occur in the genes encoding one or other of the \textit{phox} components resulting in a reduction or complete loss of NADPH oxidase activity. The genetic defects may result in either an absence or partial loss of protein expression or production of a dysfunctional protein. Furthermore the two subunits of the cytochrome \textit{b}, gp91\textsuperscript{phox} and p22\textsuperscript{phox} appear to stabilise each other. In X-linked CGD where gp91\textsuperscript{phox} is absent, a loss of p22\textsuperscript{phox} is also noted, and vice versa in autosomal recessive CGD when p22\textsuperscript{phox} is undetectable. The underlying genetic defects affecting the \textit{phox} proteins are heterogeneous, and a wide variety of mutations have been characterised as detailed in a review by Roos \textit{et al.}, (1996).

\textbf{1.5.1.1. X-linked CGD.}

The most common form of CGD, found in approximately 60\% of cases is X-linked and is caused by mutations in the \textit{CYBB} gene encoding the large subunit of the cytochrome \textit{b}, gp91\textsuperscript{phox}. Mutations in the \textit{CYBB} gene can occur from, deletions, insertions, splice site mutations, and missense or nonsense mutations, although in one patient a combination of a deletion and an insertion has been characterised. Deletions which range in size from a single base-pair to 5 000 kb, and insertions which are usually of a single base-pair, generally lead to mRNA instability and a lack of gp91\textsuperscript{phox} protein and result in the most severe form of CGD in which NADPH oxidase activity is completely lost. Splice site mutations in which a complete exon in the gp91\textsuperscript{phox} mRNA is missing account for approximately 16\% of X-linked CGD patients. gp91\textsuperscript{phox} protein is undetectable in all but one of these cases, where only part of exon 12 is missing. In this case the protein is probably dysfunctional due to a 10 amino acid deletion which is located to within the 20 amino acid region to which the NADPH binding site has been mapped. Nonsense mutations usually induce a stop codon and therefore almost always lead to a total lack of gp91\textsuperscript{phox}. In contrast missense mutations, which affect the level of gp91\textsuperscript{phox} protein and the function of the cytochrome \textit{b} in a variety of ways, often lead to the expression of a stable but non-functional protein.

Mutations that result in the production of non-functional gp91\textsuperscript{phox} protein have been used to characterise the biochemical properties of gp91\textsuperscript{phox} and the cytochrome \textit{b}. Three patients with a proline to histidine mutation at position 415 were shown to exhibit reduced 2-azido-NADPH binding, which indicated that gp91\textsuperscript{phox} was the enzymatic
subunit of the cytochrome b (Segal et al., 1992). Investigation of a solubilised mutant cytochrome b, with a mutation in gp91phox at residue 54 of arginine to serine, demonstrated a shift in optical absorbance properties, a reduction in the midpoint potential of one of the two heme groups involved and also a lack of electron transport from FAD, indicating that electron transfer from FAD to oxygen in this oxidase enzyme requires involvement of two heme groups in series (Cross et al., 1995). In both cases the cytosolic components p47phox and p67phox translocated to the membrane as required for formation of the active NADPH oxidase enzyme complex, although the activity of which was markedly reduced. Thus such mutations have enabled useful investigations of NADPH oxidase function.

1.5.1.2. Autosomal recessive CGD.
Mutations in the CYBA gene encoding the small subunit of the cytochrome b, p22phox account for a rare subgroup of autosomal recessive CGD (Dinauer et al., 1990). A variety of mutations account for this phenotype, including deletions, insertions, splice site mutations and missense mutations. With one exception p22phox protein levels are undetectable, either due to expression of an unstable protein or to a decreased interaction with gp91phox. Mutation of p22phox at position 156, proline to glutamine (P156Q) which yields a stable protein probably results in a loss of superoxide production due to a lack of translocation of the cytosolic components to the membrane, as was characterised using in vitro assays (Dinauer et al., 1991; Leusen et al., 1994).

Autosomal recessive CGD is more commonly due to deficiencies in either of the cytosolic proteins p47phox or p67phox. The majority of patients deficient of p47phox are homozygous for a dinucleotide deletion that occurs at a tandem GTGT repeat. This deletion predicts premature termination of translation after the synthesis of a 50 amino acid peptide. A few patients are heterozygous for this GT deletion in combination with a point mutation. Only a few mutations in the gene encoding p67phox have been identified, most of which despite normal amounts of mRNA result in lack of p67phox protein. Of those characterised a single base-pair deletion in the 5’ splice site in intron III results in lack of exon 3, whereas a dinucleotide insertion in exon 5 predicts loss of protein due to premature termination during synthesis. A point substitution in exon 3 of G233 to A predicts replacement of residue 78 from a glycine to glutamate residue and is suggested to result in production of an unstable protein (de Boer et al., 1994). In one patient with
half the normal amount of p67\textsuperscript{phox} a GAA codon deletion, predicted to result in deletion of lysine 58, was found in one allele whereas the other allele had an 11-13 kb deletion. It is likely that the in frame deletion of lysine 58 results in the expression of a non-functional protein, and that protein is not produced from the second allele.

1.5.2. \textit{In vitro} reconstitution of the NADPH oxidase.

Information regarding the function of the phagocytic oxidase components was acquired from the analysis of cells from patients with CGD, and the ability to reconstitute NADPH oxidase activity in a cell-free system. Electron transport can be reproduced in a mixture of cytosol and membranes from unstimulated cells by the addition of amphiphiles such as AA, (Bromberg et al., 1984; Cumette et al., 1985; McPhail et al., 1984) or SDS (Bromberg et al., 1985). Activity of the NADPH oxidase can be monitored by the level of superoxide production estimated by measuring cytochrome \textit{c} reduction in the presence of NADPH.

1.5.3. The neutrophil NADPH oxidase components.

The components are found in three distinct complexes in resting neutrophil cells, either in association with membranes or in the cytosol. gp91\textsuperscript{phox} and p22\textsuperscript{phox} are tightly associated and make up the membrane associated heterodimeric cytochrome \textit{b}. p47\textsuperscript{phox} and p67\textsuperscript{phox} exist in the cytosol in a high molecular mass complex which contains the majority of p67\textsuperscript{phox} and some p47\textsuperscript{phox}, although the latter is also found free within the cytosol. A third cytosolic \textit{phox} component p40\textsuperscript{phox} has also been identified and is found to exist in complex with p67\textsuperscript{phox} in resting cells. This component is not essential for oxidase activity reconstituted \textit{in vitro} and its function \textit{in vivo} is not clear. Rac1/2 is found in the cytosol of resting cells in complex with RhoGDI (a guanine nucleotide dissociation inhibitor). Upon activation of the respiratory burst the cytosolic \textit{phox} components and Rac1/2 translocate to the membrane, where they form an active enzyme complex with the cytochrome \textit{b}.

1.5.3.1 The cytochrome \textit{b}.

The heterodimeric membrane associated cytochrome \textit{b} which is comprised of the two components gp91\textsuperscript{phox} and p22\textsuperscript{phox}, present with a stoichiometry of 1:1, was identified as a component of the respiratory burst oxidase by Allison’s group (Segal \textit{et al.}, 1978). It has been shown that gp91\textsuperscript{phox} (β-subunit) is an FAD-containing flavoprotein dehydrogenase.
with redox function that can be reconstituted by relipidation in vitro (Segal et al., 1992; Abo et al., 1992). This \(b\)-type flavocytochrome has a typical optical absorption maximum of 558-559 nm but an unusually low midpoint potential at -245 mV, hence the cytochrome \(b\) is also referred as flavocytochrome \(b_{558}\) or \(b_{245}\) (DeLeo and Quinn, 1996). Redox titration studies of COS-7 expressing \(gp91^{phox}\) and \(p22^{phox}\) indicate that \(gp91^{phox}\) contains the binding sites for both heme groups present (Yu et al., 1998). The cytochrome \(b\) is also thought to contain the NADPH binding site. Indeed in one rare form of X-linked CGD, a missence mutation occurs in a region of \(gp91^{phox}\) with weak homology to the NADPH binding site of the ferrodoxin NADP+ reductase family. In this form of CGD normal amounts of \(gp91^{phox}\) are present and translocation of the cytosolic factors to the membrane occurs upon stimulation; however superoxide is not produced (Dinauer et al., 1989; Roos et al., 1996).

Cellular staining with anti-cytochrome \(b\) antibodies and subcellular fractionation studies have shown that \(\sim 20\%\) of the cytochrome \(b\) is located within the plasma membrane with \(\sim 80\%\) present in the membranes of the specific granules of resting neutrophils. However in neutrophils that had phagocytosed \(Staphylococcus aureus\), the cytochrome \(b\) was found mainly in the membrane of the phagocytic vacuole (Jesaitis et al., 1990). A role for the Ras related GTPase Rap1A as a chaperone for cytochrome \(b\) translocation upon activation of the NADPH oxidase has been suggested from its ability to associate and colocalise with the cytochrome \(b\) (Quinn et al., 1989 and 1992).

\(gp91^{phox}\) is a heavily glycosylated 570 residue poly-peptide that has three transmembrane helices (Imajoh-Ohmi et al., 1992). The stability of \(gp91^{phox}\) depends upon the simultaneous expression of \(p22^{phox}\). In CGD cells deficient of \(p22^{phox}\), \(gp91^{phox}\) is absent despite the presence of ample mRNA (Parkos et al., 1988).

\(p22^{phox}\) is a transmembrane protein with extracellular and cytosolic domains. The cytosolic domain (amino acids 132-195) is well conserved between human and mouse sequences (Sumimoto et al., 1989) and contains two polyproline domains, amino acids 149-162 and 174-195 (Parkos et al., 1988; Sumimoto et al., 1989). Mutation of one of the proline residues in the first polyproline domain (P156Q) results in a form of CGD (Dinauer et al., 1991).
1.5.3.2. The cytosolic component p47\textsuperscript{phox}.

p47\textsuperscript{phox} is a highly basic protein of 390 amino acids that represents approximately 0.5% of neutrophil cytosolic protein (Leto et al., 1991). This protein contains two SH3 domains (amino acids 151-214 and 227-284) and a number of proline rich sequences (amino acids 70-83, 300-346 and 360-370) (Volpp et al., 1991; Leto et al., 1990), see appendix A.6 for sequence and domain structure of p47\textsuperscript{phox}. In addition to the proline-rich regions the C-terminal half of p47\textsuperscript{phox} is highly basic and contains multiple serine/threonine phosphorylation sites. Indeed extensive phosphorylation of p47\textsuperscript{phox} occurs upon stimulation of the oxidase (see below).

1.5.3.3. The cytosolic component p67\textsuperscript{phox}.

p67\textsuperscript{phox} which represents approximately 0.3% of neutrophil cytosolic proteins (Leto et al., 1991) contains 526 amino acids and has two SH3 domains (amino acids 245-295 and 460-510) and a proline-rich region (amino acids 226-236), (Leto et al., 1990). In addition analysis of the p67\textsuperscript{phox} sequence has revealed the presence of 4 tetratricopeptide (TPR) repeats within the N-terminus (amino acids 6-36, 37-70, 71-104 and 121-154; Ponting, 1996), see section 1.2.5, and appendix A.6, for the sequence and domain structure of The importance of the TPR motifs in determining the structure of is perhaps illustrated in a form of autosomal recessive CGD in which glycine 78 is mutated to a glutamate (de Boer et al., 1994). At position 8 of the third TPR of p67\textsuperscript{phox} this residue may be involved in the formation of the non-polar ‘hole’ structure which is involved in TPR motif directed protein folding. Disruption of the association attributed to these motifs may result in destabilisation of p67\textsuperscript{phox} explaining the absence of p67\textsuperscript{phox} in the above case.

1.5.3.4. The cytosolic component p40\textsuperscript{phox}.

An additional component p40\textsuperscript{phox} was identified by its ability to coimmunoprecipitate or copurify with p67\textsuperscript{phox} from the cytosol of resting neutrophils (Wientjes et al., 1993; Someya et al., 1993; Tsunawaki et al., 1994). p40\textsuperscript{phox} is found in the high molecular mass complex with p67\textsuperscript{phox} and p47\textsuperscript{phox} in the cytosol of resting cells. p40\textsuperscript{phox} consists of 339 amino acids and contains an SH3 domain (amino acids 175-226) and shares some similarity with the N-terminus of p47\textsuperscript{phox} (Wientjes et al., 1993), see appendix A.6, for sequence and domain structure. The C-terminal of p40\textsuperscript{phox} contains a distinct region of
sequence referred to as the PC motif (amino acids 282-309) which is also found in signalling molecules including the budding yeast Cdc42-GEF, Cdc24, and human and rat MEK5 (Nakamura et al., 1998). The PC motif, has the consensus ZXYXDEGDZXXSZDEDZXZZ where Z indicates a hydrophobic residue, X is variable, and the underlined residue is actually an alanine in p40phox. Although mutation of p40phox has not been reported to occur in CGD, a dramatic loss of protein is observed in CGD cells deficient of p67phox (Wientjes et al., 1993; Dusi et al., 1996).

1.5.3.5. Rac requirement in the NADPH oxidase.

A dependence upon GTP for activation of the NADPH oxidase was observed and superoxide generation was inhibited by the addition of GDP analogues (Gabig et al., 1987; Seifert et al., 1986; Ligeti et al., 1988). The inability of p47phox and p67phox to replace cytosol in cell-free systems led to the discovery of an additional cytosolic factor termed σ1(Pick et al., 1989; Shaag and Pick, 1990). Fractionation of guinea pig macrophage cytosol used in a cell-free system identified σ1 as a heterodimer shown to be Rac1 in complex with RhoGDI (Abo et al., 1991). The active factor of this complex was identified as Rac1, as addition of Rac1 protein preloaded with GTPγS to macrophage cytosol depleted of σ1 reconstituted superoxide production in the presence of macrophage membranes. The ability of Rac2 to enhance oxidase activation in a cell-free system of cytosol and membranes taken from resting human neutrophil cells indicated that Rac2 is likely to account for the GTP-dependency in this cell type. Rac2 specific antibodies were able to inhibit superoxide production in this system (Knaus et al., 1991). Whereas Rac1 expression is ubiquitous Rac2 is found mainly in cells of haematopoietic lineage (Didsbury et al., 1989). More than 96 % of the Rac protein in human neutrophils was quantified as Rac2 (Heyworth et al., 1994).

The ability to reproduce oxidase activity in vitro in a cell-free system using recombinant proteins p67phox, p47phox and pure cytochrome b (reconstituted with lipid in the presence of FAD) with either Rac1 or Rac2-GTP (Abo et al., 1992; Rotrosen et al., 1992) suggested that these proteins represent the minimal components of the oxidase. The requirement of Rac protein for activation of the NADPH oxidase was further demonstrated in whole cells. Treatment of Epstein Barr Virus (EBV)-transformed B-lymphocytes (see below) with antisense oligonucleotides containing complementary sequence shared by both Rac1 and Rac2, reduced the Rac protein level by 60 % and
resulted in a dose-dependent reduction of superoxide production by 50-60 %, in comparison to cells treated with sense oligonucleotides (Dorseuil et al., 1992). Inhibition of superoxide generation was also observed upon expression of dominant negative Rac1-T17N (Gabig et al., 1995).

*In vitro* assays have enabled characterisation of the NADPH oxidase enzyme system. However, such studies eliminate the requirement for additional factors and particularly phosphorylation events known to be involved in the activation process. *In vivo* both p47phox and p67phox are phosphorylated during activation of the NADPH oxidase (Dusi and Rossi, 1993; Faust et al., 1995). Although cell-free systems that utilise additional factors such as the signalling molecule phosphatidic acid (PA) and that are kinase dependent have been established (McPhail et al., 1995; Park et al., 1997), the use of whole cell studies has illustrated the limitations of cell-free systems.

1.5.4. Whole cell systems.

The HL60 cell line, derived from a patient with acute myeloid leukaemia, can be maintained in cell culture independently of a leukaemia-specific growth factor, a feature which is uncommon in human leukaemic cells. These promyelocytic cells provide a useful tool, as they are capable of proliferation and can be induced to differentiate into a number of different cell types, including granulocytes, monocytes, macrophage-like cells and eosinophils. Treatment with compounds such as retinoic acid or DMSO results in differentiation of HL60 cells into cells with characteristics of mature neutrophils. These differentiated cells, which can be stimulated to produce superoxide by factors such as fMLP and PMA, provide an *in vitro* model to investigate the properties of cells such as neutrophils, which cannot be maintained in cell culture (Collins et al., 1978 and 1979).

EBV-transformed B-cell lines are capable of producing low levels of superoxide by activation of the same NADPH oxidase system as present in neutrophils. EBV-transformed cells from patients deficient of the phox factors provide a useful tool for studying oxidase activation in whole cells (de Mendez et al., 1994). Alternatively, transfection of K562 cells (an undifferentiated multipotent leukaemic cell line) with the oxidase factors p67phox, p47phox and gp91phox in episomal expression vectors, can reconstitute the complete NADPH oxidase system. K562 cells express, which
endogenous Rac and p22phox, provide a useful system in which to study genetic modifications of several of the oxidase components (de Mendez and Leto, 1995).

1.5.5. Assembly of the active NADPH oxidase enzyme complex.

In the cytosol of resting neutrophils the components p67phox, p40phox and some but not all p47phox exist in a high molecular mass complex of approximately 240 kDa, as has been described by separation of neutrophil cytosol by gel-filtration chromatography (Park et al., 1992; Someya et al., 1993). The association of p47phox with this complex appears to be readily dissociatable (Iyer et al., 1994) and is perhaps directed by an SH3-polyproline domain interaction with p67phox. The C-terminal SH3 domain of p67phox was found to interact with a proline-rich region in the C-terminal of p47phox using biotinylated probes (Leto et al., 1994), and was able to precipitate native p47phox from the cytosol of resting HL60 cell lysates, via an interaction that was blocked in the presence of proline-rich peptide from p47phox (amino acids 363-370) (Finan et al., 1994). The association of p47phox with the high molecular mass complex in resting cells may also be directed by an association with p40phox. It has been proposed that p40phox interacts with p47phox via an SH3-polyproline domain interaction (Fuchs et al., 1996). The predominant interaction between the phox components within the cytosol of resting neutrophils appears to exists between p67phox and p40phox, and takes place via an SH3-domain independent interaction (Wientjes et al., 1996).

Upon activation of the NADPH oxidase, approximately 10% of the cytosolic components p47phox and p67phox translocate to the membrane (Clark et al., 1990) where they form an active enzyme complex with the cytochrome b. Translocation of p40phox has been noted in neutrophil cells stimulated with PMA or fMLP (Wientjes et al., 1993; Dusi et al., 1996). Immunogold labelling studies have demonstrated that the cytosolic components p47phox, p67phox and p40phox colocalise with the cytochrome b at the membrane of stimulated neutrophils (Wientjes et al., 1997). Multiple intermolecular interactions have been predicted to participate in the process of translocation to the membrane and also for assembly of the active enzyme complex.

1.5.5.1 p47phox interacts with the membrane associated cytochrome b.

Experiments using cells from patients with CGD demonstrated the requirement for an interaction between p47phox and the cytochrome b in order for translocation of p47phox
and p67phox to the membrane (Heyworth et al., 1991). In addition, translocation of p40phox is abrogated in cells lacking p47phox (Dusi et al., 1996). It has been predicted that the interaction between the cytosolic factors and the cytochrome b is directed by an interaction between the first SH3 domain of p47phox and the proline-rich region in the cytoplasmic tail of p22phox (Leto et al., 1994; de Mendez et al., 1997). The involvement of the SH3 domains of p47phox was first indicated by the ability of a fusion protein containing the two SH3 domains of p47phox (amino acids 154-285) to inhibit superoxide production when added to a cell free system with cytosol and membranes (Sumimoto et al., 1994). In vitro binding assays using biotinylated probes showed that the SH3 domains of p47phox can interact with the C-terminal of p22phox (Sumimoto et al., 1994). The C-terminus of p22phox contains a proline-rich region at amino acids 149-162. The ability of the p47phox SH3 domains to interact with p22phox was abolished by the point mutation P156Q, and was inhibited by a proline-rich peptide corresponding to amino acids 149-162 of p22phox (Leto et al., 1994). Notably the P156Q mutation occurs in an autosomal recessive form of CGD (Dinauer et al., 1991), which results in the inability of the cytosolic components p47phox and p67phox to translocate to the membrane (Leusen et al., 1994). Collectively these data indicate that during assembly of the enzyme complex the fundamental interaction occurs between the first SH3 domain of p47phox and amino acids 149-162 of p22phox.

1.5.5.2. p47phox directs p67phox to the membrane.

Direction of p67phox to the membrane requires the presence of p47phox, as was demonstrated in CGD cells lacking p47phox, and occurs via a direct association between these two proteins. Two possible interactions between these two cytosolic components have been illustrated in vitro, involving either the proline-rich regions within the C-terminus or the SH3 domains of p47phox (Finan et al., 1994; Sumimoto et al., 1994). The interaction between the C-terminal halves of p67phox and p47phox mentioned above, has been demonstrated using the yeast two-hybrid system (Fuchs et al., 1996). In agreement with Finan et al., (1994), this interaction in the yeast two-hybrid system requires the proline-rich region located at amino acids 360-370, but not the proline-rich region between amino acids 299 and 346 (Hata et al., 1998). Superoxide production can be reconstituted in vitro using p67phox deleted of both SH3 domains. However, an absolute requirement of the C-terminal SH3 domain of p67phox for oxidase activity in transfected EBV-B-cells has been demonstrated (de Mendez et al., 1994). This suggests that the
predicted SH3-polyproline domain interaction between the C-terminal halves of \( p47^{phox} \) and \( p67^{phox} \) is required for assembly of an active enzyme complex \textit{in vivo}.

A second interaction between \( p47^{phox} \) and \( p67^{phox} \), involving the SH3 domains of \( p47^{phox} \), may also participate in assembly of the active enzyme complex. The isolated SH3 domains of \( p47^{phox} \) (amino acids 154-285) expressed as a GST-fusion protein precipitated \( p67^{phox} \) from human neutrophil cytosol, in the presence of AA (Sumimoto \textit{et al.}, 1994). The isolated SH3 domains of \( p47^{phox} \) can support low level superoxide production in transfected K562 cells in response to PMA, and were found to direct translocation of both full-length and an N-terminal \( p67^{phox} \) protein to the membranes, via an interaction involving the second SH3 domain of (de Mendez \textit{et al.}, 1997). That the N-terminal of \( p67^{phox} \) (amino acids 1-246) is sufficient for superoxide production in cell free systems (Diekmann \textit{et al.}, 1994; de Mendez \textit{et al.}, 1994), suggests the presence of a functional domain involved in oxidase activation. This may involve the proline-rich region of \( p67^{phox} \) (amino acids 226-236) which has been proposed as an interacting site for the second SH3 domain of \( p47^{phox} \). A requirement for the N-terminal SH3 domain of \( p67^{phox} \) has been demonstrated in EBV-B-cells, as \( p67^{phox} \) deleted of this domain was capable of generating only 10% of the level of superoxide observed with the full-length protein. However, a target for this region of \( p67^{phox} \) has not been identified.

1.5.5.3. Interactions of \( p40^{phox} \).

That \( p40^{phox} \) interacts with \( p67^{phox} \) via an interaction that is not dissociatable by the action of detergents suggests that the affinity of this interaction is high and so may persist during assembly of the oxidase enzyme complex (Tsunawaki \textit{et al.}, 1994). Indeed translocation of \( p40^{phox} \) to the membrane of neutrophils, in response to PMA, requires the presence of \( p47^{phox} \) and \( p67^{phox} \) (Dusi \textit{et al.}, 1996). It has been suggested that \( p40^{phox} \) interacts with sites within the N and C-terminal halves of \( p67^{phox} \) (Wientjes \textit{et al.}, 1996; Fuchs \textit{et al.}, 1996; Sathyamoorthy \textit{et al.}, 1997).

The SH3 domain of \( p40^{phox} \) has been reported to interact with a proline-rich region within the C-terminal of \( p47^{phox} \), which overlaps with the site defined as a target for the C-terminal SH3 domain of \( p67^{phox} \) (Fuchs \textit{et al.}, 1996; Hata \textit{et al.}, 1998). Although \( p40^{phox} \) is not required for activation of the oxidase \textit{in vitro}, an inhibitory role has been suggested for this component. Addition of \( p40^{phox} \) to cell free assays inhibited superoxide
production by $p67^{phox}$, $p47^{phox}$, Rac and cell membranes. In transfected K562 cells, the presence of $p40^{phox}$ caused a 30% reduction in oxidase activity; expression of the isolated SH3 domain was found to act as a more potent inhibitor (Sathyamoorthy et al., 1997). This suggests that the inhibitory effect was due to an SH3-polyproline domain directed interaction, which may result from $p40^{phox}$ competing with $p67^{phox}$ for $p47^{phox}$ binding.

Taken together, the observations detailed above can be used to describe a model for the interactions between the oxidase components (see Fig. 1.1).

1.5.5.4. Rac translocates to the membrane.
Following activation of neutrophil cells with either PMA or fMLP, Rac is detected in association with the membrane. Rac translocation from the cytosol has been found to correlate with oxidase activity (Quinn et al., 1993; Abo et al., 1994). In the cytosol of resting cells, Rac is found in complex with RhoGDI. PMA or fMLP stimulation of human neutrophils and SDS action in vitro lead to the dissociation of Rac from RhoGDI. An association of Rac with the membranes can occur independently of $p47^{phox}$, $p67^{phox}$ and cytochrome $b$, although the stability of this association appears to depend upon the presence of the cytochrome $b$ (Heyworth et al., 1994). This suggests that Rac acts at the membrane level during activation of the NADPH oxidase. The dissociation of Rac from RhoGDI may involve phosphorylation of RhoGDI, or the activity of lipid molecules released by phospholipases.

1.5.5.5. An intramolecular interaction within $p47^{phox}$.
The inability of an antibody raised to the SH3 domain region of $p47^{phox}$ to immunoprecipitate $p47^{phox}$ from the cytosol of resting neutrophils in the absence of amphiphiles (e.g. AA and SDS), suggests that $p47^{phox}$ adopts a tertiary conformation which renders the SH3 domains inaccessible (Sumimoto et al., 1994). The SH3 domains have been shown to interact with sequences within the C-terminal of $p47^{phox}$ (Sumimoto et al., 1994). An interaction between the first SH3 domain and a C-terminal construct containing proline-rich sequences (amino acids 322-378), has been demonstrated using the yeast two-hybrid system (Fuchs et al., 1996). The target site for this SH3 domain has also been mapped to amino acids 299-346 (Hata et al., 1998). This site is distinct from the site involved in the interaction with $p67^{phox}$. This information suggests a requirement
Fig. 1.1. A Model for the Interactions Between the \textit{phox} Components.

This diagram shows the positions of the various domains in the \textit{phox} components. The interactions proposed to occur between these proteins are also indicated. The domain structure and sequences of the \textit{phox} proteins are shown in appendix A.6 and described in section 1.5. The C-terminal of \(p47^{\text{phox}}\) contains two polyproline domains, one of which (aas 300-346) contains two proline-rich motifs, as indicated (P) (see appendix A.6). The first SH3 domain of \(p47^{\text{phox}}\) can interact with \(p22^{\text{phox}}\) and a proline-rich region in the C-terminal of \(p47^{\text{phox}}\) itself (de Mendez \textit{et al.}, 1997; Hata \textit{et al.}, 1998). Competition between these two interactions may provide the difference between the resting and activated states of \(p47^{\text{phox}}\) and explain the ‘unmasking’ of \(p47^{\text{phox}}\) caused by oxidase activators \textit{in vitro} (Sumimoto \textit{et al.}, 1994). The second SH3 domain of \(p47^{\text{phox}}\) can recruit the N-terminal of \(p67^{\text{phox}}\) to the membrane, suggesting an interaction with the proline-rich region of \(p67^{\text{phox}}\) (aas 226-236) (de Mendez \textit{et al.}, 1997). This interaction has not been shown directly hence the arrow is broken. The C-terminal SH3 domain of \(p67^{\text{phox}}\), required for oxidase activity \textit{in vivo} (de Mendez \textit{et al.}, 1994), interacts with a proline-rich motif (aas 360-370) in \(p47^{\text{phox}}\) (Finan \textit{et al.}, 1994; Hata \textit{et al.}, 1998). \(p40^{\text{phox}}\) has been shown to interact with both the N and C-terminal halves of \(p67^{\text{phox}}\), via an SH3 domain-independent interaction (Wientjes \textit{et al.}, 1996; Fuchs \textit{et al.}, 1995; Sathyamoorthy \textit{et al.}, 1997). The SH3 domain of \(p40^{\text{phox}}\) has also been shown to interact with the same site on \(p47^{\text{phox}}\) as the C-terminal SH3 domain of \(p67^{\text{phox}}\). These interactions may compete for \(p47^{\text{phox}}\) binding (de Mendez \textit{et al.}, 1996; Sathyamoorthy \textit{et al.}, 1997). aas-amino acids.
for a conformational change prior to the interaction between p47phox and the cytochrome b. Unfolding of p47phox may be due to the action of protein kinases or lipid second messengers, or is perhaps driven by the availability of a higher affinity binding partner. The conformation of p47phox could act as a negative constraint preventing non-specific activation of the oxidase.

1.5.6. Phosphorylation events

Activation of the NADPH oxidase is accompanied by multiple phosphorylation events. Upon PMA stimulation of neutrophils, phosphorylation of p47phox and p67phox and translocation of these components to the membrane was found to correlate with oxidase activity (Dusi and Rossi, 1993). Fuchs et al., (1997) reported a basal level of p40phox phosphorylation, on serine residues, that increased with oxidase activity in HL60 cells. A key role for PKC during activation of the NADPH oxidase has been identified. Neutrophils express several PKC isoforms including PKC-α, β1, β11, δ and ξ. Purified PKC can stimulate superoxide formation, by a solubilised NADPH oxidase, and is activated upon stimulation of neutrophils with fMLP. The use of isoform specific PKC inhibitors has suggested the involvement of more than one PKC isoform and also PKC-independent pathways in neutrophil NADPH oxidase activation (Pongracz and Lord, 1998; Kawakami et al., 1998).

1.5.6.1. p47phox phosphorylation.

The appearance of a 47 kDa phosphoprotein was associated with oxidase activation (Hayakawa et al., 1986), the loss of which (in certain forms of CGD) indicated that this protein was p47phox (Okamura et al., 1988a and 1988b). Multiple phosphorylation of p47phox was found to occur exclusively on serine residues (Okamura et al., 1988b; Hayakawa et al., 1986). The C-terminal of p47phox contains a number serines flanked by basic residues that provide potential sites for phosphorylation by PKC (Volpp et al., 1989; Lomax et al., 1989). Phosphopeptide mapping of p47phox from PMA and fMLP stimulated neutrophils, has suggested that phosphorylation of 9 distinct serine residues, which lie within PKC and MAPK consensus sites, can occur (El Benna et al., 1994; Faust et al., 1995). The use of PKC inhibitors indicates that PKC-dependent phosphorylation of p47phox is required for the translocation of this component and p67phox to the membrane (Nauseef et al., 1991). p47phox phosphorylated by PKC in vitro was able to act in a cell-free assay in the absence of amphiphiles (Park et al., 1997).
1.5.6. **p67phox phosphorylation.**

Following PMA or fMLP stimulation of neutrophils, phosphorylated p67phox was found in the cytosol and was associated with the membrane. The appearance of phosphorylated p67phox in the cytosol and membrane fractions following PMA stimulation, correlated with the respiratory burst measurements, which increased linearly up to 3 min (Dusi et al., 1993). This suggests that phosphorylation and translocation of p67phox correlates with NADPH oxidase activity in neutrophils, and that continuous phosphorylation and translocation of p67phox during the linear increase in oxygen consumption.

Following PMA stimulation of human neutrophils phosphorylated forms of both p67phox and p47phox were coprecipitated with antibodies directed to either protein, which suggests the ability of these proteins to interact in their phosphorylated states (El Benna et al., 1997). p47phox was also found to coprecipitate with the p47phox-p67phox phosphoprotein complex, suggesting that an association with the assembled enzyme complex may occur.

1.5.7. **Secondary messengers involved in NADPH oxidase activation.**

Activation of neutrophils by factors such as fMLP and PMA results in the stimulation of numerous signalling mechanisms. These include activation of lipid kinases, phospholipases and protein kinases. This results in increased cellular levels of second messenger molecules including Ca²⁺, AA, DAG, PA and cAMP. The production of some of these molecules has been linked to regulation of NADPH oxidase activity.

1.5.7.1. **Calcium (Ca²⁺).**

Stimulation of neutrophils with fMLP leads to a transient increase in the Ca²⁺ concentration, from 100 nM to 1 μM within 20-30s. In neutrophils, Ca²⁺ is released from intracellular stores called calciosomes by IP₃, which is present at raised levels within seconds of fMLP stimulation. Treatment of neutrophils with the Ca²⁺ chelator BAPTA or high levels of the Ca²⁺ indicator Quin-2, inhibited the production of ROS (Morel et al., 1991), indicating a requirement for Ca²⁺ for NADPH oxidase activation.

1.5.7.2. **Cyclic AMP (cAMP)**

Neutrophil activation is associated with an elevation in the intracellular concentration of cAMP. Agents that result in the elevation of cAMP levels can inhibit agonist stimulated
degranulation, chemotaxis and NADPH oxidase activity. However, these inhibitory effects are not seen when neutrophils are stimulated with PMA, indicating that the inhibition occurs prior to PKC activation (Smolen et al., 1991). In fMLP stimulated neutrophil cells treated with dibutyryl-cAMP, a reduction in the level of p$^{\text{phox}}$ phosphorylation was observed, which was reversed in the presence of the PKA inhibitor KT 5720. These effects correlated with the level of superoxide produced, and were not observed in PMA stimulated neutrophils. This data may explain the physiological mechanism by which cAMP exerts its inhibitory effect on the NADPH oxidase (Bengisgarber and Gruener, 1996).

1.5.7.3. Arachidonic acid (AA).

AA is produced by the action of cytosolic PLA$_2$ upon stimulation of neutrophils with fMLP. The kinetics of AA release correlates with the production of superoxide, indicating a role for this second messenger in NADPH oxidase activation (Dana et al., 1994). Furthermore, AA can stimulate NADPH oxidase activity in vitro (Bromberg et al., 1984; Cumette et al., 1985). PLA$_2$ inhibitors caused a loss of NADPH oxidase activity, which was rescued by the addition of exogenous AA (10 mM), indicating a requirement for AA in oxidase activation (Dana et al., 1994).

1.5.7.4. Diacylglycerol (DAG).

Formation of DAG in fMLP stimulated neutrophils is biphasic. A small transient increase in levels is seen within seconds, and coincides with IP$_3$ formation. The second, larger and more sustained increase in DAG levels is thought to result from PLD metabolism of PA. That DAG plays a role in activation of the NADPH oxidase has been suggested; phorbol esters such as PMA and other DAG analogues can activate the NADPH oxidase, and PKC which is activated by DAG (in the presence of Ca$^{2+}$), phosphorylates the NADPH oxidase component p$^{\text{phox}}$ in vitro (El Benna et al., 1994).

1.5.7.5. Phosphatidic acid (PA).

It has been suggested that PLD activation and PA generation are associated with oxidase activity in human neutrophils. Inhibition of PA formation, by treatment with propranolol or ethanol, blocked superoxide production in response to fMLP or IL-8 respectively (Agwu et al., 1991; Sozzani et al., 1994). The use of kinase-dependent cell-free assays suggests the involvement of a PA mediated kinase in p$^{\text{phox}}$ phosphorylation (Waite et
However, the action of PA in NADPH oxidase activation is not fully understood.

1.5.7.6. Phosphatidylinositol 3-kinase (PI 3-kinase).

The phospholipid product of PI 3-kinase, PIP₃, was first identified in neutrophils and is detected following cell activation (Traynor-Kaplan et al., 1988). A requirement for PI 3-kinase in neutrophil activation has been demonstrated using specific inhibitors (e.g. Wortmannin), which completely inhibited superoxide production (Arcaro et al., 1993; Vlahos et al., 1995). The PI 3-kinase activity identified in neutrophils lies downstream of G-protein coupled receptors. The regulation and kinetics of PI 3-kinase activation described in neutrophils varies greatly from the activity identified downstream of tyrosine kinase receptors, and may be due to a distinct isoform of PI 3-kinase (Braselmann et al., 1997).

1.6. Project Aims.

Identification of the requirement for the small Ras-related GTP-binding protein Rac for activation of the NADPH oxidase represented one of the first cellular processes shown to be directly regulated by a member of this superfamily of GTPases. The molecular mechanism by which Rac activates the NADPH oxidase of phagocytic leukocytes has not been characterised. For a number of reasons, the NADPH oxidase provides a model system in which to study the biological functions of Rac and related proteins. Neutrophil cells are readily available and the oxidase enzyme system has been well documented. The characteristics of the components have been extensively studied, and numerous assays have been designed to investigate the activation of the enzyme in vitro and in vivo. Production of superoxide can be monitored providing a measurable endpoint of the enzyme system.

To identify target proteins that may be involved in the signalling pathways by which Rho family proteins transmit their effector functions, Rho family interacting proteins were studied in human neutrophils.
Chapter 2

Materials and Methods
2.1. Materials.

2.1.1. Commercial laboratory reagents.
Peptides were generated by Alta Biosciences (Birmingham).

\( [\gamma^{32}\text{P}]\text{ATP} \) (5 000 Ci/mmol, 10 mCi/ml), Hyperfilm-ECL, ECL reagents, T4 DNA ligase, were from Amersham.

Centriprep concentrators were from Amicon.

General laboratory reagents were obtained from BDH.

pAS2-1 and pACT2 plasmids were from Clontech.

Heparin was from CP Pharmaceuticals Ltd.

Peroxidase-conjugated Swine Anti-rabbit immunoglobulins were from DAKO.

Bacto-tryptone, Bacto-yeast extract, Bacto-agar, and Difco yeast nitrogen base without amino acids were from Difco.

Oligonucleotides were produced by Genosys.

Foetal calf serum, L-glutamine, antibiotic/antimycotic solution, agarose, prestained protein molecular weight markers, restriction endonucleases [Eco RI and SalI], agarose, HaeIII-digested \( \Phi x174 \) and HindIII-digested \( \lambda \) bacteriophage were from Gibco-BRL.

X-Omat film was from Kodak.

\( [\gamma^{32}\text{P}]\text{GTP} \) (6 000 Ci/mmol, 10 mCi/ml; 1.6 \( \mu \text{M} \)) and \( [\alpha^{32}\text{P}]\text{GTP} \) (3 000 Ci/mmol, 10 mCi/ml; 0.8 \( \mu \text{M} \)) were from NEN.

Restriction endonuclease [Nco1] New England Biolabs.

Ficoll-Hypaque ‘lymphoprep’ was from Nycomed.

PBS tablets were from Oxoid.

G-50-Sephadex, benzamidine-Sepharose 6B, Chelating-Sepharose, ampholines, and Dextran Blue 2000 were from Pharmacia.

Wizard™ DNA purification system, and Wizard™ PCR Preps DNA purification system were from Promega.

Nitrocellulose filter was from Schleicher and Schuell.

Bis-acrylamide (30 %/0.8 %) was from Scotlab.

Dextran, ampicillin, arachidonic acid, SDS, RPMI media, amino acids [L-isoleucine, L-valine, L-adenine hemisulfate salt, L-arginine HCl, L-histidine HCl monohydrate, L-leucine, L-lysine HCl, L-methionine, L-phenylalanine, L-threonine, L-tryptophan, L-tyrosine, L-uracil], protease inhibitors [PMSF, aprotinin, leupeptin, and pepstatin A], thrombin, glutathione-Sepharose, protein A-Sepharose, guanosine triphosphate (GTP)
and adenosine triphosphate (ATP), alkaline phosphatase, BSA fraction V, Tween 20, Trizma-base, ITPG, MES, PIPES, ethidium bromide, Coomassie Brilliant Blue, bromophenol blue, myelin basic protein, xylene cyanol, and DTT were from Sigma. YRG-2 Yeast Competent cell kit, competent E. coli. cells [XL-1 Blue and BL21 strains], β-mercaptoethanol, Taqplus Long polymerase mixture and Taqplus Long low-salt reaction buffer, and dNTP mix were from Stratgene.

2.1.2. Antibodies.
p67<sup>phox</sup> and p57 (human coronin-like protein) antibodies were raised against the recombinant proteins (purified from insect Sf9 cells) and were supplied by Professor A. W. Segal (Department of Medicine, University College London). Affinity purified p67<sup>phox</sup> antibodies were produced by F. Wientjes (Department of Medicine, University College London) as described previously (Wientjes et al., 1993).

α-PAK and β-PAK antibodies were raised in rabbits using purified recombinant protein from E.coli and were supplied by E. Manser. The immunisation procedure consisted of injection of fusion protein (100 µg) in complete Freund’s adjuvant, followed by boosts (every 4 weeks) of fusion protein (100 µg) in incomplete Freund’s adjuvant. Rabbits were bled 1 week after the third boost and after subsequent injections. Antibodies were purified on affinity columns of the appropriate recombinant protein.

Ste20 antibody (anti-yeast Ste20-subdomain VI), raised to amino acids 738-752 within the kinase domain (subdomain VI) was obtained from Upstate Biotechnologies Incorporated (UBI) (see appendix A.8, for sequence alignment of PAK proteins with Ste20 protein).

2.1.3. Recombinant proteins
Full-length p47<sup>phox</sup>, p67<sup>phox</sup> and p57 human coronin-like proteins were purified from insect Sf9 cells (see appendix A.4, for p47<sup>phox</sup> purification protocol); p47-C-terminal deletion mutant (amino acids 204-390) and p67<sup>phox</sup> 1-526P174L expressed as GST fusion proteins, and p40<sup>phox</sup>, were provided by the members of Professor A.W. Segal’s laboratory.
Annexin VI, was from S. Moss (Department of Physiology, University College London).

p67phox 1-199 was from A. Hall (Medical Research Council Laboratory for Molecular Cell Biology, University College London).

p190, n-chimaerin, and phosphatidylinositol 3-kinase p85α subunit (amino acids 129-273)-GST fusion proteins were supplied by A. Best and S. Ahmed.

The p21 binding domains of p120ACK and α-PAK amino acids 1-151 (α-PAK1-151), and α-PAK-GST were supplied by E. Manser.

2.1.4. Native proteins, cell lysates, and protein fractions.
Native neutrophil cytochrome b, was provided by Professor A.W. Segal.

Separation of human neutrophils from whole blood and generation of neutrophil cytosol (large scale) were carried out in Professor A.W. Segal's laboratory, who supplied the required materials. These procedures were carried out with the help of A. Grogan, E. Reeves and L. Forbes (Department of Medicine, University College London).

Cdc42Hs binding activity was purified from neutrophil cytosol (with the exception of the Cdc42Hs-G12V affinity and the chelating-Sepharose columns), in Professor A. W. Segal's laboratory with the help of E. Reeves and A. Grogan. The materials required for both purification protocols, detailed in section 2.2.11, were provided by Professor A. W. Segal.

Insect Sf9 cell lysates were supplied by F. Wientjes, (see appendix A.4).

Fractions from human neutrophil cytosol containing the Arp2/3 complex components were generated and supplied by E. Reeves, (see appendix A.5).

2.1.5. cDNA.
β-PAK-GST was supplied by E. Manser.
Wild type p21s Rac1, Cdc42Hs, RhoA and Rac2, and mutants Cdc42Hs-Q61L, Cdc42Hs-D38A, Rac1-Q61L and Cdc42-G12V cDNAs were generated and supplied by S. Ahmed and A. Best. Point mutations were introduced using a Clontech mutagenesis kit as described previously, (Best et al., 1996). Proteins were expressed in pGEX or derivative plasmids and purified as described in sections 2.2.1.2 and 2.2.1.3.

p67phox cDNA constructs encoding amino acids; 1-526 (full-length), 1-238, 1-192, 126-238, 170-238, 192-238, 170-199, 1-131, 1-58, 1-460, 300-526, 300-460, 1-526Δ58K, 1-526Δ226-236, and 1-526Δ178-184 were supplied by F. Wientjes and S. Ahmed (see appendix A.1.3).

Rac1-Q61L-C196S cDNA inserted into the yeast expression vector pAS2-1 was provided by S. Govind.

2.1.6. Other materials and services.

HL60 cells were provided by P. Roberts (Department of Haematology, University College London).

Peptide sequence analysis was carried out by Dr J. Hsuan at the Ludwig Institute for Cancer Research (University College London).
2.2. Methods.

2.2.1. Purification of recombinant proteins.
Competent *E. coli* cells (see appendix A.1) were transformed with plasmid DNAs, (pGEX or derivative vectors) containing inserts encoding the protein of interest, for the expression of fusion proteins with an N-terminal glutathione S-transferase (GST) tag.

2.2.1.1. Transformation of competent *E. coli* cells.
(a) Transformation of competent *E. coli* strain XL-1 Blue and JM107 cells.
Plasmid DNA (10-50 ng) was mixed with 200 μl of competent *E. coli* strain XL-1Blue or JM107 cells and incubated on ice for 10 min, cells were subjected to heat shock at 42 °C for 2 min then returned to ice for 10 min. 1 ml of L-Broth (LB; Bacto-tryptone 10 g/l, Bacto-yeast extract 5 g/l and NaCl 10 g/l) was added to the cells and they were allowed to recover for 1 h at 37 °C with agitation. Aliquots of 100 μl of cells were plated on to LB-Agar (Bacto-Agar 15 % w/v) plates containing 50 μg/ml ampicillin using a glass spreader and plates were incubated at 37 °C for 16 h. Aliquots of transformed cells larger than 100 μl were pelleted at 14 000 x g for 15 s then resuspended in 100 μl of LB and plated onto LB-Agar as described above. Plates were stored at 4 °C as necessary, up to 7 days.

(b) Transformation of competent *E. coli* strain BL21 cells (Strategene).
(β-PAK-GST and p67phox deletion mutants 1-526Δ58K and 1-526Δ178-184)
100 μl of thawed competent BL21 cells were mixed and decanted into a prechilled 15 ml tube. 1.7 μl of β-mercaptoethanol was added, to a final concentration of 25 mM, and mixed. The cells were incubated on ice for 10 min, with mixing every 2 min. 10 ng of β-PAK-GST plasmid DNA or 2 μl of p67phox 1-526Δ58K and 1-526Δ178-184 miniprep DNA was added to the cells and incubated for 30 min on ice. The cells were heat shocked at 42 °C for 45 s and then returned to ice for 2 min. 0.9 ml of SOC medium (20 g/l Bacto-tryptone, 5 g/l Bacto-yeast extract, 0.5 g/l NaCl, 2.5 mM KCl, pH 7.0) were added and the cells were allowed to recover for 1 h at 37 °C with shaking. Cells were then plated on LB-Agar as described above, and incubated for 16 h at 37 °C.
2.2.1.2. Expression of recombinant GST-fusion proteins.

Single colonies of cells transformed with the appropriate plasmid DNA were picked from LB-Agar plates and used to inoculate cultures, 50-100 ml of LB containing 50 µg/ml ampicillin which were then incubated overnight for 16 h at 37 °C in an orbital shaker. These overnight cultures were then diluted 10-50 fold into 1-2 litres of prewarmed LB containing 50 µg/ml ampicillin and grown at 37 °C for 3-4 h, to an OD_{550nm} of 0.5. Unless mentioned otherwise protein expression was induced by adding 0.2 mM isopropylthio-β-D-galactoside (IPTG) to the cultures which were then grown for a further 1-2 h at 37 °C.

In some cases it was desirable to reduce the temperature of the culture prior to induction of protein synthesis, to enhance the stability or solubility of the protein being produced. In such cases cultures were removed from the incubator and allowed to cool at room temperature before being replaced in the incubator. 0.2-0.5 mM IPTG was then added and cultures were grown for 4-6 h at 26 °C or 30 °C as appropriate. This applied to a number of the p67^{phox} deletion mutants, as detailed in table 2.1, and also β-PAK-GST.

For expression of β-PAK-GST protein colonies were picked from LB-agar plates and used to inoculate overnight cultures and these were diluted as described above. For induction of protein expression the temperature of the culture was reduced to 26 °C prior to addition of IPTG (0.5 mM) and cultures were grown for 6 h prior to harvesting.

Cells were harvested by centrifugation at 3 000 x g for 20 min (J6 centrifuge, Beckman), washed in phosphate buffered saline (PBS; 140 mM NaCl, 3 mM KCl, 4 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.4 Oxoid) and pelleted by centrifugation again at 3 000 x g for 20 min (as above). Cell pellets were then frozen at -20 °C or on dry ice and stored at -20 °C as required.

2.2.1.3. Purification of recombinant GST-fusion proteins (from E. coli cell pellets).

E. coli cell pellets were thawed on ice and resuspended in PBS, 1% Triton X-100 containing protease inhibitors (phenylmethylsulphonyl fluoride (PMSF) 100 µg/ml and aprotinin, leupeptin and pepstatin at 1 µg/ml). Cells were lysed by sonication (Heat Systems Sonicator Ultrasonic processor XL) 5 x 20 s pulses (Microtip setting 5),
separated by 5 s breaks on ice. If necessary efficiency of sonication was judged by measuring the OD$_{550\text{nm}}$ before and after sonication. Lysates were cleared by centrifugation at 15 000 x g for 20 min at 4 °C (J2-21 centrifuge, Beckman).

Cleared cell lysates were incubated with 0.5 ml (per 1 of bacterial cell culture) of prewashed glutathione-Sepharose resin for 1 h at 4 °C, with rotation. The glutathione-Sepharose was collected by centrifugation at 2 000 x g at 4 °C (J6 centrifuge, Beckman), unbound material was decanted off and the glutathione-Sepharose was washed twice (10 bed volumes each) in PBS, 1% Triton X-100 and then once in 50 mM Tris-HCl, pH 8.0 to equilibrate the resin for elution with glutathione (or in Thrombin Cleavage Buffer if proteins were to be cleaved, see section 2.2.1.4).

For elution of proteins the glutathione-Sepharose resin was incubated with 10 bed volumes of freshly prepared 10 mM glutathione in 50 mM Tris-HCl pH 8.0 for 10 min at room temperature with rotation. The eluant was collected by passing the resin through a column; additionally, proteins were eluted from the resin with a further 5 bed volumes of 10 mM glutathione which were passed through the column, the eluants were pooled and then dialysed at 4 °C against 2 l of buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2.5 mM CaCl$_2$) with three changes, of at least 90 min. The proteins were then concentrated using a Centriprep concentrator. Unless otherwise stated proteins were used as GST fusion proteins.

2.2.1.4. Cleavage of recombinant GST-fusion proteins.

If the GST-fusion protein was to be cleaved from the GST moiety following purification, the third glutathione-Sepharose wash (see above) was carried out with Thrombin Cleavage Buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2.5 mM CaCl$_2$), to equilibrate the resin. Thrombin was added at a concentration of 5 units/mg of protein being cleaved in 10 bed volumes of the above buffer and incubated with the proteins on the glutathione-Sepharose resin for 90 min at room temperature. Cleaved proteins were eluted from the glutathione-Sepharose resin by collecting the resin in a column. Thrombin was removed by passing the cleaved protein eluant through a benzamidine-Sepharose (p-aminobenzamidine (PAB) coupled to Sepharose) column, which acts to isolate serine proteases, including thrombin, via binding to the PAB ligand. Cleaved proteins were then concentrated as described above.
2.2.1.5. Expression and purification of recombinant p67phox from insect Sf9 cells.

p67phox protein (expressed in insect Sf9 cells) was purified by F. Wientjes and L. Forbes (Department of Medicine, University College London), (see appendix A.4).

2.2.1.6. Preparation of p67phox cDNAs and purification of recombinant proteins.

p67phox proteins were expressed in pGEX or derivative plasmids in E. coli strain cells as indicated in Table 2.1, (also see appendix A.1.3). Protein expression and purification was carried out as indicated in sections 2.2.1.2 and 2.2.1.3. When reduction of the temperature of growing cultures was required for protein expression, induction was carried out for 4-6 h (see Table 2.1). p67phox deletion mutants were used as GST fusion proteins unless otherwise indicated. Purity of p67phox deletion mutants as judged by SDS-PAGE was variable, as illustrated in Results Chapters 4, 5, and 6.

2.2.1.7. Quantification of protein.

Protein was quantified by the method of Bradford (1976), using the Bio-Rad protein assay according to the manufacturer’s protocol.

2.2.2. Preparation of cell lysates and cellular protein fractions.

2.2.2.1. Separation of human neutrophils from whole blood. (Heyworth et al., 1991).

Blood taken from healthy volunteers was diluted with 1x Saline (0.9% NaCl) containing Heparin (CP Pharmaceuticals) as an anticoagulant to increase the yield of cells obtained. The blood was mixed with Dextran (260 kDa-1 % w/v) to aid separation of the different blood cells and left to settle for 1 h at room temperature. During this time the different blood cells separated into two layers. The white cell solution containing the neutrophils was decanted off into fresh tubes using a 50 ml syringe. To separate the neutrophils from the white blood cell solution Ficoll-Hypaque (10-20 %, v/v) was added underneath the solution using a syringe with an adapter and the cells were centrifuged at 2 000 rpm for 10 min at room temperature. During this spin the neutrophils and any residual red blood cells settled in a pellet underneath the Ficoll-Hypaque layer, whereas lymphocytes and other white cells remained above the Ficoll-Hypaque layer. The white cell solution was removed leaving a red coloured pellet containing the neutrophils and some red blood cells. Red blood cells were subjected to hypotonic lysis by adding water. Lysis of red
Table 2.1. Expression of p67phox Proteins.

<table>
<thead>
<tr>
<th>p67phox constructs Amino acids</th>
<th>Expression system and expression temperatures.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-526 (full-length)</td>
<td>Insect Sf9 cells</td>
</tr>
<tr>
<td>1-526 (full-length)</td>
<td>XL-1 Blue (26 °C)</td>
</tr>
<tr>
<td>1-238</td>
<td>XL-1 Blue (26 °C)</td>
</tr>
<tr>
<td>1-192</td>
<td>XL-1 Blue (37 °C)</td>
</tr>
<tr>
<td>126-238</td>
<td>XL-1 Blue (26 °C)</td>
</tr>
<tr>
<td>170-238</td>
<td>XL-1 Blue (26 °C)</td>
</tr>
<tr>
<td>192-238</td>
<td>XL-1 Blue (26 °C)</td>
</tr>
<tr>
<td>170-199</td>
<td>XL-1 Blue (37 °C)</td>
</tr>
<tr>
<td>1-131</td>
<td>XL-1 Blue (26 °C)</td>
</tr>
<tr>
<td>1-58</td>
<td>XL-1 Blue (26 °C)</td>
</tr>
<tr>
<td>1-460</td>
<td>JM107 (26 °C)</td>
</tr>
<tr>
<td>300-526</td>
<td>XL-1 Blue (37 °C)</td>
</tr>
<tr>
<td>300-460</td>
<td>XL-1 Blue (37 °C)</td>
</tr>
<tr>
<td>1-526Δ58K</td>
<td>BL21 (26 °C)</td>
</tr>
<tr>
<td>1-526Δ226-236</td>
<td>XL-1 Blue (30 °C)</td>
</tr>
<tr>
<td>1-526Δ178-184</td>
<td>BL21 (26 °C)</td>
</tr>
<tr>
<td>1-526 P174L</td>
<td>protein provided by F. Wientjes</td>
</tr>
</tbody>
</table>

Table 2.1. Expression of p67phox proteins. This table summarises the expression of p67phox proteins used in this study. The numbers indicate the amino acids of each deletion mutant. The E. coli cell strain in which the proteins were expressed and the temperature at which protein expression was induced, as required for production of a stable protein preparation, is also indicated.

Proteins were purified and used as GST fusion proteins unless otherwise stated. p67phox mutants 1-526Δ58K, 1-526Δ226-236 and 1-526Δ178-184 were deleted of lysine 58, amino acids 226-236 and amino acids 178-184 respectively; in p67phox 1-526P174L proline residue 174 was substituted by a leucine residue.
blood cells was complete after only a few seconds during which time the neutrophils remained intact, lysis was stopped by adding an equal volume of 2x Saline containing Heparin.

Neutrophils were collected by pelleting at 1 500 rpm for 5 min at room temperature and the supernatant was removed. The neutrophils were then washed in an appropriate volume of PBS containing 5.5 mM glucose, and collected by centrifugation at 1 500 rpm for 5 min. The supernatant was discarded and 2 mM diisopropyl flurophosphate (DIFP) was added to the pellet. DIFP was gently mixed in with the pellet which was then left on ice for 10 min to allow the protease inhibitor to enter the cells prior to lysis. The cell pellet was then resuspended in a buffer as required, either PBS or Lambert's Break Buffer (10 mM PIPES, 3 mM NaCl, 10 mM KCl, 4 mM MgCl₂, pH 7.0) depending on the following use of the cells.

2.2.2.2. Cell culture of HL60 cells.

HL60 cells were cultured in RPMI medium with 10 % foetal calf serum, 5 % (v/v) L-glutamine and 5 % antibiotic/antimycotic solution (Gibco-BRL) at 37 °C and 5 % CO₂. HL60 cell cultures were diluted twice weekly at approximately 1 in 5. Cells were harvested by centrifugation at 2 000 x g for 5 min, and the cell pellets were washed in PBS prior to fractionation, see below.

2.2.2.3. Preparation of neutrophil cytosol (large scale).

All stages in generating neutrophil cytosol were carried out at 4 °C or on ice. The neutrophil cell pellet was resuspended in Lambert's Break Buffer containing protease inhibitors: 2 mM PMSF, 1 μg/ml pepstatin and leupeptin, 10 μg/ml Nα-p-tosyl-l-lysine chloromethyl ketone (TLCK) and 5 mM EDTA. The cells were disrupted by sonication (MSE Soniprep) on ice for 3 x 5 s pulses (Microtip setting 3). The lysed cell solution was subjected to centrifugation at 2 500 rpm at 4 °C to remove unbroken cells and any cell debris. The supernatant was collected and EGTA (1.25 mM) was added prior to centrifugation at 100 000 rpm for 30 min at 4 °C. The supernatant collected contained neutrophil cytosolic proteins and from now on will be referred to as neutrophil cytosol.
2.2.2.4. Fractionation of neutrophil cellular proteins (small scale).

(a) Cytosol.

A frozen cell pellet containing $2 \times 10^9$ neutrophil cells was thawed and resuspended in 20 ml of Relaxation Buffer (100 mM KCl, 3 mM NaCl, 3.5 mM MgCl$_2$, 1.25 mM EGTA, 10 mM Tris-HCl pH 7.5 and protease inhibitors: 2 mM PMSF, and 1 µg/ml pepstatin and aprotinin) containing 6 % sucrose. Cells were homogenised, using a B4049 homogeniser (10 strokes on ice). In addition neutrophil cells were disrupted by sonication (Heat Systems Sonicator Ultrasonic Processor XL) on ice, 2 x 5 s pulses (Microtip setting 3). Unbroken cells and nuclei were pelleted by centrifugation at 250 x g (1 200 rpm), for 5 min at 4 °C (J6 centrifuge Beckman). The supernatant (S1) was collected and subjected to ultracentrifugation to further clear the cell lysates in a TLA 100.3 rotor (Beckman), at 124 000 x g (55 000 rpm) for 20 min at 4 °C. The supernatant (S2) containing the cytosolic proteins will be referred to as neutrophil cytosol. The insoluble material contained in the pellet was washed by resuspension in relaxation buffer (see above) and collected by centrifugation at 124 000 x g (55 000 rpm) for 20 min at 4 °C. The wash, supernatant (S3), was decanted off. The pellet (P1) contained membrane proteins and was further processed to fractionate membrane proteins into membrane and membrane-cytoskeletal components.

(b) Membrane (Triton X-100 soluble) and (c) membrane-cytoskeletal (Triton X-100 insoluble) protein fractions.

A Triton-X100 extraction was carried out to separate the membrane proteins from the membrane-cytoskeletal proteins. Whole membranes from $2 \times 10^9$ cells collected as described above (P1) were resuspended in 750 µl of Cytoskeletal Buffer (20 mM Tris-HCl pH 7.5, 3 mM MgCl$_2$, 8 % Sucrose, 0.5 % Triton X-100, 5 mM EGTA and protease inhibitors: 1 mM PMSF, 1 µg/ml pepstatin and aprotinin) by homogenising and then were incubated at 4 °C for 45 min. Following this incubation the Triton X-100 soluble and Triton X-100 insoluble material were separated by ultracentrifugation at 180 000 x g (65 000 rpm) for 2 h at 4 °C. The supernatant (S4) containing the membrane proteins (referred to as the membrane fraction, M) was separated from the pellet (P2) containing the Triton X-100 insoluble membrane-cytoskeletal components (referred to as the membrane-cytoskeletal fraction, S). The pellet from $1 \times 10^6$ cell equivalents was resuspended in 250 µl of 20 mM Tris-HCl, pH 7.5 by sonication on ice (1 x 5 s pulse Microtip setting 5, followed by 2 x 5 s pulses at setting 3).
2.2.2.5. Fractionation of HL60 cell proteins.
HL60 cells were harvested for fractionation (3.5 x 10^7 cells). Fractionation of HL60 cellular proteins was carried using the same protocol applied to neutrophils (section 2.2.2.4) to generate cytosol (C), Triton X-100 soluble membrane (M) and Triton X-100 insoluble membrane-cytoskeletal fractions (S).

2.2.2.6. Preparation of rat brain cytosol.
Rat brain cytosol was generated and supplied by A. Best, briefly; brains from Sprague Dawley rats were homogenised in four volumes of homogenisation buffer (10 mM Tris-HCl (pH 7.4), 5 mM DTT, 0.5 mM PMSF, 1 μg/ml aprotinin and pepstatin) and insoluble material was pelleted at 100 000 x g for 1 h (70Ti rotor, Beckman). Glycerol was added to the supernatants (cytosolic fraction) to a final concentration of 5 % (v/v).

2.2.2.7. Preparation of insect Sf9 cell lysates.
Lysates from control Sf9 cells or cells infected with p67 genomic DNA were generated by F. Wientjes (Department of Medicine, University College London) (see appendix A.4).

2.2.2.8. Purification of Arp2/3 complex components from human neutrophil cytosol.
The purification was carried out by E. Reeves, who supplied semi-purified fractions for p21-binding protein and immunoblot analysis. Details of the purification are described in Machesky et al., 1997, and in appendix A.5.

2.2.3. Analysis of proteins by electrophoresis.
2.2.3.1. 2-Dimensional (2-D) gel electrophoresis.
Proteins were separated (i) according to their isoelectric points (pI) on isoelectric focusing (IEF) tube gels and (ii) by their molecular weights in SDS-polyacrylamide gels (see below). For narrow range 2-dimensional (2-D) analysis neutrophil and rat brain cytosolic proteins (section 2.2.2.) were mixed with an equal volume of lysis buffer (9.5 M urea, 10 mM lysine, 5% β-mercaptoethanol, 2% Nonidet P-40 (NP-40)) containing ampholines pH 5-7 (4 %) and pH 3.5-10 (1 %). Samples were then loaded on to isoelectric focusing (IEF) tube gels containing the same ampholines and overlayed with
lysis buffer diluted 2-fold with ddH$_2$O. Proteins were separated by electrophoresis overnight at 300 V and then for 1 h at 1 000 V, against basic (10 mM NaOH) and acidic (20 mM H$_3$PO$_4$) running buffers, using the Bio-Rad system. IEF tube gels were then equilibrated in sample buffer for 1 h and loaded onto SDS-polyacrylamide gels, and proteins were separated according to their molecular weights as described below.

**Isoelectric focusing (IEF) tube gels;** Urea, bis-acrylamide (28.38 %/1.62 %), NP-40 and ampholines; pH 5-7 (4 %) and pH 3.5-10 (1 %).

### 2.2.3.2. Separation of proteins by SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Bio-Rad minigels 1.5 mm thick.

25-100 µg of cellular proteins or 5 µg of recombinant proteins unless otherwise stated were prepared in sample buffer (125 mM Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 0.14M β-mercaptoethanol, 0.1% bromophenol blue) and boiled for 5 min before being separated in SDS-polyacrylamide gels, containing 10 or 12% bis-acrylamide. Electrophoresis was carried out at a constant voltage of 180 V for 50 min in 1 x SDS-PAGE running buffer. Apparent molecular sizes were estimated by co-running prestained protein markers of known molecular size (Gibco-BRL).

**SDS-PAGE acrylamide solution:** 0.375 M Tris-HCl pH 8.8, 0.1% SDS, an appropriate amount of 30%/0.8% bis-acrylamide to make a gel of desired percentage, 0.05% APS, Temed and ddH$_2$O.

A stacker was applied to the top of the gel once it was polymerised, into which a 10 well comb was inserted to generate loading wells for application of protein samples or not if the gel was being used for the second dimension of 2-D gel analysis.

**The stacker solution:** 0.1% SDS, 0.125 M Tris-HCl pH 6.8, 3.9% bis-acrylamide (30%/0.8%), 0.025% APS and Temed; supplemented with Urea (3.9 g/10 ml -30% w/v) for the second dimension of 2-D-gel analysis, when IEF tubes were to overlay the polymerised stacker.
SDS-PAGE running buffer: 25 mM Tris-HCl, 0.25 M glycine, 0.1 % SDS.

2.2.3.3. Visualisation of proteins.
Proteins separated in polyacrylamide gels were visualised by staining the gel by immersing in Coomassie Blue stain (0.1 % Coomassie Brilliant Blue, 45 % methanol v/v, 10 % glacial acetic acid v/v) for 1 h followed by destaining in 10 % glacial acetic acid, 40 % ethanol with plenty of changes.

2.2.4. Immobilisation of proteins on nitrocellulose filter.
2.2.4.1. Western transfer of proteins onto nitrocellulose filter.
SDS-(1 and 2-Dimensional) gels, containing separated proteins, were equilibrated in transfer buffer (4.8 mM Tris, 3.9 mM Glycine and 0.375% SDS) for 15 min at 4 °C and transferred to nitrocellulose filters in transfer buffer using a Bio-Rad semi-dry blotter, at 4 °C for 1 h at a constant voltage of 12 V.

2.2.4.2. Dot blotting proteins directly onto nitrocellulose filter.
5-50 µg of recombinant or cytosolic proteins and 25-100 µg of synthetically produced peptides, in 10-20 µl were directly applied to 1-2 cm² nitrocellulose filters by pipetting. Larger volumes were applied by allowing the solution to dry on the nitrocellulose before subsequent aliquots were directly applied on top. Volumes in which protein samples were applied to the nitrocellulose were equalised within each experiment, to enable more accurate analysis of the data obtained.

Nitrocellulose filters were blocked in PBS containing 5 % non-fat milk or 3 % BSA depending upon the subsequent analysis.

2.2.5. p21 binding assays
p21 proteins were used as GST fusion proteins or were cleaved from the GST moiety, similar results were obtained with either protein. These proteins included Cdc42Hs, Cdc42Hs-Q61L, Cdc42Hs-D38A, Rac1, Rac1-Q61L, Rac2 and RhoA.
Proteins were separated in SDS-gels and transferred to nitrocellulose using a Bio-Rad semi-dry blotter in transfer buffer (section 2.2.4), or dot blotted directly onto nitrocellulose filters. After protein immobilisation, nitrocellulose filters were incubated in renaturation buffer: PBS containing 3% BSA, 0.1% Triton X-100, 0.5 mM MgCl₂, and 5 mM DTT, for at least 2 h at 4 °C.

2.2.5.1. Generation of [γ³²P]GTP labelled p21 probes.

p21 probes, loaded with [γ³²P]GTP, were prepared by incubating Cdc42Hs and Rac1, wild-type or mutated forms, or RhoA and Rac2 (5 µg) with 1 µl [γ³²P]GTP (NEN; 6 000 Ci/mmol, 10 mCi/ml; 1.6 µM) or 2 µl [α³²P]GTP (NEN; 3 000 Ci/mmol, 10 mCi/ml; 0.8 µM) in 50 µl of nucleotide exchange buffer (50 mM NaCl, 25 mM MES (pH 6.5), 25 mM Tris-HCl (pH 7.5), 1.25 mM EDTA, 1.25 mg/ml BSA, 1.25 mM DTT) for 10 min at room temperature. [α³²P]GDP probes were generated by loading with [α³²P]GTP as for [γ³²P]GTP and allowing the p21-GTP complex to hydrolyse the γ-phosphate group for 60-90 min at room temperature. Probes were used immediately on preparation by diluting into petri dishes containing 4 ml of binding buffer (50 mM NaCl, 25 mM MES (pH 6.5), 25 mM Tris-HCl (pH 7.5), 1.25 mM MgCl₂, 1.25 mg/ml BSA, 1.25 mM DTT, 0.5 mM GTP). Filters were incubated with p21 probes for 5 min at room temperature before being washed on ice, 6 x 5 min in wash buffer 1: (50 mM NaCl, 25 mM MES pH 6.0, 5 mM MgCl₂, 0.25% Triton X-100) or wash buffer 2: (as for buffer 1 except for 0.05% Triton X-100) depending on the p21 being used as the probe. After washing the filters were quickly dried by placing on 3 MM Whatman paper and then exposed to Kodak X-Omat film for 5-25 h, or as necessary.

2.2.5.2. Quantification of p21 binding signals.

p21 binding to dot blotted proteins, was quantified following p21 binding assays, by cutting out the 1-2 cm² of nitrocellulose and determining the amount of radioactivity by scintillation counting, using the Beckman LS 6000 series Liquid Scintillation system (Beckman Instruments). Nitrocellulose squares were placed in Bio-vials and 4 ml of scintillant, Ready Safe™ liquid scintillation cocktail were added, the vials were vortexed to ensure mixing prior to counting (Beckman LS 6000 scintillation counter). Binding signals were then expressed in counts per minute (cpm). Quantified binding signals were
analysed by subtracting the cpm obtained with control proteins from the value obtained with the protein of interest. In order to determine the existence of any adverse background by eye, filters were exposed to Kodak X-Omat film prior to this analysis. Background binding was variable and therefore it was essential to make comparisons with p21 target interactions and control proteins within the same experiment.

2.2.5.3. Competition p21 binding assays.
The specificity of p21 binding interactions was determined using competition binding assays. Proteins immobilised on nitrocellulose filters were probed with the appropriate p21s as described above in the presence of a competitor protein. The N-terminal deletion mutant of α-PAK containing the p21 binding site α-PAK(1-151), p40^{phox}, or p21 proteins themselves were used as competitors by one of two methods: 1, nitrocellulose filters were preincubated with a competitor at the appropriate concentration in binding buffer (described above) for 1 h at 4 °C and the labelled p21 was added to this incubation which was continued for a further 5 min at room temperature, or 2, the competitor was added at the appropriate concentration to the binding buffer immediately prior to addition of the labelled p21 and the incubation was carried out as above. Competitor p21s were preloaded with GTP prior to addition to the incubation, this was carried out at room temperature for 10 min in nucleotide exchange buffer, as described above, in the presence of 20 μM GTP. For p21-GDP competitors GTP loaded proteins were incubated at room temperature for 60-90 min prior to use, as above.

Alternatively competing proteins were incubated together in low salt buffer (25 mM NaCl, 50 mM Tris-HCl, 2.5 mM CaCl₂, pH 7.5), for 16 h at 4 °C with rotation, prior to immobilisation on nitrocellulose filter by dot blotting, and probing with labelled p21 probes. This method was applied to determine whether the C-terminal p67^{phox} deletion mutants or proline-rich peptides derived from either p67^{phox} or p47^{phox} affected the ability of p67^{phox} to bind Rac1.

2.2.6. GTPase activating protein (GAP) assays.
GAP assays were carried out essentially as described by Ahmed et. al., 1991.
Wild type Cdc42Hs, or Cdc42Hs-Q61L and Cdc42Hs-D38A (1-6 mg/ml; final concentration 2 μM) were labelled with [γ²P]GTP (10 mCi/ml; 6 000 Ci/mmol; 1.6 μM)
or [α³²P]GTP (10 mCi/ml; 3 000 Ci/mmol; 0.8 μM) in the presence of 4 mM EDTA in a buffer containing 16 mM Tris-HCl pH7.5, 20 mM NaCl and 80 μM DTT, for 10 min at room temperature. MgCl₂ was added (3 mM final concentration) to inhibit exchange and the labelled p21s were placed on ice prior to use. The p21s were then diluted into GAP incubation buffer (20 mM Tris-HCl pH 7.5, 0.1 mM DTT) containing 3 mM GTP and 1.3 mg/ml BSA, in the presence or absence of a GAP (or potential GAP) or another factor and placed at 15 °C.

Loss of label (γ³²P or α³²P) was measured either over a 20 min time course or after a single time point of 10 min, by pipetting 5 μl of the incubation reaction onto a 1 cm² square of nitrocellulose. The nitrocellulose squares were then washed 6 x 1 min in wash buffer (50 mM Tris-HCl pH 7.5, 50 mM NaCl, 5 mM MgCl₂) on ice and allowed to dry at room temperature. The amount of³²P remaining on the nitrocellulose was quantified by conversion to counts per minute (cpm) by scintillation counting using the Beckman LS 6000 series Liquid Scintillation system, as described above (section 2.2.5.2). The amount of label remaining at each time point was used to calculate the rate of GTP hydrolysis by expressing this as the percentage of the labelled GTP bound at time point 0 min.

2.2.7. Antibodies and immunoblot analysis

2.2.7.1. Antibodies.
Affinity purified p67phox and p57-human coronin-like protein antibodies were used for immunoblot analysis at a dilution of 1:10 000 and 1:2 000 respectively. α-PAK and β-PAK antibodies were used at a dilution of 1:500 and 1:1 000 respectively. Ste20 antibody (UBI), was used at a concentration of 5 μg/5 ml.

2.2.7.2. Immunoblot analysis.
Proteins separated in SDS-gels were equilibrated in transfer buffer (4.8 mM Tris, 3.9 mM Glycine and 0.375% SDS) for 15 min at 4 °C and then transferred to nitrocellulose filters in transfer buffer using a Bio-Rad semi-dry blotter at 4 °C for 1 h at a constant voltage of 12 V.
Nitrocellulose filters were then blocked in PBS/0.1% Tween20, 5% non-fat milk for 16 h at 4 °C or for 1 h at room temperature and then washed in PBS/0.1% Tween20 5 x 5 min at room temperature before incubation with primary antibody. Primary antibodies were diluted as listed above in PBS/0.1% Tween20, 1% non-fat milk, and incubated with nitrocellulose filters for 1 h at room temperature. Filters were washed in PBS/0.1% Tween20 (5 x 5 min) at room temperature before incubation with secondary antibody; DAKO Peroxidase-conjugated Swine anti-rabbit immunoglobulins, diluted 1 in 1 000 in PBS/0.1% Tween20, 1% non-fat milk, for 45 min at room temperature. The filters were then washed again in PBS/0.1% Tween20 5 x 5 min and staining was visualised using enhanced chemiluminescence (ECL-Amersham) according to the manufacturer’s instructions. Filters were exposed to ECL-hyperfilm as appropriate.

2.2.8. Immunoprecipitation.

Immunoprecipitation of proteins from semi-purified neutrophil material, p68 fraction and from recombinant protein preparation (α-PAK-GST) was carried out using α-PAK specific antibody.

2.2.8.1. Immunoprecipitation of kinase activity from p68 fraction and recombinant α-PAK-GST.

Recombinant α-PAK-GST and purified neutrophil material, p68 fraction, containing Cdc42Hs binding protein (section 2.2.11) were phosphorylated in an in vitro kinase assay (Method 1, section 2.2.9.1) in the presence of [γ^32P]ATP and Rac1/Cdc42Hs-GTP. Phosphorylated proteins were incubated for 60 min at 4 °C with either 5 µl of affinity-purified α-PAK antibody or preimmune serum in (50 mM Tris (pH 7.5), 25 mM NaCl, 0.5 mM MgCl2, 0.05% Triton X-100, 1 mM DTT, final volume 100 µl). A 50% slurry in the same buffer (v/v) of protein A-Sepharose beads (100 µl) was then added and incubation continued for a further 60 min at 4 °C. The protein A-Sepharose beads were sedimented by packing in a spin-column and the unbound material and free label were removed by discarding the supernatant. The protein A-Sepharose column was then washed 3 times with 750 µl PBS/0.1% Triton X-100. 50 µl of sample buffer (x2) were then added to the beads followed by incubation at 95 °C for 10 min. The beads were then spun down and the supernatant containing protein (stripped from the protein A-
sepharose in the latter two stages) was analysed by SDS-PAGE. Phosphorylated proteins were visualised by exposing the dried gel to X-Omat film as described in section 2.2.9.1.

2.2.9. *In vitro* solution kinase assays.

2.2.9.1. Method 1: Autophosphorylation of p68 fraction proteins and recombinant α-PAK-GST.

p68 fraction proteins, containing Cdc42Hs binding activity, purified from neutrophil cytosol as described in section 2.2.11, (5 µg) were incubated at 30 °C for 15 min with 50 μCi [γ32P]ATP (Amersham; 5 000 Ci/mmol, 10 mCi/ml) in kinase buffer (50 mM Hepes pH 7.0, 5 mM MgCl₂, 5 mM MnCl₂, 1 mM DTT, 0.05% Triton X-100), the reaction was stopped by addition of 2 x sample buffer and boiling for 5 min. Samples were then loaded onto SDS-gels and subjected to electrophoresis for 50 min at 180 V. The gel was then dried and exposed to Kodak X-Omat film for 10 min. This method was also applied to generate phosphorylated α-PAK-GST protein used in immunoprecipitation experiments (section 2.2.8.1).

2.2.9.2. p21 stimulation of autophosphorylation.

For stimulation of autophosphorylation by p21s, GTP loaded p21s were added to the kinase reaction. Cdc42Hs or Rac1 were loaded with GTP by incubating in nucleotide exchange buffer (as described in section 2.2.5.) in the presence of 20 μM GTP for 10 min at room temperature. 6 µg of GTP-p21 was added to each kinase assay, prior to incubation at 30 °C.

2.2.9.3. Method 2: *In vitro* kinase activity of recombinant β-PAK-GST.

β-PAK autophosphorylation and phosphorylation of recombinant proteins including p67₇phox deletion mutant proteins.

1 or 2 µl (0.125 or 0.250 µg) of recombinant β-PAK-GST protein were incubated alone or with 5µg of recombinant protein, (for example p67₇phox full-length or fragment proteins, in kinase buffer (described above) containing 20 μM ATP in the presence of [γ³²P]ATP 2μCi (Amersham; 5 000 Ci/mmol, 10 mCi/ml) for 15 min at 30 °C. The reaction was stopped by the addition of 5x sample buffer and boiling for 5 min. The
proteins were then separated by electrophoresis in SDS-gels until the dye front reached the bottom of the gel, the dye front containing 'free-label' was removed and proteins were visualised by staining with Coomassie Blue (section 2.2.3.). The staining procedure acted to focus the protein bands by fixing. The gels were then dried and exposed to Kodak X-Omat film for 10 min to 5 h, as appropriate.

2.2.10. β-PAK and neutrophil p68 fraction overlay assays.
Neutrophil or rat brain cytosols (50 μg), p47phox, annexin VI, p57 human coronin-like protein and GST (10 μg) were immobilised on nitrocellulose filter by western transfer (section 2.2.4). Filters were incubated in renaturation buffer (3 % BSA, 0.1 % Triton X-100, 0.5 mM MgCl₂, 5 mM DTT) for at least 2 h, and then washed in binding buffer (50 mM NaCl, 25 mM MES pH 6.5, 25 mM Tris-HCl pH 7.5, 1.25 mM MgCl₂, 1.25 mg/ml BSA, 1.25 mM DTT).

2.2.10.1. Preparation of ³²P-β-PAK and p68 fraction protein probes.
p68 fraction (see section 2.2.11), (67 μl, ~30 μg) was incubated in kinase buffer as described (section 2.2.9.1) in the presence of [γ³²P]ATP (10 μCi), Rac1-Q61L-GTP, and 20 μM ATP, total volume 100 μl. The incubation was carried out at 30 °C for 15 min. For β-PAK labelled probes 40 μg of recombinant β-PAK-GST protein was incubated as for p68 fraction proteins with [γ³²P]ATP (20 μCi) in the absence of GTP-p21. These reaction mixtures were diluted 2 fold in binding buffer (as above) containing 100 μM ATP. Blue Dextran 2000 was added and the mix was applied to a G-50-Sephadex column to separate the ³²P labelled protein probes from the 'free-label'. Protein was eluted from the column in 750 μl binding buffer containing 100 μM ATP.

2.2.10.2. ³²P-PAK overlay assays.
Probes were diluted in 4 ml of binding buffer containing 100 μM ATP and incubated with washed nitrocellulose filters (see above) for 10 min at room temperature. Filters were washed on ice 6 x 5 min in wash buffer (50 mM NaCl, 25 mM MES pH 6.0, 5 mM MgCl₂, 0.05 % Triton X-100), dried quickly on Whatman 3MM paper and exposed to Kodak X-Omat film at -70 °C for 70 h or as appropriate.
2.2.10.3. Analysis of $^{32}$P-PAK probes.

5 μl of the kinase assay reaction prior to dilution and loading onto G-50-Sephadex, or 20 μl of probe were mixed with 2x sample buffer, separated by SDS-PAGE and phosphorylated proteins were visualised by exposing the dried gel to Kodak X-Omat film for 1 h or 70 h respectively.

2.2.11. Purification of Cdc42Hs binding activity from human neutrophil cytosol.

2.2.11.1. Purification protocol I.

Neutrophil cytosol generated as described in section 2.2.2.3 (large scale), was separated by gel-filtration chromatography using a Superose-12 column and fractions containing Cdc42Hs binding activity were identified using either the dot blot or gel-overlay assays. Ten fractions from this column (40-50) were pooled and applied to a Q-Sepharose column from which Cdc42Hs binding activity was eluted in fractions 6-10 which were further applied to separation on a mono-Q column. Fractions 42-45 eluted from the mono-Q column contained Cdc42Hs binding activity and were subjected to affinity chromatography.

Proteins in the mono-Q column pool were loaded onto a Cdc42Hs-G12V-GTP glutathione-Sepharose affinity column in the presence of 1 mM GTP. For these experiments Cdc42Hs was not cleaved with thrombin, thus leaving the GST moiety as an affinity tag. The column was washed in 5 ml of buffer Z (100 mM NaCl, 0.5 mM MgCl$_2$, 0.05% Triton X-100) containing 25 mM MES pH 6.0. Proteins were eluted in 1 column volume of buffer Z containing 25 mM Tris pH 8.5 or freshly prepared 5 mM glutathione in 50 mM Tris pH 8.0.

2.2.11.2. Purification protocol II.

Cdc42Hs binding proteins were purified in four steps using either the dot blot or gel-overlay Cdc42Hs binding assay (section 2.2.5). (i) Neutrophil cytosol (280 ml, containing 20 mg/ml of protein) was applied to a Q-Sepharose column equilibrated with buffer (10 mM PIPES, 100 mM KCl, 3 mM NaCl, 4 mM MgCl$_2$ pH 7.0) containing 6% sucrose and protease inhibitors, (1 mM pepstatin and 100 mM TLCK). Protein was eluted with a linear gradient of NaCl (0.1-1 M) in the above buffer and 10 ml fractions collected. Protein from every third fraction was assayed for Cdc42Hs binding proteins.
which eluted at between 0.2-0.3 M NaCl. Six fractions were pooled and diluted 3-fold in ice cold water. (ii) The above pool (180 ml) was applied to a Heparin-Agarose column, equilibrated as above and proteins eluted with a linear NaCl gradient (0.1-1 M), in the same buffer. Binding protein eluted between 0.3-0.4 M NaCl. Five fractions of 5 ml were pooled. The volume was reduced to 5 ml by dialysis against polyethylene glycol, for 3 h at 4 °C. (iii) After filtration to clear the sample, protein was loaded onto a Superose-12 gel-filtration column, equilibrated as before and 1.3 ml fractions were collected in the above buffer. Five fractions (30-34) were found to contain Cdc42Hs binding activity and were pooled. (iv) A 1 ml chelating-Sepharose column was prepared by applying 10 column volumes of 100 mM ZnCl$_2$, protein sample containing fractions 30-34 from the Superose-12 column was applied, and the column was washed in buffer Z containing 25 mM Tris pH 7.5. Elutions were carried out in buffer Z containing 25 mM MES pH 6.0.

2.2.11.3. Quantification of Cdc42Hs binding and PAK protein present in the elutions from each column.

The elution profile of Cdc42Hs binding activity at each stage of purification II was estimated by converting dot blot signals, obtained when each fraction was probed with Cdc42Hs-[γ$^{32}$P]GTP probes, to cpm values as in section 2.2.5.2.

The yield of PAK protein obtained in the pool of fractions collected at each stage of the purification was estimated by converting the Cdc42Hs binding signals from dot blot experiments to cpm values. The cpm values were then converted to μg amounts of PAK protein by comparison with the cpm values obtained when a series of β-PAK protein amounts were probed with Cdc42Hs-[γ$^{32}$P]GTP probes. This analysis relies on the assumption that all the Cdc42Hs binding activity in the pool of fractions under investigation was due to presence of PAK protein and that native and recombinant PAK protein interact with Cdc42Hs in similar ways. This analysis provided a means of estimating the purification of PAK protein in the protein purification procedures carried out.

2.2.11.4. Peptide sequencing of p68.

Proteins from the fifth fraction collected from the chelating-Sepharose column (E5) were separated in 10 % SDS-gels and visualised by Coomassie Blue staining. Staining of proteins was carried out (as described in section 2.2.3.3) briefly with freshly prepared
solutions. Once destained the gel was rinsed in ddH₂O and the protein band that migrated with the 68 kDa (BSA) marker was excised using a scalpel. Protein was extracted from the gel piece and peptide sequence analysis was carried out by J. Hsuan at the Ludwig Institute for Cancer Research.

2.2.12. Subcloning of p67phox DNA constructs into yeast cell expression vectors.

2.2.12.1. Preparation of p67phox DNA constructs.

p67phox and p47phox DNA constructs were generated by polymerase chain reaction (PCR) using the appropriate sense and antisense oligonucleotide primers, as listed below. Oligonucleotides were designed with a CLAMP sequence GACGTC at the ends of each oligonucleotide in either orientation and to contain restriction enzyme sites after this sequence as indicated below. Constructs were manufactured to encode p67phox and p47phox protein fragments. p67phox protein fragments: amino acids 1-300 (which contain the proline-rich region and the N-terminal SH3 domain), amino acids 1-199 (which terminates before the proline-rich region) and amino acids 300-526 (which contain the C-terminal SH3 domain). p47phox protein fragment: amino acids 204-390.

Oligonucleotide primers.

A. p67phox amino acids 1-300 (nucleotides 67-966; 899 base pairs).

Forward 5' GACGTC\underline{CATGGTCATGTCCCTGGTGGAGGCCATC} 3'  
Reverse 5' GACGTCG\underline{AATTCTCCGCAACTCAACTGGTCCAAG} 3'  

B. p67phox amino acids 1-199 (nucleotides 67-664; 597 base pairs).

Generation of this fragment utilised a common forwards primer to construct A. (1-300).

Reverse 5' GACGTCG\underline{AATTCCTAGGTAATCCTTCTTGGCCAG} 3'  

C. p67phox amino acids 300-526 (nucleotides 965-1645; 680 base pairs).

Forward 5' GACGTC\underline{CATGGTGCGGATCCACCCTCAGCAGCAG} 3'  
Reverse 5' GACGTCG\underline{TTCACGACAGACTTCTCTCCGAGTGCTTTC} 3'  

EcoRI  
Sall
D. p47phox amino acids 204-390 (nucleotides 603-1161; 578 base pairs).

Forward 5'GACGTCCCATGGAGATGAAAGCAAAGCGAGGCTGG 3'
NcoI

Reverse 5'GACGTCGAATTACAGACGGCAGACGCCAGCTTCCG 3'
EcoRI

2.2.12.2. Polymerase chain reaction (PCR).

Polymerase chain reactions (100 μl) were carried out in 1 x Taqplus Long low-salt reaction buffer (20 mM Tris-HCl pH 8.75, 10 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1 % Triton X-100, 100 μg/ml nuclease-free BSA) with 1 μl of each oligonucleotide primer (150 ng/μl), 1 μl template DNA p67phox in pGEX or p47phox in pGEX in the presence of 0.25 mM of each dNTP and 2.5 units of Taqplus Long DNA polymerase. Using GeneAMP PCR System 9600 (Perkin Elmer).

The reactions were heated to 94 °C for 5 min for initial denaturation of the template DNA which was then amplified by 20 temperature cycles of 94 °C (40 seconds) to denature, 60 °C (1 min) to anneal, 72 °C (1 min) for extension, finally the reaction was held at 72 °C for 5 min to allow completion of DNA synthesis.

10 μl of the PCR reaction was taken and the DNA was analysed by agarose gel electrophoresis (section 2.2.14.7) to confirm production of a fragment of the desired size and also to estimate the purity of the DNA produced.

To generate p47phox C-terminal DNA construct PCR reactions were carried out as above in the presence of DMSO, which was required as the sequence of interest is GC rich. Due to the difficulties encountered, although the p47phox DNA fragment of the correct size was generated it was not inserted in to the appropriate yeast cell expression vector.

2.2.12.3. Purification of PCR products.

Wizard™ PCR Preps DNA Purification Resin (‘Clean Up’ Resin), Promega.

90 μl of the PCR reactions were mixed and incubated with 1 ml Wizard™ PCR Preps ‘Clean Up’ resin (6 M guanidine thiocyanate) for 1 min at room temperature. Resin was collected, passed through a minicolumn using a 2 ml syringe and washed with 2 ml
isopropanol (80 % v/v) before eluting the DNA in Tris-EDTA (TE; 10 mM Tris-HCl pH 7.5, 1 mM EDTA) previously heated to 75 °C.

2.2.12.4. Digestion of DNA by restriction enzymes.
Purified DNA fragments generated by PCR and circularised plasmid vectors were digested in 20-30 µl volumes with the appropriate enzymes prior to ligation reactions. PCR fragments encoding p67\textsuperscript{phox} amino acids 1-300, 1-199 and pACT2 plasmid were digested with EcoRI and NcoI in REact buffer #3 at 37 °C for 90 min. PCR fragment encoding p67\textsuperscript{phox} amino acids 300-526 and pAS2-l plasmid vector were digested sequentially with NcoI in REact buffer #3 at 37 °C for 90 min and SalI in REact buffer #2 at 37 °C for 90 min. DNA was ethanol precipitated (section 2.2.14.4.1) following digestion with the first enzyme. After each digestion reaction enzymes were inactivated by incubation at 65 °C (20 min). DNA was purified using Wizard™ 'Clean Up' resin (6 M guanidine HCl), using the method described in section 2.2.14.3.

2.2.12.4.1. Ethanol precipitation.
The volume of the digestion reaction (20-30 µl) was diluted with TE buffer to 200 µl. 2 volumes of ethanol (400 µl) and 0.1 volumes of 3 M sodium acetate pH 5.2 (20 µl) were added. This solution was mixed and incubated at -20 °C (16 h). Precipitated DNA was pelleted by centrifugation at 14 000 x g for 20 min at 4 °C. The supernatant was carefully decanted off and the pellet washed in 1 ml ethanol (70 % v/v) to remove contaminating salts. The pellet was dried under vacuum (Savant Speed-Vac) and resuspended in the buffer of choice.

2.2.12.5. Ligation of DNA fragments into plasmid vectors.
N-terminal p67\textsuperscript{phox} DNA constructs encoding amino acids 1-300 and 1-199 were inserted in to the NcoI EcoR1 site within the multiple cloning region of the pACT2 plasmid and p67\textsuperscript{phox} DNA encoding C-terminal amino acids 300-526 was inserted into the NcoI SalI site in the multiple cloning region of pAS2-l plasmid, (see appendix A.2, for details of the expression vectors used, including the multiple cloning sites).

Ligation reactions were carried out in 20 µl volumes containing 60 ng of insert DNA (4 µl: p67\textsuperscript{phox} fragments encoding amino acids 1-300, 1-199 and 300-526), and 200 ng of
cut plasmid DNA (2 μl: pACT2 and pAS2-1), generating a 3:1 molar ratio of insert: vector and T4 DNA ligase (2.5 units, 1 μl) in T4 DNA ligase buffer provided with the enzyme (66 mM Tris-HCl pH 7.6, 6.6 mM MgCl₂). Ligation reactions were incubated at 23 °C (4-5 h). To assess the efficiency of ligation 10 μl of each ligation reaction were used to transform 100 μl of *E. coli* XL-1 Blue competent cells as in section 2.2.1.1. Single colonies were picked from LB-agar plates and were used to inoculate 5 ml of ‘overnight’ culture as described (2.2.1.2). Plasmid DNA was purified from *E. coli* cells as described below.

2.2.12.6. Purification of plasmid DNA (from *E. coli* XL-1 Blue cell lysates).

(Wizard™ Minipreps DNA purification system, Promega)

Cells from 5 ml overnight cultures were pelleted by centrifugation, the supernatants were discarded. Cells were resuspended in 200 μl Cell Resuspension Solution (50 mM Tris-HCL, pH 7.5, 10 mM EDTA, 100 μg/ml RNase A) and transferred to fresh tubes. Cells were lysed by addition of 200 μl of Cell Lysis Solution (0.2 M NaOH, 1 % SDS) with gentle mixing by inversion. Cell lysates were neutralised by the addition of 200 μl of neutralising solution (1.32 M potassium acetate, pH 4.8) and cleared by centrifugation at 14 000 x g for 5 min. The supernatant was carefully pipetted off, avoiding the white genomic DNA precipitate and cleared lysates were mixed in a fresh tube with 1 ml of Wizard™ Minipreps DNA Purification Resin. Resin and bound DNA was collected by pipetting the resin/DNA solution into a 2 ml syringe and passing through a Wizard™ minicolumn. The column was washed with 2 ml of column wash solution (100 ml of 200 mM NaCl, 20 mM Tris-HCl pH 7.5, 5 mM EDTA diluted by the addition of 140 ml ethanol). The resin was dried by spinning the minicolumn in a microcentrifuge at 14 000 x g for 2 min. To elute the DNA from the resin, 50 μl of TE (10 mM Tris-HCl pH 7.5, 1 mM EDTA) preheated to 75 °C, was added to the column, allowed to stand 1 min and collected by spinning for 20 seconds.

2.2.12.7. Separation of DNA fragments by electrophoresis.

DNA samples were mixed with the appropriate volume of 6 x loading buffer (0.25 % Bromophenol Blue w/v, 0.25 % Xylene cyanol FF w/v and 30 % glycerol v/v) and were subjected to electrophoresis in 1 % agarose gels. Gels were made by dissolving the appropriate amount of agarose in 0.5 x TBE buffer (45 mM Tris, 45 mM boric acid, 1
mM EDTA). Gels were cast in horizontal gel trays (Bio-Rad), a comb was inserted into the gel solution to generate sample wells. The set gels were submerged in 0.5 x TBE buffer in a 'DNA Sub-Cell' (Bio-Rad) and samples were loaded in to the wells. Samples were subjected to electrophoresis at 150 V for 50 min. DNA was stained with ethidium bromide (0.5 µg/ml) in 0.5 x TBE for 45 min, viewed under ultraviolet light and recorded by Polaroid photography. HaeIII-digested Φx174 and HindIII-digested λ bacteriophage were run as markers, in parallel with samples, to enable estimation of DNA fragment sizes.

2.2.13. The yeast two-hybrid system.

The yeast two-hybrid system was applied in order to investigate an intramolecular interaction within p67^phox and to identify whether p67^phox and Rac1-Q61L interact in this system. p67^phox and Rac1 cDNAs were inserted into GAL4 activation domain/Target (pACT-2) or the GAL4 DNA binding domain/Bait (pAS2-1) plasmids. Subcloning of the Rac1Q61L-C196S cDNA into pAS2-1 plasmid was carried out by Sheila Govind. Plasmids were purchased from Clontech (see appendix A.2). pACT2 plasmid generates a fusion protein containing the GAL4 activation domain (AD) amino acids 768-881, an HA epitope tag and the protein of interest, the second plasmid pAS2-1 generates a fusion of the GAL4 DNA-binding domain (BD) amino acids 1-147 and the protein of interest.

The YRG-2 dual reporter *Saccharomyces cerevisiae* yeast strain was used, (see appendix A.3). This strain contains lacZ and HIS3 reporter genes for the *in vivo* detection of protein-protein interactions. YRG-2 strain carries auxotrophic markers; leucine (*leu2*) and tryptophan (*trp1*) for selection of yeast transformed with the GAL4 activation domain (AD) and GAL4 DNA-binding domain (BD) plasmids respectively, and also the auxotrophic marker histidine (*his3*), for selection of yeast that has been transformed with interacting proteins.

2.2.13.1. Solutions and media.

**Synthetic Dropout (SD) medium**

For Synthetic Dropout (SD) medium, Difco yeast nitrogen base without amino acids (6.7 g/l) and D-sorbitol (182.2 g/l) was diluted in ddH2O (pH 5.8) and sterilised by
autoclaving. Prior to use glucose (2 %) and 100 ml/l of Dropout solution (10x), lacking amino acids L-leucine and L-tryptophan, were added.

**Synthetic Dropout (SD) agar**

For synthetic dropout agar plates, agar (15 g/l) was added to synthetic dropout medium as above (pH 5.8) containing 100 ml/l of Dropout solution (10x), either lacking amino acids L-leucine and L-tryptophan or lacking amino acids L-leucine, L-tryptophan and L-histidine.

**10 x Dropout solution**

L-isoleucine (300 mg/l), L-valine (1500 mg/l), L-adenine hemisulfate salt (200 mg/l), L-arginine HCl (200 mg/l), L-histidine HCl monohydrate (200 mg/l), L-leucine (1000 mg/l), L-lysine HCl (300 mg/l), L-methionine (200 mg/l), L-phenylalanine (500 mg/l), L-threonine (2000 mg/l), L-tryptophan (200 mg/l), L-tyrosine (300 mg/l), L-uracil (200 mg/l).

2.2.13.2. Transformation and growth of yeast strain YRG2 competent cells.

Cotransformation of yeast YRG2 strain cells with pACT2 (AD) and pAS2-1 (BD) plasmids containing various combinations of p67^phox^ constructs and Rac1Q61L-C196S were carried out as summarised in Table 2.2. Both pACT2 and pAS2-1 plasmids (~100 ng) with or without insert were mixed and incubated together with 100 µl aliquots of competent YRG2 cells at 30 °C for 30 min. The cells were then treated with a 5 min heat pulse at 42 °C. Synthetic Dropout medium lacking amino acids L-leucine and L-tryptophan was added (0.9 ml) and cells were incubated at 30 °C for 3 h with shaking. 20 % of the cells from each transformation were plated on SD agar plates either lacking amino acids L-leucine (L) and L-tryptophan (W) -L/W plates (to check for correct transformation of both plasmids) or lacking amino acids L-leucine, L-tryptophan and L-histidine (H) -L/W/H plates (to determine whether interaction specific reconstitution of the GAL4 transcription factor had enabled transcription of the **his3** marker gene). Plates were incubated at 30 °C for 3-7 days.

Growth was monitored in order to determine whether interactions were occurring between the proteins of interest, expressed as fusion proteins with the GAL4 transcription factor activation domain or DNA binding domain. Colonies observed on the
-L/W/H were picked and streaked onto fresh SD agar plates lacking amino acids L-leucine, L-tryptophan and L-histidine, which were incubated at 30 °C for a further 3-7 days, in order to retest the observations.

Table 2.2. Cotransformations of YRG2 Cells with pACT2 and pAS2-1 Plasmids.

<table>
<thead>
<tr>
<th>No.</th>
<th>Activation Domain Plasmid (pACT2)</th>
<th>Binding Domain Plasmid (pAS2-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>p67phox 1-300</td>
<td>p67phox 300-526</td>
</tr>
<tr>
<td>2</td>
<td>p67phox 1-300</td>
<td>Rac1Q61L</td>
</tr>
<tr>
<td>3</td>
<td>p67phox 1-300</td>
<td>pAS2-1 (no insert)</td>
</tr>
<tr>
<td>4</td>
<td>p67phox 1-199</td>
<td>p67phox 300-526</td>
</tr>
<tr>
<td>5</td>
<td>p67phox 1-199</td>
<td>Rac1Q61L</td>
</tr>
<tr>
<td>6</td>
<td>p67phox 1-199</td>
<td>pAS2-1 (no insert)</td>
</tr>
<tr>
<td>7</td>
<td>pACT2 (no insert)</td>
<td>p67phox 300-526</td>
</tr>
<tr>
<td>8</td>
<td>pACT2 (no insert)</td>
<td>Rac1Q61L</td>
</tr>
<tr>
<td>9</td>
<td>pACT2 (no insert)</td>
<td>pAS2-1 (no insert)</td>
</tr>
<tr>
<td>10</td>
<td>SV40</td>
<td>p53</td>
</tr>
<tr>
<td>11</td>
<td>p53</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2.2. Cotransformations of YRG2 cells with pACT2 and pAS2-1 plasmids.

Plasmids (~100 ng) containing p67phox and Rac1 DNA or those with out inserts as indicated in experiments 1-9 were used to cotransform competent YRG2 strain S. cerevisiae, in the combinations indicated. Following transformations cells were plated onto -L/W and -L/W/H plates, incubated at 30 °C and growth was monitored over 3-7 days. Plasmids encoding SV40 and p53 were provided with the competent cells (Strategene) and were transformed according to the manufacturer's protocol. Experiments 10 and 11 were carried out to provide a positive control for interaction specific reconstitution of the GAL4 transcription factor, and to estimate transformation efficiency respectively.
Results

The use of p21 binding assays, whereby proteins immobilised on nitrocellulose filter are incubated with $[^{32}P]$GTP-labelled p21 probes, has identified the presence of a number of Rho family interacting proteins in brain and other tissue extracts (Manser et al., 1994). Purification of Cdc42Hs binding activity from rat brain cytosol, using p21 binding assays, led to the isolation and cloning of two brain enriched Cdc42Hs and Rac1 binding proteins, with serine/threonine kinase activity. These proteins were identified as p21-activated kinases and are referred to as α-PAK (68 kDa) and β-PAK (65 kDa) (Manser et al., 1994 and 1995a). In addition a ubiquitously expressed PAK protein with an apparent molecular mass of 62 kDa has been identified using p21 binding assays and is referred to as γ-PAK (Teo et al., 1995).

Human neutrophil cytosol was investigated for the presence of Rho family interacting proteins using p21 binding assays, in order to identify the presence of potential target proteins in this cell type.

3.1. Cdc42Hs and Rac1 binding proteins in human neutrophil cytosol.

To investigate the presence of binding partners for Rho family GTP binding proteins in human neutrophils, proteins from neutrophil cytosol were separated by SDS-PAGE, transferred to nitrocellulose filter, and probed with Cdc42Hs-$[^{32}P]$GTP probes. The nitrocellulose filters were then washed and exposed to X-Omat film for 5 to 25 h. The signals observed indicated that proteins with molecular masses of 60-68 kDa bound to the Cdc42Hs-$[^{32}P]$GTP (Fig. 3.1). This interaction was specific to the GTP-bound state of Cdc42Hs, as Cdc42Hs-$[^{32}P]$GDP did not detect the neutrophil binding proteins (Fig. 3.1). Rac1-$[^{32}P]$GTP probes also detected proteins of 60-68 kDa in neutrophil cytosol (Fig. 3.1), this reaction was also specific to GTP-bound Rac1 probes. However, the signals were approximately 5-fold weaker when Rac1 was used as a probe, suggesting that Rac1 binds less tightly to the 60-68 kDa binding proteins than Cdc42Hs in this analysis (Fig. 3.1, lane 3; note the longer exposure time for Rac1).

A very weak signal at 48 kDa was also seen with Rac1 (Fig. 3.1, lane 3) and with Cdc42Hs probes (see below) but this was variable. This signal may represent a novel
Fig. 3.1. Cdc42Hs and Rac1 Binding Proteins in Human Neutrophil Cytosol.

Neutrophil cytosol (100 µg/lane) was separated by SDS-PAGE, transferred to nitrocellulose filter and probed with GTP or GDP bound Cdc42Hs and Rac1 probes.

Probes:

- Lane 1, Cdc42Hs-[γ³²P]GTP,
- Lane 2, Cdc42Hs-[α³²P]GDP,
- Lane 3, Rac1-[γ³²P]GTP,
- Lane 4, Rac1-[α³²P]GDP.

Filters were exposed to X-Omat film for 5 h (Cdc42Hs) and 25 h (Rac1). Arrow shows the position of a faint signal at 48 kDa.
target for Cdc42Hs and Rac1 or be due to breakdown of the 60-68 kDa binding proteins. In some cases Cdc42Hs probes also detected a protein in neutrophil cytosol with a higher molecular mass of ~120 kDa (Fig. 3.2A, lane 2). To obtain maximum resolution of proteins with molecular masses between 45 and 95 kDa SDS-gels containing 10 % polyacrylamide were used, which are unsuitable for accurately determining the size of proteins larger than 100 kDa. Further investigation is required to determine the identities of the 48 and 120 kDa Rac1/Cdc42Hs and Cdc42Hs binding proteins. However, the latter protein could relate to the Cdc42Hs specific binding protein p120^ACK (Manser et al., 1993).

Cdc42Hs-[^32P]GTP probes detected proteins in rat brain cytosol (Fig. 3.2B, lane 1) of a similar size to those observed in human neutrophils, which are most probably PAK-related proteins (Manser et al., 1994). It was noted that the Cdc42Hs binding proteins in brain cytosol appeared to migrate with slightly slower mobility on SDS-gels (with apparent molecular masses of 65 and 68 kDa) in comparison to those in neutrophil cytosol (apparent molecular masses between 60 and 68 kDa). This could be explained by there being a variation in the abundance of protein species between the two tissues, supporting the idea that α and β-PAK (68 and 65 kDa) are highly enriched in brain whereas as γ-PAK (62 kDa) is ubiquitously expressed (Manser et al., 1994 and 1995a; Teo et al., 1995). Partial purification of the Cdc42Hs binding activity from neutrophil cytosol by column chromatography revealed that at least two distinct protein bands between 60 and 68 kDa were detected with the Cdc42Hs probes (Fig. 3.2B, lane 3; see section 3.3).

Proteins from neutrophil cytosol were also probed with RhoA-[^32P]GTP probes (Fig. 3.2B, lane 2). The pattern of RhoA binding proteins in neutrophil cytosol differed from that observed when either Rac1 or Cdc42Hs probes were used. RhoA probes did not bind to proteins of 60-68 kDa, but instead bound a number of proteins over a wide range of sizes, at approximately 30, 35, 43, 47, 50, 55, 75, 90, and 120 kDa (as indicated by the arrows in Fig. 3.2B). The RhoA probe used in this experiment bound weakly to proteins from brain cytosol at 40 and 50 kDa (Fig. 3.2B, lane 1). The binding signals observed in either tissue may relate to novel RhoA target proteins or to previously
Fig. 3.2. Cdc42Hs and RhoA Binding Proteins in Rat Brain and Human Neutrophil Cytosol.

Fig. 3.2. Cdc42Hs and RhoA binding proteins in rat brain and human neutrophil cytosol. A and B: -

Lane 1, brain cytosol (50 µg),
Lane 2, neutrophil cytosol (100 µg),
Lane 3, partially purified neutrophil cytosol (2 µg).

Partially purified neutrophil cytosolic proteins were taken from the chelating-Sepharose column, elution 2 (as described in the Methods section 2.2.11.2; see Fig. 3.6.).

Proteins were separated by SDS-PAGE, transferred to nitrocellulose filter by western blotting and probed with Cdc42Hs-[γ^{32}P]GTP (A) and RhoA-[γ^{32}P]GTP (B) probes. Filters were washed and exposed to X-Omat film for 5 h (Cdc42Hs) and 17 h (RhoA). Arrows indicate the high molecular mass protein in neutrophil cytosol bound by Cdc42Hs probes (A) and various proteins detected in neutrophil cytosol by the RhoA probes (B).
described RhoA binding proteins, for example the serine/threonine kinases, protein kinase N (PKN), which migrates on SDS-gels with an apparent molecular mass of 120 kDa (Amano et al., 1996), or the PKN-related protein, Rhophilin, which has a calculated molecular mass of ~71 kDa (Watanabe et al., 1996). The RhoA binding proteins detected did not purify with the Cdc42Hs binding activity from neutrophil cytosol (Fig. 3.2B, lane 3).

3.2. Human neutrophil and rat brain Cdc42Hs binding proteins are related.

The major Cdc42Hs binding proteins in brain have been shown to be PAK proteins (Manser et al., 1994). To investigate whether the neutrophil 60-68 kDa Cdc42Hs binding proteins are related to PAK, Cdc42Hs binding assays were carried out on proteins from neutrophil and brain cytosol that had been separated by 2-Dimensional (2-D) gel electrophoresis. Proteins in neutrophil and brain cytosol were separated according to their isoelectric points (pl), on narrow range isoelectric focusing tube gels, and then by size in SDS-polyacrylamide gels. Separated proteins were then transferred to nitrocellulose filter by western blotting and probed with Cdc42Hs probes (Fig. 3.3). The locations of the binding proteins in brain and neutrophil cytosol were very similar, with the proteins having similar molecular masses (around 68 kDa) and migrating with basic isoelectric points. Two major binding protein spots were detected in brain and neutrophil cytosol (Fig. 3.3). The similarity of the migration of brain and neutrophil Cdc42Hs binding proteins following 2-D gel electrophoresis supports the idea that the neutrophil proteins observed are related to brain PAK proteins.

3.3. Purification of Cdc42Hs binding proteins from human neutrophil cytosol by column chromatography.

To purify the Cdc42Hs binding proteins detected in neutrophil cytosol, a series of chromatographic columns and Cdc42Hs-[γ^{32}P]GTP binding assays were used. The fractions collected from each column containing Cdc42Hs binding proteins were pooled and applied to the subsequent column of the purification. Two different purification protocols (I and II) were followed as detailed below. The final stage of purification protocol I was a Cdc42Hs-G12V-GTP affinity column (Fig. 3.5). The yield of protein eluted from the affinity column was low and further analysis of this protein was not possible. In the second purification protocol (II) sufficient amounts of proteins for further studies were obtained.
Fig. 3.3. Two-Dimensional Localisation of Cdc42Hs Binding Proteins in Rat Brain and Human Neutrophil Cytosol.

Brain cytosol

Neutrophil cytosol

Brain and neutrophil cytosol (100 μg) was separated in narrow range isoelectric focusing tube gels, containing a discontinuous gradient of ampholines from 3.5-10 extended in the region between 5 and 7, followed by SDS-PAGE in 10 % gels (as described in the Methods section 2.2.3.1). Protein was then transferred to nitrocellulose filter and probed with Cdc42Hs-[γ32P]GTP probes. Basic to acidic (left to right), high to low molecular weights (top to bottom).
3.3.1. Purification protocol I.

Cytosolic proteins were separated using a Superose-12 (gel-filtration) column. The fractions containing Cdc42Hs binding activity were identified by probing proteins immobilised on nitrocellulose filters, either following SDS-PAGE or by the dot blotting method, with Cdc42Hs-[γ32P]GTP (Fig. 3.4A). Both methods displayed a similar elution profile of Cdc42 binding proteins from the Superose-12 column.

Superose-12 column fractions 40-50, containing Cdc42Hs binding activity, were pooled and applied to a Q-Sepharose column. Cdc42Hs binding activity was eluted in fractions 6-10 from the Q-Sepharose column, which were pooled and subjected to further purification on a mono-Q column. Fractions 42-45 from the mono-Q column were found to contain Cdc42Hs binding proteins between 60 and 68 kDa (Fig. 3.4B). Fig. 3.4B, illustrates the pool of fractions obtained from the latter two stages of the purification, in both cases the Cdc42Hs binding proteins observed, between 60 and 68 kDa, migrated in SDS-gels with similar mobility to α-PAK immunoreactivity seen in these samples (Fig. 3.4B, compare lanes 5 and 6 with lanes 8 and 9 respectively). This suggested the presence of α-PAK-related proteins.

Fractions 42-45 from the mono-Q column were pooled and applied to a Cdc42Hs-G12V-GTP glutathione-Sepharose affinity column in the presence of 1 mM GTP. After washing the affinity column proteins were eluted in buffer containing 25 mM Tris-HCl (pH 8.5), or by 5 mM Glutathione in 50 mM Tris-HCl (pH 8.0). The binding activity of the fractions eluted from this column was assessed by dot blotting and probing with Cdc42Hs-[γ32P]GTP (Fig. 3.5). The elutions were found to contain Cdc42Hs binding activity and analysis of the protein in these fractions by SDS-PAGE demonstrated the presence of a single protein band at ~68 kDa (Fig. 3.5, lanes 4 and 5). The yield of protein from the Cdc42Hs affinity column was low, with the bulk of protein either flowing through the column or remaining bound to the glutathione-Sepharose resin. As proteins could not be eluted from the Cdc42Hs affinity column further investigations using this material were not possible. Although proteins did not separate well from the glutathione-Sepharose on SDS-gels, it was demonstrated that Cdc42Hs binding activity, at ~68 kDa, was associated with the glutathione-Sepharose resin (Fig. 3.5, lane 7).
Fig. 3.4. Purification of the 60-68 kDa Cdc42Hs Binding Proteins from Human Neutrophil Cytosol, Protocol I.

A. Superose-12 (Gel-filtration)

Fig. 3.4. Purification of the 60-68 kDa Cdc42Hs binding proteins from human neutrophil cytosol, protocol I. Cdc42Hs binding proteins from neutrophil cytosol were purified by column chromatography on Superose-12 (Gel-filtration), Q-Sepharose, and mono-Q columns, (see Methods section 2.2.11.1).

A. Protein in Superose-12 column fractions 40, 43, 46, and 49 (25-50 μg) were separated by SDS-PAGE, transferred to, or directly dot blotted onto nitrocellulose filter and probed with Cdc42Hs-[γ32P]GTP probes. Dots above each lane show signals obtained from the corresponding fractions. Once washed, filters were exposed to X-Omat film for 5 h.

Lane 1, fraction 40,
Lane 2, fraction 43,
Lane 3, fraction 46,
Lane 4, fraction 49.
B. Proteins in the fractions obtained from the Q-Sepharose and mono-Q columns were separated by SDS-PAGE and stained with Coomassie Blue, lanes 1-3 or transferred to nitrocellulose filter and probed with Cdc42Hs-[\(^{32}\)P]GTP probes (as in A), lanes 4-6, or with \(\alpha\)-PAK antibody (1:500 dilution) lanes 7-9. Dots above lanes 4-6, show signals obtained from the corresponding fractions.

**Lanes 1, 4, and 7,** neutrophil cytosol (100 \(\mu\)g) (C),

**Lanes 2, 5, and 8,** Q-Sepharose fraction 10 (25 \(\mu\)g) (QS),

**Lanes 3, 6, and 9,** mono-Q fraction 42 (15 \(\mu\)g) (MQ).
Fig. 3.5. Purification of the 60-68 kDa Cdc42Hs Binding Proteins from Human Neutrophil Cytosol, Protocol I. Cdc42Hs-G12V Affinity Column.

The pool of fractions (42-45) from the mono-Q column were applied to a Cdc42Hs-G12V affinity column. Fractions collected were analysed for proteins and Cdc42Hs binding activity (see Methods section 2.2.11.1).

- **Lane 1**, mono-Q column pooled fractions, the starting material applied to this column, 50 µg.
- **Lane 2**, unbound material 40 µg (~80% of the protein applied flowed through the column),
- **Lane 3**, wash with buffer Z (pH 6.0),
- **Lanes 4 and 5**, elutions with buffer Z (pH 8.5),
- **Lane 6**, glutathione-Sepharose resin (20 µl) with Cdc42Hs-G12V-GST and bound p68,
- **Lane 7**, glutathione-Sepharose resin (20 µl), transferred to nitrocellulose filter and probed with Cdc42Hs-[γ³²P]GTP probes.

Proteins (10-20 µl) were separated by SDS-PAGE and stained with Coomassie Blue. Dot blots of the different fractions were probed with Cdc42Hs-[γ³²P]GTP and are shown above each corresponding lane. Glutathione-Sepharose resin, 20 µl was resuspended in 20 µl of 2 x sample buffer, boiled and associated proteins were separated on SDS-gels and stained with Coomassie Blue (lane 6) or transferred to nitrocellulose and probed with Cdc42Hs-[γ³²P]GTP (lane 7). The major protein bound to the resin at 48 kDa is Cdc42Hs-G12V-GST, proteins at ~26 kDa are Cdc42Hs-G12V-GST breakdown products. The 68 kDa protein seen in lanes 4, 5, and 6 (indicated by the arrow) appears to co-migrate with the Cdc42Hs binding activity seen in lane 7 although proteins were not resolved well in this analysis.
A smaller Cdc42Hs binding protein which migrated on SDS-gels with the 48 kDa marker, and was previously identified in neutrophil cytosol, was found to copurify with the 68 kDa binding protein, although its level varied between batches of neutrophil cytosol. This protein may be a breakdown product of the 68 kDa Cdc42Hs binding protein. In fractions from the Q-Sepharose and mono-Q columns a 48 kDa protein and proteins between 60 and 68 kDa were detected with α-PAK antibody, indicating that the 48 and 60-68 kDa Cdc42Hs binding proteins may be related to α-PAK (Fig. 3.4B).

3.3.2. Purification protocol II.

The chromatographic steps of the second purification are illustrated in a flow diagram (Fig. 3.7) and the details of fractions collected at each stage are tabulated in Table 3.1. Having identified the presence of α-PAK immunoreactivity in neutrophil cytosol and fractions collected during purification protocol I (Fig. 3.4), the yield of PAK protein in the pool of fractions collected at each stage of the second purification was estimated. Assuming that the Cdc42Hs binding activity was due to the presence of PAK, the amount of PAK protein present was estimated by comparing the binding activity to that observed when recombinant β-PAK protein is probed with Cdc42Hs probes, as described in the Methods section 2.2.11.3.

3.3.2.1. Q-Sepharose column chromatography.

Neutrophil cytosol was loaded onto a Q-Sepharose column. After washing the column protein in every third fraction was dot blotted and probed with Cdc42Hs-\[^{32}P\]GTP probes. Cdc42Hs binding proteins were eluted with 0.2-0.3 M NaCl and were found predominantly in fractions 18 and 19. Proteins in neutrophil cytosol and fraction 19 were separated by SDS-PAGE and analysed by Coomassie Blue staining (Fig. 3.6A, lanes 1 and 2), the associated binding activity is shown in the dot blot above each corresponding lane. Six 10 ml fractions (16-21) eluted from this column were pooled to obtain 60 ml.

Calculations from the cpm values obtained from converting the dot blot binding signals by scintillation counting, indicated that 22 % of the total Cdc42Hs binding protein from the neutrophil cytosol applied to this column was retained in this pool of fractions. This pool contained 16 % of protein applied to the column in neutrophil cytosol. These figures indicate that a slight enrichment for the Cdc42Hs binding activity was made during this stage of the purification; 3 mg of PAK protein were retained in the pool of
fractions collected with an estimated purity of 0.33% (Table 3.1). Assuming that PAK protein is solely responsible for the Cdc42Hs binding activity in this pool of fractions these data suggest that PAK protein represents approximately 0.2% of neutrophil cytosolic proteins.

3.3.2.2. Heparin-Agarose column chromatography.

The above pool of 60 ml was diluted 3-fold in ice cold water, to reduce the salt concentration; the resulting 180 ml was applied to a Heparin-Agarose column. After washing proteins, were eluted with a continuous gradient of 0.1-1.0 M NaCl, and 5 ml fractions were collected. Fractions containing Cdc42Hs binding proteins were identified using the dot blot method (Fig. 3.6B). Radioassaying of the Cdc42Hs binding signals indicated that the majority of binding activity was in fractions 6-10 (eluted with 0.3-0.4 M NaCl). To characterise the Cdc42Hs binding activity in these fractions, proteins were separated by SDS-PAGE and transferred to nitrocellulose and probed with Cdc42Hs-[γ-32P]GTP. Using this method the fractions containing binding proteins correlated with those identified using the dot blot method (Fig. 3.6B, lanes 3-7). Cdc42Hs binding proteins were identified between 60 and 68 kDa and visualisation of the binding signals after a short exposure time showed the presence of three separate bands which migrated on SDS-gels with apparent molecular masses of approximately 62, 65, and 68 kDa (Fig. 3.6B, lane 8 arrows indicate their positions), with the major binding protein being at ~65 kDa.

Five fractions of 5 ml were pooled together and this pool contained 27% of the protein obtained from the Q-Sepharose column (4.4% of the total protein in cytosol applied to the purification). The proteins applied to the column and those eluted in fraction 9, which was found to be highly enriched for Cdc42Hs binding activity, were separated by SDS-PAGE and analysed by Coomassie Blue staining (Fig. 3.6B, lanes 1 and 2). 1.8 mg of PAK protein was retained in the 25 ml pool of fractions (6-10) from the Heparin-Agarose column, with an estimated purity of 0.72% (Table 3.1).

3.3.2.3. Superose-12 (gel-filtration) column chromatography.

The pool of fractions obtained from the Heparin-Agarose column was reduced in volume from 25 ml to 5 ml by dialysis against polyethylene glycol and was passed down a Superose-12 gel-filtration column and proteins were separated on the basis of size.
1.3 ml fractions were collected and proteins were separated by SDS-PAGE and transferred to nitrocellulose filter and probed with Cdc42Hs-[γ^{32}P]GTP. A 5-fold increase in the binding activity of the start material was seen in comparison to that observed with the fractions collected from the Heparin-Agarose column (Fig. 3.6C), this corresponded with the dialysis concentration step. Binding activity was observed in fractions 30-34 and a similar pattern of binding proteins to that seen in the previous column was observed. Fractions 30-34 were combined and found to contain 10 % of the protein applied to this column and 0.80 mg of PAK protein with an estimated purity of 3.2 %.

3.3.2.4. Chelating-Sepharose column chromatography.
Fractions from the gel-filtration column were dialysed against 50 mM Tris-HCl (pH 7.5) prior to separation on a 1 ml chelating-Sepharose column, that had been saturated with ZnCl. Proteins were eluted from the chelating-Sepharose column in a buffer containing 25 mM MES (pH 6.0), seven 1 ml elutions were collected. Proteins from these elutions were separated by SDS-PAGE and analysed by Coomassie Blue staining and for Cdc42Hs binding activity, by transferring them to nitrocellulose. Cdc42Hs binding activity was detected in elutions 1-5, with the activity being most abundant in elution 2 (Fig. 3.6D, lane 3). Coomassie Blue staining demonstrated the presence of two protein bands in elution 5 at approximately 62 and 68 kDa. The larger protein appeared to migrate on SDS-gels with the Cdc42Hs binding activity observed in this elution and was excised from a Coomassie Blue stained gel for peptide sequence analysis, see below. Attempts to further purify these proteins using a Cdc42-G12V-GTP affinity column were unsuccessful due to the low levels of protein available.

Elution 2 (E2) which contained the majority of the Cdc42Hs binding activity retrieved from this column contained a number of other proteins as visualised by Coomassie Blue staining (Fig. 3.6D, lane 1). 5 μg of PAK protein was retained in this fraction with an estimated purity of 10 %. Elution 5 (E5) contained very little protein ~ 0.1 mg/ml (Fig. 3.6D, lane 2), including 25 μg of PAK protein at an estimated purity of 25 %. Both of these fractions provided useful sources of semi-purified neutrophil Cdc42Hs binding proteins for further investigations.
Fig. 3.6. Figure legend on reverse.
Fig. 3.6. Purification of the 60-68 kDa Cdc42Hs Binding Proteins from Human Neutrophil Cytosol, Protocol II

The Cdc42Hs binding activity in the fractions collected from each column of purification II was monitored and representative signals are illustrated in this figure. Proteins were separated by SDS-PAGE and visualised by staining with Coomassie Blue or transferred to nitrocellulose filter and probed with Cdc42Hs-[γ32P]GTP as indicated in each section. Dot blot signals shown correspond to the fraction in the lane below as appropriate.

A. Q-Sepharose, protein was analysed by staining with Coomassie Blue or assayed for Cdc42Hs binding by dot blotting.

Lane 1, neutrophil cytosol, 10 µl (20 mg/ml),
Lane 2, fraction 19, 10 µl (15 mg/ml).

B. Heparin-Agarose, proteins were stained with Coomassie Blue, or probed with Cdc42Hs-[γ32P]GTP, filters were exposed to X-Omat film for 17 h (lanes 3-7) or 5 h (lane 8).

Lane 1, start material (Q-Sepharose pool) 10 µl (5 mg/ml),
Lanes 2, and 6, fraction 9, 10 µl (~10 mg/ml), 10 µl
Lane 3, fraction 6, 10 µl,
Lane 4, fraction 7, 10 µl,
Lanes 5, and 8, fraction 8, 10 µl, the shorter exposure in lane 8 shows the presence of 3 Cdc42Hs binding protein bands (marked with arrows).
Lane 7, fraction 10, 10 µl. The dot blot illustrates that both p21 binding assay methods show a similar profile of binding activity.

C. Superose-12, protein was separated by SDS-PAGE, transferred to nitrocellulose filter and probed with Cdc42Hs probes.

Lane 1, start material (concentrated Heparin-Agarose pool) 10 µl, (note the binding activity is approximately 5-fold greater than fraction 8 from the Heparin-Agarose column: compare with B Lane 5).
Lane 2, fraction 30, 10 µl.

D. Chelating-Sepharose, protein was separated by SDS-PAGE and stained with Coomassie Blue (lanes 1 and 2) or transferred to nitrocellulose filter and probed with Cdc42Hs probes (lanes 3 and 4).

Lanes 1, and 3, fraction 2 (elution 2) 20 µl (0.5 mg/ml),
Lanes 2, and 4, fraction 5 (elution 5) 20 µl (0.1 mg/ml).
Fig. 3.6. Purification of the 60-68 kDa Cdc42Hs Binding Proteins from Human Neutrophil Cytosol, Protocol II.

A. Q-Sepharose
B. Heparin-Agarose
C. Superose-12
D. Chelating-Sepharose

- **A. Q-Sepharose**
  - Coomassie Stain
  - 96, 68, 43, 26 kDa
  - Lanes: 1, 2

- **B. Heparin-Agarose**
  - Coomassie Stain
  - 96, 68, 43, 26 kDa
  - Lanes: 1, 2, 3, 4, 5, 6, 7, 8
  - Cdc42Hs Binding

- **C. Superose-12**
  - Coomassie Stain
  - 96, 68, 43, 26 kDa
  - Lanes: 1, 2

- **D. Chelating-Sepharose**
  - Coomassie Stain
  - 96, 68, 43, 26 kDa
  - Lanes: 1, 2, 3, 4

Coomassie Stain

Cdc42Hs Binding
Table 3.1. Purification of Cdc42Hs Binding Activity from Neutrophil Cytosol.

<table>
<thead>
<tr>
<th>Column</th>
<th>Start Material</th>
<th>Fractions Collected</th>
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<tbody>
<tr>
<td></td>
<td>Vol. (ml)</td>
<td>Protein (mg)</td>
</tr>
<tr>
<td>Q-Sepharose</td>
<td>280</td>
<td>5600</td>
</tr>
<tr>
<td>Heparin-Agarose</td>
<td>180</td>
<td>900</td>
</tr>
<tr>
<td>Superose-12 (gel-filtration)</td>
<td>5</td>
<td>250</td>
</tr>
<tr>
<td>chelating-Sepharose</td>
<td>6.5</td>
<td>25</td>
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Table 3.1. Purification of Cdc42Hs binding activity from neutrophil cytosol. The volume (vol.) of the start material in ml and the total protein content (mg) in the samples applied to each column and that of the pool of fractions (indicated by the fraction numbers) obtained at each stage of purification protocol II are summarised here. The yield of PAK protein in the fractions collected was estimated by converting Cdc42Hs binding signals, from dot blot experiments, for each fraction or pool of fractions to counts per minute (cpm). The cpm values were converted to µg quantities of PAK by comparison to cpm values obtained with varying amounts of recombinant β-PAK protein which were dot blotted and probed with Cdc42Hs. The purification of PAK protein achieved in the fractions pooled or for those eluted from the chelating-Sepharose column individually, was calculated by expressing the amount of PAK as a percentage of the total protein present in that fraction.
Fig. 3.7. Flow Diagram of Purification Protocol II.

Start Material
280 ml Neutrophil Cytosol
5600 mg (20 mg/ml) →

5600 mg 20 mg/ml
Q-Sepharose
900 mg, 60 ml, (15 mg/ml) →

900 mg, 180 ml, 5 mg/ml
Heparin Agarose
250 mg, 25 ml 10 mg/ml →

250 mg, 5 ml, (50 mg/ml)
Superose-12 Gel-Filtration
25 mg, 6.5 ml, (3.8 mg/ml) →

Chelating-Sepharose

(i) In vitro kinase assay Fig. 3.9.
(ii) Anti-α-PAK immunoblot Fig. 3.10.
(iii) Anti-Ste20 immunoblot Fig. 3.11.
(iv) Peptide sequence analysis Fig. 3.12.
(v) $^{32}$P labelled proteins used to probe nitrocellulose filters Fig. 3.13B.
During the stages of both purification protocols carried out, the presence of more than one Cdc42Hs binding protein was demonstrated. Three proteins were consistently detected with the Cdc42Hs probes with apparent molecular masses of ~68, 65 and 62 kDa. Further work was carried out using material collected from the chelating-Sepharose column in order to establish the identities of these proteins (as indicated in Fig. 3.7). Fractions collected from the chelating-Sepharose column are designated as p68 fractions, and elutions 2 and 5 are also referred to as E2 and E5 respectively, when used in subsequent experimental procedures.

3.3.3. Proteins in the p68 fractions from the chelating-Sepharose column bind GTP-bound Cdc42Hs and Rac1.

The interaction between Cdc42Hs and proteins in the p68 fraction (E2) from purification II was investigated for the requirement of Cdc42Hs being in the GTP-bound state. This experiment was also carried out with Rac1 probes. Both p21s were loaded with [α\(^{32}\)P]GTP, half of the protein was used to probe proteins immobilised on nitrocellulose immediately, the remainder was left at room temperature for 1 h to allow hydrolysis of the bound nucleotide. Rac1-GTP bound proteins with molecular masses between 60 and 68 kDa in the E2 fraction, and the pattern binding proteins detected with this probe resembled that observed with Cdc42Hs-GTP probes. With both Cdc42Hs and Rac1 loss of signal was observed when the probes were used after 1 h (Fig. 3.8). These results suggest that (i) the Cdc42Hs interacting proteins purified from neutrophil cytosol may also bind Rac1 and (ii) that for binding to occur both p21s are required to be bound to GTP.

3.4. The p68 fraction from the chelating-Sepharose column contains a PAK-related kinase.

PAK proteins are members of a family of serine/threonine kinases (Lim et al., 1996). The kinase activity of PAK protein purified from rat brain cytosol and also recombinant α and β-PAK is stimulated in the presence of Cdc42Hs or Rac1-GTP (Manser et al., 1994 and 1995a). In vitro solution kinase assays were carried out to identify whether the p68 fractions collected from the chelating-Sepharose column contain p21-activated protein kinases.
Fig. 3.8. Rac1 and Cdc42Hs Binding to p68 Fraction Proteins.

Proteins in the second elution from the chelating-Sepharose column (p68 fraction; E2), (20 μl; 10 μg/lane) were separated by SDS-PAGE, transferred to nitrocellulose filter and probed with GTP or GDP labelled Rac1 and Cdc42Hs.

**Probes:**
- **Lane 1**, Rac1-[γ³²P]GTP,
- **Lane 2**, Rac1-[α³²P]GDP,
- **Lane 3**, Cdc42Hs-[γ³²P]GTP,
- **Lane 4**, Cdc42Hs-[α³²P]GDP.

Filters were exposed to X-Omat film for 17 h (Rac1) and 8 h (Cdc42Hs). Binding observed with the GDP[α³²P]-probes, may be due to residual levels of p21-GTP remaining after the 60 min incubation required to generate these probes, see Methods section 2.2.5.1.
3.4.1. Autophosphorylation of p68 fraction proteins.

Proteins in the p68 fraction (E5) were incubated with \([\gamma^{32}P]ATP\) in kinase buffer in the presence or absence of Rac1-GTP for 15 min at 30 °C. This reaction was stopped by the addition of sample buffer and proteins were separated in SDS-polyacrylamide gels. Gels were dried down and phosphorylated proteins were visualised by exposure of the gel to X-Omat film for 10 min. Autophosphorylated proteins in the p68 fraction with apparent molecular masses of ~68 kDa were observed; these proteins appeared to correlate with protein at ~68 kDa visualised by Coomassie Blue staining of this fraction (Fig. 3.9, compare lanes 1 and 2). The kinase activity associated with this fraction was stimulated by Rac1. The nucleotide specificity for stimulation of autophosphorylation was investigated by adding Rac1-GTP and Rac1-GDP to \textit{in vitro} solution kinase assays. Rac1 was loaded with GTP and either used immediately (for Rac1-GTP), or incubated at room temperature for 1 h to allow the bound nucleotide to become hydrolysed, by the intrinsic GTPase activity of Rac1 (for Rac1-GDP). Autophosphorylation was stimulated approximately 10-fold in the presence of Rac1-GTP (Fig. 3.9, compare lanes 2 and 4). The stimulation of autophosphorylation observed with Rac1-GDP may be due to a residual level of Rac1-GTP remaining after the 1 h incubation. Proteins with larger molecular masses probably represent hyper-phosphorylated forms of p68 fraction protein. These results indicate that p68 fraction E5 contains a p21-activated kinase.

3.4.2. Proteins in the p68 fraction react with PAK specific antibodies.

To examine whether protein in p68 fraction (E2) was related to the neutrophil NADPH oxidase component p67\(^{phox}\) or \(\alpha\)-PAK, specific affinity-purified antibodies were used. Neutrophil cytosol, p67\(^{phox}\) and semi-purified p68 fraction (E2) were separated by SDS-PAGE and transferred to nitrocellulose. p67\(^{phox}\) antibodies detected recombinant p67\(^{phox}\) and a protein of similar size in neutrophil cytosol but not p68 proteins. \(\alpha\)-PAK antibodies detected proteins in the p68 fraction and a band of similar size in neutrophil cytosol but not p67\(^{phox}\) (Fig. 3.10A). \(\alpha\)-PAK antibodies also detected a protein of approximately 68 kDa in brain cytosol. Thus p68 fraction proteins from neutrophil cytosol are closely related to \(\alpha\)-PAK.
Fig. 3.9. Kinase Activity of p68 Fraction.

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Proteins in the p68 fraction 20 µl (2 µg) were incubated with 50 µCi [γ^32P]ATP for 15 min, in the absence of additions or in the presence of GDP or GTP bound Rac1 and then separated by SDS-PAGE, the gel was dried down and exposed to X-Omat film for 10 min, in order to visualise phosphorylated proteins.

**Lane 1**, Coomassie Blue stain of partially purified p68 fraction protein (E5).

**Lanes 2-4**, autophosphorylation of a ~68 kDa protein in the partially purified p68 fraction (E5) from the chelating-Sepharose column (see Methods section 2.2.11.2).

**Lane 2**, p68 fraction 20 µl, (2 µg), in the absence of additions,

**Lane 3**, p68 fraction 20 µl, (2 µg) + Rac1-GDP,

**Lane 4**, p68 fraction 20 µl, (2 µg) + Rac1-GTP.
3.4.3. Immunoprecipitation of the kinase activity in the p68 fraction with α-PAK antibodies.

To investigate whether the kinase activity identified in the p68 fraction E2 was due to the presence of a PAK-related kinase, autophosphorylated protein in the p68 fraction was immunoprecipitated by α-PAK antibodies. Semi-purified neutrophil PAK in the p68 fraction (E2) and recombinant α-PAK-GST were incubated in parallel with \([γ^{32}P]ATP\) for 15 min, followed by an incubation with affinity purified α-PAK antibodies or preimmune serum. Proteins were precipitated using protein A-Sepharose. The α-PAK antibodies were able to precipitate an autophosphorylated ~68 kDa protein from the p68 fraction and autophosphorylated α-PAK-GST (Fig. 3.10B). That only one autophosphorylated protein at ~68 kDa was immunoprecipitated from the p68 fraction by the α-PAK antibody indicates that this antibody does not recognise hyperphosphorylated forms of p68.

3.4.4. Proteins in the p68 fraction react with Ste20 antibodies.

The kinase domain of α-PAK shares 68.1% sequence identity with *S. cerevisiae* Ste20 protein. Antibody raised against a peptide from the kinase domain of Ste20 protein was used to probe recombinant and cytosolic proteins immobilised on nitrocellulose filters (see appendix A.8, for alignment of the amino acid sequences of the kinase domains of PAK and Ste20 proteins). Ste20 antibody reacted with recombinant α and β-PAK-GST fusion proteins but not p120ACK, used here as a GST-fusion protein control (Fig. 3.11). Note that β-PAK migrates on SDS-PAGE with higher apparent molecular mass than α-PAK due to the insertion of an additional sequence during cloning of the cDNA (Manser *et al.*, 1995a; and appendix A.1.1). Ste20 antibody reacted with proteins in brain and neutrophil cytosol and three distinct protein bands in p68 fraction E5. This demonstrated that Ste20 antibody may be a useful tool for detecting PAK-like proteins and perhaps different isoforms. In correlation with the data obtained from the Cdc42Hs binding analysis the use of the Ste20 antibody suggested the presence of more than one PAK-like protein in the purified p68 fraction.
Fig. 3.10. α-PAK Specific Antibodies React with and Precipitate p68.

A

![Image]

Lane 1, and 4, purified recombinant p67<sub>phox</sub> (1 μg).

Lane 2, and 5, p68 fraction (E2) (10 μg).

Lane 3, and 6, neutrophil cytosol (100 μg).

Lane 7, brain cytosol (100 μg).

Proteins were separated by SDS-PAGE, transferred to nitrocellulose filter and then probed with either p67<sub>phox</sub> specific antibodies (dilution 1:10 000) lanes 1-3, or α-PAK specific antibodies (dilution 1:500), lanes 4-7, (as described in the Methods section 2.2.7).
B. Autophosphorylated neutrophil proteins from the p68 fraction (E2) and recombinant α-PAK-GST were immunoprecipitated with α-PAK specific antibodies (lanes 1 and 3) or preimmune serum (lanes 2 and 4), as indicated. Proteins were autophosphorylated by incubating with [$\gamma^{32}$P]ATP for 15 min at 30 °C, prior to immunoprecipitation followed by separation in SDS-gels:

- **Lane 1**, p68 fraction (E2),
- **Lane 2**, p68 fraction (E2),
- **Lane 3**, α-PAK-GST (96 kDa),
- **Lane 4**, α-PAK-GST (96 kDa).

Gels were dried down and exposed to X-Omat film for 16 h (as described in Methods section 2.2.8.).
Fig. 3.11. Ste20 Antibody Immunoreactivity.

Recombinant, or brain and neutrophil cytosol were separated by SDS-PAGE, transferred to nitrocellulose filters and probed with Ste20 antibody (UBI) at a concentration of 5 μg/ml (as described in the Methods section 2.2.7).

Lane 1, α-PAK-GST (5 μg),
Lane 2, β-PAK-GST (2.5 μg),
Lane 3, p120^{ACK}-GST (10 μg),
Lane 4, brain cytosol (25 μg),
Lane 5, neutrophil cytosol (100 μg),
Lane 6, p68 fraction E5 (3 μg).
3.5. PAK interacts with annexin VI protein.

3.5.1. Annexin VI copurifies with the 60-68 kDa Cdc42Hs binding proteins from human neutrophil cytosol.

Attempts were made to obtain peptide sequence of the Cdc42Hs binding protein purified from neutrophil cytosol in the p68 fraction. Protein in the fifth fraction from the chelating-Sepharose column (E5) comigrated on SDS-gels with the 68 kDa marker and the Cdc42Hs binding activity in this fraction at ~68 kDa (Fig. 3.6D). Proteins in this fraction were separated by SDS-PAGE and protein bands at ~68 kDa were excised from the protein gel. Protein recovered from this gel piece was digested and three of the peptides generated were sequenced. The sequence information obtained was as follows; peptide 1: KYAGSDHDT, peptide 2: kttVRPANE FNT and peptide 3: kMTNYDVELK. All three peptide sequences were found to align with the sequence of annexin VI (Fig. 3.12). The residues in lower case represent residues of which the identity is not certain; the initial lysine residues (k) result from the specificity of the enzyme used during the digestion process. This indicated the presence of annexin VI in the partially purified p68 fraction, thus suggesting that annexin VI protein copurifies with the PAK-like proteins from neutrophil cytosol.

3.5.2. Recombinant annexin VI interacts with β-PAK.

Experiments were carried out to identify whether annexin VI interacts with PAK proteins. Proteins from neutrophil cytosol and recombinant proteins were immobilised on nitrocellulose and probed with either \(^{32}\text{P}\)-β-PAK or protein in the p68 fraction (E2) labelled with \(^{32}\text{P}\). The probes were generated by incubating recombinant β-PAK-GST or p68 fraction (E2) proteins in kinase buffer with \([γ^{32}\text{P}]\text{ATP}\) for 15 min at 30 °C, as described in the Methods section 2.2.10. That β-PAK and p68 fraction proteins were labelled was confirmed by separating the probes on SDS-gels, which were then dried and exposed to X-Omat film (Fig. 3.13, lane 5 in A and B respectively). Labelled proteins were diluted into binding buffer and incubated with nitrocellulose filters on which cytosolic and recombinant proteins had been immobilised following SDS-PAGE.
Fig. 3.12. Peptide Alignment with Human Annexin VI Sequence.

A

Peptide 1  KYAGSDHDT
Peptide 2  kktVRPANEFT
Peptide 3  kMTNYDVELK

B

1   AKPAQGAKYRKSIHGDPFPFD  PNQDAEALYT  AMKGFGSDKE  AILDITRS
   KYA  GSDHD  peptide 1
51  NRQRQECQSYKSLYGKDLDKLKYTELTGK  FERLIVGLMR  PPAYCDAKEIK
101  KDAISGIGTD  EKCLIEILAS  RTNEMQHQLV  AAYKDAYERD  LEADTIGDTS
151  GHFQKMLVVL  LQGTDREDDV  VSEDLVQDVQVQD  QDLYEAGEKL  WGTDEAQPFIY
201  ILGNRSQCHLRLVFDEYLKT  TGKPIEASIR  GELGDFEKL  MLAVVCKIRS
251  TPEYFAERLKFAMKGGLGTRD  NTLIRIMVSR  SELMDLDIRIEFRTKEKSL
301  YSMIKNKTSGEYYKTLKLLSGDDDAAGQF  FPEAAQVAYQ  MWELSAVARV
351  ELKGTVRPANDEFNPADAKA  LKRAMKGLGT  DEDTIIDIT  HRSNVQRQFQ
   KTTVRPANEFTP  peptide 2
401  RQTFKSHFGRDLMTDLKSEISGLDLARLILGLMMPPAHYDKLKKAMEGA
451  GTDEKALIEI  LATRTNAEIR  AINEAYKEDY  HKSLEDALSS  DTSGHFFRL
501  ISLATGHREE  GGENLDOARE  DAQVAAEILE  IADTPSDGKT  SLETRFMTIL
551  CRTSYPHLRRVFQEFIKMTNYDVEHTIKKE  MSGDVRDAFV  AIVQSVRKNKPK
   KMTNYDVE  peptide 3
601  LFFADKLYKSMKGAGTDEKT  LTRIMVSRSE  IDLLNIRREF  IEKDYKSLHQ
651  AIEGDTSGDFLKAALLALCGGED

Fig. 3.12. Peptide alignment with human annexin VI sequence. A, Sequence of the three peptides generated from digestion of p68 protein, positions at which the residue identity is not certain are indicated by lower case letters. B, Human Annexin VI sequence showing the positions of alignment of peptides 1, 2, and 3. The positions where residues of the peptides and annexin VI sequence are identical are highlighted in yellow.
3.5.2.1. Proteins detected with the $^{32}$P -β-PAK probes.

The β-PAK probe interacted with two proteins, of approximately 62 and 65 kDa in neutrophil cytosol, recombinant p47$^{phox}$ and recombinant annexin VI but not recombinant human coronin-like protein p57, which was present here as a control (Fig. 3.13A). The apparent molecular masses of the proteins detected in neutrophil cytosol with the β-PAK probe indicated that these were not annexin VI, which migrates in SDS-gels at ~68 kDa nor did these proteins line up with p67$^{phox}$ immunoreactivity in neutrophil cytosol. The identity of these proteins is unknown. This analysis suggests that recombinant β-PAK interacts with annexin VI and p47$^{phox}$ in this assay.

3.5.2.2. Proteins detected with the $^{32}$P -p68 fraction protein probe.

Partially purified neutrophil p68 fraction proteins were also labelled with $^{32}$P and used to probe recombinant p47$^{phox}$ and annexin VI proteins and neutrophil and brain cytosol. This probe identified three proteins of approximately 65 kDa in neutrophil cytosol and a similar pattern of proteins in brain cytosol. However this probe did not detect recombinant p47$^{phox}$ or annexin VI (Fig. 3.13B).

The ability of annexin VI to interact with PAK proteins might explain its presence in the partially purified fractions collected from the chelating-Sepharose column, although the native neutrophil PAK-like proteins could not detect annexin VI in this assay. Whether annexin VI is a β-PAK substrate was also investigated using recombinant proteins, see Results Chapter 6.

3.6. p21 binding proteins in subcellular fractions from human neutrophil and HL60 cells.

Having identified the presence of a number of potential Cdc42Hs and Rac1 target proteins in neutrophil cytosol, proteins from HL60 cells, a human promyelocytic cell line, were analysed for Cdc42Hs and Rac1 binding activity. Cdc42Hs and Rac1 binding proteins identified in undifferentiated HL60 cells were compared to those found in neutrophils in order to examine whether this cell line could be useful for studying Rho family target proteins. In addition cellular proteins from both cell types were fractionated in order to investigate the subcellular localisation of Cdc42Hs and Rac1 binding proteins.
Fig. 3.13. Proteins Binding to β-PAK and p68 Fraction Probes.

Cytosolic and recombinant proteins as indicated, were separated in SDS-gels, transferred to nitrocellulose filter and incubated with $^{32}$P labelled β-PAK-GST (A; lanes 1-4) or p68 fraction protein (B; lanes 1-3) probes, (as described in Methods section 2.2.10). Nitrocellulose filters were washed and exposed to X-Omat film for 70 h.

Labelled proteins in the two probes were analysed by separation in SDS-gels, which were dried and exposed to X-Omat film for 16 h β-PAK probe (A), lane 5, or 70 h p68 probe (B), lane 4.

A.

Lane 1, neutrophil cytosol, (N; 25 μg),

Lane 2, recombinant p47$^{phox}$, (p47; 10 μg),

Lane 3, recombinant annexin VI, (A; 10 μg),

Lane 4, recombinant p57 (human coronin-like protein), (p57; 10 μg).

Probe: -

Lane 5, $^{32}$P-β-PAK probe, (20 μl),
B. Cytosolic and recombinant proteins as indicated, were separated in SDS-gels, transferred to nitrocellulose filter and incubated with \( {\text{\textsuperscript{32}P}}} \)-labelled p68 fraction proteins.

**Lane 1,** brain cytosol, (B; 25 µg),

**Lane 2,** neutrophil cytosol, (N; 25 µg),

**Lane 3,** recombinant \( p47^{\text{phos}} \), (p47; 10 µg),

**Lane 4,** recombinant annexin VI, (A; 10 µg),

**Probe:** -

**Lane 5,** \( {\text{\textsuperscript{32}P}}} \)-p68 probe, (20 µl).
Undifferentiated HL60 cells cultured in RPMI medium and neutrophil cells from human blood were harvested and proteins were separated as detailed in the Methods section 2.2.2, to obtain three different fractions containing (i) cytosolic (C), (ii) Membrane (M; Triton X-100 soluble) and (iii) membrane-cytoskeletal (S; Triton X-100 insoluble) proteins. Proteins in each fraction were separated by SDS-PAGE, transferred to nitrocellulose and probed with Rac1 and Cdc42Hs probes and antibodies raised against recombinant α-PAK-GST protein, to assay for the presence of potential p21 target proteins and PAK-like proteins. Results of the p21 binding assays and the western analysis are shown in Fig. 3.14. For a summary of the proteins detected with the different probes, in the protein fractions tested, see Table 3.2.

3.6.1. p21 binding proteins and α-PAK immunoreactivity in the cytosolic (C) and membrane (M) protein fractions from human neutrophils and HL60 cells.

Cdc42Hs probes bound proteins between 62 and 68 kDa in HL60 cell cytosol and similar binding proteins were detected with Rac1 probes (Fig. 3.14B). Filters probed with Rac1 probes required a 25 h exposure to visualise signals comparable to those seen with Cdc42Hs after 5 h, indicating that the interaction of Rac1 with proteins in HL60 cells is of lower affinity. The banding pattern of Cdc42Hs and Rac1 binding proteins observed resembled that seen when neutrophil cytosolic proteins were probed with these two p21s (Compare Fig. 3.14 parts A and B, and previous figures within this chapter e.g. Fig. 3.1). The low levels of Cdc42Hs and Rac1 binding activity associated with the protein from neutrophil cytosol in comparison to that observed in Fig 3.1, is due to there only being 5 μg of protein present rather than 100 μg. These results are shown to demonstrate that with both p21 probes the major binding protein migrated on SDS-gels with an apparent molecular mass of 65 kDa. Cdc42Hs probes also bound HL60 cell cytosolic proteins of 30 and 40 kDa and a faint band was detected with the Rac1 probe at approximately 47 kDa. These proteins may represent specific p21 interacting proteins in HL60 cells or breakdown products of the 62-68 kDa binding proteins that are not observed in neutrophil cells. Rac1 binding proteins of approximately 65 kDa and also 47 kDa were identified in the neutrophil cytosolic fraction. The 47 kDa neutrophil protein appears to be specific for Rac1 and does not bind Cdc42Hs probes.

The p21 binding assays used in the above analysis indicate that neutrophils and HL60 cells contain similar cytosolic Cdc42Hs and Rac1 binding proteins between 62 and 68
kDa. Western analysis of HL60 and neutrophil cytosolic proteins with α-PAK antibodies indicated that the ~65 kDa proteins detected with the p21 probes might be PAK-related proteins in both cell types (Fig. 3.14 A and B, lane 7). α-PAK antibodies also detected smaller proteins indicating that the HL60 cell 40 and 30 kDa Cdc42Hs binding proteins may be breakdown products of PAK-like proteins. The 35 kDa protein detected by these antibodies in HL60 cells may also be breakdown products of PAK proteins not detected by the p21 probes.

A similar pattern of Cdc42Hs and Rac1 binding proteins to that seen in the cytosolic fraction was observed in the Triton X-100 soluble membrane protein fraction of both cell types, although the binding activity was much reduced. Comparison of the p21 binding assays and western analysis of the three protein fractions probed indicates that the majority of the 62-68 kDa Cdc42Hs and Rac1 binding proteins and PAK-like proteins are located in the cytosol of these two cell types.

3.6.2. p21 binding proteins and α-PAK immunoreactivity in the Triton X-100 insoluble membrane cytoskeletal (S) protein fraction from human neutrophils and HL60 cells.

Investigation of the proteins present in the Triton X-100 insoluble membrane fraction demonstrated a difference in the distribution of Cdc42Hs and Rac1 binding proteins between the two cell types. This fraction was enriched for proteins that associate with the membrane cytoskeleton, and will be referred to here as the cytoskeletal (CSK; S) fraction.

The CSK fraction obtained from HL60 cells contained a similar pattern of Cdc42Hs binding proteins to that observed in the cytosolic and the Triton X-100 soluble membrane protein fractions obtained from this cell type. However, the quantities of these proteins were reduced in comparison to the cytosolic fraction (to less than 1 %). Rac1 probes weakly detected proteins of 62-65 kDa and a protein at 30 kDa in the HL60 CSK fraction. The 30 kDa protein was not detected in the previous fractions tested and may represent a cytoskeletal associated Rac1 target in this cell type, however 80 μg of protein from this fraction was required to see this signal and this binding interaction may not have been visible in the previous two fractions tested.
A number of different proteins in the neutrophil CSK fraction were detected with Cdc42Hs probes, at approximately 23, 27, 33, 37, 45, 57, 65, 68 and 80 kDa (Fig. 3.14A, lane 3). Upon western analysis of the CSK fraction, proteins of approximately 57 and 80 kDa were detected by α-PAK antibodies (Fig. 3.14A, lane 9), which may correspond with the Cdc42Hs binding proteins of these sizes. The identities of the proteins detected in this fraction are unknown, however the binding proteins here may represent specific cytoskeletal associated Cdc42Hs targets as they were not detected in the cytosolic fraction even following a long exposure of the experiment shown in Fig. 3.14A, lane 1. Rac1 probes detected proteins at approximately, 47 and 25 kDa in the neutrophil CSK fraction. The comparison between the proteins bound by these two p21s indicates that they may have different specific targets within the membrane cytoskeleton of neutrophil cells.

The Cdc42Hs binding proteins detected in the neutrophil CSK fraction, which were not observed in the corresponding fraction from HL60 cells, could represent proteins that are not expressed in undifferentiated HL60 cells. Analysis of protein fractions from differentiated HL60 cells is required to establish if specific p21 binding proteins are ‘turned on’ to express after induction of this cell type to differentiate. This analysis suggests that HL60 cells which unlike human neutrophils may be maintained in cell culture contain p21 binding proteins and PAK-related proteins and could be useful for investigating p21 target proteins.
Fig. 3.14. Cdc42Hs and Racl Binding Proteins and α-PAK Immunoreactivity in Fractions from Human Neutrophils and HL60 cells.

Cellular proteins of neutrophils (A) and HL60 cells (B) were fractionated as described in the Methods section 2.2.2, separated by SDS-PAGE, transferred to nitrocellulose filter and probed with Cdc42-Q61L-[γ^32P]GTP lanes 1-3, Racl-Q61L-[γ^32P]GTP lanes 4-6, and α-PAK antibody (1:500 dilution) lanes 7-9. Washed nitrocellulose filters were exposed to X-Omat film for 5-25 h, for p21 binding and as appropriate for α-PAK antibody immunoblots.

**Lanes 1, 4, and 7**, Cytosolic proteins (C)
(5 µg in A and 15 µg in B),

**Lanes 2, 5, and 8**, Triton X-100 soluble membrane proteins (M)
(5 µg in A and 15 µg in B),

**Lanes 3, 7, and 9**, Triton X-100 insoluble membrane cytoskeletal proteins (S)
(40 µg in A and 80 µg in B).
Table 3.2. Cdc42Hs and Rac1 Binding Proteins in Neutrophil and HL60 subcellular fractions.

| Proteins from neutrophil and HL60 cells were fractionated as described in Methods section 2.2.2, and separated by SDS-PAGE, transferred to nitrocellulose and probed with Cdc42Hs-GTP\[^{32}P\] and Rac1-GTP\[^{32}P\] probes or α-PAK antibodies, (see Fig. 3.14). Molecular masses of the proteins detected with the different probes were estimated from their mobility in SDS-gels, containing 10% polyacrylamide, and approximate masses are given in kDa. |
4. Rac Interacts With The Neutrophil NADPH Oxidase Component p67phox.

The Rho family GTP binding proteins Rac1 and Rac2 are required for superoxide production by the NADPH oxidase enzyme system, of guinea pig macrophages and human neutrophils respectively (Abo et al., 1991; Knaus et al., 1991). The NADPH oxidase is a multicomponent enzyme that includes the cytosolic components p47phox, p67phox, p40phox and the membrane associated cytochrome b. The molecular mechanism by which Rac acts within this enzyme system is not known. To identify the Rac target, recombinant or purified native neutrophil NADPH oxidase components, immobilised on nitrocellulose filters, were probed with Rac1-[γ32P]GTP probes. In addition Cdc42Hs binding proteins of 60-68 kDa have been identified in human neutrophil cytosol (as described in Chapter 3). To investigate whether these included the NADPH oxidase component p67phox, phox proteins immobilised on nitrocellulose filters were also probed with Cdc42Hs-[γ32P]GTP probes.

4.1. p67phox protein purified from insect Sf9 cell lysates contains Cdc42Hs and Rac1 binding activity.

The neutrophil NADPH oxidase components p67phox and p47phox were expressed as recombinant proteins in insect Sf9 cells (see Methods section 2.2.1.5), and cytochrome b protein was purified from human neutrophil cells. Proteins were separated by SDS-PAGE, transferred to nitrocellulose filter by western blotting and then probed with [γ32P]GTP labelled Rac1 and Cdc42Hs. Using this method a 67 kDa Cdc42Hs binding protein was identified in the recombinant p67phox material, which appeared to comigrate with p67phox protein on SDS-gels as judged by Coomassie Blue staining of this sample (Fig. 4.1 A, lane 3). Cdc42Hs binding activity was not associated with p47phox protein or the native cytochrome b. None of the components tested were detected with Rac1-[γ32P]GTP probes (Fig 4.1A, lanes 4-6).

To increase the sensitivity of the assay for Rac1, phox proteins were directly dot blotted onto nitrocellulose to avoid protein denaturation caused by running in SDS-gels. The NADPH oxidase component p40phox was also investigated for Rac1 activity binding in this analysis. Using this method of immobilising protein on nitrocellulose, with Rac1-[γ32P]GTP as probe, signals were obtained for p67phox (2.5-7.5 μg) but not for p40phox,
Fig. 4.1. Cdc42Hs and Rac1 bind p67phox purified from insect Sf9 cell lysates.

A. Neutrophil oxidase components; native purified human neutrophil cytochrome b, or recombinant p67phox, and p47phox purified from insect cell lysates, were separated by SDS-PAGE, transferred to nitrocellulose filter by western transfer and probed with Cdc42Hs-[γ32P]GTP (lanes 1-3) or Rac1-[γ32P]GTP (lanes 4-6).

Lanes 1, and 4, Cytochrome b (5 μg),
Lanes 2, and 5, p47phox (5 μg),
Lanes 3, and 6, p67phox (5 μg).

To visualise signals washed filters were exposed to X-Omat film for 5 h (Cdc42Hs) or 16 h (Rac1).
B. Neutrophil oxidase components; native purified human neutrophil cytochrome b, or recombinant p67\textsuperscript{phox}, and p47\textsuperscript{phox} purified from insect cell lysates, and recombinant p40\textsuperscript{phox} purified from \textit{E. coli} cells, were immobilised on nitrocellulose filter by dot blotting and probed with Rac1-[\gamma\textsuperscript{32P}]GTP probes.

Purified proteins were dot blotted as indicated at the left-hand end of each row: -
- p40\textsuperscript{phox}, 2.5, 5, and 7.5 µg (lanes 1, 2, and 3 respectively),
- Cytochrome b, 10, 20, and 40 µg (lanes 1, 2, and 3 respectively),
- p47\textsuperscript{phox}, 10, 20, and 40 µg (lanes 1, 2, and 3 respectively),
- p67\textsuperscript{phox}, 2.5, 5, and 7.5 µg (lanes 1, 2, and 3 respectively),
- \textit{Control proteins} BSA and p85\textalpha, were present at 10 µg (lane 1 only).

Signals were visualised by exposing nitrocellulose filters to X-Omat film for 16 h. Similar results were obtained in at least one other experiment.
cytochrome b, BSA or recombinant phosphatidylinositol 3-kinase p85α subunit (p85α), (Fig 4.1B). A weak signal was obtained with p47<sub>phox</sub> when it was present at high amounts (40 μg). The strength of signal obtained with the Rac1 probe increased with an increase in p67<sub>phox</sub> protein amounts between 2.5 and 7.5 μg, suggesting this was a specific interaction. That Rac1 binds p67<sub>phox</sub> using the dot blot method of immobilising p67<sub>phox</sub> on nitrocellulose but not following SDS-PAGE suggests that this interaction is sensitive to denaturation during SDS-PAGE, which perhaps disrupts the structure of the Rac1 binding site on p67<sub>phox</sub>.

Taken together these data suggest that p67<sub>phox</sub> is a potential interacting protein for both Rac1 and Cdc42Hs and may represent the Rac1 target in the NADPH oxidase enzyme system. Further studies were carried out to characterise the p21-p67<sub>phox</sub> interactions.

4.1.1. Specificity of the Rac1 and Cdc42Hs binding interactions with p67<sub>phox</sub> protein expressed in insect Sf9 cells.

The specificity of the Rac1 and Cdc42Hs-p67<sub>phox</sub> interactions was investigated using the Q61L GTPase negative mutants of Cdc42Hs and Rac1, wild-type RhoA and α-PAK(1-151). Ras-Q61L has increased effector function in terms of transformation ability and Ras-GAP interaction (Brownbridge et al., 1993). Rac2-Q61L is a more potent activator of neutrophil oxidase (Xu et al., 1994) and binds the Rac1-GAP n-chimaerin more tightly than wild type Rac1 or the GTPase negative mutant Rac1-G12V proteins (Ahmed et al., 1994).

Recombinant NADPH oxidase components p47<sub>phox</sub> and p67<sub>phox</sub> purified from insect Sf9 cells immobilised on nitrocellulose filter by dot blotting along with BSA and recombinant p85α subunit (present as controls), were probed with Rac1-Q61L-[γ<sup>32</sup>P]GTP probes. The Rac1-Q61L probe gave a stronger signal than the wild-type protein for the interaction with p67<sub>phox</sub> (compare Fig. 4.2A, lane 1 and Fig. 4.1B, lane 1; and see below). The signals observed with this Rac1 probe increased with the amount of p67<sub>phox</sub> protein applied to the nitrocellulose filter. Rac1-Q61L probe did not give signals with the two control proteins nor p47<sub>phox</sub>. Although members of the Rho family of Ras-related GTP binding proteins, which includes RhoA, Rac and Cdc42 share 50-55 % sequence similarity, RhoA probes did not bind p67<sub>phox</sub> under the conditions in this analysis (Fig. 4.2B).
Fig. 4.2. Specificity of the Rac1 and Cdc42Hs Binding Interactions with p67\textsuperscript{phox} Purified from Insect Sf9 Cell Lysates.

Neutrophil oxidase components; recombinant p67\textsuperscript{phox}, and p47\textsuperscript{phox} proteins purified from insect cell lysates, were dot blotted onto nitrocellulose as indicated at the left-hand end of each row, and probed with:

- A. Rac1-Q61L-[\gamma\textsuperscript{32}P]GTP, and
- B. RhoA-[\gamma\textsuperscript{32}P]GTP probes.

p67\textsuperscript{phox}, 2.5, 5, and 7.5 µg (lanes 1, 2, and 3 respectively, A and B), p47\textsuperscript{phox}, 10, 20, and 40 µg (lanes 1, 2, and 3 respectively, A).

Control proteins BSA and p85α, were present at 10 µg (lane 1, A).
C, D and E. Purified proteins were dot blotted onto nitrocellulose filter and probed with Cdc42Hs-Q61L-[\(\gamma^3P\)]GTP probe in the absence of additions (C), in the presence of competing Cdc42Hs-Q61L-GDP (D), and in the presence of GST or a-Pak(1-151)-GST (E).

Dot blotted proteins are indicated at the left-hand end of each row:

C. \(p47^{phax}\), 10, 20, and 40 \(\mu\)g (lanes 1, 2, and 3 respectively),
\(p67^{phax}\), 2.5, 5, and 7.5 \(\mu\)g (lanes 1, 2, and 3 respectively),

Control protein BSA and \(p85\alpha\), were present at 10 \(\mu\)g (lane 1).

D and E.
\(p67^{phax}\), 5 \(\mu\)g, was present in each lane (1, 2, and 3).

Probes:

C. Cdc42Hs-Q61L-[\(\gamma^3P\)]GTP.

D. Lane 1, Cdc42Hs-Q61L-[\(\gamma^3P\)]GTP.
   Lane 2, Cdc42Hs-Q61L-[\(\gamma^3P\)]GTP + Cdc42Hs-Q61L-GDP (equal amounts),
   Lane 3, Cdc42Hs-Q61L-[\(\gamma^3P\)]GTP + Cdc42Hs-Q61L-GDP (10-fold excess of the labelled Cdc42Hs probe).

E. Lane 1, Cdc42Hs-Q61L-[\(\gamma^3P\)]GTP,
   Lane 2, Cdc42Hs-Q61L-[\(\gamma^3P\)]GTP + GST,
   Lane 3, Cdc42Hs-Q61L-[\(\gamma^3P\)]GTP + \(\alpha\)-PAK(1-151).
   (Competing proteins were present at 500-fold excess of the labelled Cdc42Hs probe).
A binding signal was observed when dot blotted p67phox protein, purified from insect Sf9 cells, was probed with Cdc42Hs-Q61L-[γ32P]GTP (Fig. 4.2C). The signal increased with the amount of p67phox protein between 2.5 and 7.5 μg. Cdc42Hs-Q61L did not bind p47phox, BSA or p85α. The binding interaction was specific to the GTP-bound state of Cdc42 as no reduction in signal was observed when dot blotted p67phox protein was either probed with Cdc42-Q61L-[γ32P]GTP in the presence of Cdc42-Q61L-GDP (Fig. 4.2D), or probed with Cdc42-Q61L-[γ32P]GTP with Cdc42-Q61L-GDP present at equal or 10-fold higher amounts (Fig. 4.2D lanes 2 and 3 respectively).

Further evidence for the specificity of the Cdc42 interaction with protein in the recombinant p67phox material was obtained by carrying out dot blots in the presence of α-PAK(1-151) as a competitor. α-PAK(1-151) which includes the p21 binding domain, interacts specifically with GTP-bound Rac1 and Cdc42Hs with high affinity (nM). At 2 μM α-PAK(1-151) inhibited Cdc42Hs-Q61L from interacting with p67phox, as can be concluded from the loss of binding signal (Fig 4.2E, compare lanes 1 and 3), while 2 μM GST was without effect, no reduction in signal was observed in the latter case (Fig. 4.2E compare lanes 2 and 3).

4.1.2. p67phox binding to ‘effector domain’ mutants of Rac1 and Cdc42Hs.

Point mutations in the ‘effector domain’ of Ras, for example D38A in which residue 38 is mutated from asparagine to alanine, have been used to determine the specificity of interactions with potential target proteins. The Ras-D38A ‘effector domain’ mutant does not interact with Ras effectors such as Raf (Warne et al., 1993). Rac1-D38A does not induce membrane ruffling, and neither Rac1-D38A nor Rac2-D38A activates the neutrophil NADPH oxidase (Xu et al., 1994; Diekmann et al., 1994). To determine whether the interactions between p67phox and Rac1 or Cdc42Hs are functionally relevant ‘effector domain’ mutants of Rac1 and Cdc42Hs, were used as probes. The binding interactions observed when full-length p67phox protein, purified from insect Sf9 cells, and a deletion mutant containing amino acid residues 1-238 of p67phox expressed in E. coli cells, were probed with either Rac1-D38A or Cdc42-D38A probes were compared to those observed when GTPase deficient p21s were used as probes (Fig. 4.3).
Fig. 4.3. \(p67^{phox}\) Binding to ‘Effector Domain’ Mutants of Rac1 and Cdc42Hs.

![Image of binding experiment results]

**Fig. 4.3.** \(p67^{phox}\) binding to ‘effector domain’ mutants of Rac1 and Cdc42Hs. 5 µg of \(p67^{phox}\) protein purified from insect Sf9 cell lysates, and N-terminal deletion mutant (amino acids 1-238 of \(p67^{phox}\)), purified from *E. coli* XL-1 Blue cells, were dot blotted onto nitrocellulose filter as indicated at the left-hand end of each row and probed with Rac1 and Cdc42Hs \([\gamma^{32}P]GTP\) labelled probes as follows:

- **Lane 1.** Rac1-D38A-\([\gamma^{32}P]GTP\) (‘effector domain’ mutant),
- **Lane 2.** Rac1-Q61L-\([\gamma^{32}P]GTP\) (GTPase negative mutant),
- **Lane 3.** Cdc42Hs-D38A-\([\gamma^{32}P]GTP\) (‘effector domain’ mutant),
- **Lane 4.** Cdc42Hs-Q61L-\([\gamma^{32}P]GTP\) (GTPase negative mutant).

Signals were visualised as in Fig. 4.1.
Racl-Q61L probes bound full-length p67phox and the N-terminal deletion mutant (amino acids 1-238) (Fig. 4.3, lane 1), however Rac1-D38A, bound only the full-length p67phox protein (Fig. 4.3, lane 2). This suggests that either there are two Rac1 binding sites in p67phox or that an additional factor in the p67phox protein sample purified from insect Sf9 cell lysates (which is insensitive to the D38A mutation in Rac1) contributes to the Rac1 binding signal observed.

Cdc42-Q61L bound only to full-length p67phox purified from insect Sf9 cell lysates and not to the deletion mutant (amino acids 1-238) (Fig. 4.3, lane 3). The 'effector domain' mutant Cdc42-D38A did not bind either p67phox protein tested (Fig. 4.3, lane 4). This suggests that the p67phox-Cdc42Hs binding interaction is sensitive to the 'effector domain' mutation D38A and that Cdc42Hs does not interact with the same site in p67phox as Rac1. Alternatively, this result could be explained by the presence of an additional factor in the p67phox material purified from insect Sf9 cell lysates that interacts with Cdc42Hs in a manner insensitive of the D38A 'effector domain' mutation.

The above data suggest that the p67phox protein sample purified from insect Sf9 cell lysates contains both Rac1 and Cdc42Hs interacting proteins. However, the fact that Rac1 but not the Cdc42Hs binding activity was found to be sensitive to SDS-PAGE and that the Cdc42Hs but not the Rac1 binding activity was sensitive to the D38A 'effector domain' mutation, suggest that the p21 interactions observed occur with different properties. These interactions were analysed further as described below and in Chapter 5.

4.2. Characterisation of the Rac1-p67phox binding interaction.

Having established the presence of a Rac1 binding protein in the p67phox protein material purified from insect Sf9 cell lysates, whether p67phox is a Rac1 binding partner was investigated using p67phox proteins purified from E. coli cells. Full-length p67phox protein and deletion mutants, containing various p67phox protein fragments, were used to characterise the Rac1-p67phox binding interaction and to localise the Rac1 binding site.

4.2.1. The Rac1-p67phox binding interaction is GTP-dependent.

Rac protein was found to account for the GTP-dependency of activation of the neutrophil NADPH oxidase and is required to be in the GTP-bound state for reconstitution of superoxide production in vitro (Abo et al., 1991; Knaus et al., 1991
and 1992). This suggests that the Rac-target protein interaction required for activation of this enzyme system is GTP-dependent. The dependency of the nucleotide state of Rac1 for an interaction with full-length p67$^{phox}$ protein or N-terminal deletion mutant (amino acids 1-238) was investigated using two different methods: - (i) p67$^{phox}$ proteins were dot blotted and probed with Rac1-[α-$^{32}$P]GTP, either immediately following the nucleotide exchange reaction or after Rac1-[α-$^{32}$P]GTP was incubated at room temperature for an hour, to enable hydrolysis of the bound nucleotide, creating Rac1-[α-$^{32}$P]GDP probe, or (ii) p67$^{phox}$ proteins were probed with Rac1-[γ-$^{32}$P]GTP in the presence of increasing amounts of Rac1-GDP. Both methods indicated that the Rac1-p67$^{phox}$ interaction is dependent upon the GTP bound state of Rac1 (Fig. 4.4). Binding signals were not observed when either full-length p67$^{phox}$ or the N-terminal deletion mutant were probed with Rac1-GDP probes (Fig. 4.4A). The binding signals seen when p67$^{phox}$ proteins were probed with Rac1-[γ-$^{32}$P]GTP were not reduced in the presence of equal or 10-fold higher amounts of Rac1-GDP (Fig. 4.4B, lane 3), suggesting that Rac1-GDP did not compete with the Rac1-GTP probe for p67$^{phox}$ binding.

4.2.2. Quantification of the Rac1-p67$^{phox}$ binding interaction.

To quantify the binding signals observed when Rac1-[γ-$^{32}$P]GTP or Rac1-Q61L-[γ-$^{32}$P]GTP were used to probe dot blotted p67$^{phox}$, nitrocellulose squares were cut up and the amount of radioactivity associated with each nitrocellulose square was determined by conversion of the signal to cpm in scintillant. This was carried out following exposure of nitrocellulose filters to X-Omat film to eliminate the effect of high non-specific background signals on the cpm values obtained. Fig. 4.5A shows that there was an almost linear increase in Rac1-Q61L binding as the amount of p67$^{phox}$ protein was increased from 1 to 10 μg. p47$^{phox}$ bound low levels of Rac1-Q61L, just above background, but this was not dependent on the amount of p47$^{phox}$ over the range studied.

To quantify the difference between Rac1 and Rac1-Q61L in strength of binding to p67$^{phox}$, both probes were used in the same experiment and nitrocellulose filters were radioassayed in scintillant. p67$^{phox}$ protein bound approximately 13-fold more Rac1-Q61L than Rac1 (Fig. 4.5B), as calculated from the cpm values obtained. p67$^{phox}$ was also found to bind more Rac1-G12V. However this was not to the same extent as the increase seen with the Q61L mutant, indicating that the increased interaction with the Q61L mutation is not simply due to reduced GTPase activity of this protein. The
Fig. 4.4. Binding of \textit{p67}^{phox} to \textit{Rac1} in a GTP-Dependent Manner.

A

\begin{tabular}{|c|c|c|}
\hline
 & GTP & GDP \\
\hline
\textit{p67}^{phox} & & \\
\textit{p67}^{phox} (1-238) & & \\
\hline
\end{tabular}

B

\begin{tabular}{|c|c|c|}
\hline
 & Rac1-GDP & \textit{Rac1} \\
\hline
\textit{p67}^{phox} & - & x 10x \\
\textit{p67}^{phox} (1-238) & & \\
\hline
\end{tabular}

Fig. 4.4. Binding of \textit{p67}^{phox} to \textit{Rac1} in a GTP-dependent manner. \textit{p67}^{phox} proteins purified from \textit{E. coli} XL-1 Blue cells (5 \mu g) were dot blotted on to nitrocellulose filter as indicated at the left-hand end of each row: 

- \textit{p67}^{phox}, amino acids 1-526,
- \textit{p67}^{phox} (1-238), amino acids 1-238.

A. Nitrocellulose filters were probed with \textit{Rac1} probes: 

\textbf{Lane 1}, \textit{Rac1-}[\alpha-^{32}\text{P}]GTP,

\textbf{Lane 2}, \textit{Rac1-}[\alpha-^{32}\text{P}]GDP.

\textit{Rac1-GDP} probes were generated by loading \textit{Rac1} protein with \([\alpha-^{32}\text{P}]\text{GTP}\) and allowing the bound nucleotide to hydrolyse for 1 h at room temperature. For GTP-bound probes, protein was used immediately after the nucleotide exchange reaction.

B. Nitrocellulose filters were probed with \textit{Rac1-}[\gamma-^{32}\text{P}]GTP in the presence of increasing amounts of \textit{Rac1-GDP}: 

\textbf{Lane 1}, \textit{Rac1-}[\gamma^{32}\text{P}]GTP,

\textbf{Lane 2}, \textit{Rac1-}[\gamma^{32}\text{P}]GTP + \textit{Rac1-GDP} (at equal amounts),

\textbf{Lane 3}, \textit{Rac1-}[\gamma^{32}\text{P}]GTP + \textit{Rac1-GDP} (10-fold excess of the labelled probe).
**Fig. 4.5. Quantification of the Rac1-p67phox Binding Interaction.**

A

Percentage Binding (%)

<table>
<thead>
<tr>
<th>Protein (µg)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
</tr>
</thead>
<tbody>
<tr>
<td>p67phox</td>
<td>0</td>
<td>10</td>
<td>20</td>
<td>30</td>
<td>40</td>
<td>50</td>
<td>60</td>
<td>70</td>
<td>80</td>
<td>90</td>
<td>100</td>
<td>110</td>
</tr>
<tr>
<td>p47phox</td>
<td>0</td>
<td>5</td>
<td>10</td>
<td>15</td>
<td>20</td>
<td>25</td>
<td>30</td>
<td>35</td>
<td>40</td>
<td>45</td>
<td>50</td>
<td>55</td>
</tr>
</tbody>
</table>

B

Relative Binding

<table>
<thead>
<tr>
<th>Protein</th>
<th>Rac1</th>
<th>Rac1-Q61L</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 µg</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5 µg</td>
<td>14</td>
<td>20</td>
</tr>
</tbody>
</table>

**Fig. 4.5. Quantification of the Rac1-p67phox Binding Interaction.** p67phox and p47phox were dot blotted onto nitrocellulose and probed with [γ32P]GTP labelled Rac1 probes. The amount of bound radioactivity was estimated by scintillation counting the nitrocellulose squares, binding signals were converted to counts per minute (cpm) values. Background binding (cpm) determined using GST as a control protein was subtracted from all values obtained.

A. p67phox (■), and p47phox (■) were dot blotted on to nitrocellulose filter (1-10 µg) and probed with Rac1-Q61L-[γ32P]GTP. Data are presented as a percentage of cpm obtained with 10 µg of p67phox.

B. p67phox (5 µg), was dot blotted on nitrocellulose filter and probed in parallel with Rac1-[γ32P]GTP and Rac1-Q61L-[γ32P]GTP. Error bars represent ± S.D. (n=3).
increase in binding signals, observed between wild-type and Q61L Rac1 proteins, is consistent with the idea that p67phox is the Rac target within the oxidase enzyme system as the GTPase negative mutant Rac-Q61L has been shown to have a 10-fold greater activity in stimulating the oxidase enzyme in vitro (Xu et al., 1994).

4.2.3. Localisation of the Rac1 binding site in p67phox.

Deletion constructs of p67phox expressed as GST fusion proteins in E. coli cells were assayed for Rac1 binding in order to localise the Rac1 binding site (see Fig. 4.6A, for Coomassie Blue stain of proteins used). Proteins immobilised on nitrocellulose filters by direct dot blotting were probed with Rac1-Q61L-\[\gamma^{32}\text{P}\]GTP. Figure 4.6B shows a schematic representation of the positioning of the deletion constructs within the full-length p67phox protein and the corresponding binding signals obtained with each. Rac1 was found to bind to deletion constructs containing the N-terminal of p67phox. Signals were detected with N-terminal constructs containing amino acids 1-238, 1-199 and 1-192. Under these conditions Rac1 probes did not bind to deletion mutants containing the C-terminal half of p67phox, amino acids 300-526 (Fig. 4.6B). These data suggest that the Rac1-binding site is located within amino acids 1-192 of p67phox. That the Rac1 binding site lies within amino acids 1-246 of p67phox, which are sufficient for reconstitution of oxidase activity in vitro (Diekmann et al., 1994; de Mendez et al., 1994), suggests that the Rac1-p67phox interaction may be functionally relevant.

Rac1 bound to deletion proteins containing amino acids 126-238 and 170-238 but not 192-238, this indicated that residues 170-192 were important for Rac1 binding. Using this method Rac1 did not bind to N-terminal constructs containing amino acids 1-58 or 1-131 (Fig. 4.6B). The low binding signal seen with protein containing amino acids 126-238 in comparison to 170-238 could be explained by the instability of this protein purified from E. coli cells. Coomassie Blue stain analysis of this protein separated by SDS-PAGE reveals the presence of a number of breakdown products in the protein sample (Fig. 4.6A).

On the basis of the above results amino acid residues 170-199 of p67phox were expressed as a GST fusion protein and were assayed, using the dot blot method, for Rac1 binding. This small fragment of p67phox bound Rac1 probes. The amount of Rac1 bound was quantified as between 25 and 50 % of the Rac1 bound by the full-length protein and
Fig. 4.6. Localisation of the Rac1 binding site in p67^phox.

**A.** Full-length p67^phox or protein fragments (numbers indicate amino acids), (1-5 µg) were separated by SDS-PAGE in 10 % gels (lanes 1-13) or 12.5 % gels (lanes 14-15), and visualised by Coomassie Blue staining. **Lane 1,** full-length p67^phox (1-526) purified from insect Sf9 cell lysates, **Lanes 2-15,** proteins purified from *E. coli* cells contain amino acids: - 1-526 (full-length), 1-238, 1-192, 126-238, 170-238, 192-238, 300-526, 1-58, 1-131, GST, Δ226-236 (1-526Δ226-236), 1-460, GST, and 170-199 as indicated above each lane. Proteins in lanes 2-15 were used as GST fusion proteins except for full-length p67^phox and Δ226-236 (lanes 2 and 12). Proteins had differing degrees of stability, arrow heads indicate the size of the full-length proteins. Note, Δ226-236 is full-length p67^phox deleted of the proline-rich region (1-526Δ226-236).
B. The schematic representation of \( p67^{phox} \) indicates the position of the proline-rich region (P; amino acids 226-236) and the two SH3 domains (amino acids 245-295 and 460-510). \( p67^{phox} \) and deletion mutants, purified from \( E. coli \) cells (5 \( \mu \)g), were dot blotted onto nitrocellulose filter and probed with Rac1-Q61L-[\( \gamma^{32}P \)]GTP. \( p67^{phox} \) proteins contain amino acids: - 1-526, 1-238, 1-199, 1-192, 126-238, 170-238, 192-238, 170-199, 300-526, 1-131, and 1-58, as indicated and were used as GST fusion proteins except full-length \( p67^{phox} \), (amino acids 1-526), which was cleaved from the GST moiety. Nitrocellulose filters were exposed to X-Omat film for 17 h, and signals representative of at least 2-3 experiments are shown in the panel to the right of each corresponding protein. The numbers to the left of each protein indicate the lane of the gel in A, in which that protein has been separated; ns -protein was not separated in SDS-gels.
approximately 5% of the maximum binding observed, for example with the N-terminal proteins (Fig. 4.7). The data collected from all the deletion mutants applied to this analysis indicates that amino acids 170-199 contain the minimal sequence of p67phox required for Rac1 binding but that additional amino acids are required for an optimal interaction. A similar reduction in p21 binding was observed when N-terminal fragments of β-PAK expressed as GST fusion proteins were analysed using the dot blot method for Cdc42Hs binding (this work was carried out by S. Govind). Cdc42Hs binding to β-PAK fragment containing amino acids 37-149 (112 residues) was reduced to less than 5% when a smaller protein, containing just 24 residues, was assayed (Fig. 4.7), suggesting that additional sequences are required for optimal binding of Cdc42Hs to β-PAK.

4.2.4. Analysis of the Rac1 binding site in p67phox.

Taken together the results from the above experiments suggest that amino acids 170-192 of p67phox contain the Rac1 binding site. The introduction of a point mutation and a six amino acid residue deletion into this region of full-length p67phox protein were used to analyse the requirements of this sequence for Rac1 binding. Deletion of amino acids 178-184 completely abolished the Rac1-p67phox binding interaction, whereas substitution of proline 174 for a leucine residue (P174L) was without effect (Fig. 4.8). This indicated that amino acids 178-184 are required to maintain the Rac1 binding site and that proline residue 174 is not essential for a binding interaction.

During the course of this study deletion of lysine residue at position 58 in p67phox was reported to occur in a particular form of autosomal CGD. It was proposed that a loss of NADPH oxidase function in the patient harbouring this mutation was due to a loss of Rac1 binding to the mutated p67phox protein (Leusen et al., 1996). Introduction of this deletion, which does not lie within the Rac1 binding site, proposed from the above analysis, into full-length p67phox (1-526Δ58K) protein resulted in a loss of Rac1 binding (Fig. 4.8), confirming the data reported by Leusen et al., 1996. This suggests that residue lysine 58 is important in maintaining a functional Rac1 binding site, perhaps by stabilising the N-terminal structure of p67phox. Instability of p67phox protein was also observed in the deletion mutant containing amino acids 126-238, suggesting that the integrity of the N-terminus is important in maintaining the structure of p67phox and hence the Rac1 binding interaction. In addition attempts to express p67phox proteins containing amino acids 58-131 and 58-238 as GST fusion protein were unsuccessful, in both cases
Fig. 4.7. Comparison of the p21 Interaction With p67phox and PAK Fragments.

A. Schematic representation of p67phox and PAK primary structure showing the domains and positions of protein fragments expressed. T, Tetratricopeptide repeats of 34 amino acids (Ponting, 1996); P, polyproline domain (SH3 domain binding sites); RBD, Rac binding domain; CRIB, Cdc42Hs/Rac1 interactive binding motif. PAK has four polyproline sequences that may represent protein binding sites. Lines represent the fragments expressed with numbers on the left referring to the bars in B, and those on the right to amino acids expressed.

B. Purified proteins were dot blotted onto nitrocellulose filter and probed as follows: - p67phox proteins were probed with Rac1-Q61L-[γ32P]GTP (lanes 1-3), PAK proteins were probed with Cdc42Hs-Q61L-[γ32P]GTP (lanes 4-6).

Dot blot signals were converted to cpm values in scintillant. Binding activity of each p67phox or PAK protein is expressed relative to that obtained with the smallest p67phox (lane 3) and PAK (lane 6) fragments respectively, arbitrarily set at 1. Results were obtained from 2-3 experiments. Error bars are means ± S.D. (n ≥ 6).
Fig. 4. 8. Mutational Analysis of the p67phox Rac1 Binding Site.

A  p67phox sequence (amino acids 170-199)

\[
\begin{align*}
170 & \quad L Y E \boxed{\text{I I I}} V V I \boxed{P V G K L F R} P N E R Q V A Q L A K K D Y L \quad 199 \\
\end{align*}
\]

B  Rac1 binding to p67phox deletion mutants.

Rac1  Amino acids

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1-526</td>
</tr>
<tr>
<td>II</td>
<td>1-526 (P174L)</td>
</tr>
<tr>
<td>III</td>
<td>1-526 Δ178-184</td>
</tr>
<tr>
<td>(III)</td>
<td>1-526 Δ58K</td>
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</tbody>
</table>

Fig. 4. 8. Mutational analysis of the p67phox Rac1 binding site. A. p67phox sequence containing the Rac1 binding site (amino acids 170-199). This region of sequence was investigated by the introduction of an amino acid substitution at residue 174 Proline ⇒ lysine (I, P174L), and deletion of amino acids 178-184 (II, Δ178-184). In addition point mutation of lysine residue 58 (III, Δ58K), was investigated for its effect on Rac1 binding to p67phox, this residue lies outside the minimal p67phox sequence required for Rac1 binding. Mutations were introduced into the full-length p67phox protein and proteins were expressed in E. coli cells and purified as GST-fusion proteins (as described in the Methods section 2.2.1).

B. p67phox proteins (5 μg) were dot blotted onto nitrocellulose filter as indicated and probed with Rac1-Q61L-[γ32P]GTP probes. Signals were visualised by exposure of filters to X-Omat film for 16 h.
protein was completely broken down, with the majority of protein in these samples being GST.

4.2.5. Rac2 binds p67<sup>phox</sup> at the same site as Rac1.

Rac2, which shares a 92% sequence identity with Rac1, is the small GTP binding protein required for activation of the NADPH oxidase in human neutrophils (Knaus et al., 1991 and 1992). Rac2-[γ<sup>32</sup>P]GTP probes bound p67<sup>phox</sup> dot blotted onto nitrocellulose filter and was found to bind p67<sup>phox</sup> with similar characteristics as Rac1. Rac2 bound to deletion mutants containing amino acids 170-238 and 170-199 (Fig. 4.9). As with Rac1, protein containing amino acids 170-199 of p67<sup>phox</sup> bound ~5% of the Rac2 bound by the N-terminal deletion containing amino acids 1-192. These results indicate that Rac2 interacts with p67<sup>phox</sup> at the same site as Rac1.

4.2.6. The Rac1 binding site is masked in full-length p67<sup>phox</sup> protein.

It was noticed that on dot blots, p67<sup>phox</sup> N-terminal proteins 1-238, 1-199 and 1-192 bound more Rac1-Q61L than full-length p67<sup>phox</sup>, suggesting that the Rac1 binding site is further exposed upon deletion of the C-terminal of the protein (Fig. 4.6B, Rows 1 to 4). The possibility that recombinant p67<sup>phox</sup> adopts a folded conformation that partially masks the Rac1 binding site was explored. p67<sup>phox</sup> contains two SH3 domains and a proline-rich region. SH3 domains are capable of directing intermolecular and intramolecular protein interactions by interacting with target sequences rich in proline residues (Pawson, 1995). The polyproline domain of p67<sup>phox</sup> is situated at amino acids 226 to 236, and the two SH3 domains are at amino acids 245-295 and 460-520. Two deletion constructs were generated and used to assay whether deletion of these regions might effect Rac1 binding. Proteins deleted of either the C-terminal SH3 domain (and residues immediately C-terminal), containing amino acids 1-460, or the proline-rich region (1-526Δ226-236), were applied to Rac1 binding studies. A stimulation of Rac1 binding was observed with both of these proteins when binding signals were compared to those obtained with the full-length protein within the same experiment (Fig. 4.10A). Upon quantification, p67<sup>phox</sup> proteins containing amino acid residues 1-192, 1-460 and 1-526Δ226-236 bound approximately 8-fold more Rac1 than the full-length wild-type protein (Fig. 4.10B).
Fig. 4.9. Rac2 Binding p67<sup>phox</sup> Proteins.

<table>
<thead>
<tr>
<th>p67&lt;sup&gt;phox&lt;/sup&gt; Amino acids</th>
<th>Rac2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-192</td>
<td></td>
</tr>
<tr>
<td>170-238</td>
<td></td>
</tr>
<tr>
<td>170-199</td>
<td></td>
</tr>
<tr>
<td>300-526</td>
<td></td>
</tr>
<tr>
<td>GST</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 4.9. Rac2 binding to p67<sup>phox</sup> proteins.

p67<sup>phox</sup> deletion mutants and GST protein (1, 5, and 10 μg, columns 1-3 respectively) were immobilised on nitrocellulose filter by dot blotting (as indicated at the left-hand end of each row) and were probed with Rac2-[γ<sup>32</sup>P]GTP probes. p67<sup>phox</sup> fragment proteins were purified from <i>E. coli</i> cells as GST fusion proteins (see Fig. 4.6 for SDS-PAGE analysis) and contain amino acids: 1-192, 170-238, 170-199, and 300-526. Binding signals were visualised by exposure of nitrocellulose filters to X-Omat film for 17 h. Representative signals of results obtained in at least 2-3 experiments are shown in the panel to the right of each corresponding protein.
The above data suggest that the \( p67^{phox} \) Rac1 binding domain is masked within the full-length protein and that removal of either the polyproline domain or the C-terminal SH3 domain changes \( p67^{phox} \) protein conformation, further exposing the Rac1 binding site. Although these mutations could introduce instability in the conformation that causes extension of the structure, unfolding might be a result of disrupting an intramolecular interaction involving the C-terminal SH3 domain and the proline-rich region.

The above data confirm the initial observations, using \( p67^{phox} \) protein purified from insect Sf9 cells, that \( p67^{phox} \) is a Rac1 interacting protein. That the binding site lies within the N-terminal piece of \( p67^{phox} \), which is sufficient for reconstitution of oxidase activity \textit{in vitro}, and that the interaction observed requires GTP-bound Rac1 and is sensitive to the 'effector domain' mutation D38A, indicates that the interaction between Rac1 and \( p67^{phox} \) may be functionally relevant. In addition quantification of the binding interaction demonstrates that Rac1-Q61L has, increased affinity for \( p67^{phox} \) compared to wild-type Rac1 correlating with the enhanced ability of this GTPase negative mutant to stimulate superoxide production \textit{in vitro} in comparison to the wild-type Rac1 protein. That Rac2 interacts with \( p67^{phox} \) with similar characteristics to Rac1 suggests that these p21s interact with \( p67^{phox} \) at a common binding site and that \( p67^{phox} \) represents a potential target for both Rac1 and Rac2. That the Rac1 binding site is masked in the full-length protein could suggest a possible role for mediating the Rac-\( p67^{phox} \) interaction \textit{in vivo}.

4.3. Rac1 and \( p40^{phox} \) interact with \( p67^{phox} \) at distinct sites.

It has been reported that the NADPH oxidase components \( p40^{phox} \) and \( p67^{phox} \) interact within the cytosol of resting neutrophil cells \textit{in vivo}. An interaction between these proteins using \textit{in vitro} binding assays and the yeast two-hybrid system has been shown. It has been proposed that \( p40^{phox} \) interacts with sites within both the N and C-terminal halves of \( p67^{phox} \) (Wientjes \textit{et al.}, 1996; Fuchs \textit{et al.}, 1995; Sathyamoorthy \textit{et al.}, 1997). Dot blot competition experiments were carried out to identify whether \( p40^{phox} \) and Rac1 compete for \( p67^{phox} \) binding.
Fig. 4.10. Figure legend on reverse.
Fig. 4.10. The Rac1 binding site in p67phox is masked in the full-length protein. Rac1 binding to full-length p67phox was compared with binding to p67phox fragment proteins. 5 μg of proteins purified from E. coli cells, as GST-fusion proteins except for full-length p67phox (see Fig. 4.6. for SDS-PAGE analysis) were dot blotted onto nitrocellulose filter and probed with Rac1-Q61L-[^32P]GTP probes, binding was quantified as described in Methods section 2.2.5.2.

Proteins contain amino acids: -

1, 1-526,
2, 1-192,
3, 300-526,
4, 1-526 Δ226-236
5, 1-460, rows 1-5.

Numbers 1-5 correspond to rows in A and bars in B.

A. The schematic representation indicates the position of deletion mutant proteins within full-length p67phox; RBD, Rac Binding Domain; P, polyproline domain; SH3, SH3 domains. Representative binding signals of results obtained from 2-3 different experiments are shown to the right of each corresponding protein.

B. Following visualisation the binding signals were quantified. Binding activity is expressed relative to that obtained with full-length p67phox which was arbitrarily set at 1. Error bars represent mean ± S.D (n ≥ 6).
Fig. 4.10. The Rac1 Binding Site in p67phox is Masked in the Full-Length Protein.
4.3.1. Racl and p40phox do not compete for p67phox binding.

p67phox proteins, full-length and N-terminal deletion mutant (amino acids 1-526 and 1-192 respectively) were dot blotted on to nitrocellulose filter and probed with Racl-Q61L-[γ32P]GTP in the presence of increasing amounts of recombinant p40phox. No decrease in signal for Rac1 binding to p67phox was observed even when p40phox was present at a 10-fold higher amount than the Rac1 probe (Fig. 4.11A). This data indicates that p40phox and Rac1 interact with p67phox at distinct sites and that p40phox binding to p67phox does not interfere with the Rac1-p67phox binding interaction.

4.3.2. p40phox interacts with the C-terminal half of p67phox.

A direct protein-protein interaction assay was carried out by F. Wientjes (Department of Medicine, University College London) to determine the region of p67phox with which p40phox interacts. Different p67phox-GST fusion proteins containing amino acids: 1-526, 1-526Δ226-236 (deleted of the polyproline domain), 1-526Δ58K (deleted of lysine residue 58), 1-238, 300-526, 300-460, or GST protein as a control, were bound to glutathione-Sepharose resin and incubated with recombinant p40phox. After washing, the material associated with the glutathione-Sepharose resin was separated by SDS-PAGE and transferred to nitrocellulose filter. The amount of p40phox retained by the resin in each case was assessed by immunoblotting with p40phox antibody. p40phox bound to p67phox proteins containing amino acids 300-526 and 300-460 (Fig. 4.11B). Binding of p40phox to the N-terminal deletion mutant of p67phox (amino acids 1-238) was not above background levels observed with the control GST glutathione-Sepharose resin. These results suggest that the major binding site for p40phox lies within the C-terminal half of p67phox between the two SH3 domains, and thus confirms the data obtained using the yeast two-hybrid system (Fuchs et al., 1995).

Taken together these observations indicate that p40phox and Rac1 interact with p67phox at distinct sites, within the N and C-terminal halves of p67phox respectively. That p40phox binding to p67phox does not interfere with Rac1 binding to the site mapped within the N-terminal of p67phox, suggests that these proteins do not compete for p67phox binding and may interact simultaneously with p67phox.
Fig. 4.11. Distinct Binding Sites for p40\textsubscript{phox} and Racl on p67\textsubscript{phox}.

A

$$\begin{array}{c|c|c|c|c}
\text{p40}\textsubscript{phox} & \text{Racl} & \text{p67}\textsubscript{phox} \\
\hline
& \text{Amino acids} & \\
1-2 & 1-192 & \\
2 & & 1-526 \\
3 & & \\
4 & & \\
\end{array}$$

Fig. 4.11. Distinct binding sites for p40\textsubscript{phox} and Racl on p67\textsubscript{phox}. A, p67\textsubscript{phox} full-length protein (amino acids 1-526) or N-terminal deletion mutant (amino acids 1-192), (5 µg) were incubated in the absence of additions or with increasing amounts of p40\textsubscript{phox}. Proteins were then dot blotted onto nitrocellulose filter and probed with Racl-Q61L-[\gamma\textsuperscript{32}P]GTP probes:

Lane 1, in the absence of additions,

Lane 2, + p40\textsubscript{phox} at 1 times the amount of Racl,

Lane 3, + p40\textsubscript{phox} at 5 times the amount of Racl,

Lane 4, + p40\textsubscript{phox} at 10 times the amount of Racl.

Filters were washed and exposed to X-Omat film for 16 h.
B. Different p67<sup>phox</sup>-GST fusion proteins were bound to glutathione-Sepharose beads and incubated with purified p40<sup>phox</sup>. Beads were washed and associated proteins were separated in SDS-gels and transferred to nitrocellulose filter. The amount of p40<sup>phox</sup> retained by the beads was determined by probing nitrocellulose filters with p40<sup>phox</sup> antibody. Proteins bound to glutathione-Sepharose were:

Lane 1, GST;
Lane 2, p67<sup>phox</sup> (1-526);
Lane 3, 1-526Δ226-236;
Lane 4, 1-526Δ58K;
Lane 5, 1-238;
Lane 6, 300-526;
Lane 7, 300-460;
Lane 8, no protein.

Numbers indicate amino acids. Approximately equal amounts of p67<sup>phox</sup> protein were used, except for the fragment containing amino acids 1-238 which was in 5-fold excess. Experiments in part B were carried out by F. Wientjes, and similar results were obtained in at least two other experiments.
4.4. Investigation of an intramolecular interaction within recombinant p67\textsuperscript{phox}.

The data obtained from analysis of the Rac1-p67\textsuperscript{phox} binding interaction (section 4.2.) and also \textit{in vitro} phosphorylation of p67\textsuperscript{phox} by β-PAK (see Results Chapter 6) suggests that an intramolecular, SH3-polyproline domain interaction may exist within p67\textsuperscript{phox} protein. \textit{In vitro} binding assays were used to investigate whether the N and C terminal halves of p67\textsuperscript{phox} interact via their polyproline and SH3 domains respectively. The methods applied to this investigation included direct protein-protein interaction studies including use of the yeast two-hybrid system, and also indirect methods in which the ability of Rac1 to interact with the p67\textsuperscript{phox} proteins was assayed for under different conditions.

4.4.1. Investigation of an interaction between the N and C-terminal halves of p67\textsuperscript{phox} using the yeast two-hybrid system.

The yeast two-hybrid system (Fields and Song, 1989) was utilised to assay for an interaction between the N and C-terminal halves of p67\textsuperscript{phox} in an \textit{in vivo} situation. That this method can be used to detect interactions between the different \textit{phox} proteins has been demonstrated previously. For example, proteins containing the C-terminal halves of p47\textsuperscript{phox} and p67\textsuperscript{phox} have been shown to interact in the yeast two-hybrid system (Fuchs \textit{et al.}, 1996). In addition, the yeast two-hybrid system has been used to investigate the intramolecular interaction within p47\textsuperscript{phox}, and experiments have suggested that the SH3 domain region of p47\textsuperscript{phox} directs an interaction with the C-terminal half of this protein (Fuchs \textit{et al.}, 1996). Further investigations of the above interactions using the yeast two-hybrid system demonstrated that the interaction detected between the C-terminal halves of p67\textsuperscript{phox} and p47\textsuperscript{phox} was abrogated upon deletion of the C-terminal SH3 domain of p67\textsuperscript{phox} or a particular proline rich region in the C-terminal of p47\textsuperscript{phox}. The intramolecular interaction between the SH3 domains and C-terminal of p47\textsuperscript{phox} required a distinct proline rich region in the C-terminal fragment (Hata \textit{et al.}, 1998). This suggests that the yeast two-hybrid system is a suitable tool for detecting SH3-polyproline domain directed intermolecular or intramolecular interactions of \textit{phox} proteins.

To investigate an interaction between the N and C-terminal halves of p67\textsuperscript{phox}, using the yeast two-hybrid system, plasmids were generated to encode p67\textsuperscript{phox} protein fragments as fusion proteins with the GAL4 activation domain (AD) or the GAL4 DNA binding domain (BD). N-terminal sequences, with (amino acids 1-300) or without (amino acids 1-199) the polyproline domain, as fusion proteins with the GAL4 AD, or p67\textsuperscript{phox} C-
terminal sequence, (amino acids 300-526) as a fusion protein with the GAL4 BD. It should be noted that p67^phox residues 1-300 also include the N-terminal SH3 domain. The yeast two-hybrid system was also used to test whether the interaction between the N-terminal of p67^phox (amino acids 1-199) and Rac1, shown using dot blot p21 binding assays (section 4.2), could be demonstrated using this technique. Rac1 was cloned into the GAL4 DNA BD plasmid by S. Govind. Cotransformations of the GAL4 AD and BD plasmids into yeast strain YRG2 cells were carried out in a number of different combinations to investigate these interactions (Table 4.1).

Interaction dependent reconstitution of the GAL4 transcription factor was assessed by monitoring the ability of transformed cells to grow on agar plates lacking amino acids leucine, tryptophan and histidine (-L/W/H). Growth on such plates would indicate the correct transformation of both plasmids and expression of the reporter gene encoding histidine downstream of the promoter region, suggesting an interaction between the two proteins of interest. Table 4.1 summarises the data collected from analysis of the growth of transformed YRG2 cells (-L/W/H) plates.

Growth of YRG2 cells on plates in the absence of leucine and tryptophan (-L/W) was observed for each experiment, confirming the correct transformation of each plasmid. However interaction specific reconstitution of the reporter gene transcription factor was not observed in any of the experimental situations, either initially on the first set of plates or following re-streaking of cells from colonies that grew, onto fresh plates. The second test was carried out as a high number of small colonies were observed on the original agar plates, which may suggest that the spreading technique used was insufficient to ensure correct plating of the cells.

YRG2 cell growth on -L/W/H plates was observed for cells transformed with p67^phox proteins containing amino acids 1-300 and 300-526 (experiment 1) however this growth was not dependent on the presence of either protein as was suggested by the growth observed in the empty vector control experiments (3 and 7). This was also found to be the case with p67^phox proteins containing amino acids 1-199 and 300-526, (compare experiments 4, 6 and 7).
Growth of YRG2 cells on -L/W/H plates was not observed with cells transformed with the Rac1-Q61L plasmid (experiments 2, 5 and 8), although correct transformation of this plasmid was confirmed in each case by the growth observed on -L/W plates. This may be due to this protein exerting a toxic effect in the yeast strain cells used. A similar observation has been previously reported when plasmids encoding Rac1-Q61L and p67phox were used to cotransform yeast strain L40 cells (Dorseuil et al., 1996).

The data collected suggest that the conditions employed by the yeast two-hybrid system in this analysis were not sufficient to select for specific reconstitution of the GAL4 transcription factor in a manner dependent upon an interaction between the two halves of p67phox. The simultaneous use of an appropriate positive control would have enabled a full assessment of the suitability of the system used for investigating such an interaction. For example cotransformation of plasmids encoding the C-terminal of p47phox (amino acids 204-390) with the C-terminal of p67phox (amino acids 300-526) proteins, as intended, would have provided such a control. It has been reported that these protein fragments interact in the yeast two-hybrid system, in a manner dependent of the presence of an intact SH3 domain or polyproline domain respectively (Hata et al., 1998). In further investigations of an intramolecular interaction within p67phox it should be taken into consideration that amino acids 1-300 of p67phox contain the N-terminal SH3 domain, thus this protein fragment may adopt a conformation that obscures the polyproline domain. In addition further investigation is required to determine whether the choice of plasmid (i.e. AD or BD) for the two proteins of interest is important.

4.4.2. Indirect methods applied to investigate whether an intramolecular interaction exists within p67phox.

4.4.2.1. An investigation into whether disruption of the proposed tertiary structure of p67phox could be induced by the use of proline-rich peptides.

SH3 domains bind to target peptide sequences rich in proline residues (Pawson, 1995). Both the NADPH oxidase components p67phox and p47phox contain proline-rich sequences as well as two SH3 domains each. The proline-rich sequence at the C-terminal of p47phox interacts with the C-terminal of p67phox in vitro and a requirement for this interaction for activation of the oxidase enzyme system in whole cell systems has been suggested (de Mendez et al., 1996). A proline-rich peptide based on sequence of p47phox (amino acids
<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>pACT2 (AD)</th>
<th>pAS2-l (BD)</th>
<th>Description of the interaction under investigation</th>
<th>Plate 1 -L/W/H</th>
<th>Plate 2 -L/W/H</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>p67\textsuperscript{phox} 1-300</td>
<td>p67\textsuperscript{phox} 300-526</td>
<td>An interaction between the N and C-terminal halves of p67\textsuperscript{phox}.</td>
<td>++ (■)</td>
<td>++</td>
</tr>
<tr>
<td>2</td>
<td>p67\textsuperscript{phox} 1-300</td>
<td>RacQ61L</td>
<td>An interaction between the N-terminal of p67\textsuperscript{phox} and RacQ61L.</td>
<td>-</td>
<td>NA</td>
</tr>
<tr>
<td>3</td>
<td>p67\textsuperscript{phox} 1-300</td>
<td>Empty vector pAS2-1</td>
<td>Empty vector control for interactions 1 and 2.</td>
<td>++ (♦)</td>
<td>+++</td>
</tr>
<tr>
<td>4</td>
<td>p67\textsuperscript{phox} 1-199</td>
<td>p67\textsuperscript{phox} 300-526</td>
<td>An interaction between the N-terminal of p67\textsuperscript{phox} lacking the proline rich region and the C-terminal.</td>
<td>++ (♦)</td>
<td>++++</td>
</tr>
<tr>
<td>5</td>
<td>p67\textsuperscript{phox} 1-199</td>
<td>RacQ61L</td>
<td>An interaction between RacQ61L and the N-terminal of p67\textsuperscript{phox} shown to interact on dot blots.</td>
<td>-</td>
<td>NA</td>
</tr>
<tr>
<td>6</td>
<td>p67\textsuperscript{phox} 1-199</td>
<td>Empty vector pAS2-1</td>
<td>Empty vector control for interactions 4 and 5.</td>
<td>+ (■)</td>
<td>+++</td>
</tr>
<tr>
<td>7</td>
<td>Empty vector pACT2</td>
<td>p67\textsuperscript{phox} 300-526</td>
<td>Empty vector control for interactions 1 and 4.</td>
<td>+ (■)</td>
<td>+++</td>
</tr>
<tr>
<td>8</td>
<td>Empty vector pACT2</td>
<td>RacQ61L</td>
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<td>NA</td>
</tr>
<tr>
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<td>pACT2</td>
<td>pAS2-l</td>
<td>Empty vector control.</td>
<td>+ (■)</td>
<td>-</td>
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<tr>
<td>10</td>
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<td>Positive interaction.</td>
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<td>NA</td>
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<tr>
<td>11</td>
<td>p53</td>
<td>-</td>
<td>Transformation efficiency.</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Table 4.1. A summary of the interactions investigated using the yeast two-hybrid system. Activation domain (AD) and Binding domain (BD) plasmids encoding proteins as indicated were cotransformed into yeast YRG2 strain cells. Cells were plated onto agar plates lacking leucine, tryptophan and histidine (-L/W/H) and were incubated at 37 °C for 3-7 days. The growth observed was analysed, + indicates approximately 12 large colonies were counted. In most cases a large number of small colonies were also observed, either in approximately equal numbers or in excess of the number of large colonies, as indicated by ♦ or ■, respectively. In the cases where growth was observed on plate 1, experiment numbers (Exp. No.) 1, 3, 4, 6 and 7, four colonies were picked and streaked onto a fresh -L/W/H plate (plate 2), of these colonies the number that grew is indicated (+).
360-373) inhibited the ability of the p67\(^{\text{phox}}\) C-terminal SH3 domain to precipitate p47\(^{\text{phox}}\) from HL60 cell lysates. This indicated that the proline-rich region of p47\(^{\text{phox}}\) is required for this interaction. This effect required the presence of a high concentration of the peptide added (750 \(\mu\)M) (Finan et al., 1994). The proline rich region of p67\(^{\text{phox}}\) situated in the N-terminal of the protein in proximity to the Rac binding domain provides a potential SH3 domain target site.

Whether the proposed SH3-polyproline domain intramolecular interaction in p67\(^{\text{phox}}\) could be disrupted by ‘competing’ proline-rich peptides was investigated. p67\(^{\text{phox}}\) protein was incubated with the ‘competing’ peptides and Rac1 binding was assayed as a measure of the ‘folded’ state of p67\(^{\text{phox}}\). Peptides based upon the polyproline sequences of p67\(^{\text{phox}}\) and p47\(^{\text{phox}}\) were used.

**Peptide 1:** p67\(^{\text{phox}}\) amino acids 224-235  
J A A E P P P R P K T P E

**Peptide 2:** p47\(^{\text{phox}}\) amino acids 360-373  
K P Q P A V P P R P S A D L.

p67\(^{\text{phox}}\)-GST and p67\(^{\text{phox}}\) 1-460, deleted of the C-terminal SH3 domain, were incubated at 2 \(\mu\)M with or without peptides 1 or 2, which were present in vast excess (0.5 -1.5 mM). Incubations were carried out for 16 h at 4 °C in a low salt buffer and were then dot blotted on to nitrocellulose filter and probed with Rac1 probes.

In the presence of the p67\(^{\text{phox}}\) proline-rich peptide, Rac1 binding to both full length p67\(^{\text{phox}}\) or amino acids 1-460 was reduced with increasing amounts of peptide. In the presence of the p47\(^{\text{phox}}\) proline rich peptide Rac1 binding to p67\(^{\text{phox}}\) amino acids 1-460 was consistently reduced, and the extent of this effect increased with the amount of peptide added. However, Rac1 binding to the full-length protein appeared unaffected by the presence of the p47\(^{\text{phox}}\) peptide, although results were not consistent with all concentrations of peptide applied (Fig. 4.12).

The inconsistency of results obtained illustrates the complicated nature of this experimental technique. For example the effects on Rac1 binding to p67\(^{\text{phox}}\) observed may not be due to the incubations with the peptides tested, as the p67\(^{\text{phox}}\) proteins may have resumed a folded conformation once dot blotted on to nitrocellulose or perhaps
Fig. 4.12. Rac1 Binding to p67\textsuperscript{phox} Proteins in the Presence of p67\textsuperscript{phox} or p47\textsuperscript{phox} Proline-Rich Peptides.

**A**

<table>
<thead>
<tr>
<th>Percentage binding (%)</th>
<th>Peptide 1 (mM) p67\textsuperscript{phox}</th>
</tr>
</thead>
<tbody>
<tr>
<td>120</td>
<td>0</td>
</tr>
<tr>
<td>100</td>
<td>0.5</td>
</tr>
<tr>
<td>80</td>
<td>0.75</td>
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</tr>
<tr>
<td>40</td>
<td>1.5</td>
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</table>

**B**

<table>
<thead>
<tr>
<th>Percentage binding (%)</th>
<th>Peptide 2 (mM) p47\textsuperscript{phox}</th>
</tr>
</thead>
<tbody>
<tr>
<td>120</td>
<td>0</td>
</tr>
<tr>
<td>100</td>
<td>0.5</td>
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<tr>
<td>80</td>
<td>1.0</td>
</tr>
<tr>
<td>60</td>
<td>1.5</td>
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</tbody>
</table>

Fig. 4.12. Rac1 binding to p67\textsuperscript{phox} proteins in the presence of p67\textsuperscript{phox} or p47\textsuperscript{phox} proline-rich peptides. p67\textsuperscript{phox} proteins; full-length p67\textsuperscript{phox} (blue bars) or deletion mutant containing amino acids 1-460 (red bars) were incubated at 4 °C for 16 h, either in the absence of additions or in the presence of proline-rich peptides based upon the sequence of p67\textsuperscript{phox} (A), or p47\textsuperscript{phox} (B) at the indicated concentrations. Proteins were dot blotted onto nitrocellulose filter and probed with Rac1-Q61L-[\gamma\textsuperscript{32}P]GTP.

The percentage of Rac1 binding in each case was calculated regarding the binding observed following the incubation without addition of either peptide as 100%.
adopted an alternative conformation as a result of the incubation. In addition testing a control peptide in simultaneous incubations may have aided interpretation of the results obtained.

4.4.2.2. Rac1 binding to the N-terminal of p67phox in the presence of the C-terminal.

Whether 'masking' of the Rac1 binding site on p67phox could be reconstructed by an SH3-polyproline domain dependent interaction between proteins from the N and C-terminal halves of p67phox was explored.

Protein containing amino acids 1-238 of p67phox, which includes the Rac1 binding site and the polyproline domain was incubated with p67phox C-terminal proteins prior to dot blotting onto nitrocellulose and probing with Rac1 probes. The N-terminal deletion mutant, cleaved from the GST moiety, was incubated (for 3 h at 4 °C) with C-terminal proteins containing amino acids 300-526 or 300-460 (the latter being deleted of the C-terminal SH3 domain), which were present in equal amounts or 2 or 5-fold excess.

The data collected from two separate experiments were variable (Table 4.2). The presence of protein containing amino acids 300-526 reduced Rac1 binding to the N-terminal (amino acids 1-238) in experiments 1 and 2. The presence of protein containing amino acids 300-460 led to an increase in Rac1 binding to the N-terminal protein in experiment 1 but a decrease in experiment 2. These observations could suggest that an inhibition of Rac1 binding to the N-terminal p67phox occurs due to an interaction between the N and C-terminal fragment proteins dependent upon the C-terminal SH3 domain. However, the inconsistency of the results obtained make the data difficult to interpret. In addition the presence of GST also affected the Rac1 interaction with N-terminal amino acids 1-238 to some extent, indicating that the observations may not be specific. This suggests that this method is not suitable for investigating whether an interaction between the N and C-terminal halves of p67phox mask the Rac1 binding site in the full-length protein.
SH3 domain-polyproline interactions occur with low affinity, <100 µM, and it is likely that an intramolecular SH3-polyproline domain interaction may occur with lower affinity. Further analysis is required to determine a suitable direct or indirect approach for investigating the proposed p67phox intramolecular interaction. One possibility would be to introduce point mutations within the C-terminal SH3 domain of p67phox, at sites known to participate in interactions with polyproline target sequences, and monitor the Rac binding activity of such mutants. A similar analysis could be applied to the polyproline region of p67phox.
Table 4.2. Rac1 binding to the N-terminal of p67phox (amino acids 1-238) in the presence of C-terminal proteins.

<table>
<thead>
<tr>
<th>p67phox C-terminal protein incubated with N-terminal 1-238. (amino acids)</th>
<th>µg</th>
<th>Experiment 1 % Binding</th>
<th>Experiment 2 % Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>300-526</td>
<td>5</td>
<td>x1</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>x2</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>x5</td>
<td>-</td>
</tr>
<tr>
<td>300-460</td>
<td>5</td>
<td>x1</td>
<td>111</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>x2</td>
<td>142</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>x5</td>
<td>-</td>
</tr>
<tr>
<td>GST</td>
<td>5</td>
<td>x1</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>x2</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>x5</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 4.2. Rac1 binding to the N-terminal of p67phox (amino acids 1-238) in the presence of C-terminal proteins. Rac1 binding to amino acids 1-238 of p67phox observed following incubation with the C-terminal proteins as indicated is expressed as a percentage of the binding interaction observed without addition. 5 µg 1-238 protein was incubated in the presence of either 300-526-GST, 300-460-GST or GST proteins (amounts as indicated) for 3 h at 4 °C prior to dot blotting and probing with Rac1 probes. The inconsistency observed suggests that the affect upon the Rac1-p67phox binding interaction is not specifically related to the presence of additional protein in this assay.
5. Characterisation of the Cdc42Hs Binding Activity Associated With p67phox Protein Purified from Insect Sf9 Cell Lysates.

The p67phox protein sample purified from insect Sf9 cell lysates was found to contain Cdc42Hs binding activity, as demonstrated by probing recombinant p67phox material immobilised on nitrocellulose filter (see Chapter 4, Fig. 4.1). Further investigation of this interaction indicated the presence of a specific Cdc42Hs binding protein, as binding signals were abrogated in the presence of protein containing the p21 binding domain of α-PAK and were dependent upon Cdc42Hs being in the GTP-bound state. However, that this interaction was distinct from that observed between Rac1 and p67phox, was suggested by the use of ‘effector domain’ mutants of these two p21s. Further analysis was carried out to characterise the Cdc42Hs binding interaction observed.

It was noted that, unlike GTPase negative Cdc42Hs-Q61L, wild-type Cdc42Hs probes did not bind to p67phox protein that had been dot blotted directly on to nitrocellulose. However, this latter probe detected p67phox following denaturation by SDS-PAGE (Fig. 4.1A, lane 3). This indicated that denaturation of p67phox is required for Cdc42Hs binding. A similar result was obtained when proteins known to have Cdc42Hs-GAP activity were dot blotted and probed with Cdc42Hs probes (Fig. 5.1). These results suggest that the lack of binding signal, when Cdc42Hs-[γ32P]GTP was used to probe dot blotted p67phox, could be due to stimulation of the hydrolysis of the bound labelled GTP by p67phox protein. This activity may be lost following SDS-PAGE, thus explaining the signal observed when p67phox immobilised on nitrocellulose filter by western blotting was probed with Cdc42Hs (Fig. 4.1, lane 3). Further experiments were carried out to investigate whether p67phox might be a Cdc42Hs-GAP.

5.1. p67phox protein purified from insect Sf9 cells stimulates the GTPase activity of Cdc42Hs.

To examine whether p67phox has Cdc42Hs-GAP activity, Cdc42Hs was loaded with [γ32P]GTP and its GTPase activity was measured over a 20 min time course in the presence or absence of p67phox; the GAP domain of p190 was used a control GAP (Fig. 5.2A). p67phox stimulated the intrinsic GTPase activity of Cdc42Hs. This activity was eliminated upon denaturation of p67phox, by boiling for 5 min (Fig. 5.2A).
Fig. 5.1. Cdc42Hs binding interactions with p67^phox purified from insect Sf9 cell lysates, n-chimaerina and p190.

Proteins: -
p67^phox (purified from insect Sf9 cells),
n-chimaerin (purified from E. coli as a GST fusion protein),
p190 (purified from E. coli as a GST fusion protein).

5-10 µg of protein were dot blotted onto nitrocellulose filter as indicated at the left-hand end of each row and probed with: -

Lane 1, wild-type Cdc42Hs-[γ^32P]GTP (WT),
Lane 2, GTPase negative Cdc42Hs-Q61L-[γ^32P]GTP (Q61L).

Signals were visualised by exposure of the nitrocellulose filters to X-Omat film for 5 h.
Fig. 5.2. Figure legend on reverse.
Fig. 5.2. p67phox purified from insect Sf9 cell lysates has Cdc42Hs-GAP activity.

GTPase activating protein (GAP) assays were carried out as described in the Methods section 2.2.6, with Cdc42Hs proteins bound to ³²P labelled GTP nucleotides as substrates (γ³²P-GTP in A and B, or α³²P-GTP in C). Substrates were incubated at 15 °C in the presence or absence of additions and the loss of γ³²P or α³²P was monitored over a 20 min time course.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Additions</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Cdc42Hs-[γ³²P]GTP</td>
<td>- + denatured p67phox (5 μM) + p67phox (5 μM) + p190 (0.1 μM)</td>
</tr>
<tr>
<td>B. Cdc42Hs-Q61L-[γ³²P]GTP</td>
<td>- + p67phox (5 μM)</td>
</tr>
<tr>
<td>C. Cdc42Hs-[α³²P]GTP</td>
<td>- + p67phox (5 μM) + p190 (0.1 μM)</td>
</tr>
</tbody>
</table>

p67phox (purified from insect Sf9 cell lysates) was added at 5 μM and p190 GAP domain (purified as a GST fusion protein from E. coli) at 0.1 μM. p67phox protein was denatured by heating to 100 °C for 5 min prior to addition to the GAP assay. The amount of ³²P remaining at each time point was quantified by conversion to cpm and expressed as a percentage of ³²P present at t = 0 min. Similar results were obtained in 2-3 other experiments.
Fig. 5.2. \( p67^{phox} \) Purified from Insect Sf9 Cell Lysates has Cdc42Hs-GAP activity.
To demonstrate that p67phox was stimulating the GTPase activity of Cdc42Hs and not accelerating dissociation of labelled \([\gamma^{32}\text{P}]\text{GTP}\), Cdc42Hs-Q61L-[\gamma^{32}\text{P}]\text{GTP} and Cdc42Hs-[\alpha^{32}\text{P}]\text{GTP} were used as substrates (Fig. 5.2B and C, respectively). The Cdc42Hs-Q61L GTPase negative mutant has no detectable GTPase activity over a 20 min time course at 15 °C. If p67phox were accelerating dissociation of GTP from Cdc42Hs, loss of label from either substrate would occur over the 20 min time course studied. However, in this case the amount of labelled GTP remaining over the 20 min period did not change significantly, indicating that p67phox was not stimulating loss of GTP (Fig. 5.2B and C). This is consistent with the idea that p67phox is a Cdc42Hs-GAP.

5.2. Characteristics of the Cdc42Hs-GAP activity of p67phox purified from insect Sf9 cells.

In order to characterise the Cdc42Hs-GAP activity of p67phox, the amount of Cdc42Hs-[\gamma^{32}\text{P}]\text{GTP} remaining after a 10 min incubation was measured in the presence of increasing amounts of p67phox. p67phox was less active than the GAP domain of p190 (Fig. 5.2A). However, the concentration dependence of p67phox in stimulating Cdc42Hs GTPase activity was similar to that of n-chimaerin (Fig. 5.3). K_{activity} of p67phox Cdc42Hs-GAP activity was 5 μM versus 2 μM for n-chimaerin (Ahmed et al., 1994). [Note: K_{activity} is defined as the amount of protein required to reduce the half-life of p21-GTP by 50%.]

The GAP domain of p190 stimulates the GTPase activity of Rac1-D38A ‘effector-site’ mutant (Xu et al., 1994). p67phox was able to stimulate the GTPase activity of Cdc42Hs-D38A, but not that of the GTPase negative mutant Cdc42Hs-Q61L (Fig. 5.3A and B). This may help to explain the lack of binding signal when p67phox protein, dot blotted on to nitrocellulose, was probed with Cdc42Hs-D38A probes (Fig. 4.3, lane 4). PAK and p120^{ACK} bind to Rac1/Cdc42Hs and Cdc42Hs respectively, with high affinity and have strong preference for the GTP-bound form of the p21. When present at 2 μM, α-PAK(1-151) inhibited the ability of Cdc42-Q61L to interact with p67phox (Fig. 4.2E). Addition of α-PAK(1-151) or p120^{ACK} (2 μM) inhibited the intrinsic GTPase activity of Cdc42Hs as previously reported (Manser et al., 1994) and completely inhibited the Cdc42Hs-GAP activity of p67phox (Fig. 5.3C). Addition of GST (2 μM) did not affect the intrinsic GTPase activity of Cdc42Hs or the ability of p67phox to act as a Cdc42Hs-GAP.
Fig. 5.3. Figure legend on reverse.
Fig. 5.3. Characterisation of the Cdc42Hs-GAP activity of p67phox purified from insect Sf9 cell lysates.

A. GAP assays were carried out with different amounts of p67phox (10 nM to 10 μM) with either Cdc42Hs-[γ32P]GTP or Cdc42Hs-Q61L-[γ32P]GTP as substrates (as described in the Methods section 2.2.6). The amount of 32P remaining after 10 min was quantified by conversion to cpm.

B. GAP activity of p67phox on ‘effector domain’ mutant Cdc42Hs-D38A compared with wild-type Cdc42Hs.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Additions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cdc42Hs-[γ32P]GTP</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>+ p67phox (5 μM)</td>
</tr>
<tr>
<td>Cdc42Hs-D38A-[γ32P]GTP</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>+ p67phox (5 μM)</td>
</tr>
</tbody>
</table>

C. The Cdc42Hs-GAP activity of p67phox purified from insect Sf9 cells was measured in the absence or presence of additions:

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Additions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cdc42Hs-[γ32P]GTP</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>+ p67phox (5 μM)</td>
</tr>
<tr>
<td></td>
<td>+ Addition</td>
</tr>
<tr>
<td></td>
<td>+ p67phox (5 μM) + Addition</td>
</tr>
</tbody>
</table>

Additions: -
Experiment 1, p120ACK (2 μM),
Experiment 2, α-PAK-GST (2 μM),
Experiment 3, SDS (100 μM),
Experiment 4, Arachidonic acid (10 μM, AA).

For A, B, and C, similar results were obtained in at least 1 other experiment.
Fig. 5.3. Characterisation of the Cdc42Hs-GAP Activity of p67\textsuperscript{phox} purified from Insect Sf9 Cell Lysates.
Since reconstitution of oxidase activity by the NADPH components \textit{in vitro} requires the addition of SDS or AA, the Cdc42Hs-GAP activity of p67\textsuperscript{phox} was measured in their presence. While SDS (100 µM) inhibited the GAP activity of p67\textsuperscript{phox} to some extent, AA (10 µM) was without effect (Fig. 5.3C). Higher concentrations of SDS (200 µM) strongly inhibited the GAP activity of p67\textsuperscript{phox}. This might explain why Cdc42Hs probes bound p67\textsuperscript{phox} following SDS-PAGE and transfer to nitrocellulose but not when protein had been dot blotted (Fig. 4.1A and Fig. 5.1).

Although consistent results describing the Cdc42Hs-GAP activity of p67\textsuperscript{phox} as detailed here, were obtained in at least 3 separate experiments, these results were not replicated between batches of recombinant p67\textsuperscript{phox} protein purified from insect Sf9 cell lysates. In addition p67\textsuperscript{phox} protein does not share sequence similarity with other known Rho family-GAP proteins. Whether the Cdc42Hs-GAP activity observed was affected by post-translational modification of p67\textsuperscript{phox}, during expression in insect Sf9 cells, or was due to the presence of an additional factor that copurified with p67\textsuperscript{phox} from Sf9 cell lysates, was explored by studying the Cdc42Hs interaction with p67\textsuperscript{phox} proteins purified from \textit{E. coli} cells.

\textbf{5.3. Cdc42Hs does not interact with recombinant p67\textsuperscript{phox} protein expressed in \textit{E. coli} cells.}

Deletion constructs of p67\textsuperscript{phox} expressed as GST fusion proteins and purified from \textit{E. coli} strain cells (see section 4.2, Fig. 4.6A) were assayed for Cdc42Hs binding and GAP activity, in an attempt to localise the region of p67\textsuperscript{phox} protein that contains the Cdc42Hs binding or GAP activity.

N and C-terminal proteins (amino acids 1-238 and 300-526 respectively) did not affect the GTPase activity of Cdc42Hs, suggesting that the whole of p67\textsuperscript{phox} is required for this activity. However, full-length p67\textsuperscript{phox} protein purified from \textit{E. coli} cells was also unable to stimulate the GTPase activity of Cdc42Hs.
Fig. 5.4. Cdc42Hs Binding to p67\textsuperscript{phox} Proteins.

Proteins in lanes 2-6 were purified from \textit{E. coli} cells. Deletion mutants (lanes 2-5), were expressed as GST fusion proteins whereas full-length p67\textsuperscript{phox} was cleaved from GST (see Fig. 4.6, for SDS-PAGE analysis). Filters were exposed to X-Omat film for 16 h, signals represent results of at least 3 separate experiments.
All previous analyses of the Cdc42Hs-p67phox interaction were carried out using recombinant protein purified from insect Sf9 cells. Cdc42Hs binding signals were only associated with this p67phox protein. Unlike Rac1 (Fig. 4.6), Cdc42Hs did not bind to full-length p67phox protein nor the deletion constructs containing amino acids: 1-238, 1-192, 126-238, 300-526 and 1-526, purified from E. coli cells (Fig. 5.4). Cdc42Hs did not bind deletion mutants of p67phox deleted of the polyproline region or the C-terminal SH3 domain (1-526Δ226-236 and 1-460 respectively). These observations could be explained by post-translational modification of p67phox during expression in insect Sf9 cells, which favoured Cdc42Hs binding and GAP activity, in comparison with bacterial cell protein expression. Alternatively, the binding interaction and Cdc42Hs-GAP activity observed, could be caused by additional factors that copurify with p67phox from insect Sf9 cell lysates.

5.4. Cdc42Hs binding proteins in insect Sf9 cell lysates.
As the major species of Cdc42 binding proteins with molecular masses ~65 kDa have been identified as PAK family proteins in a variety of different tissues, a study was carried out to investigate whether there are PAK proteins present in insect Sf9 cell lysates, or p67phox protein preparations purified from insect Sf9 cells that had been infected with p67phox cDNA.

5.4.1. A PAK-related protein copurifies with p67phox protein purified from insect Sf9 cell lysates.
Proteins from insect Sf9 cell lysates prepared from cells infected with p67phox cDNA, or control cells (uninfected) were separated by SDS-PAGE and transferred to nitrocellulose filter. Proteins were analysed for Cdc42Hs binding activity, or anti-p67phox and anti-Ste20 immunoreactivity. Cdc42Hs binding protein(s) of ~68 kDa were detected in insect Sf9 cell lysates prepared from control cells. This binding activity was also detected in lysates prepared from p67phox infected cells but the extent of the signal was much reduced (Fig. 5.5, compare lanes 1 and 2). This protein also appeared to react with anti-Ste20 antibodies (Fig. 5.5, lanes 7 and 8), suggesting the presence of a PAK-like protein. Anti-p67phox immunoreactivity was only detected in insect cell lysates from infected cells. Together these results suggest that the Cdc42Hs binding activity in insect Sf9 cell lysates was due to the presence of a protein other than p67phox.
Fig. 5.5. Analysis of Proteins from Insect Sf9 Cell Lysates.

Fig. 5.5. Analysis of proteins from insect Sf9 cell lysates. A. Proteins from insect Sf9 cells, prepared as described in Appendix A.4, were separated in SDS-gels and transferred to nitrocellulose filters. Proteins were analysed for Cdc42Hs binding activity (lanes 1-3), anti-p67<sup>phox</sup> (lanes 4-6) and anti-Ste20 (lanes 7-9) immunoreactivity.

**Lanes 1, 4, and 7**, Sf9 cell lysates from uninfected cells (50 µg),

**Lanes 2, 5, and 8**, Sf9 cell lysates (50 µg), from cells infected with p67<sup>phox</sup> cDNA,

**Lanes 3, 6, and 9**, p67<sup>phox</sup> protein (5 µg) purified from insect Sf9 cell lysates that had been infected with p67<sup>phox</sup> cDNA.

**Probes:**

- Lanes 1-3, Cdc42Hs-Q61L-[γ<sup>32</sup>P]GTP,
- Lanes 4-6, p67<sup>phox</sup> antibody,
- Lanes 7-9, Ste20 antibody.

Signals were visualised by exposure of nitrocellulose filters to X-Omat film 5 h, lanes 1-3 or immunoreactivity was visualised as described in Methods section 2.2.7, lanes 4-9.
protein preparations purified from infected insect Sf9 cells contained a 68 kDa protein that was detected with anti-Ste20 antibodies. This immunoreactivity comigrated in SDS-gels with the Cdc42Hs binding activity and the anti-p67phox immunoreactivity in this sample (Fig. 5.5, lanes 3, 6 and 9). This suggested that the Cdc42Hs binding activity associated with purified p67phox protein was in fact due to the presence of a PAK-related protein.

5.5. p67phox protein purified from insect Sf9 cells contains contaminating proteins.
The above data suggests that p67phox itself is not a target for Cdc42Hs and that the binding activity observed is due to the presence of a PAK-like protein, which can not be distinguished from p67phox protein following SDS-PAGE. This is consistent with the findings that Cdc42Hs has a low capacity for activation of the NADPH oxidase and is not required for in vitro reconstitution of NADPH oxidase activity (Kwong et al., 1993).

Further investigation of the Cdc42Hs-GAP activity associated with the insect cell purified p67phox was not possible, as this activity was not consistent between all protein preparations. That the Cdc42Hs-GAP activity associated with the p67phox protein preparation was not inhibited by the presence of an endogenous insect Sf9 cell PAK protein (as might be expected, see section 5.2), could be explained by the PAK protein being present at low levels. The amount of PAK protein in the p67phox protein preparation was estimated by comparing the Cdc42Hs binding signals with those of recombinant PAK protein (see methods section 2.2.11). These calculations indicated that PAK was present at a concentration of approximately 15 nM, when p67phox was added to GAP assay incubations at a final concentration of 5 μM.

Subjecting p67phox protein, purified from Sf9 cells, to analysis using the GAP-overlay technique (Manser et al., 1995b), would have established the size of the Cdc42Hs-GAP and may have aided identification of the protein responsible for this activity. The material for such an experiment was not available. As no sequence homology between p67phox protein and known Cdc42Hs GAPs could be identified, and that this activity was not observed with proteins purified from E. coli cells, it seems likely that both the Cdc42Hs binding and GAP activity observed are due to the presence of proteins which copurify with p67phox from Sf9 cell lysates.
6. In Vitro Phosphorylation of p67phox by β-PAK.

The cytosolic oxidase components p47phox and p67phox are phosphorylated upon stimulation of the neutrophil NADPH oxidase (Faust et al., 1995; Dusi and Rossi, 1993). However, the kinases responsible for these phosphorylation events have not been identified. Analysis of the phospho-amino acid content of phosphorylated NADPH oxidase components p47phox and p67phox has suggested the involvement of serine/threonine kinases (El Benna et al., 1994; El Benna et al., 1997). That p67phox and a PAK-like protein copurify from insect Sf9 cell lysates, through a series chromatographic stages, could suggest that these proteins associate via a binding interaction. Whether the copurifying PAK-like protein possess kinase activity and is able to phosphorylate p67phox was investigated using in vitro solution kinase assays. Kinase assays were carried out as described in the Methods section 2.2.9; briefly, proteins were incubated with [γ32P]ATP in kinase buffer for 15 min at 30 °C. Reactions were stopped and proteins were separated by SDS-PAGE. The gels were then dried and the incorporation of 32P into the proteins present was visualised by exposure of the dried gels to X-Omat film.

6.1. The PAK-related protein in the p67phox protein sample purified from insect Sf9 cell lysates has kinase activity.

Analysis of the p67phox protein sample purified from insect Sf9 cell lysates indicated the presence of a kinase. The appearance of (at least 2) autophosphorylated protein bands was observed following incubation of this protein sample in kinase buffer in the presence of [γ32P]ATP (Fig. 6.1, lane 3). Two autophosphorylated proteins were positioned either side of the p67phox protein band following separation in SDS-gels (Fig. 6.1, compare lanes 1 and 3). This suggests that the kinase activity was not due to p67phox protein itself, but instead to the presence a contaminating factor. Whilst tests were not carried to identify whether the autophosphorylation observed was stimulated by either Cdc42Hs or Rac1, evidence from the data presented in Chapter 5 indicates that the kinase activity in this protein sample could be due to the presence of a PAK-like protein.

Whether p67phox is a PAK substrate was investigated using in vitro solution kinase assays. As p67phox and the PAK-like protein in the insect Sf9 cell protein sample have very similar molecular masses, recombinant β-PAK, expressed as a GST fusion protein
Fig. 6.1. *In Vitro* Phosphorylation of p67\textsuperscript{phox} Purified from Insect Sf9 Cell Lysates

**Proteins:**
- **Lane 1**, p67\textsuperscript{phox} (Coomassie Blue stain),
- **Lane 2**, p67\textsuperscript{phox} + β-PAK (0.1 μg),
- **Lane 3**, p67\textsuperscript{phox}, no additions.
(~96 kDa) was used as the source of PAK kinase for this analysis. A 67 kDa phosphorylated protein, which comigrated with Coomassie Blue stained p67phox protein in SDS-gels, was observed following incubation of β-PAK and p67phox protein (purified from insect Sf9 cells) in kinase buffer in the presence of [γ32P]ATP (Fig. 6.1, lane 2). This suggests that p67phox is a potential PAK substrate. This observation was investigated further using p67phox protein purified from E. coli cells (see sections 6.3 onwards). Phosphorylated p67phox and the autophosphorylated proteins seen in the protein sample purified from insect Sf9 cells did not comigrate in SDS-gels, suggesting that the autophosphorylation activity is not due to p67phox itself (Fig. 6.1, compare lanes 2 and 3).

6.2. Kinase activity of recombinant β-PAK.

Recombinant β-PAK-GST was expressed in E. coli BL21 strain cells and purified from bacterial cell lysates as a GST-fusion protein using glutathione-Sepharose. Analysis of recombinant β-PAK protein samples, isolated from BL21 cell lysates, by SDS-PAGE demonstrated the presence of a large number of breakdown products, as illustrated in Fig. 6.2A, lane 1. It was estimated that full-length β-PAK-GST protein represented less than 50 % of the total protein present in such samples. Amounts of β-PAK protein quoted as being used in the following experiments refers approximately to the quantity of full-length β-PAK protein used in each assay, for example 5 μg of β-PAK (~96 kDa) were present in the amount of protein analysed on SDS-gels in Fig. 6.2A, lane 1.

6.2.1. Autophosphorylation of recombinant β-PAK.

Following incubation of β-PAK with [γ32P]ATP in kinase buffer, phosphorylated proteins were observed at 96, 50 and 40 kDa (Fig 6.2A, lane 2). The 96 kDa phosphorylated protein comigrates with the full-length β-PAK protein in SDS-gels (compare Fig. 6.2A, lanes 1 and 2), indicating that autophosphorylation of β-PAK occurs in this in vitro assay. The 40 and 50 kDa proteins are either breakdown products of β-PAK or E. coli cell proteins that copurify with the β-PAK-GST fusion protein, which become phosphorylated in this assay.

6.2.2. GTP-bound Cdc42Hs or Rac1 do not stimulate β-PAK autophosphorylation.

The autophosphorylation of β-PAK observed in this assay did not require and was not stimulated by the addition of GTP-bound Cdc42Hs or Rac1 (Fig. 6.2A, compare lane 2
with lanes 3 and 4). In some cases the appearance of a 60 kDa phosphorylated protein was observed on addition of either p21 however this was variable between protein preparations. The above result differed from the characteristics of the kinase activity described for PAK proteins purified rat brain PAK or human neutrophil cytosol, which were stimulated to autophosphorylate by at least 10-fold in the presence of GTP-Cdc42Hs or Rac1 (Manser et al., 1994 and Chapter 3 in this study). The lack of p21 requirement for stimulation of β-PAK autophosphorylation activity suggests that β-PAK protein becomes activated during expression in *E. coli* BL21 cells.

β-PAK was also found to phosphorylate substrate protein in the absence of Cdc42Hs or Rac1-GTP. Incubation of β-PAK with myelin basic protein (MBP) in the presence of $^{32}$P lead to the appearance of an 18 kDa phosphorylated protein which comigrated with Coomassie Blue stained MBP (Fig. 6.2B). A low exposure of this experiment is shown for a clearer picture of the phosphorylated MBP so only a weak β-PAK autophosphorylation band is seen, highlighting the extent of MBP phosphorylation by β-PAK in this assay. The above evidence suggests that β-PAK protein is in an active form when purified from *E. coli* BL21 cell lysates. It has been suggested that activation of PAK family proteins involves autophosphorylation of residues within the kinase domain and the N-terminal regulatory region (Manser et al., 1997). Studies were carried out to investigate whether β-PAK purified from *E. coli* is in a phosphorylated state.

### 6.2.3. Alkaline phosphatase treatment of β-PAK.

β-PAK-GST isolated from *E. coli* BL21 cell lysates, bound to glutathione-Sepharose resin, was incubated with or without alkaline phosphatase in phosphatase buffer for 1 h at 30 °C, at a concentration of 500 units of enzyme per protein from 1 litre of *E. coli* cell culture (10 units of alkaline phosphatase per 1 µg of β-PAK protein). The alkaline phosphatase was removed and protein collected from the glutathione-Sepharose was mixed with sample buffer and separated by SDS-PAGE. Treatment with alkaline phosphatase caused a mobility shift of the β-PAK protein on SDS-gels as visualised by Coomassie Blue staining (Fig. 6.3, compare lane 1, untreated protein with lane 2, phosphatase treated protein). The increased mobility of phosphatase treated β-PAK protein in SDS-gels may be due to the removal of phosphate groups by the alkaline
Fig. 6.2. *In Vitro* Phosphorylation of Recombinant β-PAK.

A. Purified recombinant β-PAK protein (12 μg) was separated by SDS-PAGE and protein was visualised by Coomassie Blue staining (lane 1). *In vitro* solution kinase assays; 1 μg of β-PAK was incubated in kinase buffer with 5 μCi of \([\gamma^{32}P]ATP\) for 15 min at 30 °C, either in the absence of any additions (lane 2), or with GTP loaded Cdc42Hs and Rac1 (lanes 3 and 4 respectively). The reactions were stopped by mixing with 5x sample buffer and proteins were subjected to SDS-PAGE, gels were dried down and exposed to X-Omat film for 30 min in order to visualise phosphorylated proteins.

Lane 1, β-PAK (12 μg), Coomassie Blue Stain,
Lane 2, β-PAK (1 μg), in the absence of additions (Kinase Assay),
Lane 3, β-PAK (1 μg) + Rac1-GTP (Kinase Assay),
Lane 4, β-PAK (1 μg) + Cdc42Hs-GTP (Kinase Assay).

B. *In vitro* solution kinase assays; 0.5 μg of β-PAK was incubated in kinase buffer with 1.5 μCi of \([\gamma^{32}P]ATP\) for 15 min at 30 °C, either in the absence of any additions (lane 1) or in the presence of 1 μg myelin basic protein (MBP, lane 2). Phosphorylated proteins were visualised as in A. Dried gels were exposed to X-Omat film for 5 min.

Lane 1, β-PAK (0.5 μg), in the absence of additions (Kinase Assay),
Lane 2, β-PAK (0.5 μg) + MBP (1 μg) (Kinase Assay).
phosphatase enzyme. These data suggest that β-PAK purified from *E. coli* is in a phosphorylated form.

Alkaline phosphatase treated β-PAK appeared to become more heavily phosphorylated in *in vitro* kinase assays than untreated protein (Fig. 6.3, lane 3). However, the apparent elevation in $^{32}$P incorporation could be explained by the presence of more available phosphorylation sites made accessible by the removal of phosphate by prior enzyme treatment. Following an *in vitro* kinase assay alkaline phosphatase treated protein still appeared to have increased mobility under SDS-PAGE. This suggests that the extent of phosphorylation that occurs in solution is not as great as that which occurs in *E. coli* BL21 cells during β-PAK protein expression. This might explain the reduced ability of the alkaline phosphatase treated protein to phosphorylate substrate protein in comparison to the untreated protein (Fig. 6.3, lanes 5 and 6). Further stimulation of phosphorylation by the addition of Cdc42Hs-GTP or Rac1-GTP might be required to achieve the level of phosphorylation observed in the untreated purified protein.

### 6.2.4. Cdc42Hs and Rac1 binding to recombinant β-PAK.

β-PAK protein produced in *E. coli* BL21 cells was found to bind weakly to $[^{32}\text{P}]$GTP labelled Rac1 and Cdc42Hs probes (Fig. 6.4). However, an increase in the binding signals observed was seen following alkaline phosphatase treatment of β-PAK, suggesting an increase in the binding to Rac1 and Cdc42Hs (Fig. 6.4). These data suggested that removal of phosphate from β-PAK protein increased binding to either p21 tested. This result is in agreement with the finding that autophosphorylation upon activation of recombinant β-PAK leads to a decrease in Cdc42Hs binding (Manser *et al.*, 1995a). Also this observation is consistent with the model for Cdc42Hs or Rac1 binding to PAK proposed by Manser *et al.*, 1994, which suggested that the affinity of GTP-Cdc42Hs for PAK was reduced upon phosphorylation of the PAK protein resulting in a down regulation of the Cdc42Hs-PAK interaction, once the kinase activity of PAK is stimulated.
**Fig. 6.3. Alkaline Phosphatase Treatment of β-PAK.**

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<th>Coomassie stain</th>
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**Fig. 6.3. Alkaline phosphatase treatment of β-PAK.**

β-PAK protein treated with alkaline phosphatase (+) was analysed in SDS-gels (lanes 1 and 2), or phosphorylated in *in vitro* solution kinase assays (lanes 3-6) and compared to untreated protein (−). β-PAK-GST bound to glutathione-Sepharose resin was incubated with (+) 500 units of alkaline phosphatase or without (−) additions, for 1 h at 4 °C; protein bound to resin was washed and analysed as described below.

β-PAK protein was separated in SDS-gels and visualised by Coomassie Blue staining:

- **Lane 1**, β-PAK protein (5 µg) + alkaline phosphatase (+),
- **Lane 2**, β-PAK protein (5 µg) - alkaline phosphatase (−).

Note the increased mobility of the phosphatase treated protein, compare lanes 1 and 2.

β-PAK was incubated in the presence of 1 µCi [γ-32P]ATP, in the absence of additions (lanes 3 and 4) or in the presence of p67phox protein, amino acids 1-192 (lanes 5 and 6). Proteins were separated in SDS-gels, dried gels were exposed to X-Omat film for 2 h.

- **Lane 3**, β-PAK (+) alkaline phosphatase, in the absence of additions,
- **Lane 4**, β-PAK (−) alkaline phosphatase, in the absence of additions,
- **Lane 5**, β-PAK (+) alkaline phosphatase, + p67phox 1-192 (5 µg),
- **Lane 6**, β-PAK (−) alkaline phosphatase, + p67phox 1-192 (5 µg).
Fig. 6.4. Alkaline Phosphatase Treatment of β-PAK Increased Binding to Rac1 and Cdc42Hs $\gamma^{32}$P|GTP Labelled Probes.

**Fig. 6.4. Alkaline phosphatase treatment of β-PAK increased binding to Rac1 and Cdc42Hs $\gamma^{32}$P|GTP labelled probes.**

Analysis of Rac1 and Cdc42Hs binding to β-PAK ± alkaline phosphatase treatment.

1 μg of β-PAK protein untreated (−, lanes 1 and 3) or treated with alkaline phosphatase (+, lanes 2 and 4) was separated by SDS-PAGE. Protein was transferred to nitrocellulose filter by western transfer and probed with either Rac1-Q61L or Cdc42Hs-Q61L probes, (lanes 1 and 2 or 3 and 4 respectively). Nitrocellulose filters were washed and exposed to X-Omat film for 16 h.

**Lane 1,** β-PAK (1 μg) (−) alkaline phosphatase,
**Lane 2,** β-PAK (1 μg) (+) alkaline phosphatase,
**Lane 3,** β-PAK (1 μg) (−) alkaline phosphatase,
**Lane 4,** β-PAK (1 μg) (−) alkaline phosphatase.

Probe

- **Rac1-Q61L-$\gamma^{32}$P|GTP**
- **Cdc42Hs-Q61L-$\gamma^{32}$P|GTP**
6.3. *In vitro* phosphorylation of recombinant p67<sub>phox</sub> by β-PAK.

Recombinant β-PAK was used as a source of PAK kinase to investigate whether a PAK protein can phosphorylate p67<sub>phox</sub> *in vitro*. On addition of 5 µg of recombinant p67<sub>phox</sub> (expressed and purified from *E. coli* cells) to an *in vitro* solution kinase assay in the presence of β-PAK a 67 kDa phosphorylated protein was detected, (Fig. 6.5A, lane 2). This phosphorylated protein aligned with p67<sub>phox</sub> protein on a Coomassie Blue stained gel (Fig. 6.6B). This suggests that p67<sub>phox</sub> is a substrate for β-PAK phosphorylation *in vitro*. This *in vitro* phosphorylation was not stimulated by the addition of either Cdc42Hs or Rac1 proteins in their GTP-bound states (Fig. 6.5B).

6.3.1. *In vitro* phosphorylation of p67<sub>phox</sub> deletion mutants by β-PAK.

In order to identify the region of p67<sub>phox</sub> that is phosphorylated by β-PAK and to define potential phosphorylation sites, various p67<sub>phox</sub> deletion mutants were added to *in vitro* kinase assays in the presence of β-PAK. Kinase reactions were stopped by the addition of sample buffer and phosphorylated proteins were visualised by exposing the dried down gels to X-Omat film (Fig. 6.6A).

In the presence of β-PAK a number of p67<sub>phox</sub> deletion mutants became phosphorylated, including proteins containing amino acids: 1-238, 1-192, 126-238, 170-238 but not 192-238, 300-526, 1-131, 1-58 or GST protein (Fig. 6.6A, lanes 3-11 respectively). This was suggested by the appearance of phosphorylated protein bands correlating with the position of these proteins on SDS-gels stained with Coomassie Blue (compare appropriate lanes in Fig. 6.6A and B).

These data suggest that potential *in vitro* β-PAK phosphorylation sites lie within the N-terminal half of p67<sub>phox</sub>, however this analysis does not rule out there being other phosphorylation sites in the C-terminal half of the protein that require N-terminal sequences to become phosphorylated. These data indicate that the major *in vitro* phosphorylation sites are located in the region of p67<sub>phox</sub> containing amino acids 170-238. However it was noted that the sequence 170-192 does not contain any potential β-PAK phosphorylation sites and examination of the location of the deletion mutants within the full-length protein and their levels of phosphorylation (qualitatively estimated)
Fig. 6.5. Phosphorylation of p67$^{phox}$ by β-PAK.

A. The ability of β-PAK to phosphorylate p67$^{phox}$ was investigated. Full-length protein purified from E. coli cells was added to in vitro solution kinase assays in the presence of recombinant β-PAK. Proteins were separated by SDS-PAGE and phosphorylated proteins were visualised by exposing the dried gel to X-Omat film.

Lane 1, β-PAK, in the absence of additions,

Lane 2, β-PAK + p67$^{phox}$ (5 μg).

B. The ability of GTP-p21s to stimulate phosphorylation of p67$^{phox}$ by β-PAK was investigated. β-PAK (1 μg) and p67$^{phox}$ (5 μg) were incubated in in vitro solution kinase assays as in A, either alone (lane 1), or in the presence of Cdc42Hs or Rac1-GTP (5 μg; lanes 2 and 3 respectively).

Lane 1, β-PAK + p67$^{phox}$ (5 μg), in the absence of additions (-).

Lane 2, β-PAK + p67$^{phox}$ (5 μg) + Cdc42Hs-GTP (C),

Lane 3, β-PAK + p67$^{phox}$ (5 μg) + Rac1-GTP (R).
Fig. 6.6. *In vitro* Phosphorylation of p67<sup>phox</sup> Deletion Mutants by β-PAK.

A. *In vitro* phosphorylation of p67<sup>phox</sup> deletion mutants by β-PAK. Numbers indicate amino acids of p67<sup>phox</sup> proteins added to each assay.

Lane 1, no additions,
Lane 2, GST,
Lane 3, 1-238,
Lane 4, 1-192,
Lane 5, 126-238,
Lane 6, 170-238,
Lane 7, 192-238,
Lane 8, 300-526,
Lane 9, 1-131,
Lane 10, 1-58,
Lane 11, GST.

β-PAK (100 ng/lane) was incubated with [γ<sup>32</sup>P]ATP as in previous figures in the absence of addition or in the presence of p67<sup>phox</sup> proteins or as a control GST protein (5 μg). The degree of stability differed between proteins; protein amounts added to the kinase assays were equalised so that ~5 μg of the full-length fusion protein was present in each case. Phosphorylated proteins were visualised by exposure of dried gels to X-Omat film for 5-10 min. This figure shows results representative of at least 3 different experiments. Arrow heads mark the positions of the full-length proteins used; see B for Coomassie Blue stain of the p67<sup>phox</sup> proteins used in these kinase assays.
Fig. 6.6. B Coomassie Blue Stain of p67phox proteins.

B. Coomassie stain of p67phox protein used in the in vitro solution kinase assays shown in A. p67phox proteins purified from E. coli cells, containing amino acids as indicated, were separated in SDS-gels and stained with Coomassie Blue. Proteins purified as GST fusion proteins are indicated in the panel on the right, others were cleaved from GST: - full-length p67phox (lane 1), 1-238 and 1-192 (lanes 2 and 3), 1-526 Δ226-236 (lane 11), and 1-460 (lane 13). Note, the amounts of protein in each lane correlate with those used in the kinase assays in A, and were equalised to ~5 µg of the full-length protein in each case.
indicates that there may be sites between amino acids 126 and 170 that are phosphorylated by β-PAK.

The GST fusion protein containing amino acids 126-238 of p67phox is particularly unstable when produced in E. coli. This is illustrated by the number of breakdown products observed on Coomassie Blue stain analysis of this protein (Fig. 6.6B, Lane 4). The instability of this protein could explain the reduction in the level of phosphorylation by β-PAK in comparison to 170-238 protein. Alternatively the 126-238 fusion protein may adopt conformation due to the additional sequences between this and 170-238, which block the phosphorylation sites. This result correlates with the reduction in Rac1 binding observed when proteins containing amino acids 126-238 and 170-238 of p67phox were compared (see Results Chapter 4). Residues in the region deleted between these proteins should be considered in the analysis of potential β-PAK phosphorylation sites on p67phox.

6.3.2. Identification of potential β-PAK phosphorylation sites on p67phox.

Potential β-PAK phosphorylation sites on p67phox were identified by analysing the protein sequence between amino acids 126 and 238 (Fig. 6.7). Members of the PAK family of protein kinases phosphorylate serine and threonine residues. Figure 6.7, shows the sequence of this region of p67phox (serine and threonine residues are highlighted). Within this sequence there are three threonine residues (at positions 148, 203 and 233) and six serine residue (at positions 149, 152, 157, 207, 213 and 215) that may provide potential phosphorylation sites for β-PAK. Deletion of amino acids 170-192, of the 170-238 mutant, abolished phosphorylation by β-PAK completely (Fig. 6.6A, lanes 5 and 6). Analysis revealed that this sequence (amino acids 170-192) does not contain any serine or threonine residues (Fig. 6.7), suggesting that this region of p67phox is important for the interaction with β-PAK for phosphorylation to occur. The nature of the β-PAK-p67phox interaction is unknown. The potential β-PAK phosphorylation sites and the site suggested to be required for an interaction with p67phox, are situated in close proximity to the Rac binding site on p67phox.
Fig. 6.7. Potential β-PAK phosphorylation sites in p67<sup>phox</sup>. Analysis of p67<sup>phox</sup> sequence from amino acid 126-238. The predicted amino acid sequence of p67<sup>phox</sup> is displayed in single letter code. The serine and threonine residues are shown in blue. The potential SH3 domain target sequence (proline-rich region) is underlined. Note the lack of potential β-PAK phosphorylation sites between residues 170 and 192.
The potential serine and threonine residues in the region of p67<sup>phox</sup> between amino acids 126 and 238 do not lie within regions of sequence with homology to those in which PAK autophosphorylation sites have been mapped (Manser <i>et al.</i>, 1997), as analysed using DNASTAR protein sequence alignment software.

### 6.3.3. β-PAK phosphorylation sites on p67<sup>phox</sup> are masked in the full-length protein.

Comparison of the level of phosphorylation of full-length p67<sup>phox</sup> and the various deletion mutants, by qualitative analysis of the autoradiographical data, suggests that β-PAK phosphorylation sites are masked in the full-length p67<sup>phox</sup> protein. Deletion of the C-terminal part of p67<sup>phox</sup> (amino acids 239-526) stimulated phosphorylation by β-PAK (compare Fig. 6.6A, lane 1 with Fig 6.6A, lane 3). β-PAK phosphorylation of p67<sup>phox</sup> was also stimulated upon deletion of the C-terminal SH3 domain and residues immediately C-terminal (protein containing amino acids 1-460). The level of stimulation observed with this deletion mutant was not consistent between protein preparations and varied between 4 and 20-fold in comparison to the level of phosphorylation observed with the full-length protein (Fig. 6.8, lanes 4 and 6). Deletion of the proline-rich region between amino acids 226 and 236 (Δ226-236) also resulted in a stimulation of phosphorylation by ~4-fold (Fig. 6.8, lane 3). These results indicate that the β-PAK phosphorylation sites are masked by the tertiary conformation of the full-length protein, perhaps by an intramolecular interaction between the SH3 domain and polyproline regions. Quantification of the phosphorylation of deletion proteins 1-460 and Δ226-236, by placing rehydrated gel pieces in scintilant was not successful, all quantification mentioned above was qualitatively assessed from the autoradiographical data.

The potential β-PAK phosphorylation sites on p67<sup>phox</sup>, as mapped by <i>in vitro</i> kinase assays lie within a region of sequence adjacent to the Rac1 binding site and the also within the polyproline domain (threonine 233). Phosphorylation of p67<sup>phox</sup> by a PAK might affect the local structure of p67<sup>phox</sup> and hence alter the Rac1 binding interaction. Further studies are required to identify whether the phosphorylated state of p67<sup>phox</sup> influences the interaction with Rac. Information obtained would be particularly useful for understanding the mechanism by which Rac acts within the NADPH oxidase enzyme system as it has been reported that p67<sup>phox</sup> becomes phosphorylated <i>in vivo</i> during assembly of the active enzyme complex (Dusi and Rossi, 1993).
Phosphorylation of $p67^{phox}$ by $\beta$-PAK is stimulated by deletion of the C-terminal SH3 domain or the polyproline domain. Simultaneous in vitro solution kinase assays were carried out to investigate the effect of deleting either the C-terminal SH3 domain or the polyproline region of $p67^{phox}$ on phosphorylation by $\beta$-PAK. $\beta$-PAK (100 ng) was incubated alone (lanes 1 and 5), or in the presence of $p67^{phox}$ (lane 2), or deletion mutants: - deleted of the polyproline region amino acids 226-236 ($\Delta 226$-236; lane 3), or deleted of the C-terminal SH3 domain (lanes 4 and 6). Two different preparations of $p67^{phox}$ (amino acids 1-460) were applied to this analysis in a separate assays, (lanes 4 and 6).

**Proteins:**

**Lane 1**, $\beta$-PAK, in the absence of addition,

**Lane 2**, $\beta$-PAK + $p67^{phox}$ (1 µg), (lane 1),

**Lane 3**, $\beta$-PAK + $1-526\Delta 226$-236 (1 µg), (lane 11),

**Lane 4**, $\beta$-PAK + 1-460-GST (1 µg), (lane 12),

**Lane 5**, $\beta$-PAK, in the absence of addition,

**Lane 6**, $\beta$-PAK + 1-460 (5 µg), (lane 13).

Phosphorylated proteins were visualised exposing dried gels to X-Omat film for 1 h (lanes 1-4) or 10 min (lanes 4-6). The different exposure times illustrate the different levels of phosphorylation that occur on the two $p67^{phox}$ proteins containing amino acids 1-460. Italics indicate lanes in Fig. 6.6B in which proteins are separated in SDS-gels and stained with Coomassie Blue.
6.4. Recombinant $p_{47}^{phox}$ is phosphorylated by chelating-Sepharose p68 fraction proteins.

Whether $p_{47}^{phox}$, which is also phosphorylated upon activation of the neutrophil NADPH oxidase, is a PAK substrate, was investigated using native human neutrophil PAK-like protein kinase, present in the p68 fraction purified from human neutrophil cytosol (see Chapter 3), as the source of PAK kinase. Addition of recombinant $p_{47}^{phox}$ to a solution kinase assay in the presence of p68 fraction proteins and Rac1-GTP saw the appearance of phosphorylated protein bands correlating with the position at which $p_{47}^{phox}$ protein migrates on SDS-gels (Fig. 6.9, lane 3, for Coomassie Blue stain of $p_{47}^{phox}$ protein). This suggests that $p_{47}^{phox}$ is a substrate for the protein kinases identified within the p68 fraction.

6.4.1. $\beta$-PAK phosphorylates recombinant $p_{47}^{phox}$ protein.

Although protein fractions purified from neutrophil cytosol containing PAK-related kinase activity were found to phosphorylate recombinant $p_{47}^{phox}$ protein, owing to the nature of the p68 fraction used in this analysis, the action of a kinase unrelated to PAK or other copurifying factor can not be eliminated. As recombinant $\beta$-PAK probe was found to interact with $p_{47}^{phox}$ immobilised on nitrocellulose filter (Chapter 3, section 3.5.2.1), whether a PAK-related kinase could phosphorylate $p_{47}^{phox}$ in \textit{in vitro} kinase assays, was investigated using recombinant $\beta$-PAK protein.

A deletion mutant of $p_{47}^{phox}$ (containing amino acids 204-390) expressed as a GST fusion protein was used for this analysis. The major sites that become phosphorylated on $p_{47}^{phox}$ \textit{in vivo} have been mapped to a number of serine residues within the C-terminal half of $p_{47}^{phox}$ (El Benna \textit{et al.}, 1994). Incubation of $p_{47}^{phox}$ 204-390 protein in kinase buffer with $[\gamma^{32}\text{P}]\text{ATP}$, in the presence of $\beta$-PAK, led to the appearance of phosphorylated protein bands that correlated with the position of $p_{47}^{phox}$ (204-390) on SDS-gels (Fig. 6.10, compare lane 4 in A, with lane 5 in B). The level of phosphorylation observed with $p_{47}^{phox}$ resembled that seen when equivalent amounts of $p_{6}^{phox}$ protein (amino acids 170-238) were added to such an \textit{in vitro} assay (see Fig. 6.6A).
Fig. 6.9. *In Vitro* Phosphorylation of p47<sup>phox</sup> by p68 Fraction Proteins.

p68 fraction (E5) proteins (20 µl; 2 µg) were incubated with [γ<sup>32</sup>P]ATP (10 µCi), with Rac1-GTP; in the absence of additions (lane 1) or in the presence of p47<sup>phox</sup> (lane 2). Proteins were separated by SDS-PAGE and the dried gel was briefly exposed to X-Omat film in order to observe individual phosphorylated protein bands.

**Lane 1**, p68 fraction (2 µg) + Rac1-GTP, in the absence of additions.

**Lane 2**, p68 fraction (2 µg) + Rac1-GTP, in the presence of p47<sup>phox</sup> (5 µg).

**Lane 3**, p47<sup>phox</sup> (5 µg), Coomassie Blue stain. The mobility of phosphorylated protein in lane 2 resembled that of the recombinant p47<sup>phox</sup> which separates as a doublet in SDS-gels, as shown in lane 3. Note, see Chapter 3 for generation of p68 fractions and autophosphorylation of p68 fraction (E5) proteins.
Fig. 6.10. In Vitro Phosphorylation of p47phox and Annexin VI by β-PAK.

A. Kinase Assay

Lane 1, β-PAK-GST (100 ng) was incubated in kinase buffer with [γ32P]ATP (2 μCi) for 15 min at 30 °C, in the absence of additions (lane 1), or in the presence of p57 human coronin-like protein (lane 2), recombinant annexin VI (lane 3) and p47phox C-terminal deletion mutant (amino acids 204-390) as a GST fusion protein (lane 4); 5 μg of each protein was added. To visualise phosphorylated proteins, samples were separated by SDS-PAGE and the dried gels were exposed to X-Omat film for 1 h.

Lane 1, β-PAK
Lane 2, β-PAK + p57 (p57; 5 μg).
Lane 3, β-PAK + annexin VI (A; 5 μg).
Lane 4, β-PAK + p47 (204-390)-GST (p47; 5 μg).

B. Recombinant proteins used in the kinase assays shown in A, were separated by SDS-PAGE and stained with Coomassie Blue.

Lane 1, p57-human coronin-like protein (5 μg),
Lane 2, annexin VI (5 μg),
Lane 3, p47phox (204-390)-GST (5 μg).
6.5. β-PAK phosphorylates recombinant annexin VI protein.

Copurification of annexin VI protein from neutrophil cytosol with PAK related proteins and the ability of $^{32}$P-β-PAK probes to interact with annexin VI immobilised on nitrocellulose filter, suggested that annexin VI might be a substrate for PAK kinases (see Chapter 3). Incubation of recombinant annexin VI in an *in vitro* kinase assay in the presence of β-PAK led to the appearance of phosphorylated protein bands correlating with the position of annexin VI on SDS-gels (Fig. 6.10), suggesting that annexin VI is a potential PAK substrate.

Taken together the results presented in this chapter suggest that $p67^{phox}$, $p47^{phox}$ and annexin VI are potential PAK substrates. That the region of $p67^{phox}$ required for an interaction with β-PAK and the location of the potential β-PAK phosphorylation sites in $p67^{phox}$, lie within close proximity to the Rac binding domain, may indicate that the phosphorylation state of $p67^{phox}$ is important in controlling the $p67^{phox}$-Rac interaction. The stimulation of $p67^{phox}$ phosphorylation by β-PAK upon deletion of the C-terminal half, the polyproline domain or the C-terminal SH3 domain, correlates with the Rac binding data and further suggests that $p67^{phox}$ protein adopts a folded structure *in vitro*, perhaps directed by an intramolecular SH3-polyproline domain interaction.
7. p21 Binding Proteins Associated with the Arp2/3 Complex.

The Rho family proteins RhoA, Rac1 and Cdc42 mediate reorganisation of the actin cytoskeleton in response to various stimuli in many different cell types. These functions are most clearly seen in mammalian fibroblast cell lines. RhoA, Rac1 and Cdc42 regulate the formation of distinct F-actin containing structures, for example stress fibres, lamellipodia and filopodia respectively and also the formation of focal adhesions (RhoA) and focal complexes (Rac1 and Cdc42) at points where cells adhere to the substrate and also where actin filaments meet the cell membrane (Ridley and Hall 1992; Ridley et al., 1992; Nobes and Hall, 1995; Kozma et al., 1995). The mechanism of Rac1 or Cdc42 activity in these processes is unknown. It has been indicated that activation of Rac leads to rapid actin polymerisation as measured by incorporation of monomeric actin into filamentous actin containing structures including lamellipodia and membrane ruffles (Machesky and Hall, 1997). Addition of Cdc42-GTP to cell lysates induced actin polymerisation in an in vitro assay (Zigmond et al., 1997) and Cdc42-GTPγS was able to restore membrane dependent actin polymerisation to cell lysates that had been depleted of Rho family proteins by prior incubation with RhoGDI (Moreau and Way, 1998). This suggests that Rac and Cdc42 can stimulate actin polymerisation.

The Arp2/3 complex components are localised to areas of fibroblast cells undergoing dynamic actin cytoskeletal reorganisation, for example at the leading edge of motile fibroblasts and lamellipodia of PDGF stimulated stationary cells (Welch et al., 1997b; Machesky et al., 1997). The Arp2/3 complex purified from human platelets can direct actin polymerisation at the surface of L. monocytogenes in vitro and is thought to be involved in the generation of polymerised actin required for propulsion of this pathogen through the cytoplasm of an infected cell, by acting in collaboration with a number of host cell proteins including the microfilament and focal adhesion associated protein VASP (Chakraborty et al., 1995; Welch et al., 1997a). The Arp2/3 complex has been shown to stimulate actin filament nucleation and to cap the pointed ends of actin filaments, by using in vitro actin filament polymerisation assays (Mullins et al., 1998a). These observations suggest that the cellular functions of the Arp2/3 complex maybe to promote actin filament polymerisation and possibly provide a link between actin polymerisation and focal complexes.
Studies were carried out to investigate whether the Rho family proteins Cdc42Hs or Rac1 mediate reorganisation of the actin cytoskeleton and formation of focal complexes via the Arp2/3 complex. The seven polypeptide components of the Arp2/3 complex, which share a high level of conservation between yeast, amoebae and mammals (Welch et al., 1997b), were purified from human neutrophil cytosol by column chromatography to near homogeneity, and the presence of associated Cdc42Hs or Rac1 binding proteins was investigated using p21 binding assays.

7.1. Association of Cdc42Hs and Rac1 binding proteins with the Arp2/3 complex components purified from human neutrophil cytosol.

Proteins in fractions from the mono-S column (10-18) or the Superose-12 column (ii) (36-48), in which the Arp2/3 complex components were present with 37 and 58 % purity respectively (see appendix A.5), were separated by SDS-PAGE and transferred to nitrocellulose by western blotting. Filters were probed with Rac1-Q61L-[γ32P]GTP and Cdc42Hs-Q61L-[γ32P]GTP probes, or anti-p57 (human coronin-like protein) and β-PAK antibodies, (anti-p57 and anti-β-PAK respectively), see Fig. 7.1.

7.1.1. Rac1 binding proteins.

Three Rac1 binding proteins were detected in the mono-S column fractions that contain the Arp2/3 complex proteins. These proteins were predominantly observed in fraction 10, and migrated on SDS-gels with apparent molecular masses of approximately 65, 60 and 47 kDa. The 65 kDa protein which also appeared to bind Cdc42Hs might be a PAK-like protein not detected with the β-PAK antibody used in this study. The migration of this protein, in SDS-gels, resembles that of PAK-like proteins identified in neutrophil cytosol, see Chapter 3. Alternatively the 65 kDa protein may represent a novel Rac1 and Cdc42Hs target protein. That fraction 10 does not appear to contain β-PAK immunoreactivity could be explained by insufficient transfer of protein to nitrocellulose. Upon close inspection of the western analysis shown in Fig. 7.1, anti-β-PAK signals, that may be due to the presence of partial protein bands can be seen. This could also explain the unusual elution profile of β-PAK immunoreactivity, observed during this analysis.

The 60 kDa Rac1 binding protein detected in fraction 10 migrated with similar mobility on SDS-gels to the anti-p57 immunoreactivity identified in this fraction. The p57 antibody recognised a single band in neutrophil cytosol, mono-S column fractions (9-13)
and Superose-12 column fractions (42-48) at ~60 KDa. This data suggested that p57 may be a target for Rac1. Binding of Rac1 to the 60 kDa protein was weak and the absence of Rac1 signals in the Superose-12 column fractions containing p57 immunoreactivity, could be explained by there being insufficient protein present. That p57 is a Rac1 target protein was investigated further using recombinant protein (see section 7.2). The 47 kDa Rac1 binding protein may be a breakdown product of PAK-like kinases as previously identified in neutrophil cytosol (Chapter 3), further characterisation is required to identify this protein.

7.1.2. Cdc42Hs binding proteins.
Cdc42Hs probes detected proteins between 60 and 68 kDa in the mono-S column fractions 10, 12 and 13. Although not all the mono-S column fractions were tested with Cdc42Hs probes due to limited availability of samples, the data collected indicates that the majority of Cdc42Hs binding activity was in the mono-S column fraction 10. A shorter exposure time of the experiment shown in Fig. 7.1, demonstrates that this binding activity is predominantly due to a ~65 kDa protein and to a lesser extent to a 60 kDa protein that interacted with significantly lower affinity in this assay. A single protein band in Superose-12 fractions 42-46 was detected with Cdc42Hs probes at approximately 65 kDa. This analysis suggests that a Cdc42Hs binding protein copurifies with the Arp2/3 complex components from human neutrophils. Although further analysis is required to establish the nature of the association of this 65 kDa Cdc42Hs binding protein with the Arp2/3 complex, this protein could provide the link between Cdc42Hs and the actin cytoskeleton.

7.1.3. β-PAK immunoreactivity.
Western analysis of mono-S column fractions 9-13 using antibody raised against recombinant β-PAK protein detected proteins of 68, 65, 62 and 47 kDa in fraction 11 and predominantly the 68 kDa protein in fractions 12 and 13. The presence of a human isoform of rat brain β-PAK in neutrophil cells has recently been suggested using PAK isoform specific antibodies (K. Marler and S. Ahmed unpublished observations). The 62 kDa β-PAK immunoreactive protein was also observed in the Superose-12 column fraction 44. This suggests that the β-PAK antibody preparation used in this analysis detects either proteins present in differently phosphorylated states, or multiple degradation products of PAK protein. However, that the 62 kDa protein detected by this
Fig. 7.1. Purification of Rac1 and Cdc42Hs Binding Proteins with the Arp2/3 Complex from Human Neutrophils.

<table>
<thead>
<tr>
<th></th>
<th>Mono-S</th>
<th>Superose-12 (Gel-filtration)</th>
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</thead>
<tbody>
<tr>
<td>Cyt</td>
<td>9</td>
<td>36</td>
</tr>
<tr>
<td></td>
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<td></td>
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<td>46</td>
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<td></td>
<td></td>
<td>48</td>
</tr>
</tbody>
</table>

### Coomassie stain

### Rac1

### Cdc42Hs

### Anti-p57

### Anti-β-PAK

**Right Panel; Mono-S column fractions: - 9, 10, 11, 12, and 13.** Note fraction 9 and 11 were not probed with Cdc42Hs probes.

**Left Panel; Superose-12 (gel-filtration) column fractions: - 36, 38, 40, 42, 44, 46, and 48.** Note fraction 36 was not probed with Rac1 probes.
antibody was the only protein detected in the Superose-12 column fraction 44, possibly indicates a specific purification of this protein with the Arp2/3 complex components. This suggests that PAK-like proteins associate with Arp2/3 complex proteins in human neutrophils.

7.2. Rac1 interacts with a human coronin-like protein (p57). Fractions from the mono-S column, of the series of chromatographic stages followed to purify the Arp2/3 complex components from human neutrophil cytosol, were found to contain Rac1 binding proteins (section 7.1). Fractions from the mono-S column contain stoichiometric amounts of each of the Arp2/3 complex components with an approximate purity of 37%, with the human coronin-like protein p57 representing the most abundant copurifying factor (Machesky et al., 1997). The 60 kDa Rac1 binding activity in the mono-S column fraction 10 was found to comigrate in SDS-gels with anti-p57 immunoreactivity suggesting that the human coronin-like protein was a Rac1 binding protein (see section 7.1). Further investigations were carried out to determine whether p57 is a Rac1 binding protein using recombinant p57 protein purified from insect Sf9 cells.

Rac1 probes bound recombinant p57 protein separated by SDS-PAGE and immobilised on nitrocellulose filter (Fig. 7.2). The signal observed with Rac1 probes from analysis of the autoradiograph comigrated with anti-p57 immunoreactivity in this sample and was qualitatively found to be of similar strength to that observed with p67phox protein (Fig. 7.2). Recombinant p57 protein was also found to bind Rac1 probes when dot blotted onto nitrocellulose (Fig. 7.4). As with p67phox proteins the binding interaction was more easily detected when protein was applied to nitrocellulose using the latter technique. This indicated that the structural requirements of p57 for an interaction with Rac1 were susceptible to denaturation during the process of SDS-PAGE. Recombinant p57 protein did not interact with Cdc42Hs probes, indicating that it represents a potential Rac1 specific binding partner.

Analysis of the sequence of human p57 and Dictyostelium coronin proteins identified a region of sequence that could be aligned with the p21 binding domains of PAK and p120ACK, that contain the Cdc42/Rac interactive binding ‘CRIB’ motif (Burbelo et al., 1995) (Fig. 7.3). Interestingly, the sequence of p57 shared some similarity with the Rac1
Fig. 7.2. Racl Binding to Recombinant p57 (Human Coronin-Like Protein).

**Fig. 7.2. Racl binding to recombinant p57 (human coronin-like protein).**

Recombinant p67<sup>phox</sup> purified from E. coli (lane 1; 5 µg), or p57 protein purified from insect cells (lanes 2-4; 5 µg) were separated by SDS-PAGE and transferred to nitrocellulose filter. Following renaturation (lanes 1, 2, and 3) or blocking in 5 % milk (lane 4), filters were probed with Rac1-Q61L-[γ<sup>32</sup>P]GTP (lanes 1 and 2), Cdc42-Q61L-[γ<sup>32</sup>P]GTP (lane 3), or anti-p57 antibodies (α-p57) diluted 1:2000 (lane 4). Signals were visualised by exposure of filters to X-Omat film for 16 h lanes 1-3, or using ECL reagents (Amersham), lane 4.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Probe</th>
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<tr>
<td>Lane 1, p67&lt;sub&gt;phox&lt;/sub&gt;</td>
<td>Rac1-Q61L-[γ&lt;sup&gt;32&lt;/sup&gt;P]GTP,</td>
</tr>
<tr>
<td>Lane 2, p57</td>
<td>Rac1-Q61L-[γ&lt;sup&gt;32&lt;/sup&gt;P]GTP,</td>
</tr>
<tr>
<td>Lane 3, p57</td>
<td>Cdc42Hs-Q61L-[γ&lt;sup&gt;32&lt;/sup&gt;P]GTP,</td>
</tr>
<tr>
<td>Lane 4, p57</td>
<td>Anti-p57 antibody.</td>
</tr>
</tbody>
</table>
Fig. 7.3. Sequence Alignment of Coronin, p57 and p67phox.

PAK ISLP S . . FEHTIHVGFDAVTGEF
ACK ISQPLQNSFIHTGHGDSDPRHCWG
Coronin 118 ISTPLQTLSGHKRKVGTISFGPVV

Consensus

120 LREPVVTLEGHTKRVGIWAHTTA
p57 120 LREPVVTLEGHTKRVGIWAHTTA143

p67phox 171 LYE PVVIPVGKLFRPNEROVAQLA194

Fig. 7.3. Sequence alignment of coronin, p57 and p67phox. Sequence alignment of coronin with the Cdc42/Rac interactive binding ‘CRIB’ motif of PAK and p120ACK (ACK) showing the consensus (as described by Burbelo et al., 1995). Coronin sequence (amino acids 118-141) contains six of the ‘CRIB’ motif consensus residues (which are shown in bold) and additional identity with the sequence of ACK (underlined residues).

The sequence of human coronin-like protein, p57 (amino acids 120-143) contains four of the consensus residues of the ‘CRIB’ motif (shown in bold). In addition amino acids 120-143 of p57 share 8 common residues with the sequence of p67phox (amino acids 171-194), in which the Rac binding site has been located, these residues are highlighted in yellow.
Fig. 7.4. Rac1 Binding to Dot Blotted p57 and Coronin Peptide (A1).

A. Recombinant p57, human coronin-like protein (5 μg, 90 pmoles) was dot blotted onto nitrocellulose filter and probed with Rac1-Q61L-[γ32P]GTP probes (p57 was present at 22 nM in the binding incubation).

B. Coronin peptide A1, bradykinin and GST 25, 50 and 100 μg (columns 1-3 respectively) were dot blotted onto nitrocellulose filter, as indicated at the left-hand end of each row. Following renaturation for 2 h at 4 °C filters were incubated with Rac1-Q61L-[γ32P]GTP probes.

A1 peptide was present in the probing incubations at 2.9, 5.8, and 11.5 μM, columns 1-3 respectively. Filters were exposed to X-Omat film for 16 h, to visualise signals. Data shown is representative of at least two separate experiments.
binding sequence of p67phox (Fig. 7.3). Peptides of 16 amino acid residues based on the sequences at equivalent sites in coronin (amino acids 118-133) and p57 (amino acids 120-135) were tested for Rac1 binding. Peptide A1 was based upon the sequence of Dictyostelium coronin and A2 upon the equivalent sequence of the human homologue of coronin (p57).

**Peptides**

**A1 Dictyostelium (coronin)**

<table>
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<tr>
<th>118</th>
<th>I</th>
<th>S</th>
<th>T</th>
<th>P</th>
<th>L</th>
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**A2 Human (p57)**

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<th>120</th>
<th>L</th>
<th>R</th>
<th>E</th>
<th>P</th>
<th>V</th>
<th>V</th>
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Peptide A2 could not be dissolved in the various solutions tested so the Rac1 binding properties could not be investigated. Peptide A1 (coronin) was found to interact with Rac1 when dot blotted on to nitrocellulose. Detection of a signal required the presence of at least 25 μg (~11 nmoles) of peptide, in comparison with 90 pmoles of recombinant p57. Signals increased with the amount of peptide applied and were not detected when equivalent μg amounts of either GST protein or control peptide (Bradykinin) were used, suggesting that the interaction observed was specific. As with the full-length recombinant human p57 protein, signals were not seen when Cdc42Hs probes were applied. Taken together, these data imply that coronin and human p57 contain sequences able to interact specifically with Rac1.

This work has identified the presence of Rac1 and Cdc42Hs binding proteins, that associate with the Arp2/3 complex during chromatographic purification from human neutrophil cytosol. These p21 binding proteins which include PAK-like protein and p57 could provide a link between Cdc42Hs or Rac1 and the Arp2/3 complex, that may be involved in the signalling mechanism by which these Rho family proteins mediate changes in the organisation of the actin cytoskeleton.
Chapter 8
Discussion
8.1. The Rho family of Ras-related GTPases.

Members of the Ras superfamily of GTP-binding proteins act as molecular switches, by cycling between GTP and GDP-bound states, to control a diverse array of cellular functions in response to extracellular signals. Rho family p21s, which includes RhoA, Cdc42Hs, Rac1 and Rac2, have been implicated in controlling a wide variety of cellular processes. These include organisation of the actin cytoskeleton, transcriptional activation, cell cycle progression and activation of the NADPH oxidase of phagocytic cells (Hall, 1998; Van Aelst and D'Souza-Schorey, 1997). Rac protein was found to account for the GTP-dependency of the NADPH oxidase enzyme system of phagocytic leukocytes, and is required to be in the GTP-bound state to stimulate superoxide production \textit{in vitro} (Abo et al., 1992; Knaus et al., 1992). This suggests that the downstream Rac effect within the NADPH oxidase is GTP-dependent.

Members of the Ras superfamily share approximately 30% sequence identity, with marked conservation observed in regions of sequence involved in GTP-binding. These regions include the ‘effector domain’, which participates in the binding of downstream effectors of Ras such as Raf (Marshall, 1993). The sequence similarity between the ‘effector domain’ regions of Rho family proteins and Ras, suggests that the effector function of this region is conserved in Rho proteins. However, information obtained from comparing the crystal structures of Rac1 and H-Ras, suggests that the effector domain loops of Rac1 and H-Ras are structurally different (Hirschberg et al., 1997). Members of the Rho family also contain a region of sequence not found in other Ras-related proteins, known as the ‘insert region’. This region spans amino acids 120-137 and is predicted to form a highly charged surface loop (loop 8) near the GTP binding site (Valencia et al., 1991; Hirschberg et al., 1997).

The structural differences of the two regions mentioned above may confer effector specificity of Ras-related proteins. Chimaeric proteins of Ras and Rho were used to investigate the role of their ‘effector domains’ in downstream effects. Rho, with residues 23-46 replaced with the corresponding sequence of Ras, was able to induce transformed foci in NIH-3T3 fibroblast monolayers, albeit to a fraction of the extent seen with Ras-G12V. However, Ras substituted with amino acids 25-48 of Rho did not induce stress fibre formation when injected into MDCK cells (Self et al., 1993), thus suggesting that Rho protein has a second ‘effector domain’ required for biological activity. Site directed
mutagenesis and deletion analysis have suggested that the 'insert region' of Rac is important for the effector function of Rac involved in the activation the NADPH oxidase (Freeman et al., 1996).

8.2. Rho family interacting proteins.

In addition to interacting with regulatory proteins (GAPs, GEFs and GDIs), Rho family members interact with proteins that bind specifically to the GTP-bound form. These binding partners, which include PAKs (Manser et al., 1994), can act to inhibit the intrinsic GTPase activity and may function as downstream effectors. In order to understand the mechanisms by which Rho family proteins function in signalling pathways, it is important to identify targets for these GTP-binding proteins. Target proteins that interact preferentially with the GTP-bound forms of these p21s could represent effector molecules. Different approaches including p21 binding assays, yeast two-hybrid screening, affinity chromatography, database searches and genetic analysis have identified proteins that interact with Rho family members (see below; Table 8.2). Binding assays which utilise \[^{32}P\]-GTP-p21 probes have enabled the identification of Rho family target proteins in different rat tissue extracts, including members of the PAK family of protein kinases (Manser et al., 1994). A large family of PAK-related proteins has now been identified, which includes S. cerevisiae Ste20, three different mammalian PAK isoforms and members in a variety of different organisms (Lim et al., 1996; table 8.1(ii)). PAK proteins are activated in vitro upon interaction with Rac and Cdc42Hs in a GTP-dependent manner, and thus may represent downstream effectors for these p21s.

8.3. p21 binding proteins in human neutrophils.

In this study a number of binding partners for Rho family GTPases were identified in human neutrophils using p21 binding assays. Neutrophil cytosol contains Rac1 and Cdc42Hs binding proteins between 60 and 68 kDa that preferentially bind to the GTP-bound state of these p21s. Fractionation of neutrophil proteins identified (i) Cdc42Hs binding partners that associate with membrane-cytoskeletal proteins, and (ii) Cdc42Hs and Rac1 binding proteins that associate with the Arp2/3 complex purified from neutrophil cytosol. Whether the neutrophil cytosolic 60-68 kDa Cdc42Hs and Rac1 binding proteins relate to PAK proteins was investigated using a number of different methods, including purification of the Cdc42Hs binding activity from neutrophil cytosol.
8.3.1. PAKs in human neutrophils.

The major Cdc42Hs binding proteins in rat brain have been shown to be PAKs (Manser et al., 1994). The relationship between the 60-68 kDa Cdc42Hs binding proteins in neutrophil cytosol and those identified in brain cytosol was investigated by carrying out 2-dimensional gel analysis. An almost identical pattern of Cdc42Hs binding protein spots was observed when proteins from neutrophil and brain cytosol were compared. This suggests that the 60-68 kDa Cdc42Hs binding proteins in neutrophil cytosol are related to PAK proteins in brain.

Purification of the Cdc42Hs binding activity from neutrophil cytosol yielded protein fractions that contained 60-68 kDa Cdc42Hs and Rac1 binding proteins. Protein in fraction E5 from the chelating-Sepharose column, visualised by Coomassie Blue staining, migrated on SDS-gels with the 68 kDa (BSA) molecular mass marker. Hence this and similar fractions were referred to as p68 fractions. p68 fractions contained kinase activity at ~68 kDa that was stimulated approximately 10-fold upon the addition of Rac1-GTP. The autophosphorylated 68 kDa protein was immunoprecipitated from p68 fractions by α-PAK specific antibodies. This suggests that the 68 kDa Cdc42Hs and Rac1 binding protein was PAK-related. To confirm that the p68 fraction contained an α-PAK-related protein, western analysis was carried out using affinity purified antibodies. Proteins in the p68 fraction were detected by antibodies raised against recombinant α-PAK protein but not by p67\textsuperscript{phox} antibodies, demonstrating that p68 fraction proteins are related to α-PAK. Thus neutrophil cytosol contains proteins related to the previously described brain specific α-PAK. It was estimated that PAK proteins were present in the p68 fractions obtained in the chelating-Sepharose column fractions with 10-25 % purity (Table 3.1).

Three protein bands were detected in the p68 fraction with antibodies raised against a peptide from the kinase domain of Ste20 protein (amino acids 738-752). The kinase domains of Ste20 and α-PAK share ~70 % sequence identity, and the peptide sequence used for Ste20 antibody generation differs from rat brain α, β, and γ-PAK sequences at only three residues (see appendix A.8). Therefore it is likely that this antibody may recognise different isoforms of PAK. In addition to detecting three proteins at ~62, 65 and 68 kDa in neutrophil cytosol and the p68 fraction, the Ste20 antibody detected recombinant α and β-PAK-GST proteins. The three Ste20 immunoreactive proteins appeared to comigrate on SDS-gels with the Cdc42Hs binding activity detected in the
p68 fraction. Ste20 antibody also detected proteins in rat brain cytosol, which appeared to migrate at ~65 and 68 kDa in SDS-gels. These findings correlated with the report that α and β-PAK (68 and 65 kDa) isoforms are highly abundant in brain (Manser et al., 1994), whereas γ-PAK (62 kDa) is ubiquitously expressed (Teo et al., 1995). That the Ste20 antibody recognised 3 proteins in the p68 fraction may suggest the presence of different isoforms of PAK, which purify from neutrophil cytosol with similar characteristics through the columns used in this study. Alternatively the multiple protein bands detected by the Ste20 antibody could represent differently phosphorylated forms or degradation products of the same protein.

Cellular fractionation of neutrophil proteins to generate fractions enriched for cytosolic, membrane and membrane-cytoskeletal associated proteins was carried out in an attempt to localise Cdc42Hs and Rac1 binding proteins. The distribution of p21 binding proteins in neutrophils was compared to that of undifferentiated HL60 cells, to determine whether these promyelocytic cells (which can be differentiated to resemble the characteristics of a mature neutrophil) would be useful for the study of p21 binding proteins. The 60-68 kDa Cdc42Hs and Rac1 binding proteins were found predominantly in the cytosolic fraction of both neutrophils and HL60 cells, and this distribution correlated with α-PAK immunoreactivity. This suggests that both neutrophils and HL60 cells contain PAK proteins, which mainly reside in the cytosol of resting cells, and that HL60 cells would provide a useful tool for the investigation of p21 interacting proteins. Further analysis is required to identify whether the distribution of these proteins varies upon cellular stimulation.

Martin et al., (1995) reported the presence of three Cdc42Hs binding proteins in neutrophil cytosol between 60 and 68 kDa. Peptide sequence obtained from a 65 kDa Cdc42Hs binding protein purified from neutrophil cytosol was used to isolate a clone from a human placental cDNA library. Sequence obtained from this clone was found to have homology to the kinase domain of PAK and the yeast protein Ste20. This protein was designated as hPAK65 and appears to represent the human homologue of the ubiquitously expressed rat γ-PAK (Martin et al., 1995; Teo et al., 1995).

The presence of two PAK isoforms, α-PAK (PAK1) and γ-PAK (PAK2), in human neutrophils has also been reported (Knaus et al., 1995). Antibodies raised to a GST
fusion protein containing amino acids 175-306 of rat α-PAK (a region of sequence which is divergent between PAK proteins), or to the N-terminal of rat γ-PAK (amino acids 1-252) recognised distinct proteins in neutrophil cytosol (Knaus et al., 1995). This suggests the presence of more than one human neutrophil PAK protein. Western analyses of neutrophil cytosol using isoform specific antibodies have demonstrated the presence of α, β and γ-PAK isoforms in human neutrophils (K. Marler and S. Ahmed, unpublished observations).

8.3.2. The PAK family.

Isoforms of rat α, β and γ-PAK from other mammalian species have been cloned and sequenced, including two human PAK isoforms, designated PAK1 and hPAK65/PAK2 (see Table 8.1(i) for nomenclature). Human PAK1 differs in sequence from α-PAK at just 8 residues and shares an overall identity of 98 % with α-PAK (Brown et al., 1996), whereas hPAK65/PAK2 shares 78 % identity with rat brain α-PAK and is more similar to the ubiquitous γ-PAK (Martin et al., 1995). A PAK isoform isolated from rabbit tissues, designated PAKI, was found to share 95 % sequence identity with hPAK65 and 78 % with α-PAK (Jakobi et al., 1996). An isoform from mouse (mPAK3), which was found to share 81 % identity with α-PAK, was considered to be most similar to β-PAK (Bagrodia et al., 1995a).

The PAK family also includes many non-mammalian members in a variety of organisms including S. cerevisiae and S. pombe, Acanthamoeba, Dictyostelium, C. elegans, and Drosophila (Table 8.1(iii)) and also members of the germinal centre kinase (GCK) family in both humans and yeast (Sells and Chernoff, 1997). Across these species, PAK proteins share high sequence identity within their kinase domains and also within a 60 amino acid region termed the PAK N-terminal (PAN) motif (Zhao et al., 1998). The PAN motif contains the p21 binding site, encompassing the Cdc42/Rac interactive ‘CRIB’ motif (Burbelo et al., 1995). However in some cases divergence from this domain structure is observed. For example, the human homologue of Ste20 (MST1) lacks the PAN motif (Creasy and Chernoff, 1995), and S. cerevisiae Cla4 contains an N-terminal PH domain (Cvrckova et al., 1995).
Table 8.1. on reverse.
Table 8.1(i). PAK Isoforms

<table>
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<th>Nomenclature given to PAK isoforms in different mammalian species</th>
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<tr>
<td>rat</td>
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<tr>
<td>α-PAK</td>
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<tr>
<td>β-PAK</td>
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<tr>
<td>γ-PAK</td>
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<tr>
<th>PAK Isoform</th>
<th>Kinase domain aas (% ID)</th>
<th>PAN Motif aas (% ID)</th>
<th>Notes</th>
<th>Reference</th>
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<td>71-131 (100)</td>
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<td>Manser et al., 1994</td>
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<td>PAK1 human</td>
<td>270-516</td>
<td>71-132</td>
<td>Differs from α-PAK at only 8 residues</td>
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<td>β-PAK Rat</td>
<td>254-528 (96)</td>
<td>70-126 (94.7)</td>
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<td>Manser et al., 1995a</td>
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<td>mPAK3 mouse</td>
<td>254-527</td>
<td>70-127</td>
<td>81% identity to α-PAK, most similar to β-PAK</td>
<td>Bagrodia et al., 1995a</td>
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<td>γ-PAK Rat</td>
<td>235-509 (92.7)</td>
<td>74-130 (94.7)</td>
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<td>78 % identity to α-PAK</td>
<td>Sells et al. 1997</td>
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<td>52-113 (92)</td>
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<td>Martin et al., 1995</td>
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<td>74-130</td>
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### Table 8.1(ii).

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<td>606-881 (68.1)</td>
<td>337-393 (52.6)</td>
<td>N-terminal PH domain (62-178)</td>
<td>Ramer and Davis, 1993</td>
</tr>
<tr>
<td>Cla4 (Sc)</td>
<td>551-834 (58)</td>
<td>184-240 (49.1)</td>
<td>N-terminal PH domain</td>
<td>Cvrckova et al., 1995</td>
</tr>
<tr>
<td>ScPAK (Sc)</td>
<td>363-648 (55.6)</td>
<td>123-179 (49.1)</td>
<td>N-terminal PH domain</td>
<td>Cvrckova et al., 1995</td>
</tr>
<tr>
<td>DPAK (Dm)</td>
<td>416-690 (82.5)</td>
<td>83-139 (78.9)</td>
<td></td>
<td>Chen et al., 1996</td>
</tr>
<tr>
<td>CePAK (Ce)</td>
<td>278-552 (76)</td>
<td>67-123 (71.9)</td>
<td></td>
<td>Otitilie et al., 1995</td>
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<tr>
<td>Pak1/Shk1 (Sp)</td>
<td>372-646 (62.5)</td>
<td>149-203 (61.4)</td>
<td>N-terminal PH domain</td>
<td>Sells et al., 1998; Yang et al., 1998</td>
</tr>
<tr>
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<tr>
<td>MIHCK (Ac)</td>
<td></td>
<td></td>
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<td>Brzeska et al., 1996</td>
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<tr>
<td>MIHCK (Dd)</td>
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<td></td>
<td>Myosin I heavy chain kinase</td>
<td>Lee et al., 1996</td>
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<tr>
<td><strong>Germinat Centre Kinase subfamily</strong></td>
<td></td>
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<tr>
<td>GC kinase (Hs)</td>
<td>16-290 (43)</td>
<td>none</td>
<td>Germinal centre (GC) kinase</td>
<td>Katz et al., 1994</td>
</tr>
<tr>
<td>hMST1 (Hs)</td>
<td></td>
<td>none</td>
<td>mammalian Ste20-like</td>
<td>Creasy and Chernoff, 1995</td>
</tr>
<tr>
<td>SpS1 (Sc)</td>
<td>4-281</td>
<td>none</td>
<td></td>
<td>Friesen et al., 1994</td>
</tr>
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</table>

Table 8.1. PAK isoforms. (i) Mammalian PAK isoforms, the top panel shows the nomenclature ascribed to the various proteins identified, the bottom panel lists the mammalian PAK proteins, grouped according to sequence identity. (ii) The PAK family includes non-mammalian PAKs in a variety of organisms ranging from yeast to worms as indicated. In (i) and (ii), the second and third columns show details of the kinase domain and PAK N-terminal (PAN) motif. The position of each domain is indicated (aas- amino acid residues) and the percentage identity (ID) of that region in comparison to rat α-PAK is given in brackets. HMSTI (human mammalian ste20 like protein) SpS1 and GC kinase do not contain PAN motifs and some proteins contain an N-terminal pleckstrin homology (PH) domain as indicated. Hs, human; Sc, S. cerevisiae; Dm, Drosophila; Sp, S. pombe; Ce, C. elegans; Ac, Acanthamoeba; Dd, Dictyostelium.
8.3.2.1. PAK activation.

Binding to either GTP-Rac or Cdc42 stimulates autophosphorylation of PAK on serine and threonine residues and leads to subsequent kinase activation \textit{in vitro} (Manser \textit{et al.}, 1994). Two lines of evidence suggest that PAK activation involves detachment of an N-terminal inhibitory element. Firstly, proteolytic cleavage of S6/H4 kinase (which shares similarity with \(\gamma\)-PAK) followed by autophosphorylation, yields an active kinase fragment \textit{in vitro} (Benner \textit{et al.}, 1995). Secondly, a truncation allele of Ste20, deleted of the N-terminal was found to activate the mating response in \textit{S. cerevisiae} in the absence of exogenous mating pheromone, and was able to suppress the mating defect of a strain in which the Ste20 kinase domain had been deleted (Ramer \textit{et al.}, 1993), suggesting that this mutant is constitutively active.

Mutational analysis of the PAN motif has identified the presence of a conserved negative regulatory region in \(\alpha\)-PAK, and a model for PAK activation by p21 binding has been suggested. Mutation of residues within the C-terminal of the PAN motif of \(\alpha\)-PAK (amino acids 101-137) which resulted in the production of a constitutively active kinase, and the use of deletion constructs of \(\alpha\)-PAK (containing sequences from the PAN motif) that blocked PAK activation \textit{in vitro}, has located the presence of an inhibitory domain, which lies immediately C-terminal to the p21-binding domain (Zhao \textit{et al.}, 1998). That the deletion constructs used in this analysis were unable to inhibit the activity of autophosphorylated PAK suggests that the inhibitory domain does not function by acting as a pseudosubstrate site for the kinase domain, but instead forms a complex with the kinase domain rendering it inactive. Thus mutations in the \(\alpha\)-PAK PAN motif that reduce the requirement for p21 binding for activation and result in a constitutively active kinase, could act by altering the conformation of the inhibitory domain. It could be suggested that p21 binding induces a conformational change in the inhibitory motif that relieves the interaction with the kinase domain, resulting in autophosphorylation of particular residues in the regulatory region of the kinase domain, shown to occur prior to subsequent kinase activity (Manser \textit{et al.}, 1997).

Further analysis has suggested that PAK proteins may be activated by p21-independent mechanisms. Over expression of PAK1 with the adapter protein Nck in 293T-cells led to direction of PAK to the membrane, and resulted in a 6-10 fold increase in kinase activity that was independent of exogenous Cdc42 (Lu \textit{et al.}, 1997). Thus it was considered that
PAKs may be activated by the action of biologically active lipids. Sphingosine was found to stimulate PAK activation in a concentration dependent manner in vitro, and treatment of COS-7 cells expressing PAK1 with factors such as sphingosine, fumonisin B, or sphingomyelinase, which act to increase the cellular level of sphingosine was found to increase the kinase activity of PAK (Bokoch et al., 1998). The biologically inactive sphingosine derivative N,N-dimethylsphingosine, inhibited the ability of either sphingosine or Cdc42 to activate PAK, suggesting that the action of lipids may resemble the p21 mechanism of PAK activation. In vitro stimulation of PAK1 was also induced by phosphatidic acid and phosphatidylinositol (Bokoch et al., 1998).

In vitro analysis of the p21 binding activities of PAK proteins perhaps indicates important differences between the functions of PAK isoforms. Like rat brain purified PAK, recombinant β-PAK has decreased affinity for p21 binding once phosphorylated, suggesting that both are released from the p21 once activated (Manser et al., 1995a). In contrast, recombinant α-PAK or purified rat γ-PAK and hPAK65 do not exhibit reduced p21 binding following activation (Manser et al., 1995a; Teo et al., 1995; Martin et al., 1995). This suggests that the distinct isoforms of PAK may act differently downstream of Cdc42 or Rac.

8.3.2.2. PAK functions.
Ste20 is required for pheromone mating in S. cerevisiae (Ramer et al., 1993; Leberer et al., 1992), and was found to interact with Cdc42 via a sequence with similarity to the p21 binding motifs of p120CαK and PAK (Manser et al., 1994). Cdc42 has an important role in the yeast pheromone response pathway and has been shown to act upstream of Ste20 (Zhao et al., 1995; Simon et al., 1995). Ste20 acts as the first kinase in the pheromone response kinase cascade, upstream of Ste11, Ste7 and the MAP kinase Fus3, which phosphorylates and activates the transcription factor Ste12, leading to the expression of pheromone response elements (Herskowitz, 1995). Expression of PAK protein in Ste20-null yeast restored the mating defects of this phenotype (Brown et al., 1996), suggesting that PAK proteins might act within MAP kinase cascades that signal to the nucleus. The use of constitutively active PAK mutants have suggested that PAK proteins are involved in the signalling pathways downstream of Rac and Cdc42, leading to activation of the stress activated JNK/SAPK and p38 MAP kinases in mammalian cells (Bagrodia et al., 1995b; Zhang et al., 1995). Expression of the constitutively active
PAK1(L107F) in COS-7 cells stimulated the kinase activity of JNK1 towards c-jun to levels comparable to those observed upon expression of active Cdc42 or Rac1, or following treatment of cells with ultraviolet light (Brown et al., 1996).

The Rho family proteins Cdc42, Rac and RhoA have been shown to mediate cytoskeletal organisation in cultured cells in response to extracellular stimuli, for example growth factors. Many groups have investigated the involvement of PAKs in coordinating control of the actin cytoskeleton and there are a number of reports that suggest a role for this family of kinases in regulating cell morphology.

Injection of PAK1 into Swiss 3T3 fibroblasts rapidly induces the formation of polarised actin structures, such as filopodia and membrane ruffles, resembling structures documented to be induced by Cdc42 and Rac1 respectively (Sells et al., 1997). The morphological changes promoted by expression of active PAK1 in Swiss 3T3 cells resembled those seen in motile fibroblasts. The simultaneous induction of filopodia and retraction fibres observed mimicked the phenotype seen upon injection of Swiss 3T3 cells with active Cdc42 (Kozma et al., 1995). PAK was found to localise to focal complex structures induced by active Cdc42 and Rac1 in HeLa cells. Additionally, active PAK induced loss of stress fibres, caused disruption of focal adhesion complexes and led to cell retraction, with similar activities as seen upon injection of active Cdc42 and Rac1 proteins in this cell type (Manser et al., 1997). Collectively the evidence strongly suggests a function for PAK proteins in Rho family signalling pathways involved in the reorganisation of the actin cytoskeleton.

Both PAK1 and PAK2 proteins were activated following fMLP stimulation of human neutrophils, and this activation was found to be downstream of pertussis toxin sensitive G-protein coupled receptors (Knaus et al., 1995). PAK proteins immunoprecipitated from unstimulated or fMLP stimulated guinea pig neutrophil lysates, using antibodies raised against rat α-PAK, were found to possess activities that resembled those of a group of renaturable kinases, between 60 and 70 kDa, that participate in neutrophil NADPH oxidase activation (Ding et al., 1996; Lian et. al., 1997). This indicates that PAK proteins may play a role in the activation of the neutrophil NADPH oxidase.
8.3.3. p21 binding proteins associated with membrane-cytoskeletal proteins in neutrophils.

A number of Cdc42Hs binding proteins were identified in neutrophil fractions that were enriched for membrane-cytoskeletal proteins at approximately 23, 27, 33, 43, 57, 65, 68 and 80 kDa. These proteins did not bind Rac1 probes and were not detected in the equivalent fraction of HL60 cells with Cdc42Hs probes. This suggests that these proteins represent specific cytoskeletal-associated Cdc42Hs target proteins in neutrophils that may not be expressed in undifferentiated HL60 cells. Analysis of proteins from HL60 cells differentiated towards mature neutrophils, is required to determine whether specific p21 target proteins are expressed following differentiation.

8.3.4. p21 binding proteins associate with the Arp2/3 complex from neutrophils.

Members of the Rho family of GTPases are involved in regulating the organisation of the actin cytoskeleton. In fibroblasts, activation of Rho leads to the formation of stress fibres and focal adhesion complexes (Ridley and Hall, 1992; Nobes and Hall, 1995), whereas activation of Rac induces the formation of peripheral F-actin rich structures such as lamellipodia and membrane ruffles (Ridley et al., 1992), and activation of Cdc42 leads to the generation of filopodia (Kozma et al., 1995). In addition, the cytoskeletal changes induced by Rac and Cdc42 are associated with the formation of focal complexes. The mechanism of action of these p21 proteins in controlling the actin cytoskeleton is unknown. Activation of Rac led to the incorporation of monomeric actin into F-actin rich structures at the cell periphery, including lamellipodia and membrane ruffles (Machesky and Hall, 1997). Monomeric actin was not incorporated into Rho stimulated stress fibres, which suggests that rather than induce actin polymerisation, Rho stimulates the bundling of actin filaments into the thick F-actin cables (Machesky and Hall, 1997). Addition of Cdc42-GTP to cell lysates induced actin polymerisation in an *in vitro* assay (Zigmond et al., 1997). Cdc42-GTPyS was able to restore membrane dependent actin polymerisation to cell lysates that had been depleted of Rho family proteins by prior treatment with RhoGDI (Moreau and Way, 1998). These observations suggest that Rac and Cdc42 could mediate the organisation of the actin cytoskeleton by directing actin polymerisation.

Protein fractions from human neutrophils known to contain the Arp2/3 complex components with an estimated purity of 38 %, contained Rac1 binding proteins at ~47,
60 and 68 kDa, Cdc42Hs binding proteins between 60 and 68 kDa, p57 immunoreactivity at 60 kDa and β-PAK immunoreactivity between 60 and 68 kDa. Fractions containing Cdc42Hs binding activity and proteins that reacted with β-PAK antibody did not correlate exactly, indicating that the Cdc42Hs binding activity in these fractions was due to the presence of either another PAK isoform or additional Cdc42Hs target proteins, for example WASP, which has been previously identified as a Cdc42Hs binding protein in human neutrophils (Symons et al., 1996). The 60 kDa Rac1 binding protein appeared to comigrate on SDS-gels with the p57 immunoreactive protein, suggesting that p57, the human coronin-like protein, is a Rac1 target. This was investigated using recombinant p57 material (see below). Fractions containing the Arp2/3 components, with estimated purity of 54 %, were found to contain Cdc42Hs binding proteins at ~68 kDa, p57 immunoreactivity at 60 kDa and β-PAK immunoreactivity at ~65 kDa. The association of Cdc42Hs and Rac1 binding proteins, including a β-PAK-like protein, with the Arp2/3 complex may provide the link between these GTPases and the actin cytoskeleton.

The Arp2/3 complex purified from human platelets has been identified as sufficient to promote actin filament assembly on the surface of *L. monocytogenes* in vitro. In an *in vitro* assay the Arp2/3 complex purified from Acanthamoeba, accelerated spontaneous actin polymerisation in a concentration dependent manner, by increasing the rate of actin filament nucleation (Welch et al., 1997a; Mullins et al., 1998a). The Arp2/3 complex may also regulate the organisation/stability of F-actin containing structures by its ability to cap the pointed ends of actin filaments, and bind to the sides of and cross link actin filaments. These characteristics enable the Arp2/3 complex to generate a network of actin filaments that resembles structures found in the lamellipodia of fibroblasts (Svitkina et al., 1997; Mullins et al., 1998a and 1998b). Indeed, the Arp2/3 complex becomes redistributed to cellular regions of dynamic actin filament assembly, for example peripheral lamellipodia, upon stimulation of Swiss 3T3 cells with PDGF, and the leading edge lamellae of motile fibroblasts (Welch et al., 1997b; Machesky et al., 1997).

The ability of Rac1 and Cdc42Hs binding proteins and PAK-like proteins to associate with the Arp2/3 complex could suggest a mechanism by which these Rho family p21s act to regulate organisation of the actin cytoskeleton. Evidence suggests that the mechanism by which the Arp2/3 complex is involved in actin polymerisation on the surface of *L.
monocytogenes, is similar to that observed during generation of dynamic actin structures at the surface of fibroblasts. Addition of actin monomers to the barbed ends of actin filaments abutting either the pathogen surface or the plasma membrane, generates the force required to propel the pathogen through the cytoplasm or force the plasma membrane out from the cell, forming structures such as filopodia and lamellipodia. It could be speculated that Cdc42 and Rac act through the Arp2/3 complex to direct changes in the actin cytoskeleton. In addition the Arp2/3 complex which functions to drive L. monocytogenes motility in collaboration with other host cell proteins, including the focal adhesion associated protein VASP, could provide a link between Cdc42 and Rac and the generation of focal complexes.

8.3.5. p57 (human coronin-like protein) is a Rac1 binding protein.

As mentioned above, analysis of the neutrophil protein fractions containing the Arp2/3 complex suggested that p57, the human coronin-like protein, is a target for Rac1. Rac1 probes bound recombinant p57 protein immobilised on nitrocellulose following SDS-PAGE or by direct dot blotting. In addition Rac1 probes bound a coronin peptide of 16 amino acid residues which shared some sequence similarities to the p21 binding domains of PAK and p120^c}

Interestingly a similar region in p57 was found to share some sequence similarity with the Rac binding site of p67^{pax}, suggesting that Rac1 may interact with p57 and p67^{pax} via similar binding sites. p57 was found to copurify with the Arp2/3 complex from human neutrophils, although substoichiometric amounts were present in the final stage of the purification (separation on a sucrose gradient) (Machesky et al., 1997). As a Rac1 target, p57 may provide the link between Rac and the Arp2/3 complex and the actin cytoskeleton.

Dictyostelium coronin is a cytosolic actin binding protein and was named because of its association with crown-shaped cell surface projections of growth-phase cells (DeHostos et al., 1991). Coronin associates with actin-myosin complexes and has been implicated in a number of processes that involve dynamic changes in the actin cytoskeleton. For example, coronin is required for cell motility, cytokinesis and phagocytosis in Dictyostelium (DeHostos et al., 1991 and 1993; Maniak et al., 1995). In aggregating Dictyostelium moving towards a cAMP source, coronin is relocated from the cytoplasm to the leading edge in dynamic cell surface protrusions (DeHostos et al., 1991). Redistribution of coronin also occurs upon the attachment of particles to the cell surface.
of *Dictyostelium*, whereby coronin becomes enriched in the phagocytic cup and is equally as important as F-actin for efficient particle uptake and macropinocytosis (Maniak *et al.*, 1995; Hacker *et al.*, 1997). Sequence information obtained from calf spleen and human HL60 cell cDNA libraries revealed that p57 shares 40 % similarity with *Dictyostelium* coronin, and human p57 was also found to be an actin binding protein (Suzuki *et al.*, 1995).

Coronin shares sequence similarity with the β-subunits of heterotrimeric G-proteins (DeHostos *et al.* 1991). Coronin and p57 contain five WD repeat motifs (Suzuki *et al.*, 1995). WD repeat motifs are found in many proteins that participate in the formation of multiprotein complexes, for example heterotrimeric G-proteins (Neer *et al.*, 1994). The presence of five WD repeat motifs in coronin or p57 suggests a potential involvement in directing protein-protein interactions. PH domains, which are found in a wide variety of proteins (including a large number of cell signalling and cytoskeletal molecules) are ligands for WD repeat motifs. PH domains, which direct protein-protein interactions and can simultaneously bind phospholipids, have been implicated in directing signalling proteins to the membrane (Lemmon *et al.*, 1996; Shaw *et al.*, 1996). Thus PH domains may link WD containing proteins, for example coronin and p57, to the membrane or cytoskeleton.

Human p57 contains a leucine zipper motif at its C-terminus which is not found in *Dictyostelium* coronin, but which is conserved between human and bovine p57 proteins (Suzuki *et al.*, 1995). The leucine zipper motif of p57 contains a basic region but it does not share the conserved sequence of other basic leucine zipper (bZIP) motif containing proteins, which include transcription factors. This suggests that p57 is unlikely to be a transcription factor or to bind directly to DNA. Leucine zipper motifs also participate in the formation of homopolymers (Vinson *et al.*, 1993), further suggesting that p57 protein may be involved in the formation of multiprotein complexes.

Analysis of the distribution of p57 in murine tissues, by immunoblotting using an antibody raised against a peptide sequence of p57 (amino acids 449-460), demonstrated that p57 protein is highly expressed in brain and immune tissues, including thymus, spleen, lymph nodes and bone marrow, with low levels detected in lung and gut. Northern analysis of various human tissues demonstrated that IR10, a five WD repeat
motif containing protein with structural similarity to coronin and p57, has a high level of expression in brain, lung and placenta. IR10 protein was identified (upon screening a human epidermal cDNA library) during investigation of expressed sequences in the candidate genomic region of the nevoid basal cell carcinoma syndrome (9q22.3). In addition to p57 and Dictyostelium coronin, IR10 shares similarity with C. elegans protein R01H10.3. All four proteins contain five WD repeat motifs and the α-helical structure in the C-terminal extension of the fifth motif, found to be important for actin binding, is conserved (de Hostos et al., 1991; Zaphiropoulos and Toftgard, 1996).

The tissue distribution of p57 may indicate that like coronin, it is important in cell motility and chemotaxis, as neuronal cells are highly migratory during development and immune tissues contain cells that migrate in response to chemoattractant. p57, which binds actin (Suzuki et al. 1995), has recently been suggested to be involved in the actin cytoskeletal changes that occur during the respiratory burst of human neutrophils (Grogan et al., 1997). An involvement of the membrane-cytoskeleton in the oxidase enzyme system has been suggested as the cytosolic oxidase components have been found to associate with membrane-cytoskeletal proteins upon oxidase activation (Woodman et al., 1991). In an in vitro reconstitution assay, superoxide production was found to be enhanced when monomeric actin was added to the cytosol used in this study and was inhibited by the addition of DNase I resin (Morimatsu et al., 1997). Human p57 has been found in association with p47phox, p67phox and p40phox in the cytosol of PMA stimulated neutrophil cells (Grogan et al., 1997). Solubilisation of p57 upon activation of neutrophil cells appears to require the presence of p47phox and p67phox. This may indicate a role for the cytosolic oxidase components in directing cytoskeletal changes associated with oxidase activation. Stimulation of polymorphonuclear leukocytes with chemoattractant results in a two-fold increase in the level of F-actin (Devreotes and Zigmond, 1988). F-actin distributed in the perinuclear region of unstimulated human neutrophils was shown to become condensed around the nucleus of PMA stimulated human neutrophils (Grogan et al., 1997). Lack of p47phox and p67phox led to abrogation of the reorganisation of F-actin during the respiratory burst. However the oxidase components are not required for chemotaxis of neutrophils (Zicha et al., 1997), indicating that dynamic actin cytoskeletal reorganisation, which occurs during these two processes, is regulated by differing mechanisms.
As a Rac1 interacting protein, p57 may link this p21 to organisation of the actin cytoskeleton, either directly or by mediating an association with the Arp2/3 complex.

8.3.6. Rho family target proteins.

In addition to PAK proteins, the Wiskott-Aldrich Syndrome Protein (WASP) in human neutrophils has been reported to bind Cdc42Hs. WASP was purified from human neutrophils by its ability to interact with Cdc42Hs using p21 binding assays, and its identity was confirmed by western blotting and peptide sequence analysis (Symons et al., 1996). Overexpression of WASP in PAE cells led to the induction of F-actin rich clusters to which WASP was localised. Coexpression of WASP and dominant negative Cdc42Hs protein blocked the effects of WASP on the actin cytoskeleton, and suggested that WASP acts as a target for Cdc42Hs in controlling organisation of the actin cytoskeleton (Symons et al., 1996).

Rho family interacting proteins that may act to regulate the effects of these p21s on the organisation of the actin cytoskeleton in addition to PAKs (mentioned above), have been identified by a number of different methods. Some of these proteins are listed in Table 8.2. The Rac and Cdc42-GAP n-chimaerin induced the simultaneous formation of lamellipodia and filopodia when injected into fibroblast or neuroblastoma cell lines. These actions were selectively inhibited when n-chimaerin was coinjected with either dominant negative Rac-T17N (blocked lamellipodia formation) or Cdc42-T17N (blocked filopodia formation), thus suggesting that n-chimaerin functions downstream of these p21s to mediate control of the actin cytoskeleton (Kozma et al., 1996). The Rac interacting protein POR1 was found to synergise with active Ras to induce membrane ruffles when injected into REF-52 cells, and a truncated POR1 protein interfered with Rac-induced membrane ruffling, thus suggesting that POR1 may play a role in Rac stimulated actin cytoskeletal changes (Van Aelst et al., 1996). In addition, the Cdc42 and Rac interacting protein IQGAP and the Rac specific target p140sra have been localised to membrane ruffles (Brill et al., 1996; Kobayashi et al., 1998). Both these targets have been suggested to interact with F-actin. IQGAP contains a potential actin binding domain and p140sra was found to cosediment from cells with F-actin, thus they may act to link Rho proteins to the actin cytoskeleton. The ability of RhoA and Rac to interact with and activate PIP 5-kinase, which catalyses the production of PIP$_2$, may mediate the actions of these Rho family proteins on the actin cytoskeleton. PIP$_2$ regulates the activities of a
### Table 8.2. Rho Family Target Proteins.

<table>
<thead>
<tr>
<th>Name</th>
<th>Rac</th>
<th>Cdc42</th>
<th>Rho</th>
<th>Possible Effector Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>p120^ACK</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>?</td>
<td>Manser et al., 1993</td>
</tr>
<tr>
<td>PAK</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>Actin CSK organisation</td>
<td>Manser et al., 1997; Sells et al., 1997; Lim et al., 1996.</td>
</tr>
<tr>
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<td>-</td>
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</tr>
<tr>
<td>MLK2/3</td>
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<td>+</td>
<td>-</td>
<td>JNK, ERK, p38 activation</td>
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</tr>
<tr>
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<td>+</td>
<td>-</td>
<td>Actin CSK organisation</td>
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</tr>
<tr>
<td>MRCK</td>
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<td>+</td>
<td>-</td>
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<tr>
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<td>-</td>
<td>+</td>
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</tr>
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<td>-</td>
<td>+</td>
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<td>?</td>
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<td>-</td>
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</tr>
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<td>+</td>
<td>-</td>
<td>?</td>
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<td>+</td>
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<td>Link to Actin CSK</td>
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<td>+</td>
<td>-</td>
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<td>JNK activation</td>
<td>Tapon et al., 1998</td>
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<td>p140mDia</td>
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<td>Actin CSK organisation</td>
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<td>p140sra</td>
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<td>kinectin</td>
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Table 8.2. Rho family target proteins. This table includes some of the Rho family binding proteins identified, which may represent downstream effector molecules for these p21s. Excluding PIP-5K, these proteins interact with Rac, Cdc42 or RhoA as indicated in a GTP-dependent manner. Possible effector functions are indicated where possible. However, not all these proteins have been implicated in mediating biological functions associated with Rho family p21s. ACK, activated Cdc42 kinase; PAK, p21 (Cdc42/Rac)-activated kinase; POR, partner of Rac; MEKK4, MEK kinase 4 (MAP kinase kinase kinase 4); MLK, mixed lineage kinase; WASP, Wiskott-Aldrich Syndrome protein; MRCK, myotonic dystrophy kinase related Cdc42-binding kinase; PKN, protein kinase N; PI 3-K, phosphatidylinositol 3-kinase; PIP-5K, phosphatidylinositol 4-phosphate 5-kinase; pp70S6K, 70 kDa ribosomal S6 kinase; MBS, myosin binding subunit (of myosin light chain phosphatase); POSH, plenty of SH3 domains; CSK, cytoskeleton, JNK, c-jun N-terminal kinase; ERK, extracellular-signal-regulated kinase; SRF, serum response factor.
number of actin binding proteins. Stimulation of PIP 5-kinase in response to active Rac was accompanied by an increase in the cellular levels of PIP$_2$ and uncapping of actin filaments, suggesting that Rho family proteins could regulate changes in the actin cytoskeleton via inositol phospholipids (Ren et al., 1998).

In addition to PAK protein, Cdc42 and Rac targets that may participate in downstream effects leading to control of transcriptional activation have been identified. Mixed lineage kinases (MLKs), which are closely related to the MAPKKK family, were initially suggested to be Cdc42 and Rac targets from database searches using the 'CRIB' motif sequence of PAK (Burbelo et al., 1995). Transfection of MLK2, which interacts with both Cdc42 and Rac in a GTP-dependent manner, into COS cells leads to a strong activation of JNK and also activation of ERK and p38 (Nagata et al., 1998). The scaffold protein POSH, identified as a Rac target using the yeast two-hybrid system, was found to induce JNK activation and translocation of the transcription factor NFkB to the nucleus when expressed in COS-1 cells (Tapon et al., 1998).

8.4. PAK proteins interact with annexin VI.
The presence of annexin VI protein in the p68 fraction, as was indicated by peptide sequence analysis, suggests that annexin VI copurifies with PAK proteins from neutrophil cytosol. The ability of β-PAK labelled probes to interact with recombinant annexin VI in vitro was demonstrated, and recombinant β-PAK was found to phosphorylate annexin VI. Taken together these results suggest that a direct interaction occurs between annexin VI and PAK-like proteins.

Annexin VI, which binds phospholipids and monomeric actin (in a calcium dependent manner), has also been shown to cosediment with F-actin from bovine brain lysates and to colocalise with stress fibres (Hosoya et al., 1992). This suggests a role for annexin VI in linking the actin cytoskeleton to the membrane. An interaction between the PAK and annexin VI may link Cdc42 or Rac to the actin cytoskeleton. Annexin VI has been reported to suppress tumour growth of A431 cells cultured subcutaneously in nude mice (Theobald et al., 1995). A requirement for Rac and Cdc42 for cell transformation by Ras has been indicated (Kosravi et al., 1995; Qiu et al., 1995a and 1997; Lin et al., 1997b). Expression of a kinase deficient mutant of PAK1 in Rat-1 fibroblasts has indicated that PAK acts in the pathway downstream of Ras leading to cellular transformation. Further
analysis is required to describe a role for an interaction between PAK and annexin VI in either of the two processes mentioned above.

8.5. The neutrophil NADPH oxidase component p67\textsuperscript{phox} is a Rac target.
The NADPH oxidase of neutrophils is a multicomponent enzyme that lies dormant in resting cells and catalyses the production of superoxide upon stimulation of neutrophils by factors such as fMLP, in response to invading pathogens. Activation of the NADPH oxidase involves the translocation of the cytosolic components p47\textsuperscript{phox}, p67\textsuperscript{phox} and p40\textsuperscript{phox} to the membrane (Clark \textit{et al.}, 1990; Wientjes \textit{et al.}, 1993), where they form the active enzyme complex with the membrane associated cytochrome \textit{b} (DeLeo and Quinn, 1996). The Ras-related GTPase Rac, which is required for activation of the NADPH oxidase, resides in the cytosol of resting neutrophil cells in complex with the guanine nucleotide dissociation inhibitor RhoGDI. \textit{In vitro} investigation has demonstrated that upon stimulation of neutrophils with fMLP or PMA, Rac dissociates from RhoGDI and translocates to the membrane. The translocation of Rac, which is agonist dependent, coincides with the translocation of p47\textsuperscript{phox} and p67\textsuperscript{phox} to the membrane, and correlates with NADPH oxidase activity (Abo \textit{et al.}, 1994). Immunogold labelling of the NADPH oxidase components has demonstrated that Rac colocalises with the cytosolic components on the cytoplasmic surface of the plasma membrane of unroofed neutrophils, as visualised by electron microscopy (Wientjes \textit{et al.}, 1997). Translocation of Rac to the membranes has been reported to occur independently of the cytosolic components p47\textsuperscript{phox} and p67\textsuperscript{phox}. However, in X-linked CGD cells Rac association with the membrane was reduced to 25\% of that seen in control cells, suggesting that the cytochrome \textit{b} plays a role in stabilising the association of Rac with the membranes (Heyworth \textit{et al.}, 1994). It is therefore likely that Rac participates in the activation of the NADPH oxidase at the membrane level.

8.5.1. p67\textsuperscript{phox} is the Rac target in the NADPH oxidase system.
To investigate the Rac target within the neutrophil NADPH oxidase system, neutrophil oxidase components immobilised on nitrocellulose by dot blotting were probed with Rac1-GTP[\gamma\textsuperscript{32}P] probes. The human neutrophil NADPH oxidase component p67\textsuperscript{phox} was found to bind specifically to Rac1 in a GTP dependent manner. The other NADPH oxidase components, p47\textsuperscript{phox}, p40\textsuperscript{phox} and the cytochrome \textit{b}, did not bind Rac1 in this assay. In addition, p67\textsuperscript{phox} proteins were also found to bind Rac2 probes, indicating that
This NADPH oxidase component is a target for both Rac1 and Rac2, the latter being the major Rac protein species in human neutrophils. Other groups have also reported a specific interaction between Rac and \( p67^{phox} \). \( p67^{phox} \) was found to bind specifically to Rac1 using \( p67^{phox} \)-GST fusion proteins immobilised on glutathione-Sepharose and also using the yeast two-hybrid system (Diekmann et al., 1994; Dorseuil et al., 1996).

\( p67^{phox} \) bound approximately 13-fold more GTPase negative mutant Racl-Q61L than the wild-type Rac1 protein, correlating with the increased ability of this Rac1 mutant to stimulate superoxide production \textit{in vitro} (Xu et al., 1994). Neither \( p67^{phox} \) purified from insect cells or the N-terminal deletion mutant of \( p67^{phox} \) containing amino acids 1-238 bound the 'effector domain' mutant Rac1-D38A. Introduction of an equivalent point mutation in the 'effector domain' of Ras protein blocked its ability to interact with c-Raf \textit{in vitro} (Warne et al., 1993), and inhibited the biological activity of Ras when introduced into cells (Zhang et al., 1993). Rac1-D38A does not activate the NADPH oxidase \textit{in vitro}, thus suggesting that Rac1 activates the NADPH oxidase enzyme system through an interaction with \( p67^{phox} \).

8.5.2. Amino acid residues 170-199 of \( p67^{phox} \) bind Rac1 and Rac2.

Deletion mutant proteins of \( p67^{phox} \) were used to localise the Rac1/2 binding site. Rac1 probes bound \( p67^{phox} \) proteins containing N-terminal sequences (amino acids 1-238, 1-199 and 1-192), and did not bind to deletion mutants containing C-terminal sequences (amino acids 300-526). Rac2 probes also interacted with \( p67^{phox} \) N-terminal deletion constructs (amino acids 1-192). This indicates that the Rac binding site in \( p67^{phox} \) is located within the N-terminal sequence containing amino acids 1-192. The use of a series of deletion mutants was applied to analyse the Rac binding interaction with the N-terminal of \( p67^{phox} \). Rac1 bound to deletion constructs containing amino acids 126-238 and 170-238 but not to proteins containing amino acids 1-58, 1-131 or 192-238, suggesting that amino acid residues 170-192 were important for Rac1 binding. Both Rac1 and Rac2 probes bound to protein containing amino acid residues 170-199, indicating that this region of \( p67^{phox} \) contains the Rac binding site and that both Rac proteins interact with \( p67^{phox} \) at the same site. In addition, full-length \( p67^{phox} \) protein deleted of amino acid residues 178-184 did not bind Rac1 probes. The N-terminal of \( p67^{phox} \), which is able to reconstitute oxidase activity \textit{in vitro}, does not interact with...
Racl-D38A, this suggests that the Rac binding site located within N-terminal sequences may be functionally relevant in terms of NADPH oxidase activation.

Identification of the Rac1/2 binding site in p67phox suggests that the interaction with this target occurs via a sequence distinct from the recently described ‘CRIB’ motif, which has been identified in a number of Rac and Cdc42 target proteins (Burbelo et al., 1995). The Rac binding sequence of p67phox does not align with the binding regions of other Rac specific targets, for example POR1, tubulin or p140Sra-1 (Van Aelst et al., 1996; Best et al., 1996; Kobayashi et al., 1998). The identification of further Rac targets is required in order to define a specific Rac binding motif.

Amino acids 170-199 of p67phox expressed as a GST-fusion protein bound approximately 5% of the Rac1 bound by amino acids 1-192, suggesting that N-terminal sequences are required to stabilise the Rac binding site for an optimal binding interaction to occur. Similar observations were made for Cdc42Hs binding to recombinant β-PAK (Fig. 4.7) and a novel Cdc42 specific binding partner, CBP5 (S. Govind and S. Ahmed unpublished observations).

*In vitro* binding techniques have been used to investigate the sequence requirements for the interactions between Rac and Cdc42, and ‘CRIB’ motif containing target proteins, α-PAK and WASP. These studies demonstrated that the ‘CRIB’ motif (amino acids 75-88) was necessary, but not sufficient, for p21 binding to α-PAK, whereas amino acids 75-105 were sufficient for a high-affinity p21 binding interaction (Thompson et al., 1998). Similar characteristics have been described for the interaction between Cdc42 or Rac and WASP. Proton and 15N NMR measurements indicated that the ‘CRIB’ motif of WASP does not possess secondary sequence, whereas the region immediately C-terminal is α-helical (Rudolph et al., 1998). This could suggest that the residues of the ‘CRIB’ motif are required for recognition of the p21 whereas the sequence C-terminal is important for stabilising the p21-target interaction. A similar situation may exist for p67phox whereby sequences N-terminal to the Rac binding site are required for an optimal interaction.

**8.5.3. Functions of the p67phox N-terminal tetratricopeptide repeat motifs.**

p67phox immobilised on nitrocellulose filter following separation in SDS-gels, did not bind Rac1 probes, suggesting that the Rac1 binding site in p67phox is denatured during SDS-
PAGE. Analysis of Rac1 binding to p67phox deletion mutant proteins, and also of p67phox mutations that occur in autosomal recessive forms of CGD, have indicated that the stability of p67phox depends upon the integrity of the N-terminal half of the protein. The N-terminal sequence of p67phox contains 4 tetratricopeptide repeat (TPR) motifs; amino acids 6-36 (TPR1), 37-70 (TPR2), 71-104 (TPR3), and 121-154 (TPR4) (Ponting, 1996). TPR protein motifs are thought to form amphipathic α-helices and to be involved in protein folding. Disruption of TPR3 might account for the absence of p67phox protein in an autosomal recessive CGD patient who is homozygous for a mutation causing substitution of amino acid 78 from glycine to glutamic acid (de Boer et al., 1994). This residue maps to position 8 in TPR3, which is predicted to be involved in forming the non-polar 'hole' into which the bulky hydrophobic 'knob' residue at position 24 (leucine 94 in TPR3) fits, to form the antiparallel α-helical folded structure. Mutation of glycine 78 is likely to destabilise p67phox structure, as has been predicted for a similar substitution, of glycine to aspartic acid, at position 8 of a TPR motif, which is thought to account for temperature sensitive nuc2 and CDC23 mutants (Hirano et al., 1990; Sikorski et al., 1990).

TPR motifs are found in more than 25 proteins with a wide variety of functions, including cell cycle control, transcription control, stress response, protein kinase inhibition, mitochondrial and peroxisomal protein transport and neurogenesis, and have been implicated in directing protein-protein interactions via TPR-TPR and TPR-non-TPR interactions. TPR motifs are found individually or as tandem arrays of 3-16 motifs (Lamb et al., 1995). Proteins containing multiple TPR motifs can act as scaffolding proteins and direct the assembly of multiprotein complexes. For example the TPR containing proteins cdc16, cdc23 and cdc27, are subunits of the anaphase promoting complex (APC). Mutations within the TPR motifs of these APC proteins cause mitotic arrest at the metaphase to anaphase transition stage of the cell cycle, suggesting that the functional form of the APC is lost (Hirano et al., 1990; Sikorski et al., 1990; King et al., 1995). Perhaps in addition to defining the N-terminal structure of p67phox, the TPR motifs may participate in the formation of the multiprotein NADPH oxidase enzyme complex.

Deletion of lysine 58 in p67phox, a mutation described in a particular form of autosomal recessive CGD, abolished Rac1 binding to dot blotted p67phox. Loss of this interaction is
predicted to contribute to abrogation of oxidase activity (Leusen et al., 1996). However, this mutation lies outside the minimal sequence required for Rac1 binding. The effect upon Rac1 binding observed could be due to destabilisation of the N-terminal structure of p67phox, as this mutation falls within the second TPR motif of p67phox (Ponting, 1996), further supporting the idea that the structure of the Rac1 binding site requires the integrity of the N-terminal half of the protein.

Attempts to generate GST-fusion proteins containing fragments of the N-terminal of p67phox, for example residues 58-238 or 58-131, were unsuccessful and resulted in a highly degraded protein. Degradation was also observed when amino acids 126-238 were expressed as a GST fusion protein. The inability to produce soluble GST-fusion proteins containing N-terminal fragments of p67phox (amino acids 1-59, 1-105, 1-135 and 30-150) has been noted previously (Diekmann et al., 1994). These observations further indicate the importance of N-terminal sequences to maintain the structure of p67phox.

8.5.4. Rac1 and p40phox interact with p67phox at distinct sites.

Data from in vitro binding assays and the yeast two-hybrid system suggest that p40phox interacts with sites within the N and C-terminal of p67phox (Wientjes et al., 1996; Sathyamoorthy et al., 1997; Fuchs et al., 1995 and 1996; Nakamura et al., 1998). It is therefore possible that p40phox and Rac1 might compete for p67phox binding. However even at amounts 10-fold in excess of Rac1 protein, the presence of p40phox did not affect the in vitro Rac1-p67phox binding interaction. This suggests that p40phox and Rac1 can interact with p67phox simultaneously. A direct protein-protein interaction study carried out by F. Wientjes (shown in Chapter 4), demonstrated that the major p40phox binding site lies between the two SH3 domains of p67phox, in agreement with studies using the yeast two-hybrid system, which mapped the p40phox interacting site to within the C-terminal of p67phox (Fuchs et al., 1996). This suggests that Rac and p40phox interact at distinct sites in p67phox, and may be able to interact simultaneously during assembly of the active NADPH oxidase enzyme.

Analysis has indicated that the majority of p40phox is in tight complex with p67phox in the cytosol of resting neutrophil cells (Someya et al., 1993; Tsunawaki et al., 1994), and that an interaction with p67phox is required for stabilisation of p40phox, as p40phox protein levels are markedly reduced in CGD cells deficient of p67phox (Wientjes et al., 1993; Dusi et al.,
The interaction with p67<sup>phox</sup> requires a region in the C-terminal of p40<sup>phox</sup> (amino acids 282-309) containing a distinct sequence referred to as a PC motif, which is also found in proteins involved in signalling pathways, including the yeast Cdc42-GEF Cdc24, and human and rat MEK5 (Nakamura <i>et al.</i>, 1998). The p40<sup>phox</sup> and p67<sup>phox</sup> complex is maintained in the presence of amphiphiles and is not disrupted by DTT or ethanol treatment (Someya <i>et al.</i>, 1993; Nakamura <i>et al.</i>, 1998), suggesting that it may persist during assembly and activation of the NADPH oxidase. Indeed, p40<sup>phox</sup> translocates to the membrane in a manner dependent upon the presence of p40<sup>phox</sup> and p67<sup>phox</sup> upon fMLP stimulation of human neutrophils (Dusi <i>et al.</i>, 1996). A functional role for the interaction between p40<sup>phox</sup> and p67<sup>phox</sup> has been suggested, as antibodies raised to a polypeptide from the C-terminal of p40<sup>phox</sup> (amino acids 325-339), which disrupted the association of p40<sup>phox</sup> with p67<sup>phox</sup> <i>in vitro</i>, inhibited superoxide production when added to neutrophil cytosol prior to cell free reconstitution of NADPH oxidase activity. Antibodies raised to the N-terminal amino acids 1-15 of p40<sup>phox</sup> were without effect (Tsunawaki <i>et al.</i>, 1996). Thus the finding that p40<sup>phox</sup> and Rac1 interact with p67<sup>phox</sup> at distinct sites, is an important observation for understanding the mechanisms of assembly of the neutrophil NADPH oxidase.

8.6. <i>p67</i><sup>phox</sup> is a PAK substrate.

8.6.1. A PAK-like kinase copurifies with p67<sup>phox</sup> from insect Sf9 cells.

Lysates from control insect Sf9 cells and cells infected with p67<sup>phox</sup> cDNA were analysed for Cdc42Hs binding activity and were compared to the Cdc42Hs binding activity associated with the purified p67<sup>phox</sup> protein sample. A ~68 kDa protein in control or infected insect cell lysates and the purified p67<sup>phox</sup> protein sample, bound Cdc42Hs probes. However, the binding activity was reduced in the latter two samples in which p67<sup>phox</sup> protein was present, indicating that a protein other than p67<sup>phox</sup> was responsible for the Cdc42Hs binding activity associated with p67<sup>phox</sup>

The purified p67<sup>phox</sup> protein sample contained a ~68 kDa protein that reacted with Ste20 antibody. This immunoreactivity comigrated with the Cdc42Hs binding activity in this sample on SDS-gels, indicating the presence of a PAK-like protein. This suggests that a PAK-like protein was responsible for the Cdc42Hs binding activity associated in this protein preparation. Indeed, incubation of p67<sup>phox</sup> protein sample with [γ<sup>32</sup>P]ATP saw the appearance of autophosphorylated protein bands. These results suggest copurification of
a PAK-like kinase with p67\textsuperscript{phox} from insect Sf9 cell lysates. This may be due to a direct interaction.

The presence of a Cdc42Hs-GAP was also identified in the p67\textsuperscript{phox} protein sample purified from insect Sf9 cell lysates. The characteristics of the Cdc42Hs-GAP activity resembled that of n-chimaerin (Ahmed et al., 1994), and like RhoGAP p190, was active towards the 'effector domain' mutant Cdc42Hs-D38A. The GAP activity of the p67\textsuperscript{phox} protein sample was inhibited in the presence of α-PAK and p120\textsuperscript{CK}, which have been found to inhibit the GTPase activity of Cdc42Hs (Manser et al., 1993 and 1994), and also in the presence of SDS (100 μM). However, the GAP activity was not conserved to p67\textsuperscript{phox} proteins purified from E. coli cells and p67\textsuperscript{phox} does not share sequence homology with other known Cdc42Hs-GAP proteins (Lamarche and Hall, 1994), suggesting that the activity in the insect cell p67\textsuperscript{phox} sample was due to the presence of a contaminating factor. Identification of the Cdc42Hs-GAP present may have been aided using GAP-overlay assay analysis (Manser et al., 1995b), but material was not available to carry out such studies.

**8.6.2. In vitro phosphorylation of p67\textsuperscript{phox} by β-PAK.**

That a PAK-like protein copurifies from insect Sf9 cells with p67\textsuperscript{phox} suggests that p67\textsuperscript{phox} interacts with a PAK-like kinase. To determine whether p67\textsuperscript{phox} is a PAK substrate, p67\textsuperscript{phox} full-length protein and deletion mutants were added to \textit{in vitro} kinase assays in the presence of β-PAK. Recombinant β-PAK-GST protein was found to phosphorylate the C-terminal of p47\textsuperscript{phox} (amino acids 204-390) and recombinant p67\textsuperscript{phox} \textit{in vitro}.

Recombinant β-PAK protein phosphorylated full-length p67\textsuperscript{phox} and deletion mutants containing amino acids 1-238, 1-192, 126-238 and 170-238 but not proteins containing amino acids 192-238, 300-526, 1-131, 1-58 or GST. This suggested that p67\textsuperscript{phox} represents a potential PAK substrate, and that \textit{in vitro} β-PAK phosphorylation sites lie within the N-terminal of p67\textsuperscript{phox} protein. The major β-PAK phosphorylation sites were located within the p67\textsuperscript{phox} deletion mutant containing amino acids 170-238. In comparison, protein containing amino acids 192-238 was not phosphorylated by β-PAK. As amino acids 170-192 do not contain either serine or threonine residues, this indicates that this region of p67\textsuperscript{phox} is essential for an interaction with PAK. This analysis also
indicates that amino acids 126-170 contain β-PAK phosphorylation sites, and cannot be used to eliminate the presence of additional PAK phosphorylation sites in the C-terminal of the protein that require N-terminal sequences to become phosphorylated. Sequence of p67phox between amino acids 126 and 238 contains 6 serine and 3 threonine residues. The major in vivo phosphorylation site on p67phox, mapped to threonine 233 by Forbes et al., (1997), represents a potential in vitro β-PAK phosphorylation site, and is located within the proline-rich region (amino acids 226-236). The potential β-PAK phosphorylation sites lie within close proximity to the Rac binding site and are adjacent to the proline-rich region. This could indicate that the phosphorylation state of p67phox is important in regulating the Rac binding interaction and protein interactions directed by the polyproline domain.

The NADPH oxidase components p47phox and p67phox are phosphorylated upon activation of the NADPH oxidase (Hayakawa et al., 1986; Okamura et al., 1988a and 1988b; Dusi and Rossi, 1993). Phosphorylation of p47phox occurs on a number of serine residues in the C-terminal half of the protein. The requirement for p47phox phosphorylation for oxidase activity has been demonstrated by mutational analysis of serine residues in this region of p47phox (Faust et al., 1995). Substitution of serine residue 379 to alanine, or dual substitution of serine residues 303 and 304 to alanine, resulted in loss of oxidase activity in EBV-transformed B-cells (Faust et al., 1995; Inanami et al., 1998). This suggests that phosphorylation of these residues is required for oxidase activity. Of the serine residues within the C-terminal 100 amino acids of p47phox, 4 are located near basic amino acid residues in sites that resemble consensus substrate sequences for protein kinase C (PKC) [(K/R)X(S/T) and (S/T)X(R/K)] or protein kinase A (PKA) [RRXS]. Serine residues 345 and 348 are found within the sequence PXSP, which is recognised by proline directed kinases, including MAP kinase. In vitro phosphorylation of p47phox by PKC and MAP kinase has been demonstrated (El Benna et al., 1996), and p47phox phosphorylated in vitro by PKC was active in a cell-free assay in the absence of amphiphiles (Park et al., 1997). This suggests a role for PKC during activation of the NADPH oxidase. However, the use of PKC inhibitors has suggested the involvement of PKC-independent pathways leading to neutrophil NADPH oxidase activation (Kawakami et al., 1998).
Kinase activity in p68 fractions containing neutrophil PAK proteins with ~25 % purity, was found to phosphorylate p47\textsuperscript{phox} in vitro, which in agreement with other studies (Knaus et al., 1995) suggests that p47\textsuperscript{phox} represents a PAK substrate. Human PAK1 and PAK2, immunoprecipitated from fMLP stimulated human neutrophils, phosphorylated p47\textsuperscript{phox} but not p67\textsuperscript{phox} in the presence of Rac-GTP\textsubscript{yS} (Knaus et al., 1995). Constitutively active PAK1 was shown to phosphorylate p47\textsuperscript{phox} and a peptide from the C-terminal of p47\textsuperscript{phox} (amino acids 324-331) known to contain sites phosphorylated in vivo during activation of the NADPH oxidase. This indicates that PAK phosphorylates a physiologically relevant site in p47\textsuperscript{phox} and may play a role in activation of the NADPH oxidase (Knaus et al., 1995). That p67\textsuperscript{phox} was not phosphorylated by PAK1 (the human isoform of \(\alpha\)-PAK) or PAK2 (the human isoform of \(\gamma\)-PAK) immunoprecipitated from human neutrophils or recombinant PAK1 (Knaus et al., 1995), but was found to be a substrate for \(\beta\)-PAK in vitro, may suggest a difference in the substrate specificity of the different isoforms of PAK proteins.

Immunoprecipitation of p67\textsuperscript{phox} from fMLP or PMA stimulated neutrophils has demonstrated that this cytosolic component is phosphorylated on serine residues during oxidase activation (El Benna et al., 1997). In contrast, another group have described the major phosphorylation site on p67\textsuperscript{phox} to be threonine residue 233 (Forbes et al., 1997). Phosphopeptide analysis of p67\textsuperscript{phox} from cells stimulated with PMA or fMLP, and also of p67\textsuperscript{phox} phosphorylated by PKC in vitro, revealed one major phosphopeptide which located to a similar position in the phosphopeptide maps in all three cases. However, the PKC inhibitor GF109203X, which strongly inhibited the phosphorylation of p67\textsuperscript{phox} induced by PMA, only mildly inhibited p67\textsuperscript{phox} phosphorylation induced by fMLP. This suggests the involvement of PKC dependent and independent pathways leading to p67\textsuperscript{phox} phosphorylation (El Benna et al., 1997).

Analysis of phosphorylated p47\textsuperscript{phox} from normal and CGD cells has suggested that p47\textsuperscript{phox} phosphorylation occurs both in the cytoplasm and also at the membrane of stimulated cells (Okamura et al., 1988a and 1988b; Heyworth et al., 1991). This stepwise phosphorylation of p47\textsuperscript{phox} might suggest the involvement of more than one kinase, and perhaps an analogous situation can be extended to p67\textsuperscript{phox}. Identification of the protein kinases that phosphorylate both p47\textsuperscript{phox} and p67\textsuperscript{phox} would provide important information for understanding the control of the NADPH oxidase.
In neutrophils PAK activation by fMLP occurs rapidly. Active PAK was detected 30 s to 1 min following fMLP stimulation (Knaus et al., 1995), which is consistent with the rapid translocation of Rac to the membrane and with superoxide production in fMLP stimulated neutrophil cells (Quinn et al., 1993; Abo et al., 1994). However, PMA stimulation of neutrophils which induces Rac translocation to the membrane and superoxide production, did not lead to the activation of PAK proteins (Knaus et al., 1995). This suggests a role for PKC in oxidase activation. Indeed, PKC has been shown to phosphorylate both p47phox and p67phox in vitro (Park et al., 1997; El Benna et al., 1997). Taken together these results suggest that PAK proteins may be involved in the activation of the neutrophil NADPH oxidase. However, PAK activation may require signals in addition to p21-GTP binding, which could result from p21-independent mechanisms, for example the actions of phospholipids (Bokoch et al., 1998).

8.6.3. PAK substrates.
Recombinant human PAK1 has been shown to have a similar substrate specificity to Acanthamoeba Myosin I heavy chain kinase (MIHCK). MIHCK has been identified as a member of the PAK family of kinases, and in addition to the sequence similarity shared between the members of this family, PAK1 was able to phosphorylate myosin IC heavy chain in vitro. The kinetics and the extent of phosphorylation and activation of myosin IC induced by either kinase suggested that, like MIHCK, PAK1 phosphorylates one specific site on myosin IC (Brzeska et al., 1997). A synthetic peptide based on the sequence of Acanthamoeba myosin IC (GRGRSSVYS) was phosphorylated on a serine residue (underlined) by MIHCK and PAK1 in vitro, suggesting that these kinases phosphorylate the same site in myosin IC. Neither kinase phosphorylated the peptide substrate when the tyrosine residue, at the position 2 residues C-terminal (+2) to the phosphorylated serine residue, was mutated to leucine (Brzeska et al., 1997). Myosin ID isolated from Dictyostelium has also been shown to be phosphorylated by PAK family proteins including the yeast cell proteins Ste20 and Cla4, and recombinant mammalian PAK (mPAK3) (Wu et al., 1996). The phosphorylated residues responsible for activation of myosin I isoforms have been identified as either serine or threonine residues within the sequence motif (K/R)(S/T)XY, which is conserved in the heavy chains of myosin I isoforms in organisms from amoeba to mammals. This motif resembles sequence found in γ-PAK substrates using a series of synthetic peptides (Tuazon et al., 1997). This study
further determined that the residue, at the position 1 residue N-terminal (-1) to the phosphorylated serine residue, could be either basic, acidic or neutral, and that the presence of an acidic residue at this position within a peptide substrate was most favourable for phosphorylation by γ-PAK (with the sequence AKRESAA). Neither PKC nor PKA can phosphorylate substrate sites with an acidic residue at the -1 position. Thus peptides containing the sequence KRES may be helpful in identifying γ-PAK substrates.

A serine residue in the C-terminal peptide of p47\(^{phax}\), phosphorylated by recombinant PAK1 \textit{in vitro}, lies within the sequence RRNSVR. The residues mapped as potential phosphorylation sites for β-PAK on p67\(^{phax}\) (Fig. 6.7) do not lie within regions of sequence with similarity to the consensus mentioned above. Further identification of PAK family substrates, and also of the sites phosphorylated in substrates other than myosin I isoforms, is required to determine the full range of PAK substrate recognition sequences. For example, PAK1 has been shown to phosphorylate the extracellular signal regulated (ERK) kinase MEK1, \textit{in vivo} and \textit{in vitro}, on serine residue 298, which lies within a polyproline insert within the catalytic domain (GRPLSSYG, serine residue 298 is underlined). Although this region is not required for an interaction with Raf, it is necessary for efficient activation of downstream ERKs, as assessed from the activities of MEK1 deletion mutants in cells (Frost \textit{et al.}, 1997; Dang \textit{et al.}, 1998). In addition, the actin-binding protein severin is phosphorylated by a PAK family protein isolated from Dictyostelium, which was designated as a member of the germinal centre kinase (GCK) subfamily of PAKs by sequence analysis (Eichinger \textit{et al.}, 1998). Analysis of substrates from all PAK family members may aid defining a consensus for substrate recognition.

8.7. Crypticity of the Rac1 and PAK interactions with p67\(^{phax}\).

8.7.1. The Rac1 binding site in p67\(^{phax}\) is cryptic.

Deletion of the C-terminal of p67\(^{phax}\) (amino acids 239-526) saw an 8-fold increase in Rac1 binding, suggesting that the Rac1 binding site is masked within the full-length protein. A similar stimulation of Rac1 binding was also observed upon deletion of the C-terminal SH3 domain (amino acids 461-526) or the polyproline domain (amino acids 226-236). In addition, deletion of the C-terminal of p67\(^{phax}\) (amino acids 239-526) resulted in a stimulation of p67\(^{phax}\) phosphorylation by β-PAK. This was also noted upon deletion of the C-terminal SH3 domain (amino acids 461-526) or the polyproline domain (amino acids 226-236). Taken together these observations suggest that an SH3-
polyproline domain interaction may exist within recombinant p67phox, which acts to mask the binding sites for Rac1 and β-PAK. The cryptic nature of these two interactions may represent a point of control of the oxidase *in vivo*.

Regulatory intramolecular interactions control catalytic activity and the ability of proteins to direct protein-protein interactions. An intramolecular interaction within Src family tyrosine kinases, involving the SH3 domain, participates in ‘holding’ Src proteins in a repressed state (Xu *et al.*, 1997; Williams *et al.*, 1997). This has also been observed in c-Abl (Barila *et al.*, 1998), and it has been suggested that regulators act to disrupt or favour these intramolecular interactions. The ability of vinculin, an abundant cytoskeletal protein found in focal adhesions and adherens junctions, to interact with F-actin or talin, requires relief of an intramolecular interaction between the ‘head’ and ‘tail’ regions of this protein (Johnson and Craig, 1994 and 1995). Suppression of the enzyme activity of protein phosphatase 5 (PP5), which contains a C-terminal catalytic domain and 3 TPR motifs within its N-terminus, is stimulated >25-fold by AA interacting with the TPR motif domain (Chen and Cohen, 1997). The interaction of AA with the TPR domain induces an allosteric conformational change within PP5 and the resulting enzyme activity resembles that of the isolated phosphatase domain proteolytically cleaved from the TPR domain. That AA induces conformational change within p67phox during NADPH oxidase activation has been indicated from investigations using cell-free oxidase assays (Hata *et al.*, 1998; see below for further discussion). This may result from an interaction of AA with the N-terminal TPR motif region of p67phox.

### 8.7.2. An intramolecular SH3-polyproline domain interaction exists within p67phox.

The results presented here collected from two distinct *in vitro* assays suggest that p67phox adopts a tertiary structure partially masking the Rac1 binding site and β-PAK phosphorylation sites, and that this occurs via an SH3-polyproline domain interaction.

Evidence that p67phox adopts a closed conformation, relief of which requires the action of either AA or SDS, has been found in studies examining interactions with p47phox. Sumimoto *et al.*, (1994) described an *in vitro* interaction between native p67phox and recombinant p47phox protein (containing just the SH3 domains) that required pretreatment with AA. In retrovirally transduced K562 cells, p67phox N-terminal protein (amino acids 1-246) translocated to the membrane when cotransfected with p47phox.
protein encoding the SH3 domain core region (amino acids 151-284), whereas translocation of the full-length p67phox protein required PMA stimulation (de Mendez et al., 1996). This indicates that full-length p67phox requires additional signals for an interaction to occur with the SH3 domains of p47phox. Furthermore, reconstitution of oxidase activity has recently been achieved in vitro using N-terminal proteins from both p47phox and p67phox in the absence of amphiphilic agents. Analysis of this phenomenon produced evidence to suggest that the action of amphiphiles was directed towards both of these oxidase components, as opposed to just p47phox as was previously understood (Hata et al. 1998).

An intramolecular SH3-polyproline domain interaction in p67phox could act to inhibit oxidase complex formation in vivo. The use of deletion mutants has suggested that both SH3 domains of p67phox are required for activation of enzyme activity in an in vivo system (de Mendez et al. 1994). The strongest interaction between p67phox and p47phox in vitro, occurs between the C-terminal regions of these two proteins, involving the C-terminal SH3 domain of p67phox and a proline-rich region in p47phox. This interaction is predicted to participate in assembly of the oxidase enzyme complex in vivo (Finan et al., 1994; Leto et al., 1994; Fuchs et al., 1996; de Mendez et al., 1996). The isolated SH3 domains of p47phox support low level superoxide production in whole cells and are able to recruit the N-terminal of p67phox (amino acids 1-246) to the membranes, suggesting that an interaction can also occur between the SH3 domains of p47phox and the polyproline domain of p67phox (de Mendez et al., 1994 and 1996). These observations indicate that an intramolecular interaction between the C-terminal SH3 and the polyproline domains would prevent the interactions required for assembly of an active oxidase enzyme complex. In vitro binding assays presented here describe that an ‘open’ structure of p67phox is also required for an optimal interaction with Rac, further suggesting that relief of an intramolecular interaction within p67phox would be required to occur prior to, or during oxidase enzyme assembly.

The yeast two-hybrid system has been useful in defining the interactions that occur between the oxidase components (for example Fuchs et al., 1995 and 1996). The yeast two-hybrid system was used to investigate a polyproline domain dependent interaction between the N and C-terminal halves of p67phox. However, results from this study and in vitro binding assays were not conclusive. SH3 domains direct intermolecular protein-
protein interactions with polyproline targets with low affinity (dissociation constants ranging between 5 and 100 μM) (Pawson, 1995) and it is likely that intramolecular interactions of this type are of lower affinity. This suggests that the methods applied were not suitable for investigating an SH3-polyproline domain interaction within p67phox.

Proline-rich peptides were also used to investigate whether an SH3-polyproline domain intramolecular interaction occurs in p67phox. Incubation of proline-rich peptides based upon sequences of p47phox (amino acids 360-373) and p67phox (amino acids 224-235) with full-length p67phox protein or deletion mutant containing amino acids 1-460, was carried out prior to probing p67phox proteins with Rac1 probes. It was considered that the presence of these peptides at high concentrations (0.5-1.5 mM) might bind the p67phox C-terminal SH3 domain and thereby relieve an interaction directed by this domain, resulting in an increase in Rac1 binding to p67phox. The p47phox peptide used here had been used previously to inhibit an interaction between the C-terminal SH3 domain of p67phox and native p47phox from HL60 cells (Finan et al., 1994). The presence of either peptide reduced or had no effect upon Rac1 binding to either full-length or the deletion mutant of p67phox (amino acids 1-460). The inconsistency of data obtained from this assay illustrates the complex nature of this experimental technique. For example, the effects observed on Rac1 binding may not occur as a direct result of the incubation with the proline-rich peptide, as the p67phox proteins may have resumed a folded conformation or perhaps adopted an alternative conformation once dot blotted onto nitrocellulose following the incubation with peptide. Maintenance of the peptide concentration throughout the experimental procedure may provide more useful information. Further analysis is required to determine a suitable direct or indirect approach for investigating the proposed p67phox intramolecular interaction.

It has been suggested that the oxidase component p47phox adopts a tertiary conformation in the cytosol of resting cells that masks the SH3 domains, as an antibody directed specifically to the SH3 domains was unable to react with p47phox protein in the absence of amphiphiles such as AA or SDS (Sumimoto et al., 1994). Biotinylated p47phox SH3 domain probes interacted with the C-terminal of p47phox, and this interaction has also been demonstrated using the yeast two-hybrid system (Sumimoto et al., 1994 and Fuchs et al., 1996). The C-terminal of p47phox contains two regions rich in proline residues, which could act as SH3 domain target sites. Deletion of amino acids 229-346, which
contain a polyproline domain, abolished the interaction between the SH3 domains and
the C-terminal of p47phox, demonstrated using the yeast-two hybrid system (Hata et al.,
1998), suggesting that an SH3-polyproline domain intramolecular interaction exists
within p47phox. The SH3 domains of p47phox are thought to be required for an interaction
of this cytosolic component with the membrane bound cytochrome b (Leto et al., 1994;
de Mendez et al., 1997), indicating that p47phox undergoes conformational change during
assembly of the oxidase enzyme.

Conformational change within both these cytosolic components may result from the
action of amphiphilic agents such as AA, the release of which occurs with similar
kinetics to superoxide production in fMLP stimulated neutrophils (Dana et al., 1994), or
by phosphorylation events on p47phox and p67phox that accompany oxidase enzyme
assembly and superoxide production (Faust et al., 1995; Inanami et al., 1998; Dusi and
Rossi, 1993). Interestingly the enzyme activity of protein phosphatase 5 is stimulated
>25-fold upon an interaction between AA and the N-terminal region of this protein
(which contains 3 TPR motifs) by inducing a conformational change (Chen and Cohen,
1997). A similar event may alter the conformation of p67phox.

8.8. Conclusions.
The work presented in this study, some of which has been reported elsewhere (Prigmore
et al., 1995; Ahmed et al., 1998), identifies the presence of a number of Cdc42Hs and
Rac1 interacting proteins in human neutrophils. Purification of the major Cdc42Hs
binding activity from neutrophil cytosol (proteins with relative molecular masses of 60-
68 kDa) demonstrated the presence of distinct binding proteins at approximately 62, 65,
and 68 kDa, which were found to bind Cdc42Hs in a GTP-dependent manner. A similar
pattern of proteins were also found to interact specifically with Rac1-GTP probes.
Purified protein fractions (p68 fractions) contained three Ste20 immunoreactive proteins
that appeared to comigrate on SDS-gels with the Cdc42Hs and Rac1 binding activity.
p68 fraction proteins included an α-PAK-related kinase, which was stimulated to
autophosphorylate in the presence of Rac1-GTP. Quantification of the Cdc42Hs binding
activity in the p68 fractions suggested that PAK proteins were separated from neutrophil
cytosol to approximately 25 % purity. These observations suggest the existence of
multiple isoforms of PAK proteins in human neutrophil cells.
Fractionation of neutrophil cell proteins indicated that the 60-68 kDa Cdc42Hs and Rac1 binding activity is mainly found in the cytosol of these cells, which appeared to correlate with the localisation of α-PAK immunoreactivity. A similar pattern and distribution of p21 binding proteins and correlating α-PAK immunoreactivity was noted in undifferentiated HL60 cells. This finding suggests HL60 cells could be used as a model cell system to study p21 binding proteins in neutrophil cells. A distinct pattern of Cdc42Hs binding proteins was associated with neutrophil membrane-cytoskeletal proteins, which did not correlate with either Rac1 binding proteins or α-PAK immunoreactivity. This perhaps suggests the presence of cytoskeletal-associated proteins that specifically interact with Cdc42Hs and could act as binding partners through which Cdc42Hs mediates changes in the cytoskeleton.

Cdc42Hs and Rac1 interacting proteins were found to associate with the Arp2/3 complex purified from human neutrophil cytosol. These included a β-PAK-like protein and p57 (human coronin-like protein). The latter was shown to interact specifically with Rac1 and not Cdc42Hs, using recombinant protein in in vitro p21 binding assays. In addition, Rac1 was shown to interact with a 16 residue peptide from Dictyostelium coronin. These observations may suggest links between these Rho family proteins and the actin cytoskeleton. The Arp2/3 complex can direct actin polymerisation and perhaps acts to mediate the formation of dynamic actin structures in cells. Coronin and p57 are cytosolic actin binding proteins. Coronin has been implicated in a number of cellular processes that involve dynamic changes in the actin cytoskeleton of Dictyostelium. p57 has been implicated in mediating the actin cytoskeletal changes that occur during activation of the NADPH oxidase of human neutrophils, and was found to copurify with the Arp2/3 complex from human neutrophil cytosol. Hence, PAK or p57 proteins may link Cdc42/Rac and Rac respectively to the actin cytoskeleton, either via the Arp2/3 complex or directly.

The NADPH oxidase component p67phox was identified as a Rac1 and Rac2 interacting protein. Rac1 and Rac2 bound to amino acid residues 170-199 within the N-terminal half of p67phox, thus indicating that this region of p67phox contains the Rac binding site and that both Rac proteins interact with p67phox at the same site. p67phox bound 13-fold more Rac1-Q61L than wild-type Rac1 protein, which correlated with the increased ability of this GTPase mutant to stimulate oxidase activity in vitro. The N-terminal half of p67phox,
which is sufficient for reconstitution of oxidase activity in vitro, did not interact with the Rac1-D38A ‘effector domain’ mutant, which is unable to stimulate superoxide production in vitro. Taken together these observations suggest that p67\textsuperscript{phox} is the Rac target within the NADPH oxidase enzyme system.

A Cdc42Hs interacting protein, with a molecular mass of ~67 kDa, was identified in p67\textsuperscript{phox} protein samples purified from lysates of insect Sf9 cells that had been infected with p67\textsuperscript{phox} cDNA. Cdc42Hs binding activity was not associated with p67\textsuperscript{phox} proteins expressed and purified from E. coli cells, suggesting that p67\textsuperscript{phox} is not a Cdc42Hs interacting protein, in contrast to initial predictions. Ste20 immunoreactivity and a kinase of similar molecular mass were identified in p67\textsuperscript{phox} protein samples purified from insect Sf9 cells. This indicated that the Cdc42Hs binding activity associated with such samples was due to the presence of a PAK protein that copurified with p67\textsuperscript{phox}. These observations implied that p67\textsuperscript{phox} and PAK may interact directly. The use of in vitro kinase assays demonstrated that p67\textsuperscript{phox} is phosphorylated by recombinant β-PAK, suggesting that p67\textsuperscript{phox} is a potential PAK substrate. Phosphorylation of p47\textsuperscript{phox} was seen when recombinant proteins were mixed with either p68 fractions (containing neutrophil PAK proteins with ~25 % purity) or recombinant β-PAK. Hence PAK proteins, which are activated upon stimulation of neutrophils with fMLP, may participate in the phosphorylation of both p47\textsuperscript{phox} and p67\textsuperscript{phox} that occurs during oxidase activation.

Deletion of the C-terminal (amino acids 239-526), the C-terminal SH3 domain (amino acids 461-526) or the polyproline domain (amino acids 226-236) of p67\textsuperscript{phox} stimulated Rac1 binding by approximately 8-fold and also β-PAK phosphorylation of p67\textsuperscript{phox}. These observations suggested that an intramolecular SH3-polyproline domain interaction may exist within p67\textsuperscript{phox} and that the Rac and β-PAK interacting sites in p67\textsuperscript{phox} are cryptic.

Taken together the information discussed above can be used to describe a model for assembly of the active NADPH oxidase enzyme complex (Fig. 8.1). In resting neutrophils, the NADPH oxidase components are present in one membrane and two cytosolic complexes. The transmembrane proteins gp91\textsuperscript{phox} and p22\textsuperscript{phox} form a tightly associated heterodimer that constitutes the cytochrome b. In the cytosol, p67\textsuperscript{phox} and p40\textsuperscript{phox} are in tight association, whereas p47\textsuperscript{phox} associates with the p67\textsuperscript{phox}-p40\textsuperscript{phox} complex more loosely. Rac1 (or Rac2 in human neutrophils) is found in association with
RhoGDI in the cytosol of resting cells. From the data presented here, it could be proposed that p67\textsuperscript{phox}, like p47\textsuperscript{phox}, exists in a closed conformation in the cytosol of resting cells via an intramolecular SH3-polyproline domain interaction. Unfolding of p67\textsuperscript{phox} and p47\textsuperscript{phox} may occur upon stimulation of neutrophils, by factors that lead to NADPH oxidase activation (for example fMLP), due to the action of second messengers (for example AA) or as a result of phosphorylation events. This may induce the formation of new SH3-polyproline domain interactions promoting the assembly of a more stable complex that can translocate to the membrane and interact with the cytochrome \(b\). These events would be accompanied by the dissociation of Rac1 from RhoGDI, promoting the association of Rac1-GTP with the membrane (Fig. 8.1; Step 1).

In the proposed model Rac1, which has been found to translocate to the membrane independently of the cytosolic \(phox\) components, would interact with the p22\textsuperscript{phox}-p47\textsuperscript{phox}-p67\textsuperscript{phox}-p47\textsuperscript{phox} complex assembled at the membrane. Rac1 could interact with p67\textsuperscript{phox} to induce conformational changes in p67\textsuperscript{phox}, supporting oxidase activity, or to recruit PAK kinases to the membrane-associated complex, facilitating phosphorylation of p67\textsuperscript{phox} or p47\textsuperscript{phox} (Fig. 8.1; Step 2). The major \textit{in vivo} phosphorylation site in p67\textsuperscript{phox} has been mapped to threonine 233 (Forbes \textit{et al.}, 1997), which represents a potential PAK phosphorylation site \textit{in vitro}. As the potential PAK phosphorylation sites (mapped in this study using \textit{in vitro} kinase assays) were located adjacent to the Rac1 binding domain and the polyproline domain, phosphorylation by PAK may regulate the activities of these two domains in p67\textsuperscript{phox}. PAK phosphorylation of p47\textsuperscript{phox} may contribute to phosphorylation of p47\textsuperscript{phox} in collaboration with other kinases (for example PKC).

This work indicates that PAK, p67\textsuperscript{phox} and p57 are targets for Rho family p21s in human neutrophils. Characterisation of the p21 binding partners identified in this study will be important for understanding the signal transduction pathways through which Rho family proteins carry out their cellular functions.
Fig. 8.1. Figure legend on reverse.
Fig. 8.1. Model for Assembly and Activation of the NADPH Oxidase. In this diagram the phox components are illustrated schematically, see appendix A.6, for details regarding amino acid sequences and domain structures of p67<sup>phox</sup>, p47<sup>phox</sup> and p40<sup>phox</sup>.

Resting cells: p67<sup>phox</sup> and p47<sup>phox</sup> are in closed conformation via intramolecular SH3-polyproline interactions; in p67<sup>phox</sup> the C-terminal SH3 domain (aas 460-510) binds the polyproline sequence (aas 226-236); in p47<sup>phox</sup> the first SH3 domain (aas 151-214) binds a polyproline sequence (within aas 299-346) (Hata et al., 1998). The C-terminal of p40<sup>phox</sup> (PC motif; aas 282-309) binds tightly to p67<sup>phox</sup> (aas between the two SH3 domains). The SH3 domain of p40<sup>phox</sup> may interact with the proline-rich region in the C-terminal of p47<sup>phox</sup> (aas 361-369) (Ito et al., 1996).

**Step 1**: Activation of neutrophils leads to the phosphorylation of p47<sup>phox</sup> and p67<sup>phox</sup> (Okamura et al., 1988b; Dusi and Rossi, 1993), and the release of second messengers e.g. AA (Dana et al., 1994). These two events may cause the unfolding of p47<sup>phox</sup> and p67<sup>phox</sup> and trigger conformational changes within the complex that resides in the resting cell. This may promote an interaction between the C-terminal polyproline domain of p47<sup>phox</sup> (aas 360-370) and the C-terminal SH3 domain of p67<sup>phox</sup> (aas 460-510) (Finan et al., 1994; Leto et al., 1994) thus exposing the first and second SH3 domains of p47<sup>phox</sup> allowing them to direct interactions with the polyproline region of p22<sup>phox</sup> (aas 149-162) and p67<sup>phox</sup> (aas 226-236) respectively, (de Mendez et al., 1997). These changes in SH3-polyproline domain interactions may lead to a more stable complex which can translocate to the membrane. During this time Rac1 dissociation from RhoGDI occurs, promoting association of Rac1-GTP with the membrane.

**Step 2**: At the membrane Rac1 associates with the assembled complex via an interaction with the RBD in p67<sup>phox</sup> (aas 170-199), leading to oxidase activation, which may occur as a result of conformation changes and protein phosphorylation events (on both p67<sup>phox</sup> and p47<sup>phox</sup>), by recruiting PAK to the membrane. The 9 p47<sup>phox</sup> residues phosphorylated in vivo include S303/304 and S379 as indicated (+). Potential PAK phosphorylation sites on p47<sup>phox</sup> have been mapped to sites that also become phosphorylated in vivo (S324 and S331) (Knaus et al., 1995). Potential PAK phosphorylation sites on p67<sup>phox</sup> have been mapped to residues 148, 149, 152, 167, 203, 207, 213, 215 and 233, locations indicated (+). Phosphorylation of T233 has also been reported by Forbes et al., 1997. These sites on p67<sup>phox</sup> lie in proximity to the RBD and the polyproline domain, suggesting that PAK phosphorylation of p67<sup>phox</sup> may influence the activities of these two domains within the assembled complex. PAK phosphorylation of p47<sup>phox</sup> may contribute to phosphorylation of p47<sup>phox</sup>, in collaboration with other kinases e.g. PKC (El Benna et al., 1994). aas-amino acids; GDI-guanine nucleotide dissociation inhibitor; PKC and PKA, protein kinase C and A respectively.
Fig. 8.1. Model for Assembly and Activation of the NADPH Oxidase.

1. Resting

2. Complex Formation

3. Active Oxidase

Membrane Cytosol

Step 1

Step 2

O$_2$-


Appendices
A.1. Generation of DNA fragments and subcloning into expression vectors.

A.1.1. α- and β-PAK DNA constructs.
α- and β-PAK DNA constructs were supplied by E. Manser. α-PAK cDNA was cloned by introducing a BamH1 site immediately preceding the initiating methionine and then cloning a BamH1/EcoR1 fragment into pGEX-2T; β-PAK cDNA was engineered to contain a Smal linker sequence at the 5’ end and a Smal/XhoI fragment was cloned into pGEX-4T (Manser et al., 1995a). Note, that the β-PAK protein contains additional polylinker derived sequence and has slower mobility than recombinant α-PAK in SDS-gels.

A.1.2. β-PAK deletion mutant constructs.
β-PAK deletion mutants were generated from β-PAK cDNA by S. Govind, who subcloned the DNA fragments into pGEX vectors. The NcoI fragment of β-PAK, was cloned into the NcoI site of a pGEX-3X derivative vector (p261), and encoded amino acids 37-149 (112 residues). This NcoI clone was digested with BamHI and BglII and religated, generating a plasmid that encoded amino acids 70-149 (79 residues) of β-PAK. This clone starts at the first residue (isoleucine) of the Cdc42/Rac interactive binding (CRIB) motif (Burbelo et al., 1995). The NcoIΔBamHI/BglII plasmid was digested with EcoRI and religated, deleting amino acids 93-149. The resulting plasmid (NcoIΔBamHI/BglIII) encoded amino acids 70-93 (24 residues) of β-PAK.

A.1.3. p67phox cDNA constructs.
p67phox deletion mutants were generated by digestion of p67phox full-length cDNA in pGEX or a derivative vector, or by polymerase chain reaction (PCR) with the appropriate oligonucleotide primers, using p67phox cDNA in pGEX as the template. p67phox cDNA constructs as listed in Table A.1, were supplied by F. Wientjes and S. Ahmed.

For p67phox mutants generated by PCR the sense primer contained a BamH1 site and the antisense primer an EcoRI site. In all cases the correct sequence was confirmed by nucleotide sequencing using an ABI automated sequencer.
Table A.I. \textit{p}67^{phox} cDNA Constructs.

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>Generation of cDNA</th>
<th>Expression Vector</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-526(full-length)</td>
<td>PCR of human \textit{p}67^{phox} cDNA</td>
<td>pGEX-2T</td>
</tr>
<tr>
<td>1-238</td>
<td>EcoRI/BglII digest of full-length \textit{p}67^{phox} in pGEX-2T</td>
<td>pGEX-2T</td>
</tr>
<tr>
<td>1-192</td>
<td>EcoRI/Bpu11021 digest of full-length \textit{p}67^{phox} in pGEX-2T</td>
<td>pGEX-2T</td>
</tr>
<tr>
<td>126-238</td>
<td>PCR of \textit{p}67^{phox} cDNA in pGEX</td>
<td>pGEX</td>
</tr>
<tr>
<td>170-238</td>
<td>PCR of \textit{p}67^{phox} cDNA in pGEX</td>
<td>pGEX</td>
</tr>
<tr>
<td>192-238</td>
<td>PCR of \textit{p}67^{phox} cDNA in pGEX</td>
<td>pGEX</td>
</tr>
<tr>
<td>170-199</td>
<td>AvrII/EcoRI digests of 170-238 cDNA in pGEX</td>
<td>pGEX</td>
</tr>
<tr>
<td>1-131</td>
<td>PCR of \textit{p}67^{phox} cDNA in pGEX</td>
<td>pGEX</td>
</tr>
<tr>
<td>1-58</td>
<td>EcoRI/Stul digest of \textit{p}67^{phox} cDNA in pGEX</td>
<td>pGEX</td>
</tr>
<tr>
<td>1-460</td>
<td>PCR of \textit{p}67^{phox} cDNA in pGEX</td>
<td>pGEX</td>
</tr>
<tr>
<td>300-526</td>
<td>BamHI/EcoRI digest of \textit{p}67^{phox} cDNA in pGEX-2T</td>
<td>pGEX-3X</td>
</tr>
<tr>
<td>300-460</td>
<td>BamHI/EcoRI digest of 1-460 in pGEX</td>
<td>pGEX-3X</td>
</tr>
<tr>
<td>1-526\Delta58K</td>
<td>USE kit Amersham (using \textit{p}67^{phox} in pGEX)</td>
<td>pGEX</td>
</tr>
<tr>
<td>1-526\Delta226-236</td>
<td>USE kit Amersham (using \textit{p}67^{phox} in pGEX)</td>
<td>pGEX</td>
</tr>
<tr>
<td>1-526\Delta178-184</td>
<td>USE kit Amersham (using \textit{p}67^{phox} in pGEX)</td>
<td>pGEX</td>
</tr>
<tr>
<td>1-526P174L</td>
<td>USE kit Amersham (using \textit{p}67^{phox} in pGEX)</td>
<td>pGEX</td>
</tr>
</tbody>
</table>

Table A.I. \textit{p}67^{phox} cDNA constructs. A summary of the \textit{p}67^{phox} proteins used in this study. Numbers indicate the amino acids encoded by each construct. The method used to generate the cDNAs, and the vector into which the cDNAs were cloned are indicated. Mutant \textit{p}67^{phox} constructs 1-526\Delta58K, 1-526\Delta226-236 and 1-526\Delta178-184 are deleted of lysine 58, amino acids 226-236 and amino acids 178-184 respectively; in 1-526P174L proline residue at position 174 was substituted by a leucine residue.

**pACT2 AD Vector**

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**A.2.1. Structure of the pACT-2 vector.** Structure of the pACT2 activation domain (AD) expression vector, showing the location of the multiple cloning site. The sequence of the multiple cloning site is shown in the lower panel.
A.2.2. Structure of the pAS2-1 Vector.

pAS2-1 DNA-BD Vector

ACT GTA TCG CTA TTA CTT CCA CAC CTG ACT

CAT ATG GCC ATG GAG GCC GAA TTC CCA GGG ATC

GT CTA CCT GCC GAA TTA ATT CGG GCC GAA TTT

CT TAT GAT TTT TAT TAT TAA ATA

A.2.2. Structure of the pAS2-1 vector. Structure of the pAS2-1 DNA binding domain (BD) expression vector, showing the location of the multiple cloning site. The sequence of the multiple cloning site is shown in the lower panel.

A.3.1. *E. coli* cell strains.

**XL-Blue:** recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac, [F’ proAB, lacI\^ZAM15, Tn10, (Tet')].

**BL21 (DE3):** B F- , dcm, ompT, hsdS(rB- mB-), gal, λ(DE3).

**JM107:** supE44, endA1, hsdR17, gyrA96, relA1, thi Δ(lac-proAB).

A.3.2. Yeast cell strain.

**YRG2:** Matα, ura3-52, his3-200, ade2-101, lys2-810, trp1-901, leu2-3 112, gal4-542, gal80-538, LYS2::UASGAL1-TATA\_GAL1-HIS, URA3::UASGAL4 17mer(x3)-TATA\_CYC1-lacZ.

A.4. Expression and purification of p67\textsuperscript{\text{hox}} from insect Sf9 cells, and isolation of insect Sf9 cell lysates.

Sf9 cells in IPL-41 medium containing 10 % foetal calf serum were grown in suspension in an orbital shaker. 800 ml of cell culture were infected to saturation with p67\textsuperscript{\text{hox}} cDNA containing baculovirus (or control, not infected) and were harvested after 72 h. The cells were washed with 50 mM Tris-HCl, pH 7.5, 50 mM NaCl and 1 mM EDTA supplemented with 1 mM DTT, 1 mM DIFP, 1 μg/ml leupeptin, 1 μg/ml pepstatin and 1 μg/ml TLCK. Cells were disrupted by sonication (MSE Soniprep) and cell debris was removed by centrifugation (30 000 x g, 15 min). The supernatant collected at this stage was either used for experiments to compare proteins of infected and control cell lysates or for purification of p67\textsuperscript{\text{hox}} protein by column chromatography; cell extracts were applied to a Hi-load Q-Sepharose column and protein eluted with a 50-500 mM NaCl gradient was then applied to a phenyl-Sepharose column from which protein was eluted with a 1 to 0 M NH\textsubscript{4}SO\textsubscript{4} gradient yielding a p67\textsuperscript{\text{hox}} preparation of ~95 % purity as judged by SDS-PAGE.
A.5. Purification of the Arp2/3 complex components from human neutrophil cytosol.

The purification was carried out by E. Reeves (Department of Medicine, University College London), who kindly supplied semi-purified fractions for p21-binding protein and immunoblot analysis. Details of the purification are described in Machesky et al., 1997, and briefly below.

Cytosol (approximately 1 400 mg of protein) from $10^{10}$ human neutrophils that had been stimulated with PMA for 3 min was chromatographed successively as follows; (1) Fast Flow S-Sepharose, (2) Heparin-Agarose, (3) Superose-12 (i), (4) mono-S, (5) Superose-12 (ii) and finally (6) Sucrose gradient. Fractions from the last two columns (steps 4 and 5) of this chromatographic series were investigated for the presence of Rac1 and Cdc42Hs binding proteins, β-PAK and p57 human coronin-like protein immunoreactivity.

Fractions from the mono-S and the second gel-filtration column, Superose-12 (ii), were shown to contain the Arp2/3 complex proteins (with approximate stoichiometry of 1), which were present with estimated purity of 37 and 58 % in the fractions collected from the mono-S and Superose-12 columns respectively (Machesky et al. 1997). Arp3 antiserum was raised to the human sequence homologous to the predicted loop region of *Acanthamoeba* Arp3, and was used to monitor the elution profile of the Arp2/3 complex during this purification.

Arp2/3 complex components were found to be eluted from the mono-S column with approximately 250 mM NaCl. The peak of this elution was found in fraction 13, fractions 10-18 were pooled and passed down a Superose-12 column. The Arp2/3 complex proteins were eluted at approximately 300 kDa (fractions 42-50) and were found to be the major constituents along with p57 (human coronin-like protein) of fraction 44. This latter fraction was also found to have 60 kDa kinase activity.
A.6. phox protein sequences and domain structures.

A.6.1. p67\textsuperscript{phox} Amino Acid Sequence and Domain Structure.

The predicted amino acid sequence of p67\textsuperscript{phox}, in single letter code (upper panel), and the domain structure of p67\textsuperscript{phox} (lower panel). The domain structure illustrates the positions of the tetratricopeptide repeat (TPR) motifs 1-4, the location of the Rac binding domain (RBD), the proline-rich region (P), and the two SH3 domains.

<table>
<thead>
<tr>
<th>TPR1</th>
<th>TPR2</th>
<th>TPR3</th>
<th>TPR4</th>
<th>RBD</th>
<th>P</th>
<th>SH3</th>
<th>SH3</th>
</tr>
</thead>
</table>

The predicted amino acid sequence of p67\textsuperscript{phox}, in single letter code (upper panel), and the domain structure of p67\textsuperscript{phox} (lower panel). The domain structure illustrates the positions of the tetratricopeptide repeat (TPR) motifs 1-4, the location of the Rac binding domain (RBD), the proline-rich region (P), and the two SH3 domains.

TPR: TPR motifs at amino acids: 6-36 (TPR1), 37-70 (TPR2), 71-104 (TPR3), and 121-154 (TPR4).

P: Polyproline domain at amino acids 226-236.

RBD: Rac binding domain at amino acids 170-199.

SH3: SH3 domains at amino acids 245-295 and 460-510.
A.6.2. \(\text{p}47^{\text{phox}}\) Amino Acid Sequence and Domain Structure.

The predicted amino acid sequence of \(\text{p}47^{\text{phox}}\), in single letter code (upper panel), and the domain structure of \(\text{p}47^{\text{phox}}\) (lower panel). The domain structure illustrates the position of the SH3 domains and the polyproline domains.

- **P**: polyproline domains at amino acids 70-83, 300-346 and 360-370. Note, the region between amino acids 300 and 346 contains two proline-rich motifs (amino acids 300-305 and 339-343), indicated (PP).
- **SH3**: SH3 domains at amino acids 151-214 and 227-284.
A.6.3. \( p40^{phox} \) Amino Acid Sequence and Domain Structure.

MAVAQQLRAE SDFEQLPDVV AISANIADIE EKRGFTSHFV FVIEVKTKGG 50
SKYLIIYRRYR QFHALQSKLLE ERFGPDSKSS ALACTLPTRL AKVYVGVKQE 100
IAEMRIPALN AYMKSLLSLP VWVLMDEDVR IFFYQSPYDS EQVPQAIRRL 150
RPRTRKVKSV SPQGNSVDRM AAPRAEALFD FTGNSKLELN FKAGDVIFLL 200
SRINKDWLEG TVRGATGIFP LSFVKLKDPE PEEDDPNWLR CYYEDTIS 250
TIKDIAVEED LSSTPLLKDL LEILRREFQR EDIALNYRDA EGDLVRLSD 300
EDVALMVRQA RGLPSQKRLF PWKLHITQKD NYRVYNTMP 339

SH3
PC

A.6.3. \( p40^{phox} \) amino acid sequence and domain structure.
The predicted amino acid sequence of \( p40^{phox} \) in single letter code (upper panel), and
the domain structure (lower panel). The domain structure illustrates the position of the
SH3 domain and the PC motif.

SH3: SH3 domain at amino acids 175-226.
PC: PC motif amino acids 282-309.
A.7. **p57 Human Coronin-Like Protein Amino Acid Sequence and Domain Structure.**

MSRQVRSSK FRHVFQPAK ADQYEDVRV SQTTWDGFC AVNPKFVALI 50
CEASHGAFL VPLGLKTGRV DKNAPTVGH TAPVLDIAC PHNHNVIAEG 100
SEDCTVMWE IPDGGLMLPL REPVVTLEGH TKRQGIVAVH TTAVNVLLSA 150
GCDNVIMWD VGTGAAMTL GPEVHPDTITY SVDCSRDGGQL ICTSCRDVRV 200
RIEPRKGTG VAEKDRPHG TRPVRAVFS EKGILGFGS RMSENQVALW 250
DTHKLLEPLS LQELDTSSGV LLPFDPEDTN IVYLCGKGDS SIRYFEITSE 300
APFLHYLSMF SSKEQAGMG YMPKQGELVN KCEIARFYKL HERRCEPIAM 350
TVPRKSDLFO EDLPPPTAGP DPALTAEWL GGRDGPLLIV SLKDGYVPPK 400
SRELVRNRL DTGRRRAAPE AGTSPSSAV SRLBEBMKL QATVQELEOKR 450
LDRLEETVQA K 461

A.7. **p57 human coronin-like protein amino acid sequence and domain structure.**

The predicted amino acid sequence of p57 human coronin-like protein in single letter code (upper panel). The locations of the five WD repeat motifs are underlined and the leucine residues, of the leucine zipper motif are highlighted in yellow. The potential Rac1 binding site (amino acids 120-143) is shown in bold type-face. The position of the Rac1 binding site was deduced from the sequence similarity with the coronin peptide that was found to bind Rac1 (see Fig. 7.3). The domain structure shown in the lower panel indicates the position of the WD repeat motifs and the C-terminal leucine zipper motif.


| leucine residues of the leucine zipper motif at positions 433, 440, 447, and 454. |
### A.8. Alignment of the Kinase Domains of PAK and Ste20 Proteins.

<table>
<thead>
<tr>
<th></th>
<th>Alpha-PAK RAT</th>
<th>Beta-PAK RAT</th>
<th>Gamma-PAK RAT</th>
<th>STE20 Sc</th>
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</tbody>
</table>

### A.8. Alignment of the kinase domains of PAK and Ste20 proteins.

Alignment of the amino acid sequences of rat brain alpha-PAK, beta-PAK, gamma-PAK and yeast (Sc) Ste20 proteins. Matches to alpha-PAK protein sequence have a shaded background. Numbers to the right hand side indicate the amino acid position in each sequence. Underlined sequence indicates the region of sequence of Ste20 protein to which the anti-Ste20 antibody (ubi), was raised (amino acids 738-752). The alignment was performed using DNASTAR Megalign software.