Phosphorylation of the $p67^{phox}$ component
of the NADPH oxidase in human phagocytes

by

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Abstract

p67phox is a component of the NADPH oxidase, the multicomponent enzyme that causes the production of superoxide in phagocytes. The phosphorylation of p67phox upon cellular activation was studied in neutrophils and B lymphocytes. p67phox showed low basal phosphorylation which increased up to three-fold upon stimulation. Phosphopeptide mapping revealed one major tryptic peptide, for p67phox from the cytosol of resting and stimulated cells. The same phosphopeptide was produced by different agonists.

In vitro phosphorylation provided insight to the phosphorylation of p67phox in the intact cell. Neutrophil cytosol or mitogen-activated protein (MAP) kinase produced the same phosphopeptide. Identification of the phosphorylation site threonine 233, involved high pressure liquid chromatography-mass spectrometry and site mutagenesis. In vitro phosphorylation of recombinant p67phox was lost by the mutation of threonine 233 to alanine. The structural significance of this site was pursued by attempts at crystallisation, but these were unsuccessful.

A series of studies into the mechanism of p67phox phosphorylation were performed. Although a MAP kinase pathway inhibitor (PD098059) reduced the in vitro phosphorylation of p67phox by cytosol, intact cell experiments did not clearly provide evidence for MAP kinase in native p67phox phosphorylation. The inhibitors staurosporine and okadaic acid gave results similar to those for p47phox, in that phosphorylation of p67phox correlates with activity, and that dephosphorylation may be involved in deactivation of the NADPH oxidase.

Experiments using p47phox-deficient cells showed that p67phox phosphorylation is independent of p47phox and is unlikely to require membrane interaction. Tyrosine phosphorylation was found to occur very weakly but was not associated with the formation of active NADPH oxidase.

These results form a foundation of research into the phosphorylation of p67phox. The functional importance of this event in the regulation of the NADPH oxidase is the subject of further study. Models are proposed for a structure-function relationship of p67phox phosphorylation within the NADPH oxidase.
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<th>Prefix</th>
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<tr>
<td>aa</td>
<td>amino acid</td>
<td>adenine, cytosine, guanine, thymine</td>
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<td>A, C, G, T</td>
<td>adenosine 5'-diphosphate</td>
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<td>adenosine 5'-triphosphate</td>
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<td>alanine</td>
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k  kilo
kb  kilobase
l  litre
Lys  lysine
m  milli
mA  milliamp
M  molar
MALDI-TOF  matrix-assisted laser-desorption ionisation-time of flight
MAP kinase  mitogen-activated protein kinase
MBP  myosin basic protein
MEK  MAP kinase / ERK kinase
Met  methionine
min  minutes
mol  mole
Mr  molecular weight markers
MS  mass spectrometry
mV  millivolt
n  nano
NADPH  nicotinamide adenine dinucleotide phosphate, reduced form
NMR  nuclear magnetic resonance
N-terminal  amino terminal
PAGE  polyacrylamide gel electrophoresis
PAK  p21-activated kinase
PAS  protein-A sepharose
PBS  phosphate-buffered saline
PCR  polymerase chain reaction
phox  phagocytic oxidase
P_i  orthophosphate
PKC  protein kinase C
PLC  phospholipase C
PMA  12-phorbol myristate 13-acetate
PNS  post-nuclear supernatant
RNA  ribonucleic acid
RP-HPLC  reverse phase high pressure liquid chromatography
rpm  revolutions per minute
RT  room temperature
S, T, Y  serine, threonine, tyrosine
SDS  sodium dodecyl sulphate
secs  seconds
Ser  serine
SH3 domain  Src-homology 3 domain
S/N  supernatant
S.O.Zym  serum-opsonised zymosan
TCA  trichloro-acetic acid
Thr  threonine
µ  micro
µF  miroFarrad
UV  ultra violet
Val  valine
v/v  volume to volume ratio
w/v  weight to volume ratio
Acknowledgements

I would like to thank my supervisors, Professor Tony Segal and Steve Moss for their help during these studies. I am also very grateful for the support I have received from many people in the Rayne Institute, particularly in the Department of Medicine, and especially from my colleagues in Professor Segal’s group. Beyond the Rayne Institute, I must mention the generous advice and expertise offered by Oanh Truong, Justin Hsuan and Alistair Stirling at the Ludwig Institute, Laurence Pearl in the Department of Biochemistry, University College London, and Adrian Thrasher and Gaby Brouns at the Institute of Child Health. My thanks also go to Elena Prigmore at the Institute of Neurology, and Angela Scott in the Medical Illustrations Department of Middlesex Hospital.

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Publications

At the time of submission of this thesis, the manuscripts derived from this work were as follows:

Forbes LV, Truong O, Moss SJ, Segal AW (1997) Identification of the major phosphorylation site of the NADPH oxidase component \( p67^{\text{phox}} \). Blood 90(10) Supp 1: 410a

Forbes LV, Truong O, Moss SJ, Segal AW The major phosphorylation site of the NADPH oxidase component \( p67^{\text{phox}} \) is threonine 233. (submitted to Biochemical Journal, 1998:)

Chapter 1

General introduction

1.1 The neutrophil

Leukocytes form the cellular basis of the body’s defence against the numerous pathogens presented by the environment. They can be divided into the lymphocytes, basophils and phagocytes. The word phagocyte comes from Greek “φαγό”, meaning “eating”, aptly describing the behaviour and function of this cell type which engulfs, destroys, and digests invading micro-organisms and cellular debris. The predominant phagocytic cell is the neutrophil, which accounts for approximately 60% of total blood leukocytes in adulthood (Altman & Dittmer, 1961). The neutrophil is often called a polymorphonuclear leukocyte due to its irregularly shaped, lobed nuclei, or a granulocyte referring to the presence of multiple granules in the cytoplasm.

The neutrophil life cycle passes through three phases; beginning in the bone marrow, followed by release into the blood, and finally migration into tissue. Bone marrow is the site of proliferation and terminal maturation following a sequence of myeloblast, myelocyte, band cell, to the mature neutrophil (Bainton et al., 1971). This mature neutrophil is a fully differentiated cell and has only a short life span. Still in the bone marrow, the proliferated population of neutrophils enters a large storage pool, and after 5 days is released into the blood where the cells circulate for a few hours before entering tissues (Cronkite & Vincent, 1969; Jamuar & Cronkite, 1980). Neutrophils in the peripheral blood consist of a freely circulating pool and an equal number of neutrophils in a marginated pool, where there is loose adherence to the vascular endothelium (Athens, 1981). This loose interaction with the endothelium allows a rolling
migration of the neutrophil. Upon stimulation this interaction becomes strong adherence and leads to the neutrophil penetrating both the endothelium and the basement membrane to enter the tissue (Figure 1.1). The signalling for this directed migration to sites of infection or inflammation involves chemotaxis, movement along an increasing gradient of chemoattractants.

![Figure 1.1](image)

**Figure 1.1  Electron micrograph of a neutrophil.** A neutrophil (N) is pictured leaving a blood vessel and entering tissue. The neutrophil has a granular appearance and a large, three-lobed, polymorphic nucleus. A neutrophil (centre) is passing between endothelial cells (E), and another neutrophil and macrophage (M) are temporarily arrested in the vessel wall between the endothelial layer and the basement membrane (arrow). Bar = 1μm. Micrograph by I. Joris and J.M. Underwood (Newburger & Parmley, 1995).

Phagocytosis is initiated by interaction between receptors on the neutrophil surface and the microbe. Particles of fungi or bacteria often undergo opsonisation (from Greek “to prepare for dining”) with immunoglobulin or fragments of complement, which renders them more susceptible to ingestion (Baggiolini & Wymann, 1990). Once the neutrophil has internalised the particle into a sealed phagocytic vacuole (or phagosome),
the killing and digestion takes place, involving the release of granule contents into the vacuole, termed degranulation.

The neutrophil granules form two major categories; the primary (or azurophilic) granules and the secondary (or specific) granules. The primary granules are produced early in neutrophil maturation and contain mostly myeloperoxidase, and also lysozyme, cationic proteins, proteases and acid hydrolases (Bainton & Farquhar, 1966). The secondary granules also contain lysozyme but otherwise have a different profile of constituents, including collagenase, lactoferrin, vitamin B$_{12}$-binding protein, chemoattractant receptors, adhesion receptors and the cytochrome of the NADPH oxidase (Borregaard et al., 1993). The granules serve as intracellular protein stores, but their main function is to fuse with the phagosome membrane and provide the armoury for effective destruction of the engulfed particle. The range of granule contents provides scope for killing a variety of pathogens which differ greatly in their susceptibility to oxidative and non-oxidative mechanisms.

1.2 Killing mechanisms:

(a) non-oxidative

The antimicrobial systems which can operate in the absence of oxygen, are limited to some proteases, hydrolytic enzymes, and cationic proteins, and are effective for certain microbes only (Odell & Segal, 1991; Elsbach & Weiss, 1992). On a molar basis, the bactericidal/permeability-increasing protein (BPI) of the primary granules is the most potent of the known antimicrobial granule proteins (Gabay et al., 1989). It is strongly cationic, a characteristic shared by many of the agents of non-oxidative killing, which aids in the firm adherence to the negatively charged surfaces of their targets. Defensins are also cationic and highly abundant in the primary granules. They form a
family of cytotoxic peptides with a broad spectrum of activity against bacteria, fungi and viruses (Lehrer et al., 1991). The other key agents of non-oxidative mechanisms are cathepsin G ("chymotrypsin-like cationic protein") (Dewald et al., 1975), the iron-binding glycoprotein lactoferrin (Arnold et al., 1982) and lysozyme (Iacono et al., 1980).

(b) oxidative

Oxidative mechanisms refer to the killing and digestion which is dependent on, or significantly enhanced by, the production of oxidants in the phagocytic vacuole. The chain of reactions that produce oxidants is triggered by an enzyme called the NADPH oxidase, which causes the single electron reduction of oxygen to superoxide (Figure 1.2). When the superoxide anion O$_2^-$ was shown to be produced by the activation of neutrophils (Babior et al., 1973), it was proposed that the production of reactive oxygen species may directly destroy engulfed microbes (Babior et al., 1975). Myeloperoxidase from primary granules can react with hydrogen peroxide to produce the highly toxic hypochlorous acid (Klebanoff, 1975), forming part of a complex array of oxidative reactions (Test et al., 1984). However, there is also evidence that the oxidase activity generates an environment suitable for the activation of certain proteases in the vacuole. That is, the cationic degradative enzymes in the granules are active at pHs above 7.0 (Odeberg & Olsson, 1975), and within the vacuole of normal neutrophils, there is a rise in the pH to above 7.0 (Segal et al., 1981b).

Although the purpose of the phagocytic oxidase is to remove infectious agents, foreign particles and damaged tissue from the body, reactive oxygen species can also damage host tissue in the vicinity of an inflammatory site (Weiss, 1989). Oxidative injury is thought to be causally associated with a number of inflammatory diseases such as arthritis (Boxer et al., 1990; Kitsis & Weissmann, 1991; Zimmerman & Granger, 1994).
Hence, the NADPH oxidase of phagocytes is a powerful immune defence mechanism, which needs tight regulation of its activity. Interestingly, the NADPH oxidase or closely related isoenzymes have been identified in other cell types where its function is not understood. These include B lymphocytes (Morel et al., 1993), fibroblasts (Jones et al., 1994) and mesangial cells (Jones et al., 1995). Superoxide produced in these cells is at a rate of only 1-5% of that produced in the neutrophil.

![Figure 1.2 Schematic representation of the phagocytosis of a microbe.](image)

1.3 Chronic granulomatous disease

The importance of oxidative mechanisms in the neutrophil’s defence against infection is proven by the serious manifestations of a defective NADPH oxidase. In an inherited disorder which is characterised by a nonfunctional neutrophil NADPH oxidase, chronic granulomatous disease (CGD), there is severely impaired killing of some microorganisms. The neutrophils of these patients exhibit normal chemotaxis and motility (Zicha et al., 1997), ingestion of invading micro-organisms, but fail to produce superoxide and an increase in the vacuolar pH (Segal et al., 1981b). This results in the accumulation of neutrophils, macrophages and lymphocytes (granuloma formation)
without clearance of infection. Severe and recurrent fungal and bacterial infections predominate in the lymph nodes, subcutaneous tissues, lungs, liver and bones, with gastrointestinal manifestations also (Segal et al., 1983; Clark et al., 1989; Roos et al., 1996). CGD is a rare disorder, with an estimated incidence of approximately 1/250,000 (Roos et al., 1996). The molecular basis for this disease is now understood, as discussed later, and its monogenic pathogenesis makes it a candidate for gene therapy which is currently being pursued (Malech et al., 1997).

1.4 Discovery of the NADPH oxidase

A large increase in oxygen consumption by neutrophils during phagocytosis was first observed in 1933 (Baldridge & Gerard, 1933). Originally it was thought to represent a surge in the normal respiration processes in the cell, but it was discovered that this burst of oxygen intake was not inhibited by mitochondrial toxins (Sbarra & Karnovsky, 1957). It was also observed that phagocytosis was accompanied by an increase in glucose catabolism through the hexose monophosphate shunt (Staehelin et al., 1957; Quie et al., 1967). Then, in the 1960s it was revealed that phagocytes produced hydrogen peroxide during the burst of oxygen consumption (Iyer, G.Y.N. et al., 1961), and that NADPH (nicotinamide adenine diphosphate, reduced form) which is a product of the hexose monophosphate shunt, was a substrate for this enzymatic process (Rossi & Zatti, 1964). Three decades after the first description of the “respiratory burst”, this oxidase function was recognised as a requirement for the efficient killing of bacteria by neutrophils (Selvaraj & Sbarra, 1966). This led to the finding that the leukocytes of patients with a severe predisposition to chronic infections (CGD) did not present a respiratory burst during phagocytosis (Baehner & Nathan, 1967; Holmes et al., 1967). Phagocytes from these patients were shown to be defective in bactericidal activity (Quie et al., 1967).
Major advances in the understanding of the biochemistry of phagocytosis then came from the discovery of a novel b-type cytochrome system in the vacuoles of phagocytes (Segal & Jones, 1978). The cytochrome is localised in the plasma membrane and in the membrane of the secondary granules, and upon stimulation of neutrophils it becomes incorporated in the phagosome membranes (Segal & Jones, 1979b, 1980b). It normally undergoes reduction upon the stimulation of neutrophils (Segal & Jones, 1979a). The absence of this reduction in CGD neutrophils (Segal & Jones, 1980a) led to the deduction that the microbicidal oxidase involved a cytochrome-based electron transport chain.

1.5 Cytochrome b_{558}

The cytochrome of the NADPH oxidase has a very low midpoint potential, initially thought to be -245 mV (Cross et al., 1981), and an α-band absorption maximum of 558 nm (Cross et al., 1982), hence the use of the names cytochrome b_{245} and cytochrome b_{558}. It is composed of two subunit proteins, one of 22 kDa which is referred to as the α subunit, and the other is 91 kDa, the β subunit (Parkos et al., 1987; Segal, 1987). These proteins are also called p22^{phox} and gp91^{phox} (where phox stands for the phagocytic oxidase) and they exist in a 1:1 molar ratio (Wallach & Segal, 1996). The β subunit is heavily glycosylated (Harper et al., 1985), and the identification of the (extracellular) glycosylation sites has aided in the understanding of the topology of this membrane-spanning protein (Wallach & Segal, 1997). A series of hydrophobic transmembrane segments in both the α and β subunits proteins anchors the cytochrome in the membrane (Taylor et al., 1993; Roos et al., 1996).

The transfer of electrons from cytosolic NADPH to oxygen in the vacuole is believed to be via flavin adenine dinucleotide (FAD) and haem cofactors in the
cytochrome. The cytochrome is a flavoprotein (Rotrosen et al., 1992; Segal et al., 1992; Sumimoto et al., 1992) with the non-covalent FAD binding site in the C-terminus of the β subunit. The FAD to haem ratio appears to be 1:2 (Segal et al., 1992) with two non-identical haem centres, with midpoint potentials of -225 mV and -265 mV, involved in the electron transport (Cross et al., 1995). The position of the haems remains to be fully elucidated. The consensus is that the cytochrome directly binds NADPH for oxidation. This is supported by the identification of NADPH binding site motifs (Rotrosen et al., 1992; Sumimoto et al., 1992) and demonstration of NADPH binding to the β subunit (Segal et al., 1992; Doussiere et al., 1993; Ravel & Lederer, 1993). Under certain relipidating conditions (phosphatidylcholine together with FAD and lithium dodecyl sulphate), isolated cytochrome can generate superoxide (Koshkin & Pick, 1994), providing further evidence that it bears the complete electron-transporting apparatus of the oxidase. Also, the kinetics of cytochrome reduction correlate with the observed rate of superoxide generation (Cross et al., 1985). Thus the cytochrome oxidase catalyses the following reaction:

\[
\text{NADPH} + 2\text{O}_2 \rightarrow \text{NADP}^+ + \text{H}^+ + 2\text{O}_2^-.
\]

The cytochrome exists in phagocytic cells; neutrophils, monocytes, macrophages and eosinophils (Segal et al., 1981a), and in the myeloid cell lines HL60 (Roberts et al., 1982) and U937 (Garcia et al., 1986), amongst others. Increased expression of the cytochrome corresponds to the amount of oxidase activity (Roberts et al., 1982). The total absence of the cytochrome in neutrophils was the first molecular deficiency identified as causing CGD (Segal et al., 1983). The genetic loci are on different chromosomes for the α and β subunits; 16q24 (Dinauer et al., 1990) and Xp21.1 (Franke et al., 1985; Baehner et al., 1986; Royer-Pokora et al., 1986), respectively. Mutations in the gene encoding the β subunit account for the CGD patients presenting with X-linked inheritance. This is the most common form of CGD amounting to about
two thirds of all cases (Clark et al., 1989; Casimir et al., 1992). Both subunits are usually missing in X-linked CGD (Segal, 1987) despite normal mRNA encoding the α subunit, suggesting that protein stability of the cytochrome depends on the interaction of the two subunits. This is supported by the observation that the α subunit shows ubiquitous expression of mRNA although the protein is only detectable in phagocytic cells where the β subunit is expressed (Parkos et al., 1988). Genetic lesions in the α subunit account for approximately 5% of all CGD cases (Clark et al., 1989; Casimir et al., 1992), and is associated with autosomal recessive inheritance.

A Ras-related GTP-binding protein, Rap1A, has been copurified with the cytochrome (Quinn et al., 1989), but the role this might play in the oxidase is not known. Another feature of the cytochrome remaining to be understood in terms of its effect on oxidase activity, is the phosphorylation of both subunits (Garcia & Segal, 1988). This occurs in activated neutrophils but with a timecourse differing from that of oxidase activation. It has been suggested that phosphorylation may play a regulatory role in facilitating electron transfer or perhaps in the termination of activity.

1.6 Introduction of the cytosolic factors

In addition to the CGD patients who lacked the cytochrome, it was discovered that there were some patients who were not deficient in the cytochrome, but the cytochrome did not undergo reduction (Segal & Jones, 1980a). The cytochrome appeared to be present at normal levels and exhibit normal cytochrome properties (unchanged midpoint potential and ability to bind CO₂), suggesting the abnormality lay elsewhere (Segal et al., 1983). The first indication that the cytosol contained factors that were necessary for activation of the cytochrome, came from cell-free studies on whole cell homogenates (Bromberg & Pick, 1985; McPhail et al., 1985). These experiments showed that NADPH oxidase activity could be induced in a mixture of membranes and
cytosol prepared from resting neutrophils by the addition of an amphiphile in micromolar concentration. The neutrophil cytosol of some patients with autosomally inherited CGD, was deficient of this activity (Curnutte et al., 1988). This opened the investigation of cytosol fractions for components that played a role in the activation of the NADPH oxidase.

The absence of the phosphorylation of a 47 kDa cytosolic protein was the first clearly defined biochemical abnormality in autosomal CGD (Segal et al., 1985; Hayakawa et al., 1986). The identification of this 47 kDa protein together with a 67 kDa cytosolic protein was achieved by raising an antibody against a cytosolic fraction that could complement the membrane in a cell-free system (Volpp et al., 1988). This led to the cloning of p47phox (Volpp et al., 1989) and demonstration that it could restore the activity of some autosomal recessive CGD cytosol (Lomax et al., 1989). The other protein recognised by the antibody was shown to be able to reconstitute activity in CGD cytosol which was unresponsive to added p47phox (Nunoi & Malech, 1988). The cloning of this 67 kDa molecule (Leto et al., 1990) led to the characterisation of the rare CGD phenotype caused by p67phox-deficiency.

It was observed that p47phox and p67phox exist in the cytosol of resting neutrophils in a large complex of approximately 240 kDa (Park, J.-W. et al., 1992; Jouan et al., 1993). Immunoprecipitation of p67phox revealed a third protein of 40 kDa in this complex (Someya et al., 1993; Wientjes et al., 1993) which was cloned and named p40phox (Wientjes et al., 1993). A proportion of these three proteins, p47phox, p67phox and p40phox were shown to become membrane-bound upon stimulation of neutrophils (Clark et al., 1990; Heyworth et al., 1991; Nauseef et al., 1991; Park, J.-W. et al., 1992; Wientjes et al., 1993). It was observed that once this translocation had occurred, the cytosol had diminished activity in a cell-free assay (Ligeti et al., 1989).
A further cytosolic requirement for NADPH oxidase activation was identified by fractionation of neutrophil cytosol. A cell-free mixture of isolated membranes and cytosol fractions was only able to support oxidase activity if p47<sup>phox</sup> and p67<sup>phox</sup> and a Rac-containing fraction were all present (Abo et al., 1991). Rac (also known as p21<sub>rac</sub>) is a small GTP-binding protein, which also translocates to the membrane upon activation (Quinn et al., 1993). It is an essential ingredient in cell-free oxidase systems (Fuchs et al., 1994; Kreck et al., 1994). The cell-free system has therefore been central in the identification of the cytosolic proteins of the multicomponent NADPH oxidase. The minimal requirements for cell-free activity have now been recognised as lipid-reconstituted cytochrome (neutrophil-extracted or recombinant (Rotrosen et al., 1993)), p47<sup>phox</sup>, p67<sup>phox</sup>, Rac-GTP, FAD, NADPH and an amphiphile such as sodium dodecyl sulphate (SDS) (Abo et al., 1992; Abo & Segal, 1995).

In the following sections, an overview of the signalling pathways is given, and then each of the cytosolic protein components is described in terms of their intermolecular interactions, phosphorylation, and other regulatory events that occur in the activation of the NADPH oxidase.

### 1.7 Signal transduction pathways

The neutrophil NADPH oxidase is triggered during the process of recruitment to sites of infection by chemotaxis. Stimulation can be brought about by a variety of agonists in vitro (reviews, Rossi, 1986; Morel et al., 1991; McPhail & Harvath, 1993; Thrasher et al., 1994). The range of physiological stimuli which have been used in experiments include opsonised particles, chemoattractants such as the complement fragment C5a and the formylated peptide f-methionyl-leucyl-phenylalanine (fMLP), and activating molecules including platelet activating factor (PAF), leukotriene B4 and
interleukin-8. These activators initiate signalling via seven membrane-spanning receptors coupled to heterotrimeric guanine nucleotide binding proteins (G-proteins). Signal transduction through these receptors involves stimulation of phospholipase C (PLC) and phosphatidylinositol 3-kinase (Snyderman & Pike, 1984). This leads to the mobilisation of Ca\(^{2+}\) which can cause activation of phospholipases A2 and D, releasing arachidonic acid and phosphatidic acid respectively (Lambeth, 1988). Diacylglycerol is also produced by phosphoinositide activity, and brings about activation of protein kinase C (PKC) (Nishizuka, 1984).

Cell-permeable agonists, bypassing G-protein and PLC activation, are often applied in the study of the NADPH oxidase. These include phorbol esters such as phorbol 12-myristate 13-acetate (PMA), and fatty acids such as arachidonic acid. High oxidase activity is elicited by these agonists, but the initial signalling mechanisms are different from receptor-mediated stimuli (review, Thrasher et al., 1994). For example, PMA is a direct activator of PKC, and does not involve mobilisation of intracellular Ca\(^{2+}\) stores (Grzeskowiak et al., 1986), whereas the response triggered by fMLP induces Ca\(^{2+}\) flux upon which fMLP signal transduction is dependent (Lew et al., 1984). Stimulation by fMLP but not PMA activates two neutrophil p21-activated kinases (PAKs, 65 and 68 kDa) (Knaus et al., 1995).

Studies of the differences between PMA and receptor-mediated signalling have focused on the use of inhibitors. Wortmannin, primarily a phosphatidylinositol 3-kinase inhibitor, and tyrosine kinase inhibitors (Dewald et al., 1988; Naccache et al., 1990; Vlahos et al., 1995), reduce the response from fMLP but not PMA stimulation. PKC inhibitors do not completely prevent the fMLP response as they do in the case of PMA stimulation (Robinson et al., 1990). It is likely that although receptor-mediated and cell-permeable agonists differ in upstream signal transduction, the pathways converge to trigger the same signals controlling NADPH oxidase activity. For example, both fMLP
and PMA cause the translocation of p67<sup>phox</sup>, p47<sup>phox</sup>, p40<sup>phox</sup> and Rac to the membrane, although to differing degrees. Quantitation of the translocation in different studies has given a range of 2-20% of each protein (DeLeo & Quinn, 1996). The higher values averaging 10-15% occur with PMA stimulation, whereas only 2-5% translocates with fMLP stimulation (Clark <i>et al.</i>, 1990; Quinn & Bokoch, 1995).

### 1.8 Priming

Superoxide production for the activation of the NADPH oxidase occurs after a delay following neutrophil stimulation (Rossi <i>et al.</i>, 1983). This lag phase represents the complex series of integrated signal transduction events taking place during activation. The duration of the lag phase, and the magnitude of the subsequent oxidase response, are dependent on the nature and dose of the stimulus. For the nonphysiological stimulus PMA, the lag phase is relatively long and superoxide production is relatively high and long-lasting (McPhail & Snyderman, 1983). The response to fMLP is highly dependent on the preactivation status of the cell. This relates to priming, a process resulting in an accelerated and elevated response of the neutrophil (Bender <i>et al.</i>, 1983; McPhail <i>et al.</i>, 1984).

Priming refers to the effect of exposing cells to substimulatory levels of agonist, or nonstimulatory agents, prior to activation. Priming does not induce superoxide generation without additional stimulation. Hence, the primed neutrophil represents a state distinct from the resting and activated states (McPhail <i>et al.</i>, 1984). It is not known whether it is a physiological phenomenon, although it has been demonstrated that subpopulations of neutrophils from subjects with acute bacterial infection have increased oxidant production upon stimulation by PMA than those from uninfected individuals (Bass <i>et al.</i>, 1986). The process is believed to be partly due to increases in intracellular
levels (Morel et al., 1991), but has also been associated with upregulation of tyrosine phosphorylation (Hallett & Lloyds, 1995) and cytoskeletal rearrangements (Crawford & Eggleton, 1991; Murkerjee et al., 1994). It is feasible that priming represents a transitional state in preparation for producing an effective response upon stimulation. Reversible priming of neutrophils has been demonstrated using PAF, suggesting that priming and depriming is a control mechanism for functional activation at sites of inflammation (Kitchen et al., 1996).

1.9 Phosphorylation

Upon activation of the NADPH oxidase, many phosphorylation events occur in the cell (Schneider et al., 1981), including the phosphorylation of the cytosolic components p67phox (Dusi et al., 1993), p47phox (Segal et al., 1985), p40phox (Fuchs et al., 1997) and both subunits of the membrane-bound cytochrome (Garcia & Segal, 1988). This suggests that the activation of protein kinases is responsible for stimulation of the oxidase, supported by the observation that oxidase activity in electroporpermeablised cells is dependent on ATP and Mg2+ (Grinstein et al., 1989; Nasmith et al., 1989). Various specific phosphorylation events have been identified such as the cAMP-dependent kinase phosphorylation of Rap1A (Quilliam et al., 1991), and the fMLP-stimulated activation of cGMP-dependent protein kinase (Pryzwansky et al., 1990). However, most of the phosphorylation research has focused on three main areas: the phosphorylation of p47phox which is discussed later (section 1.11), the involvement of tyrosine phosphorylation, and the delineation of PKC-dependent and -independent processes.

Activation of the NADPH oxidase is accompanied by tyrosine phosphorylation within the neutrophil (review, Morel et al., 1991). Although serine/threonine phosphorylation is stoichiometrically more abundant, tyrosine phosphorylation of cellular...
proteins plays an important role in signal transduction. Tyrosine phosphorylation appears to be mediated through a pertussis toxin-sensitive G-protein and occurs in priming, phorbol ester and membrane receptor-mediated stimulation. It has led to the identification of tyrosine phosphorylation-triggered Ras-signalling pathways in the activated neutrophil, such as the mitogen-activated protein (MAP) kinase cascade (Worthen et al., 1994). In addition to the significant tyrosine phosphorylation of MAP kinase proteins of 40 and 42 kDa (Grinstein & Furuya, 1992; Torres et al., 1993), the phosphorylation of an unidentified ~70 kDa protein appears to be associated with oxidase stimulation (Nasmith et al., 1989; Dusi et al., 1994; Green & Phillips, 1994; Rollet et al., 1994). Tyrosine phosphorylation is also responsible for the activation of phospholipases Cγ2 and D, two processes involved in transmembrane signal transduction for the activation of the NADPH oxidase (Dusi et al., 1994). It is thought that the mechanism by which vanadate (a potent inhibitor of tyrosine phosphatases) induces oxidase activity, involves the activation of phospholipases Cγ2 and D (Yamaguchi et al., 1995).

Extensive research into the involvement of PKC in the activation of the NADPH oxidase has provided evidence both for and against (Rossi, 1986; Sergeant & McPhail, 1997). The strongest argument in favour is that exposure of neutrophils to soluble and particulate stimuli induces PKC translocation to the membrane, protein phosphorylation and oxidase activation (reviewed in Sergeant & McPhail, 1997). Conflicting results have been reported from the application of various inhibitors, and from studies measuring the effect of Ca^{2+} concentrations (Rossi, 1986). This is likely to be due to lack of specificity of the inhibitors and differences in experimental procedures. The consensus is that there are both PKC-dependent and -independent pathways required for the physiological activation of the NADPH oxidase (Baggiolini & Wymann, 1990). That is, receptor agonists trigger two pathways via heterotrimeric G-protein activation; one that is Ca^{2+}-
dependent leading to the activation of PKC, and another which is Ca\(^{2+}\)-independent and does not involve PLC or PKC (Dewald et al., 1988). It has been suggested that the initial burst of superoxide production by fMLP or phagocytic stimulation is independent of PKC, but that PKC-dependent mechanisms are responsible for sustaining the oxidase activity (Watson et al., 1991).

### 1.10 p47\(^{phox}\)

The human gene locus of p47\(^{phox}\) is 7q11.23 (Francke et al., 1990). Protein expression of p47\(^{phox}\) is largely restricted to cells of haematopoietic origin (neutrophils, monocytes, eosinophils and B lymphocytes) (Rodaway et al., 1990), but has also been detected in fibroblasts and mesangial cells (Jones et al., 1994, 1995). The protein is 390 amino acids long and has a highly cationic C-terminus (Lomax et al., 1989; Volpp et al., 1989). It contains two SH3 (Src homology 3) domains, which are regions of approximately 50 amino acids of conserved homology, first described in the Src family of tyrosine kinases (Mayer et al., 1988). They are found in a variety of proteins involved in signal transduction (Dawson & Gish, 1992; Cohen et al., 1995), and appear to mediate protein-protein interaction via binding to proline-rich motifs in target proteins (Ren et al., 1993). p47\(^{phox}\), p67\(^{phox}\) and the \(\alpha\) subunit each contain such polyproline sequences, and SH3 domains exist in p47\(^{phox}\), p67\(^{phox}\) and p40\(^{phox}\).

The multimolecular complex containing p47\(^{phox}\) in resting cells has been reported to have a molar ratio of p47\(^{phox}\) to p67\(^{phox}\) to p40\(^{phox}\) of 1:1:1 (Park, J.-W. et al., 1994; Tsunawaki et al., 1994), or 1:2:2 where p47\(^{phox}\) is less abundant (Heyworth et al., 1997). It is generally agreed that there is an excess of p47\(^{phox}\) in unstimulated cytosol (Leto et al., 1991; Heyworth et al., 1994), which is free of this complex (El Benna et al., 1994a; Iyer, S.S. et al., 1994; Heyworth et al., 1997). Three different interactions between
p47phox and p67phox have been identified. These are between the C-terminal SH3 domain of p67phox and the C-terminal p47phox polyproline region (Finan et al., 1994; Leto et al., 1994; De Leo et al., 1996; Fuchs et al., 1996; Ito et al., 1996), the N-terminal half of p67phox with the C-terminal-, or both, SH3 domains of p47phox (de Mendez et al., 1996, 1997), and a third interaction involving amino acids 323-322 of p47phox (De Leo et al., 1996). The binding of p47phox to p40phox takes place between the p40phox SH3 domain and the polyproline domain of p47phox (Fuchs et al., 1995; Ito et al., 1996; Sathyamoorthy et al., 1997). This region of p47phox can also bind p67phox so not all sites are mutually exclusive or are likely to be used simultaneously in vivo.

A variety of different methods have been employed to determine these sites of interaction. These include the yeast two-hybrid system, solid phase/immobilisation interactions, and competitive binding using peptides. Cell-free oxidase systems have also been widely applied because they allow identification of the minimum requirements for superoxide production and are practically versatile. Although this allows interactions to be examined in terms of an activity output, the use of amphiphiles is a nonphysiological way to induce activation and can be both informative and misleading. For example, some findings from cell-free systems may provide insight to in vivo mechanisms. SDS and arachidonic acid have been shown to induce p47phox binding of p67phox (Sumimoto et al., 1994). That is, the p47phox SH3 domains only become available to interact with p67phox in the presence of an amphiphile (Sumimoto et al., 1994). This has been interpreted as an amphiphile-induced conformational change such that the intramolecular binding of p47phox is disrupted, unshielding the SH3 domains for intermolecular binding. In another study, binding between p67phox and the 323-332 amino acid region of p47phox only occurred after SDS was added, suggesting it also is an activation-dependent interaction (De Leo et al., 1996).
The results of cell-free studies could be misleading since certain interactions required in vivo are apparently not essential in the presence of amphiphiles. For example, the cell-free oxidase is unaffected by the deletion of the polyproline region of p47^{phox} even though it destroys the binding of p67^{phox} (Leusen et al., 1995; Hata et al., 1997). In whole cell systems the interaction between p47^{phox} and p67^{phox} is required for maximal activity (de Mendez et al., 1994, 1996, 1997). Furthermore, in some cell-free systems with elevated levels of p67^{phox} and Rac, p47^{phox} is not required at all for oxidase activity, although optimal activity is achieved with it (Freeman & Lambeth, 1996; Koshkin et al., 1996). This finding can be interpreted as indicating that p47^{phox} may play a modulating role rather than a direct role in the electron transfer. However, these findings illustrate fundamental deviations in such in vitro models from the intact cell NADPH oxidase.

Binding studies provide evidence for a direct interaction between p47^{phox} and the cytochrome which is central to the understanding of the translocation events. Their interaction seems to use the SH3 domains of p47^{phox} and a polyproline region on the α subunit (Finan et al., 1994; Leto et al., 1994; Sumimoto et al., 1994, 1996; de Mendez et al., 1997), but some studies show that both subunits are involved (Nakanishi et al., 1992; Cross & Curnutte, 1995; DeLeo et al., 1995a,b; Adams et al., 1997; Park, M.-Y. et al., 1997). Two rare CGD cases with expressed but defective cytochrome proteins, support a role for both α and β subunit sites (Leusen et al., 1994a,b). Studies of cytochrome-deficient and p47^{phox}-deficient CGD cells have shown that in the absence of cytochrome, neither p47^{phox} or p67^{phox} translocate to the membrane, and in the absence of p47^{phox}, p67^{phox} does not translocate (Heyworth et al., 1991). p47^{phox} seems to be the first cytosolic component to interact with the cytochrome (Kleinberg et al., 1990; DeLeo et al., 1995b), and their association is a requirement for p67^{phox} translocation.
Reconstitution studies on intact cells (the multiprogenitor cell line K562), show that the core region of p47\textsuperscript{phox} spanning both SH3 domains is required for translocation and oxidase activity (de Mendez et al., 1996, 1997). Mutagenesis enabled the identification of critical residues within the putative SH3 domain binding pockets of p47\textsuperscript{phox}, and indicated that the N-terminal SH3 domain binds to the \( \alpha \) subunit and the C-terminal one binds to p67\textsuperscript{phox} (de Mendez et al., 1997). Interestingly, the translocation of the N-terminal half of p67\textsuperscript{phox}, via interaction with this SH3 domain of p47\textsuperscript{phox}, is able to support 10\% of maximal activity (de Mendez et al., 1997). However, this truncated protein may behave artificially because it was reported to bind to the membranes even without PMA activation.

Kinetic studies have shown that the timecourse of translocation closely parallels the kinetics of superoxide production (Quinn & Bokoch, 1995). Also, the phosphorylation of p47\textsuperscript{phox} corresponds with the kinetics of activation (Heyworth & Segal, 1986). It has been shown that while the NADPH oxidase is active, p47\textsuperscript{phox} undergoes both continuous phosphorylation (and dephosphorylation) (Heyworth & Badway, 1990; Ding, J. \& Badway, 1992) and continuous translocation (Clark et al., 1990; Rotrosen et al., 1990; Nauseef et al., 1991; Dusi et al., 1993). Therefore the phosphorylation and translocation of p47\textsuperscript{phox} seem to be related processes (see also section 1.7). p47\textsuperscript{phox} phosphorylation starts in the cytosol (Heyworth \textit{et al}., 1989) but it becomes more heavily phosphorylated at the membrane (Okamura \textit{et al}., 1988; Rotrosen \& Leto, 1990). This seems to be dependent on binding to the \( \beta \) subunit of the cytochrome because lack of the cytochrome (Ding, J. \textit{et al}., 1993) or disruption of the interaction between p47\textsuperscript{phox} and the \( \beta \) subunit (Rotrosen \textit{et al}., 1990) causes p47\textsuperscript{phox} to become only partially phosphorylated. Inactivation of the NADPH oxidase does not appear to involve complete disassembly of the complex, as some translocated p47\textsuperscript{phox} and p67\textsuperscript{phox} remains at the membrane (Dusi \textit{et al}., 1993), and the interaction between p47\textsuperscript{phox}
and the cytochrome appears to be irreversible (Kleinberg et al., 1990; DeLeo et al., 1995b). Interestingly, a recent study showed transient translocation of p47<sup>phox</sup> in opsonised zymosan-stimulated neutrophils, but with 5% of total cellular p47<sup>phox</sup> remaining at the membrane after activation (Sergeant & McPhail, 1997).

1.11 Phosphorylation of p47<sup>phox</sup>

After stimulation of neutrophils, there are at least six p47<sup>phox</sup> phosphoprotein isoforms due to multiple phosphorylation (Kramer et al., 1988; Rotrosen & Leto, 1990). The phosphorylation in PMA-stimulated neutrophils occurs on a region of serine residues located near the C-terminus of the protein (El Benna et al., 1994b). At least six serines in the region 303-379 are phosphorylated, and possibly more. They were identified by enzymatic digestion of phosphorylated p47<sup>phox</sup> immunopurified from cytosol, followed by chromatographic resolution of phosphopeptides and amino acid sequencing (El Benna et al., 1994b). Site-directed mutagenesis of the serines, followed by transfection into p47<sup>phox</sup>-deficient Eptein Barr virus (EBV)-transformed B cells, showed that the phosphorylation of serine 379 in particular might be important in activation of the NADPH oxidase (Faust et al., 1995). The mutation of this serine to alanine significantly inhibited translocation of p47<sup>phox</sup> and oxidant production of the transfected B cells. Also, PKC-phosphorylated Ala-379 p47<sup>phox</sup> was unable to support oxidase activity in a cell-free system (Park, J.-W. et al., 1997). However, in a subsequent publication by the same group, the phosphorylation of this serine was undetectable and the mutation to alanine did not change the p47<sup>phox</sup> phosphopeptide map (El Benna et al., 1996). It would appear, therefore, that the change produced by alanine substitution at 379 was unrelated to phosphorylation.

p47<sup>phox</sup> phosphorylation is thought to induce a conformational change required for assembly and activation of the NADPH oxidase at the membrane. It seems likely that
the strongly cationic (basic) C-terminal region between amino acids 301-379 would need to be neutralised in order to become membrane-bound. Recent studies compared the ability of anionic amphiphiles and PKC phosphorylation to induce conformational changes to recombinant p47phox, assessed by spectroscopic measurements (Swain et al., 1997), and by the ability for C-terminal alkylation (Park, J.-W. & Babior, 1997). The results indicated that significant conformational change does take place and is similar by either treatment. This is consistent with the binding studies which suggest that amphiphiles lead to the unshielding of the p47phox SH3 domains (Sumimoto et al., 1994; DeLeo et al., 1996). Amphiphiles cause p47phox to bind to the membranes in cell-free oxidase systems (Uhlinger et al., 1993), where some phosphorylation occurs but is not required for activation (Uhlinger & Perry, 1992; Nauseef et al., 1993; McPhail et al., 1995).

Phosphorylation is able to replace amphiphiles in the activation of cell-free systems. For example, the addition of PKC (Cox et al., 1985; Swain et al., 1997) or phosphatidic acid with diacylglycerol (McPhail et al., 1995) to cytosol and membranes is sufficient to activate the NADPH oxidase. In the latter assay, timecourse analysis showed phosphorylation of p47phox preceded activity. The superoxide production was 70% inhibited by staurosporine or GF109203X PKC inhibitors, with a concomitant decrease in the phosphorylation of p47phox and other proteins. Recombinant p47phox which has been phosphorylated by PKC, can translocate in cell-free systems without amphiphiles (Park, J.-W. & Ahn, 1995), and can activate the oxidase (Park, J.-W. et al., 1997). This supports data from intact cell studies which are consistent with a corequirement of p47phox phosphorylation and PKC activity for oxidase activation (Heyworth et al., 1989). The phosphorylation activated cell-free systems do not elicit as much superoxide production as the amphiphile systems (Park, J.-W. et al., 1997; Swain et al., 1997), but they demonstrate that molecular rearrangements caused by amphiphiles
can at least be partially replaced by p47phox phosphorylation. It is of interest that if the cytosol of the cell-free system were ATP-depleted before the addition of pre-phosphorylated p47phox, the oxidase activity was reduced to ~13% (Park, J.-W. et al., 1997). This suggests that another kinase-dependent reaction participates in the full activation of the NADPH oxidase other than PKC phosphorylation of p47phox.

Many studies have tried to define the relationship between p47phox phosphorylation, translocation and oxidase activation. Inhibition of PMA-induced p47phox phosphorylation by staurosporine (a non-specific PKC kinase inhibitor that also exhibits some tyrosine kinase inhibition), causes inhibition of oxidase activity (Badway et al., 1989; Robinson et al., 1990; Watson et al., 1991). Enhancement of stimulated phosphorylation by phosphatase 1 and 2A inhibitors can prolong the activated state (Ding, J. & Badway, 1992; Garcia et al., 1992; Suzuki et al., 1995). The effect of the inhibitors on translocation should also be taken into account. Translocation is reduced by staurosporine in PMA or fMLP (with prior Ca²⁺ depletion) (Dusi et al., 1993), and translocation is increased by okadaic acid (Garcia et al., 1992; Quinn & Bokoch, 1995). Although translocation contributes to oxidase activity, it is not sufficient in itself to induce activation. This has been demonstrated by the calcium ionophore A23187 which can induce translocation without causing oxidase activity (Garcia et al., 1992), and 2-deoxyglucose which can inhibit oxidase activity without affecting translocation (Clark et al., 1990). Phosphorylation per se is not sufficient for oxidase activation either, as the use of inhibitors of phosphatases 1 and 2A to induce phosphorylation does not induce oxidase activity (Ding, J. & Badway, 1992; Garcia et al., 1992; Yamaguchi et al., 1996). This has led to the common belief that a modification such as phosphorylation is required in addition to translocation to bring about oxidase activation.

There are experimental data consistent with a corequirement of p47phox phosphorylation and translocation for oxidase activity. Two studies report the inhibition
of p47\textsuperscript{phox} phosphorylation, translocation and oxidase activity by the introduction of peptides to the whole cell. One study used a peptide of the \( \beta \) subunit (Rotrosen \textit{et al.}, 1990) and the other used a peptide of p47\textsuperscript{phox} (Labadia \textit{et al.}, 1996). This amounts to circumstantial evidence that phosphorylation of p47\textsuperscript{phox} is necessary for translocation and oxidase activation.

In the case of receptor-mediated stimulation however, the phosphorylation process can be dissociated from translocation and oxidase activity. That is, staurosporine used with fMLP stimulation (without Ca\textsuperscript{2+} depletion) significantly inhibits the phosphorylation of p67\textsuperscript{phox} and p47\textsuperscript{phox} but translocation and oxidase activity proceed almost unaffected (Robinson \textit{et al.}, 1990; Dusi \textit{et al.}, 1993). Another paper reports that optimal amounts of the synthetic diglyceride 1,2-dioctanoyl-sn-glycerol, can stimulate approximately 80% normal superoxide production in the presence of staurosporine which blocked all stimulated phosphorylation of p47\textsuperscript{phox} (Badway \textit{et al.}, 1989). These studies suggest that there is heterogeneity in the activation of the NADPH oxidase depending on the circumstances of stimulation. Alternatively, there may be a modification other than phosphorylation which is required for translocation. Another explanation is that only a threshold amount of phosphoprotein may be needed, emphasising that inhibitor studies with only partial effects must be interpreted with caution. In support of this, there is a study where 20% phosphorylation of p47\textsuperscript{phox} was sufficient to obtain maximal translocation and oxidase activity (Levy \textit{et al.}, 1994). This was observed by the use of PKC inhibitors with opsonised zymosan stimulated neutrophils. In summary, the only studies to show a strict correlation between levels of phosphorylation, translocation and activity are those with PMA stimulation.

There is a body of evidence supporting the involvement of multiple kinases in the phosphorylation of p47\textsuperscript{phox}. In one study, comparisons were made between the phosphopeptide maps from PMA-stimulated EBV-B cell p47\textsuperscript{phox}, and p47\textsuperscript{phox}.
phosphorylated in vitro by PKC, protein kinase A (PKA) and MAP kinase (El Benna et al., 1996). PKC phosphorylated almost all the sites except the serines phosphorylated by MAP kinase. PKA phosphorylated some of the sites that were also phosphorylated by PKC. Recombinant p47^{phox} which has been phosphorylated in vitro by PKA or MAP kinase was unable to support oxidase activity in the cell-free system which was active with PKC-phosphorylated p47^{phox} (Park, J.-W. et al., 1997). Interestingly, a role for PKA has been proposed such that its activation downregulates the phosphorylation (or promotes dephosphorylation) of p47^{phox} for deactivation of the oxidase (Bengis-Garber & Gruener, 1996). PAK has been shown to phosphorylate p47^{phox} in vitro (Knaus et al., 1995). It has recently been suggested that p47^{phox} is phosphorylated on tyrosine residues in addition to serine, and that a cytosolic phosphatidic acid activated kinase other than PKC, MAP kinase or PAK, may be involved (Waite et al., 1997). Correlations have been observed between the kinetics of activation of several unidentified kinases and the kinetics of p47^{phox} phosphorylation (Ding, J. & Badway, 1993).

It should be noted that the phosphorylation sites of intact cell phosphorylated p47^{phox} have been analysed by PMA activation only. Although p47^{phox} phosphopeptide maps have been shown to be similar by PMA and fMLP stimulation (El Benna et al., 1994b), as are the numbers of phosphorylated isoforms (Okamura et al., 1988), the physiological significance of the p47^{phox} phosphorylation would only be properly evaluated by physiological stimuli. It is known that stimulation induced by opsonised particles causes fewer proteins to be phosphorylated, and phosphorylation is more transient, than for PMA stimulation (Heyworth & Segal, 1986). All published p47^{phox} phosphorylation site data relates to cytosolic p47^{phox}, so no delineation of the cellular localisation of individual phosphorylation events has been made. Thus, the p47^{phox} results to date do not provide definitive proof that phosphorylation is a prerequisite for
activation, and the exact sites, kinases and translocation requirements remain to be
determined for physiological stimulation of the oxidase.

1.12 \textit{p67}^{*}^{*}

\textit{p67}^{*}^{*} was the second cytosolic component discovered to be required for a
functional NADPH oxidase (Nunoi \& Malech, 1988). Its absence accounts for the
defective microbicidal activity of neutrophils from autosomal recessive CGD patients
which are both \textit{p47}^{*}^{*} and cytochrome-positive (Leto \textit{et al.}, 1990). \textit{p67}^{*}^{*}-deficiency
is a rare phenotype of CGD, amounting to only 5\% of all cases, compared with about
25\% for \textit{p47}^{*}^{*}-deficiency (Clark \textit{et al.}, 1989; Casimir \textit{et al.}, 1992). The expression of
\textit{p67}^{*}^{*} appears to be restricted to the same cell lineages as for \textit{p47}^{*}^{*} (see section 1.6),
but the gene is located on a different chromosome, 1q25 (Francke \textit{et al.}, 1990; Kenney \textit{et
al.}, 1993).

\textit{p67}^{*}^{*} is the subject of this study. The \textit{p67}^{*}^{*} gene, locus name NCF-2
(neutrophil cytosolic factor), is 37 kb with 2.4 kb mRNA (Leto \textit{et al.}, 1990). There are
16 exons (Kenney \textit{et al.}, 1993), coding for a 526 amino acid protein. Six CGD patients
with the rare phenotype of \textit{p67}^{*}^{*}-deficiency have been analysed for the genetic defect
(Table 1.3). Unlike \textit{p47}^{*}^{*}-deficiency where a dinucleotide deletion is common to the
majority of cases analysed (Casimir \textit{et al.}, 1991; Aoshima \textit{et al.}, 1996), the genetic basis
of \textit{p67}^{*}^{*}-deficiency is highly heterogeneic.

This phenotype of CGD was originally described as total lack of \textit{p67}^{*}^{*} protein,
but with normal levels of mRNA (Leto \textit{et al.}, 1990). Three cases (Table 1.3; numbers
4-6) have now been shown to have a defect in transcription or mRNA stability. The
genetic defects of cases 1-3 presumably render an unstable protein since the mRNA is
transcribed to a normal level. Case 6 has raised significant interest because half normal
levels of \textit{p67}^{*}^{*} protein have been reported to be detected, with inability to translocate
to the membrane (Leusen et al., 1996). Recombinant p67<sup>phox</sup> with the Lys 58 deletion was shown unable to bind Rac. However, the researchers have communicated that sometimes neutrophils collected from this patient did not contain any detectable p67<sup>phox</sup> protein. This case remains particularly unusual. However, collective analysis of the genetic defects underlying the molecular deficiency in CGD can provide some insight to the crucial amino acids or regions essential for protein stability and normal function. Also, there are three polymorphisms reported within the coding region of p67<sup>phox</sup>, which are either silent mutations or conservative amino acid substitutions (de Boer et al., 1994; Nunoi et al., 1995).

Table 1.3  **Known genetic defects causing p67<sup>phox</sup>-deficient CGD.**

<table>
<thead>
<tr>
<th>(reference)</th>
<th>mRNA</th>
<th>genetic defect</th>
<th>predicted effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>case 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>normal level</td>
<td>homozygous</td>
<td>G233 to A in exon 3</td>
</tr>
<tr>
<td>case 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>normal level</td>
<td>heterozygous; in exon 5</td>
<td>A279 to T and A 481 to G</td>
</tr>
<tr>
<td>case 3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>normal level</td>
<td>homozygous</td>
<td>AG insertion 399 in exon 5</td>
</tr>
<tr>
<td>case 4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>reduced amount</td>
<td>homozygous</td>
<td>T to C in splice site intron 3</td>
</tr>
<tr>
<td>case 5&lt;sup&gt;e&lt;/sup&gt;</td>
<td>smaller size</td>
<td>homozygous</td>
<td>G to A in splice site intron 9</td>
</tr>
<tr>
<td>case 6&lt;sup&gt;f&lt;/sup&gt;</td>
<td>normal level</td>
<td>heterozygous,GAA deletion and 11-13 kb deletion</td>
<td>Lys 58 deletion from one allele, unstable mRNA from other allele</td>
</tr>
</tbody>
</table>

<sup>a</sup>: (de Boer et al., 1994), <sup>b</sup>: (Bonizzato et al., 1997), <sup>c</sup>: (Nunoi et al., 1995), <sup>d</sup>: (Tanugi-Cholley et al., 1995), <sup>e</sup>: (Aoshima et al., 1996), <sup>f</sup>: (Leusen et al., 1996).

There is less p67<sup>phox</sup> than p47<sup>phox</sup> in neutrophils. The abundance of these proteins has been estimated at approximately 0.3-0.5% total cytosolic protein (Leto et al., 1991). Three studies in which the proteins have been quantified, report a range of 1.1-4.9 pmol
per 10^6 cells for p67^{phox} with a 6-, 2- and 1.5-fold molar excess of p47^{phox} (Leto et al., 1991; Heyworth et al., 1994, 1997). The molar ratio of p67^{phox}:Rac2 is reported as 2:1 (Heyworth et al., 1994), and p67^{phox}:p40^{phox} as 1:1 (Tsunawaki et al., 1994; Heyworth et al., 1997). In B cells, the amount of p67^{phox} is even lower than in neutrophils, and together with the reduced amount of the cytochrome, this accounts for the low NADPH oxidase activity in these cells (Chetty et al., 1995). p67^{phox} has also been shown to be the limiting factor for the oxidase activity of promyeloid cell line HL60 cells, which are differentiated to the neutrophil phenotype by dimethylsulphoxide (DMSO) (de Mendez & Leto, 1995). Post-differentiation detection of p67^{phox} was later (a few days) than p47^{phox} (16 hours), and p67^{phox} expression was always at a low level. The superoxide production only approached normal neutrophil levels when p67^{phox} reached a certain threshold level.

The cell-free oxidase system has helped to define the role of p67^{phox}. In a system where the activity of diluted cytosol was reconstituted with recombinant p67^{phox} and p47^{phox}, p67^{phox} was shown to have a much greater effect than p47^{phox}, and an EC_{50} value less than half that of p47^{phox} (Uhlinger et al., 1992). Adding extra p67^{phox} has been shown to increase cell-free activity in a number of studies (Park, J.-W. et al., 1994; Uhlinger et al., 1994). It has been suggested that p67^{phox} is not required in the first step of NADPH oxidase assembly involving p47^{phox} and cytochrome binding (Kleinberg et al., 1990; De Leo et al., 1996). Aside from the indications that p47^{phox} provides the docking site for p67^{phox} as discussed, this could be explained by a role for p67^{phox} in initiating electron transfer at the membrane. Absorbance spectra of neutrophil membranes reacted with CGD cytosol showed that in the absence of p47^{phox}, p67^{phox} is capable of inducing limited electron flow between NADPH and FAD, but not from FAD to the haem of the cytochrome (Cross & Curnutte, 1995). Therefore it seems that p67^{phox} controls the first step of electron transfer, with the next step requiring p47^{phox}.
It has been proposed that p67\textsuperscript{phox} is an NADPH binding component of the oxidase (Smith, R.M. \textit{et al.}, 1996). This was developed from an observation that a 66 kDa protein in the oxidase bound labelled NADPH (Umei \textit{et al.}, 1986; DeLeo & Quinn, 1996). p67\textsuperscript{phox} is capable of binding NADPH, has weak homology with pyridine nucleotide binding sequences, and can reconstitute an NADPH dialdehyde-blocked cell-free oxidase system (Smith, R.M. \textit{et al.}, 1996). p67\textsuperscript{phox} does not bind flavin, GTP or ATP (Leto \textit{et al.}, 1991; Iyer, S.S. \textit{et al.}, 1994). As discussed, there is strong evidence that the \(\beta\) subunit is the significant NADPH binding factor of the oxidase. Therefore, the data suggest that p67\textsuperscript{phox} and the cytochrome might together control the substrate binding for electron transfer. This, however, has yet to be conclusively established.

Phosphorylation research has been less extensive for p67\textsuperscript{phox}. Unlike for p47\textsuperscript{phox} phosphorylation, lack of a 67 kDa phosphoprotein was not noticeable in p67\textsuperscript{phox}-deficient CGD. In fact, one publication challenged whether there is any phosphorylation of p67\textsuperscript{phox} (Heyworth \textit{et al.}, 1996). Another study showed, by immunoprecipitation, that phosphorylated p67\textsuperscript{phox} was found in the cytosol and membrane fractions of PMA-stimulated neutrophils (Dusi & Rossi, 1993). PMA- or fMLP-stimulated phosphorylation of p67\textsuperscript{phox} (although not purified by immunoprecipitation) was shown to be inhibited by staurosporine (Dusi \textit{et al.}, 1993). Conversely, a recent paper reported that p67\textsuperscript{phox} phosphorylation was inhibited by a PKC inhibitor (GF109203X) in PMA but not fMLP stimulation, suggesting PKC-dependent and -independent regulation (El Benna \textit{et al.}, 1997). This phosphorylation was detected as serine phosphorylation, and it was suggested that it could be mimicked by PKC \textit{in vitro}. This would not account for the fMLP phosphorylation of the same peptide which was not inhibited by the PKC inhibitor. Therefore the published studies relating to p67\textsuperscript{phox} phosphorylation do not provide clear, consistent findings.
Kinetic studies using an amphiphile-stimulated cell-free system suggest that activation of the NADPH oxidase is dependent on an alteration of p67phox (Fujimoto et al., 1989). This conclusion is based on the similarity in the rates of oxidase activation and p67phox inactivation. Such an alteration of p67phox could result from a change in binding or a chemical modification such as phosphorylation. Various findings indicate that the structure of p67phox is critical to its stability. These include the fact that single amino acid substitutions coded by the mRNA can be the cause of p67phox deficiency in CGD. In addition to p67phox being prone to proteolysis in broken cells, it is unstable in SDS (Fujimoto et al., 1989), sensitive to sulfhydryl and amino group modifying reagents (Leto et al., 1991), and thermolabile. The thermolability is characterised by a loss of activity in the cell-free assay after intact cells are incubated at 46°C, but the protein remains 67 kDa in size and detectable by immunoblotting (Erickson, R.W. et al., 1992). Thus, the loss of activity must be due to disorganisation of a functionally important structural feature.

The structure of p67phox is known to include two SH3 domains with a proline-rich region in the middle of the protein, adjacent to one of the SH3 domains (Leto et al., 1990). These regions are the only regions of homology shared with p47phox. The structure of several SH3 domains have been solved by X-ray crystallography, indicating a compact β-barrel made up of five antiparallel β-strands and three variable loops (Najmudin et al., 1997). p67phox also contains four tetratricopeptide domain repeats (TPR) within the N-terminal 233 amino acids (Ponting, 1996). These are 34 amino acid motifs which are predicted to form a self-associating “knob and hole” structure due to the relative placements of bulky hydrophobic residues to small residues forming depressions (Sikorski et al., 1990). The relevance of this possible structure for p67phox is not known, except the genetic defect of a p67phox-deficient CGD patient (Case 1, Table 1.3) (de Boer et al., 1994), occurs in this region. This patient’s RNA encodes for an
amino acid change that would disrupt the TPR sequence motif, perhaps destabilising the folding of p67<sup>phox</sup> (Ponting, 1996).

There is evidence for two SH3-polyproline interactions between p67<sup>phox</sup> and p47<sup>phox</sup>, plus another interaction independent of SH3 domains, as discussed for p47<sup>phox</sup>. However, no specific binding partners have been appointed to the N-terminal SH3 domain of p67<sup>phox</sup> or to the polyproline domain, although the latter region may be involved in p47<sup>phox</sup> binding to the N-terminal half of p67<sup>phox</sup>. Also, the intramolecular SH3-polyproline binding that has been demonstrated within p47<sup>phox</sup> (Leto et al., 1994; de Mendez et al., 1997), has not been shown for p67<sup>phox</sup>. Deletion studies in transfected whole cell (p67<sup>phox</sup>-deficient B cells) experiments have provided some important structure-function information for p67<sup>phox</sup>. Both SH3 domains of p67<sup>phox</sup> are required for optimal translocation and oxidase activity, with the larger dependency on the C-terminal SH3 domain (de Mendez et al., 1994). Deletion of 22 and 14 amino acids from the N- and C-termini respectively does not affect the level of reconstituted oxidase activity (Chanock et al., 1996). It should be noted that in the latter experiment the oxidase could only be reconstituted to 10-20% of normal B cell activity with full-length p67<sup>phox</sup>, and Southern blotting was used for detection of p67<sup>phox</sup> transfection as the level of expression was below detection by immunoblotting.

It must be emphasised that the intact cell requirements for p67<sup>phox</sup> differ from those for cell-free oxidase activity. N-terminal p67<sup>phox</sup> (amino acids 1-246), without either SH3 domain, can support >60% normal cell-free oxidase activity (de Mendez et al., 1994). Another study showed that the cell-free system only required a shorter p67<sup>phox</sup>, amino acids 1-212 (Hata et al., 1997). This truncated p67<sup>phox</sup> supported higher activity than full-length p67<sup>phox</sup> if the polyproline region of p47<sup>phox</sup> were simultaneously deleted. The authors claim that this implies a regulatory role for the p67<sup>phox</sup> SH3 to p47<sup>phox</sup> polyproline interaction. It is difficult to extrapolate these results to the cellular
oxidase (without μM amphiphiles) where the C-terminal half of p67phox is a proven requirement (de Mendez et al., 1994, 1997).

There are two other binding partners of p67phox; p40phox and Rac. It has been observed that there is a particular affinity between p67phox and p40phox (Wientjes et al., 1996). The two proteins remain tightly bound during purification of the p67phox-p47phox-p40phox complex from neutrophil cytosol (Someya et al., 1993; Wientjes et al., 1993). Most studies show their association is unaffected by dithiothreitol (DTT), various amphiphiles and detergents (Tsunawaki et al., 1994, 1996; Nakamura et al., 1998), the two proteins separating only upon application to SDS/PAGE. This interaction is not of the SH3-polyproline type, rather involving the region between the SH3 domains of p67phox (Fuchs et al., 1996; Sathyamoorthy et al., 1997). There is also one report of high affinity binding between the N-terminus of p67phox and p40phox (Wientjes et al., 1996). The N-terminal 199 amino acids of p67phox has been shown to interact with Rac in a GTP-dependent manner (Diekmann et al., 1994; Prigmore et al., 1995; Dorseuil et al., 1996). This is of particular interest as Rac is a key signalling component of the NADPH oxidase. The interaction of p67phox with p40phox and Rac is examined in more detail in the following sections.

1.13 p40phox

p40phox is an 339 amino acid protein with one SH3 domain and a high degree of homology with p47phox (Wientjes et al., 1993). The N-termini of p40phox and p47phox display the most homology of any of the phox proteins, with 67% similarity (including conservative substitutions) over the N-terminal 70% of their sequence (Wientjes et al., 1993). This region has been termed the PX (phox) domain, and this homology is also shared with many other proteins including phosphatidylinositol 3-kinases and several yeast proteins (Ponting, 1996). p40phox also contains a 28 residue motif called an
octicosapeptide repeat (OPR) within the C-terminal 282-310 amino acids. This motif is present in PKC isoforms \( \mu/\lambda \) and \( \zeta \), and possesses four conserved acidic residues suggesting the ability to bind divalent cations such as \( \text{Ca}^{2+} \) (Ponting, 1996). In p40\(_{phox} \), the OPR sequence is involved in the high affinity binding with p67\(_{phox} \), although the flanking regions are also required (Nakamura et al., 1998). There is strong agreement that the entire C-terminal end of p40\(_{phox} \), not including the SH3 domain, is the binding site for p67\(_{phox} \) (Fuchs et al., 1995, 1996; Tsunawaki et al., 1996; Wientjes et al., 1996; Sathyamoorthy et al., 1997).

The interaction between p40\(_{phox} \) and p67\(_{phox} \) appears to incur stability for p40\(_{phox} \), because in p67\(_{phox} \)-deficient CGD cells there are significantly reduced amounts of p40\(_{phox} \) (Wientjes et al., 1993; Tsunawaki et al., 1994; Dusi et al., 1996). This is not due to a co-existing defect in the p40\(_{phox} \) gene, which is located separately at 22q13.1 (Zhan et al., 1996). There are no known cases of CGD caused by a deficiency of p40\(_{phox} \), which is in fact non-essential in the cell-free NADPH oxidase. Unlike for p47\(_{phox} \) and p67\(_{phox} \), p40\(_{phox} \) RNA is widely expressed in haematopoietic lineages. That is, in addition to phagocytic cells, p40\(_{phox} \) has been detected in basophils, mast cells, megakaryocytes and T lymphocytes (Zhan et al., 1996; Mizuki et al., 1998). The detection of RNA in the leukaemia cell line PLB-985 (before differentiation and without increasing after differentiation) (Zhan et al., 1996), and in brain neuronal cells (Mizuki et al., 1998), has led to speculation that p40\(_{phox} \) may participate in cell functions other than the NADPH oxidase.

While its function in the NADPH oxidase remains unclear, there is growing evidence for an important role for p40\(_{phox} \). It has been shown to translocate to the membrane upon activation but not in p47\(_{phox} \)-deficient cells, suggesting it binds via p47\(_{phox} \) or p67\(_{phox} \) (Dusi et al., 1996). It is not known whether it translocates independently or in a bound heterodimer with p67\(_{phox} \). Although most experiments have
demonstrated the resting state interaction between p40phox and p67phox is almost unbreakable, one immunoprecipitation study showed that arachidonic acid treatment at 20°C of the p47phox-p67phox-p40phox complex caused dissociation of p40phox (Someya et al., 1996). Whether this indicates that separation of p40phox and p67phox occurs during in vivo activation is not clear.

The polyproline region on p47phox that binds the C-terminal SH3 of p67phox can also bind the p40phox SH3 domain (Ito et al., 1996). This suggests that competition in the intermolecular interactions may play a regulatory role in activation. The concept that p40phox and p47phox have related roles in the regulation of p67phox action is consistent with p40phox and p47phox being so homologous.

Further support for a moderating role in controlling superoxide production comes from oxidase activity experiments. Although the NADPH oxidase can be reconstituted in vitro without p40phox, antibodies against p40phox which disrupt the binding between p40phox and p67phox, can significantly inhibit the cell-free SDS-activated oxidase of cytosol plus membranes (Tsunawaki et al., 1996). A C-terminal peptide of p40phox was shown to inhibit both the translocation of all three cytosolic phox proteins and the cell-free oxidase activity (Someya et al., 1996). An intact cell model of the neutrophil NADPH oxidase using the erythroleukaemic cell line K562, has provided further information. This cell line produces low levels of endogenous Rac and the α subunit of the cytochrome, and transfection of the β subunit, p67phox and p47phox is sufficient to generate an NADPH oxidase (de Mendez & Leto, 1995). If p40phox, or even just the SH3 domain which binds to p47phox, is cotransfected in these cells, the oxidase activity decreases by at least 40% (Sathyamoorthy et al., 1997). This may indicate that p40phox plays a moderating role in controlling superoxide production. These results from different studies suggest that p40phox is a key element of oxidase activation, probably by
regulating the interactions between p47\textsuperscript{phox}, p67\textsuperscript{phox} and the cytochrome. It has now been postulated that phosphorylation plays a role in p40\textsuperscript{phox} modulation (Fuchs et al., 1997).

The phosphorylation of p40\textsuperscript{phox} is the most recently described phosphorylation event (Fuchs et al., 1997). The phosphorylation sites have not been determined, but after stimulation by PMA or fMLP at least four new phosphorylated forms of p40\textsuperscript{phox} were resolved by isoelectric focusing. Dephosphorylation corresponded with the cessation of oxidase activity after fMLP stimulation. This study was carried out in DMSO-differentiated HL60 cells. Co-immunoprecipitation of p40\textsuperscript{phox}, p47\textsuperscript{phox} and p67\textsuperscript{phox} did not change upon stimulation, but the incorporation of radiolabelled phosphate increased three-fold for p40\textsuperscript{phox} and p47\textsuperscript{phox}, and only by 25% for p67\textsuperscript{phox}. This was interpreted as the proteins all becoming phosphorylated in a complex as an activating step leading to co-translocation.

1.14 Rac

Rac is a member of the p21 GTP-binding Ras superfamily of regulatory proteins which have been implicated as molecular switches in other signalling pathways (Didsbury et al., 1989). These proteins are bound to GDP in their resting state, which upon activation is exchanged for GTP as the first step in a cycle of regulated GTP binding and hydrolysis (Grand & Owen, 1991). Rac was identified as a component of the NADPH oxidase by complementation studies analysing the minimal requirements for activating the NADPH oxidase of cytochrome-containing membranes (Abo et al., 1991). The cytosol fraction containing Rac showed that it was present in a complex with the GDP-dissociation inhibitor, RhoGDI (see Figure 1.4).

As its name implies, RhoGDI inhibits the dissociation of GDP therefore controlling the activation of Rac. The process of GTP binding of Rac is also likely to involve interaction with a GEF (GDP/GTP exchange factor). Rac-GTP is inactivated by
intrinsic hydrolytic activity and the action of GTPase activating proteins (GAPs). The particular GEF and GAP that interact with Rac in the NADPH oxidase are not known. The mechanism of Rac activation is also thought to involve interaction with the cytoskeletal proteins ezrin, radixin and moesin, which can reduce RhoGDI activity (Takahashi et al., 1997).

There are two isoforms of Rac which share 92% amino acid identity. Rac1 was originally isolated from guinea pig macrophages (Abo et al., 1991) and is ubiquitously expressed, whereas Rac2 is restricted to the myeloid lineage and has been reported to constitute 96% of neutrophil Rac (Heyworth et al., 1994). Rac1 and Rac2, but not the closely related Cdc42, are capable of activating the NADPH oxidase in the GTP-bound state (Kwong et al., 1993). Constitutively active Racs, or Racs containing nonhydrolysable GTP-\gamma-S, sustain superoxide production in recombinant cell-free systems at a rate similar to that if whole cytosol is used (Heyworth et al., 1993b; Kreck et al., 1994). Further support for the role of Rac in regulation of oxidase activity, is provided by the observation that the cell-free system is inactivated by the addition of a neutrophil GAP (Heyworth et al., 1993a).

Isoprenylation is a post-translational modification of Rac which appears to promote the nucleotide exchange for GTP-binding (Heyworth et al., 1993b) and is probably involved in the membrane-binding of Rac (Kreck et al., 1996). The isoprenyl group binds to a pocket in GDI (Keep et al., 1997) from which it is displaced by activating lipids. In its active GTP-bound form, Rac translocates to the membrane with the same kinetics as the phox proteins (Quinn et al., 1993). There is debate over whether the translocation of Rac is dependent (El Benna et al., 1994a; Dusi et al., 1995) or independent (Heyworth et al., 1994; Dorseuil et al., 1995; Dusi et al., 1996) of the translocation of p67\textsuperscript{phox} and p47\textsuperscript{phox} to the membrane. Despite the discrepancies in the literature, the most convincing data suggests Rac2 is the physiological binding partner of
p67\textit{phox} (Dorseuil \textit{et al.}, 1996), and they translocate independently to the membrane (Dusi \textit{et al.}, 1995, 1996). Once at the membrane, p67\textit{phox}, p47\textit{phox}\textit{ and} Rac appear to be present in equimolar amounts (Quinn \textit{et al.}, 1993; Nisimoto \textit{et al.}, 1997).

\textbf{Figure 1.4 Model of the activation and deactivation process of the NADPH oxidase.} The formation of the multimolecular complex at the flavocytochrome in phagocyte membranes is shown in the A: inactive, B: active and C: deactivated states. Before activation, p47\textit{phox}, p67\textit{phox}, and p40\textit{phox} are in a complex in the cytosol, and Rac-GDP is bound by RhoGDI. Upon activation there is protein phosphorylation, and Rac, which is isoprenylated, is released from RhoGDI and becomes GTP-bound. There is translocation of the cytosolic components to the flavocytochrome. Electron transfer is carried out from intracellular NADPH to oxygen in the vacuole. There is then protein dephosphorylation, and inactivation of Rac through GAP activity.

It is likely that Rac carries out its regulatory role once situated at the membrane after activation. Nonisoprenylated Rac2 does not bind to the membrane and does not fully activate the oxidase (Kreck \textit{et al.}, 1996), although nonisoprenylated Racs preloaded with GTP-\textit{\gamma}-S can support oxidase activity under some cell-free conditions (Heyworth \textit{et al.}, 1993b). Isoprenylation does not appear to be a requirement for p67\textit{phox} binding.
(Kreck et al., 1996). In the cell-free study where oxidase activity was inhibited by addition of GAP, it was noticed that GAP was only effective if added before the stimulus (Heyworth et al., 1993a). This suggests that either the GAP had no access to Rac in the assembled oxidase, or that Rac-GTP is not required to maintain activity once stimulation is triggered. A role for Rac in oxidase assembly and the initiation of activity is also implicated by a similar experiment using a competitive peptide which inhibited superoxide production only if added prior to the activating amphiphile (Kreck et al., 1994).

\[ p67^{phox} \] appears to be a target molecule for activated Rac. Rac interacts with the N-terminal half of \( p67^{phox} \) in a GTP-dependent manner (Diekmann et al., 1994; Prigmore et al., 1995; Dorseuil et al., 1996), and mutations to the effector sites of Rac abolish the binding of \( p67^{phox} \) and NADPH oxidase activity in cell-free systems (Diekmann et al., 1994, 1995). The effector site which binds \( p67^{phox} \) is the N-terminal region of residues 26-45 (Diekmann et al., 1994; Dorseuil et al., 1996; Nisimoto et al., 1997). One paper implicates an additional C-terminal (143-175) site of interaction (Diekmann et al., 1995). There is another effector-like domain, residues 124-135, whose target is not known but it has been shown to be crucial for Rac activity in the NADPH oxidase (Freeman et al., 1996; Nisimoto et al., 1997). This is called the insert region because it is an amino acid sequence of the Rho subfamily of p21 proteins that does not exist in Ras (Freeman et al., 1996). It is not known if \( p67^{phox} \) and Rac interact in the cytosol, or only after assembly of the components at the membrane. Affinity studies have led to the proposal of a model such that Rac binds \( p67^{phox} \) and the cytochrome via different domains, while anchored at the membrane by the isoprenylated C terminus (Nisimoto et al., 1997).

Another effector target of Rac-GTP in neutrophils is the p21-activated protein kinase, PAK (Diekmann et al., 1995; Prigmore et al., 1995). The Ras-like effector region is involved with PAK activation, but not the insert region (Freeman et al., 1996).
The same binding sites of Rac appear to be required for PAK as for p67phox (Diekmann et al., 1995). It has been speculated that the activation of PAK-related phosphorylation is a function of Rac in the regulation of the NADPH oxidase (Uhlinger et al., 1993; Knaus et al., 1995). Although it has been demonstrated that PAK can phosphorylate p47phox in vitro, the relevance of this has yet to be determined in vivo (Knaus et al., 1995).

1.15 Summary of protein-protein interactions

There is a wealth of data on the protein-protein interactions within the multicomponent NADPH oxidase. A range of techniques have been used to determine the binding sites, and some of these are quite artificial, involving the use of truncated proteins and cell-free environments with amphiphilic agents. The discrepancies in the literature (especially cell-free versus intact cell observations) have been discussed in earlier sections (see 1.10, 1.12). These have highlighted the limitations of using nonphysiological stimuli and isolated peptides which are likely to have different tertiary structure from full-length molecules. However, these studies do have value in the initial identification of possible regions of affinity between proteins.

Although the yeast two-hybrid expression system is an intact cell method where it is feasible that the proteins fold in their native conformation, it is an unrelated cell type and is restricted to resting cell conditions only. The system of choice therefore is the reconstitution of intact cells with modified NADPH oxidase components. These have been mentioned in the description of interaction studies to date (sections 1.10, 1.12, 1.13). EBV-immortalised B cell lines of CGD patients are valuable research tools, but superoxide production by reconstituted B cells is very low. K562 cells have the advantage of being easily transfected, efficient at protein expression, and capable of eliciting higher and more reproducible superoxide production by PMA stimulation.
Reference list for Figure 1.5:

a: Nakanishi et al., 1992
b: Leusen et al., 1994a
c: Sumimoto et al., 1994
d: DeLeo et al., 1995a
e: DeLeo et al., 1995b
f: Nauseef et al., 1993
g: Kleinberg et al., 1992
h: Leusen et al., 1994b
i: Sumimoto et al., 1996
j: de Mendez et al., 1997
k: Leto et al., 1994
l: Fuchs et al., 1996
m: Finan et al., 1994
n: Ito et al., 1996
o: DeLeo et al., 1996
p: de Mendez et al., 1996
q: Sathyamoorthy et al., 1997
r: Fuchs et al., 1995
s: Tsunawaki et al., 1996
t: Nakamura et al., 1998
u: Wientjes et al., 1994
v: Diekmann et al., 1994
w: Leusen et al., 1996
Figure 1.5 Diagram of p67\textsuperscript{phox}, p47\textsuperscript{phox} and p40\textsuperscript{phox} depicting the documented sites of protein-protein interactions. p67\textsuperscript{phox} has 526 amino acids with 2 SH3 domains (Leto et al., 1990), p47\textsuperscript{phox} has 390 amino acids with 2 SH3 domains (Volpp et al., 1989), and p40\textsuperscript{phox} has 339 amino acids with one SH3 domain (Wientjes et al., 1993). The positions of PX, TPR and OPR domains are also shown (Ponting, 1996). See facing page for reference list (a-w) to each interaction.
Figure 1.5 summarises the reports in the literature of the interactions between the components of the NADPH oxidase, as discussed in the previous sections. All of these interactions have been implicated as playing a role in the regulation of superoxide production, either by cell-free or intact cell methods.

1.16 The cytoskeleton

The submembranous cytoskeleton in neutrophils is a mesh of F-actin (filamentous) fibres that is implicated as having a key role in signal transduction. The evidence for involvement of the cytoskeleton comes from the following observations. Superoxide generation is confined to the phagocytic vacuole and this localisation may be controlled by the reorganisation of the cytoskeleton that occurs in the formation of a vacuole (Valerius et al., 1981). Activators of the NADPH oxidase including fMLP and PMA cause changes to the shape of neutrophils (Wymann et al., 1989), suggesting cytoskeletal rearrangements occur even in the absence of phagocytosis. In an SDS-activated cell-free system, the addition of G-actin (globular) enhances activation, and the prevention of polymerisation of endogenous G-actin suppresses activation (Morimatsu et al., 1997).

The involvement of multiple SH3 domains in the regulation of the NADPH oxidase, also supports a signalling role for the cytoskeleton. SH3 domains have been associated with the targeting of proteins to the cytoskeleton in many systems (Cohen et al., 1995). By detergent fractionation of neutrophils, it appears that a proportion of the SH3 domain containing NADPH oxidase proteins are localised at the cytoskeleton during activation (Nauseef et al., 1991; Woodman et al., 1991; Curnutte et al., 1994; El Benna et al., 1994a; Grogan et al., 1996). p67phox has been shown by immunohistochemistry to be colocalised with an actin binding protein, coronin (Grogan...
et al., 1997), and occurs in clusters on the cytoskeleton attached to the plasma membrane (Wientjes et al., 1997). Coronin copurifies with p67phox, and it can also bind one of p67phox's binding partners, C-terminal p40phox. Coronin is important for movement and phagocytosis in Dictyostelium. This provides further evidence that the cytoskeleton performs a combined role of signal transduction for the activation of the NADPH oxidase and control of morphological changes.

Another factor integral to both cytoskeletal events and signalling in the NADPH oxidase is Rac. By microinjection into culture fibroblasts, small GTP-binding proteins of the Rho family have been shown to regulate cytoskeletal rearrangements of F-actin, such as inducing the formation of stress fibres (Rho), filopodia (Cdc42) and lamellipodia (Rac) (Nobes & Hall, 1995). Furthermore, the cytoskeleton is implicated in signal transduction for the activation of Rac. The family of proteins which crosslink the cytoskeleton and plasma membrane, ezrin/radixin/moesin, has been shown to interact with RhoGDI (Takahashi et al., 1997). It is suggested that this interaction is the initiating signal for the GTP-binding of Rac, leading to activation.

The NADPH oxidase is regulated by a complicated network of signal transduction events and as such is a model system for examining the role of the cytoskeleton. Elucidation of the mechanisms of interplay between small GTP-binding proteins, the SH3-containing phox proteins, and the cytoskeleton in the neutrophil, would provide insight to the signal transduction of many biological systems.

1.17 Scope of this thesis

In summary, p67phox is an essential, labile, component of the NADPH oxidase. It is known to bind p47phox and p40phox, and is an effector target of Rac. It normally resides in the cytosol and a small proportion becomes membrane-localised upon activation. There are reports of activation-induced phosphorylation of p67phox. It seems to play a
direct role in electron transfer from the oxidase substrate NADPH to FAD in the cytochrome. It contains SH3 domains, and has been shown to bind to the cytoskeleton. This forms a substantial foundation of information on this key component of the NADPH oxidase. The modulating events controlling its translocation and intermolecular interactions leading to catalytic enzyme activation remain to be fully elucidated.

This project aimed to examine the phosphorylation of p67^{phox} in the NADPH oxidase system. As described, p67^{phox} phosphorylation is an issue of debate and the limited published data contain some inconsistencies. The first section of work in this project was concerned with detecting and characterising the phosphorylation of p67^{phox}. It had to be established that it was a native phenomenon in activation of the neutrophil. Phosphorylation in intact cells (termed \textit{in vivo}) and recombinant systems (\textit{in vitro}) were used to derive information about the activation of the reaction. This led to the determination of the site of phosphorylation and preparation of a mutant lacking this site. Finally, a series of investigative trials were carried out to elucidate the control mechanisms of p67^{phox} phosphorylation.
Chapter 2

Materials and methods

2.1 Preparation of neutrophils

Neutrophils were prepared from either buffy coat residues, buffy coats, or freshly
drawn heparinised blood from healthy volunteers. For experiments involving
measurement of activation or other metabolic studies, fresh blood (from healthy
volunteers) was the chosen source. For bulk fractionation or large scale
immunoprecipitation of p67phox, buffy coats or buffy coat residues were used (North
London Blood Transfusion Service). The blood was diluted by adding 1/3 volume 0.9%
(w/v) NaCl with 5 units/ml heparin (1×Saline), and the erythrocytes sedimented by
making it 1% (w/v) dextran (using a solution 10% (w/v) in 1×Saline), and left to stand in
a tall, narrow cylinder for 1 hr, at room temperature (RT). The upper layer of enriched
leukocytes was centrifuged through a cushion of 10% volume Ficoll Hypaque
(Nycomed) at 2000 rpm (500 g), 10 min, RT. A benchtop Mistral 3000i (MSE)
centrifuge with swing-out buckets was used throughout the cell separation procedure.
The resultant pellet was gently resuspended in water for a few seconds then made to
0.9% (w/v) NaCl by adding an equal volume of 2×Saline for hypotonic lysis of the
residual erythrocytes. Finally, centrifugation at 1250 rpm (200 g), 3 min, RT, yielded a
granulocyte pellet which was >95% pure for neutrophils (Segal et al., 1980c). The cells
were resuspended in phosphate-free HEPES-buffered saline (HBS) (Cockcroft, 1984)
with 5.5 mM glucose, and counted using a microscope haemacytometer.

HEPES-buffered saline (HBS) phosphate-free buffer

137 mM NaCl
2.7 mM KCl
20 mM HEPES (N-(2-hydroxylethyl)piperazine-N’-(2-ethane-sulphonic acid))
1 mM MgSO₄
1 mM CaCl₂
pH 7.4 with NaOH
± 0.025% (w/v) BSA (bovine serum albumin)
± 5.5 mM (0.1% w/v) glucose

2.2 Cellular ATP measurements by RP-HPLC

Prepared neutrophils were resuspended in HBS with 0.025% (w/v) BSA (fraction V, BDH) at 5×10⁷ cells/ml. Cell suspensions were mixed with either 5.5 mM glucose or 5.5 mM 2-deoxy glucose (2-DG) at time zero and incubated on a roller at 37°C. After 30 min cells were centrifuged at 1500 rpm (300 g) and resuspended in HBS containing 5.5 mM glucose and 12.5 µCi/ml [³²P]orthophosphate (³²P, 10 mCi/ml aqueous solution, Amersham), and incubated for a further 2 hr. Aliquots of 7.5×10⁷ cells were taken in triplicate at 30 min intervals over 150 min, and immediately treated with chloroform-methanol for separation of nucleotides. The required final volume ratio of CHCl₃:MeOH:H₂O of 1:1:0.9 was obtained as follows: the cell suspension was gently spun to a pellet to which 3.75 volumes CHCl₃:MeOH 1:2 was added, vortexed until homogenous, then 1.25 volumes, each, of H₂O and CHCl₃ added, vortexed and spun fast for phase separation. The aqueous phase (containing nucleotides) was dried in a Speedvac and reconstituted in 10 mM Tris pH 7.5 for analysis by reverse phase high pressure liquid chromatography (RP-HPLC). ATP measurements were by UV absorbance spectra from an isocratic HPLC system using 50 mM sodium phosphate, pH 5.8 (5 mM Na₂HPO₄/ 50 mM NaH₂PO₄) and a C18 µBondapak column (Anderson & Murphy, 1976; Cockcroft, 1984). UV detection was made at 260 nm, and radioactivity was measured by Cerenkov counting on-line to the HPLC. The HPLC was a Waters Associates system with a Ramona radioactivity detector.
2.3 Protein gel electrophoresis and blotting techniques

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS/PAGE) was carried out by standard Laemmli methods (Laemmli, 1970), using 30% (w/v) acrylamide/0.8% (w/v) bisacrylamide solution (Protogel, National Diagnostics) for a final concentration of 8.0-12.5% acrylamide depending on the protein size and required resolution. Hoefer Mighty Small II apparatus was used for electrophoresis, operated at ~30 mA per gel. Gels were run more slowly for enhanced resolution on low percentage gels. Molecular weight markers were from Pharmacia or Sigma (prestained markers), and gels were stained with Coomassie stain. Gels were either dried onto chromatography paper (Whatman) or cellophane (Biorad) using a Biorad 543 gel dryer. Buffers and solutions were made to standard protocols for protein chemistry (Coligan et al., 1995).

Tricine gels (Schagger & Von Jagow, 1987) were used for cyanogen bromide digests (see section 2.12) with a 16.7% resolving gel. Acrylogel 3 solution (BDH) was used which is 40% (w/v) acrylamide/3% (w/v) bisacrylamide. The Laemmli sample buffer was made with Coomassie brilliant blue G-250 so that the dye migrated ahead of small molecular weight peptides. Tricine gels were stained using a Novex Colloidal Coomassie kit for increased sensitivity, followed by air drying in a Promega gel drying frame with transparent film.

Immunoblotting was performed with an LKB 2117 Multiphor II semidry electroblotter using reinforced cellulose nitrate membrane (Schleicher and Scheull). Generally, 100-150 mA per blot were applied for 1hr, followed by staining with Ponceau stain (Sigma). Blocking of nonspecific binding was by incubation in Superblock, 1 hr, RT. Antibodies had been raised in rabbits and goats against recombinant proteins and peptides using standard protocols (Gullick, 1983). These were affinity purified (Harlow & Lane, 1988) with the use of antigen columns prepared with recombinant protein and
HiTrap NHS-activated affinity columns (Pharmacia) used to the manufacturer’s instructions. Affinity purified antibodies were incubated with the blot at an empirically determined optimal concentration, in 1% (w/v) gelatine/ TBS/ 0.05% (v/v) Tween 20 for 1 hr, RT. After washing in TBS/ 0.05% (v/v) Tween 20, horse radish peroxidase (HRP)-conjugated species-specific antibodies against IgG were incubated as per manufacturer’s instructions (Sigma). Blots were developed using an enhanced chemiluminescence method (ECL, Amersham), and the autoradiography processed by a Velopex MD2000 automated developer (Medivance).

Phosphotyrosine immunoblotting was performed with a recombinant antibody RC20:HRPO from Transduction Laboratories. This was conjugated with HRP eliminating secondary antibody incubations. Incubations were carried out to the manufacturers instructions, including using BSA blocking solutions without milk powder.

**Tris Buffered Saline (TBS):**

- 200 mM NaCl
- 50 mM Tris
- pH 8.5

**Superblock:**

- TBS / 0.05% (v/v) Tween 20*
- 5% (w/v) fat free milk powder
- 1% (w/v) gelatin
- 1% (w/v) BSA
- 0.1% (w/v) Na Azide

*Tween 20 (polyoxyethylene(20)sorbitan monolaurate)

### 2.4 Neutrophil permeabilisation for delivery of $^{32}$P-γ-ATP

For streptolysin-O permeabilisation (Cockcroft *et al.*, 1994), a final concentration of 0.4 units/ml was added to cells at $10^7$/ml in streptolysin-O buffer with $^{32}$P-γ-ATP and incubated for 30 secs, RT. Stimulation was then carried out at 37°C, quenched with ice-cold Streptolysin-O Buffer containing protease and phosphatase inhibitors (see 2.7), and sonicated on ice.
For electro-permeabilisation (Nasmith et al., 1989), a Biorad Pulser was used to deliver 2 pulses of 5 kV with a capacitance of 25 μF to cells at 10^7/ml in ATP-Electroporation Buffer. Cells were put on ice and used immediately after 32P-γ-ATP was added, incubated 5 min, 37°C, then stimulated as required. The reactions were quenched with ice-cold ATP-Electroporation Buffer containing protease and phosphatase inhibitors, and sonicated on ice.

Generally, reactions were carried out in volumes of 250 μl with 5-25 μCi 32P-γ-ATP (3000 Ci/mmol, Amersham). When whole cell samples were to be applied to SDS/PAGE, trichloro-acetic acid (TCA) precipitations were performed by making the reaction mixture to 10% (v/v) TCA, leaving on ice up to 30 min, and centrifuging at 13k rpm (10 000 g), 15 min, 4°C. The precipitate would be washed with 100% ethanol and then boiled in Laemmli Sample Buffer (see 2.11). For fractionation or immunoprecipitation from the cells, the cells were lysed by sonication as described above.

**Streptolysin-O Buffer:** 20 mM PIPES* + 100 μM ATP
137 mM NaCl + 2 mM NADPH
2.7 mM KCl + 1 mM MgCl₂
pH 6.8 + 100 nM Ca²⁺/3 mM EGTA*

**ATP-Electroporation Buffer:** 140 mM KCl + 100 μM ATP
10 mM HEPES + 2 mM NADPH
20 mM glucose + 1 mM MgCl₂
pH 7.0 + 100 nM Ca²⁺/3 mM EGTA*

*PIPS; 1,4-piperazine diethane sulphonic acid.
EGTA; ethylene glycol-bis(β-aminoethyl ether) tetra-acetic acid. 100 nM Ca²⁺/3 mM EGTA was obtained by adding a 1/33 volume of pCa²⁺7 buffer (Gomperts & Tatham, 1992).

### 2.5 EBV-transformed B lymphocyte cell culture

EBV-immortalised B cell lines had been previously prepared from various chronic granulomatous disease (CGD) patients and were stored in liquid nitrogen. These
were grown in suspension culture at 37°C, 5% CO₂, with half the media replaced at least twice a week. Trypan blue exclusion was used as the routine method for assessing viability of the cells. If cell viability were low, dead cells and debris were removed by centrifugation of the culture through a cushion of Ficoll Hypaque, 2000 rpm (500 g), 10 min, RT. Debris spun to the bottom of the tube, and whole cells were collected from the Ficoll/media interface and washed by centrifugation at 1000 rpm (150 g), 3 min, RT in media before returning to culture. Cell stocks were frozen at 0.5-2×10⁷/ml in Storage Buffer by placing an insulated box in a -70°C freezer to allow slow freezing before storing in liquid nitrogen.

**B Cell Culture Media:**

RPMI with Glutamax (Gibco BRL, 2 mM glutamine)  
+ 10% (v/v) foetal calf serum (heat inactivated; 50°C for 30 min)  
+ 0.01 M HEPES  
+ 100 units/ml penicillin, 100 μg/ml streptomycin  
+ 1 μg/ml amphotericin B

**B Cell Storage Buffer:**

10 ml RPMI with Glutamax and 10 ml DMSO were filtered through 0.2 microns, then added to 60 ml B Cell Culture Media. This was supplemented with an additional 15 ml foetal calf serum. This buffer may be kept at 4°C for approximately one month.

### 2.6 Metabolic labelling with \(^{32}P\)\(_i\)

**Neutrophils:** \(^{32}P\)\(_i\) was added to neutrophil suspensions of 5×10⁷/ml in HBS/glucose/BSA 0.025% (w/v) at a concentration between 0.1 and 1 mCi/ml, and incubated at 30°C without agitation for 60-90 min. Titration experiments showed that \(^{32}P\)\(_i\) incorporation increased with decreased cell concentration and increased \(^{32}P\)\(_i\) concentration. The given values are the optimal conditions within the practical limitations of radioactive experiments.

**B cells:** Cells were resuspended at 1×10⁷/ml in phosphate-free RPMI (ICN) with 10% (v/v) dialysed foetal bovine serum (Gibco BRL) and incubated with \(^{32}P\)\(_i\) at 0.1-
0.5 mCi/ml, 37°C, 4 hr. Cells were spun at 1000 rpm (200 g), 3 min, RT and the supernatant (S/N) decanted. Cells were either washed once with HBS/glucose, or just resuspended in HBS/glucose for stimulation at 37°C.

2.7 Cell stimulation and fractionation

B cells were stimulated using PMA, at 1 μg/ml for 10-20 min, 37°C. Neutrophil stimulation was by various agents, but always at 5×10⁷ cells/ml, 37°C: PMA; 1 μg/ml, 5 min, or fMLP; 1 μM, 1-2 min following a preincubation of the cells with cytochalasin B at 5 μg/ml, 5 min, or serum-opsonised zymosan (S.O.Zym); 1 mg/ml, 7 min. PMA, fMLP and cytochalasin B were all purchased from Sigma and stored at -20°C in DMSO. S.O.Zym was prepared by incubating phosphate-buffered saline (PBS)-washed zymosan A (Sigma) in pooled normal human serum at 2.5 mg/ml, 37°C, 30 min, then washing it twice with PBS before storing at 20 mg/ml, -70°C. S.O.Zym stimulations were carried out with constant swirling for agitation over the course of activation.

All stimulations were quenched by addition of >5× volume excess of ice-cold HBS, with protease and phosphatase inhibitors. All subsequent processing was done on ice or at 4°C. Cells were sedimented by centrifugation at 1000 rpm (200 g), 4°C, 3 min, then resuspended in a sonication buffer (Lamberth’s or Relaxation Buffer, see 2.8) with protease and phosphatase inhibitors, at a concentration around 2×10⁸/ml. Lysis was done by 2 or 3 short (2-4 secs) sonication bursts using a MSE Soniprep 150 sonicator. Cell lysate was centrifuged at 2000 rpm (500 g), 4°C, 5 min to give a post nuclear supernatant (PNS) free of unbroken cells and nuclei. The PNS was handled in one of two ways: either cytosol was prepared by clarifying the PNS in a Beckman Optima TLX Ultracentrifuge, rotor head (fixed angle) TLA 100.2, at 50k rpm (100 000 g), 4°C, 12
min, or (when membranes were required) the PNS was applied to a sucrose gradient for resolution of cytosol and membrane fractions (0.6 volume, each, 15% and 34% (w/v) sucrose in sonication buffer) and spun at 50k rpm (150 000 g), 30 min, 4°C, rotor head TLS 55 (swing-out). The upper layer was collected as the cytosol fraction, and the sucrose interface collected for the plasma membrane fraction. This was diluted 5x with sonication buffer complemented with (final concentration) 250 mM KCl, and spun at 100k rpm (400 000 g), 10 min, 4°C, rotor head TLA 100.2 for a washed membrane pellet.

PBS (phosphate-buffered saline)  
Lamberth’s break buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Storage Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>140 mM NaCl</td>
<td>10 mM KCl</td>
<td>-20°C at 1M in DMSO</td>
</tr>
<tr>
<td>10 mM KCl</td>
<td>3 mM NaCl</td>
<td>4°C at 2 mM in ethanol</td>
</tr>
<tr>
<td>10 mM NaH₂PO₄</td>
<td>4 mM MgCl₂</td>
<td>-20°C at 10 mg/ml</td>
</tr>
<tr>
<td>to pH 7.3 with NaOH</td>
<td>10 mM PIPES</td>
<td>pH 7.0</td>
</tr>
</tbody>
</table>

Protease inhibitors
Final concentration for use is shown, plus the storage conditions of stock solutions.

1 mM DIFP*, (stock: -20°C at 1M in DMSO)
0.2 mM phenylmethylsulphonyl fluoride, (stock: 4°C at 2 mM in ethanol)
10 μg/ml TLCK, (stock: -20°C at 10 mg/ml)
10 μg/ml leupeptin, (stock: -20°C at 10 mg/ml)
10 μg/ml pepstatin, (stock: -20°C at 10 mg/ml in DMSO)
1 mM benzamidine, (stock: 4°C at 1 M, dark)
5 mM EDTA (omitted in fractionation studies)

*DIFP; di-isopropyl fluorophosphate, was added to concentrated cell pellets or slurries only because it is subject to hydrolysis in aqueous solutions.

TLCK; N-alpha-p-tosyl-L-lysine chloro methyl ketone.

Phosphatase inhibitors

100 nM microcystin (stock: 0.4 mM in 10% methanol, -20°C)
(or 1μM okadaic acid (stock: 1 mM in dimethyl formamide, -20°C)
20 mM NaF (stock: 0.5 M, -20°C)
5 mM Na pyrophosphate (stock: 0.125 M, -20°C)
200 μM Na vanadate¹ (stock: 0.2 M, -20°C)

¹Na vanadate preparation: 200 mM in water, adjust to pH 10, boil to dissolve, readjust to pH 10.
2.8 Immunoprecipitation of p67<sub>phox</sub>

From cytosol: One volume of 2×Solubilisation buffer (Dusi & Rossi, 1993) was added to cytosol (the S/N of PNS centrifuged at 50k rpm (100 000 g), 12 min, in Relaxation Buffer) to give a volume 300-600 μl. Immunoprecipitations were usually performed at 5×10<sup>7</sup> cell equivalents (eq)/500 μl with protease and phosphatase inhibitors present in the 2×Solubilisation Buffer. Nonspecific binding was reduced for radiolabelled preparations to obtain a clear phosphorimage of the immunoprecipitate, by preadsorbing the cytosol against 40 μl 50% (v/v) protein-A sepharose (PAS, from Sigma) in 1×Solubilisation Buffer, by rotation, 4°C, 1 hr. The sepharose was sedimented at 5000 rpm (1000 g), 4°C, 2 min, and the S/N incubated with 30 μl affinity purified anti-p67<sub>phox</sub> antibody raised in rabbits against full-length glutathione S-transferase (GST)-p67<sub>phox</sub> fusion protein, on ice, 1 hr. Forty μl PAS was added for rotation, 4°C, 1 hr, then the unbound S/N removed, the PAS washed 3 times with 700 μl ice-cold 1×Solubilisation Buffer, and finally boiled in 20 μl 2×Laemmli sample buffer, 5 min. The samples were centrifuged at 13k rpm (10 000 g), RT, 2 min, and the supernatant subjected to SDS/PAGE (8%). For radiolabelling experiments, gels were analysed by autoradiography using Amersham ECL Hyperfilm or the Fuji BAS1000 PhosphorImager system. Quantification of bands was done on either the PhosphorImager or an Alpha Innotech AlphaImager 2000.

From membranes: Neutrophil membranes were prepared as previously described (see 2.7). Fractionation of cell lysate to cytosol and membranes was essential, as lysis of whole neutrophils in the detergent-based RIPA buffer (Harlow & Lane, 1988) appeared to destroy p67<sub>phox</sub>. The immunoprecipitation procedure using 2×Solubilisation Buffer was the same as for cytosol, except a larger number of cells was required to obtain a
Coomassie-visible p67<sup>phox</sup> immunoprecipitate. Membrane preparations of 7.5×10<sup>8</sup> cell eq/500 μl were found to be suitable.

<table>
<thead>
<tr>
<th>Relaxation Buffer</th>
<th>2x Solubilisation Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mM KCl</td>
<td>50 mM Tris, pH 7.5</td>
</tr>
<tr>
<td>3 mM NaCl</td>
<td>2% (v/v) Triton X-100*</td>
</tr>
<tr>
<td>3.5 mM MgCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>300 mM NaCl</td>
</tr>
<tr>
<td>1.25 mM EGTA</td>
<td>2% (w/v) Na deoxycholate</td>
</tr>
<tr>
<td>10 mM PIPES, pH 7.3</td>
<td>0.2% (w/v) SDS</td>
</tr>
</tbody>
</table>

*Triton X-100; t-octylphenoxypoly-ethoxyethanol.

2.9 2D phosphopeptide mapping and phosphoamino acid analysis

A CBS Scientific Hunter Thin Layer Electrophoresis 7000 system was used followed by the PhosphorImager. Tryptic digests of phosphorylated p67<sup>phox</sup> bands excised from unstained gels were carried out in one of two ways: first method; elution of p67<sup>phox</sup> from a ground-up gel slice using 5% (v/v) β-mercaptoethanol/ 0.1% (w/v) SDS/ 50 mM NH₄HCO₃ pH 7.3-7.6 followed by TCA precipitation, performic acid oxidation, and two incubations of 3-4 h at 37°C with 10 μg TPCK-treated trypsin (TPCK (N-tosyl-L-phenylalanine chloromethyl ketone) treatment destroys chymotrypsin activity, this trypsin preparation was purchased from Promega). The method was taken from the series “Cell Biology: A laboratory handbook” Volume 3 (Academic Press, San Diego). This method incorporates a carrier protein (RNAse A) for the precipitation.

The second approach was an in-gel digestion method from Mr A. Stirling, Ludwig Institute for Cancer Research, London. A gel slice chopped into small pieces was directly incubated with 0.1 μg modified trypsin (modified by reductive alkylation, Promega) in 0.1% (w/v) Thesit (Sigma)/ 10 mM Tris pH 8.8, 37°C, overnight, followed by extraction from the gel by sonication in a waterbath in 0.1% (w/v) SDS/ 10 mM Tris pH 8.8. This method was adopted when tryptic digests were applied in parallel to HPLC because it avoids the large excess of enzyme and carrier protein of the first method.
Also, the modified trypsin is resistant to autolytic digestion and the detergent Thesit does not have a UV chromophore, therefore reducing interference with the HPLC peptide trace.

The protocol for 2D mapping was followed as per instructions for the Hunter apparatus (Boyle et al., 1991; Hardie, 1993). Briefly, phosphopeptide maps were obtained by application of the sample to a cellulose-coated glass plate (from Merck), thin layer electrophoresis in pH 1.9 buffer (2.5% (v/v) formic acid/7.8% (v/v) acetic acid), 1000 V, 35 min followed by thin layer chromatography (7.5% (v/v) acetic acid/25% (v/v) pyridine/37.5% (v/v) butanol) for 6-8 h. For phosphoamino acid analysis, the tryptic digest was subjected to partial hydrolysis in 6 M HCl, 110°C, 1 h, then applied to a cellulose plate and consecutive electrophoresis steps of 1500 V, 20 min, pH 1.9, and 1300 V, 16 min, pH 3.5 (0.5% (v/v) pyridine/5% (v/v) acetic acid) were carried out. Phosphoamino acid markers were detected by ninhydrin spray (0.25% (w/v) in acetone) and their positions outlined on the phosphorimage.

Figure 2.1 2D phosphopeptide and phosphoamino acid analyses. The direction of the two steps applied to the cellulose-coated glass plate is indicated. The sample origin is at position X for both procedures, and the relative migration of free phosphate (Pi) and phospho-serine (S), -threonine (T) and -tyrosine (Y) is shown for phosphoamino acid analysis.
2.10 Recombinant protein expression and purification

All chromatography was carried out using a Pharmacia Fast Protein Liquid Chromatography (FPLC) system comprising of the following components: two pumps P-500, gradient programmer GP-250, single path monitor UV-1, control and optical units and fraction collector Frac-100 and chart recorder Rec-2. All FPLC was carried out at 4°C in the presence of protease inhibitors (see 2.7) and 1 mM DTT. p67phox was kept on ice as much as possible, and freeze/thaw cycles were minimised. Protein quantification was done with the Bradford colourimetric assay by Biorad or by band densitometry. Full-length p47phox and truncated p67phox mutants were prepared as GST-fusion proteins by Ms E. Reeves and Dr F. Wientjes in Professor Segal’s laboratory (Wientjes et al., 1996).

GST-fusion p67phox. Full-length p67phox cDNA had been previously cloned plasmid in Professor Segal’s laboratory from an induced HL60 λgt10 library and inserted into the pGEX-2T plasmid to produce an N-terminal GST-fusion protein (Abo et al., 1992). This method of producing recombinant protein involves overexpression of the introduced gene in Escherichia coli (E. coli) followed by purification using the GST tag (Smith, D.B. & Johnson, 1988).

Production of p67phox using this system was as follows: 500 ml Terrific Broth (with 100 μg/ml ampicillin) was inoculated with an overnight culture of pGEX-p67phox from a glycerol stock, such that absorbance at 600 nm was 0.05. This culture was grown in an orbital shaking incubator, 200 rpm, at 37°C until the optical density was ~0.5. The culture was then cooled in icy water and protein expression was induced by overnight incubation at 25-27°C, 200 rpm orbital shaker, with 0.2 mM IPTG (isopropyl β-D-thiogalactopyranoside). Temperatures >27°C rendered p67phox insoluble. The cells were pelleted by centrifugation at 3200 rpm (1500 g), 10 min, and washed in PBS. The cell
pellet was frozen to aid lysis, then thawed on ice in 5-10 ml *E. coli* Lysis Buffer with protease inhibitors (see 2.7). Lysozyme was added to the suspension at ~0.5 mg/ml, incubated on ice for 20 min, and followed by sonication in two 45 secs bursts. The sonicate was centrifuged at 16k rpm (10 000 g), 4°C, 20 min in a Sorvall RC-5B centrifuge with an SS-34 fixed angle rotor. Glutathione sepharose (Sigma) was added to the S/N at a concentration of 1 ml bead volume/500 ml grown culture, and the suspension was rotated at 4°C, 1-2 hr. The beads were collected by centrifugation at 2000 rpm (500 g), 4°C, 2 min and washed 3× with the Lysis Buffer plus inhibitors, and 2× with Thrombin Buffer. GST was cleaved by incubation of the beads in a minimal volume of Thrombin Buffer (~1 ml) with thrombin (bovine, Sigma) at 20 units/1 ml beads, 10 min rotation, RT, then centrifuged at 2000 rpm (500 g), 2 min to collect p67<sup>phox</sup>-containing S/N. Thrombin was removed by incubation of the S/N with antithrombin III agarose beads (Sigma) on ice for 2 min, followed by centrifugation at 2000 rpm (500 g), 2 min, and finally any residual thrombin in the S/N was quenched with 10 µg/ml TLCK. The glutathione sepharose beads were carried though this procedure twice again, and the S/Ns combined after checking for p67<sup>phox</sup> by SDS/PAGE. Glutathione beads can be regenerated for repeated use as detailed below.

Purification of p67<sup>phox</sup> was performed by anion exchange chromatography, Mono Q sepharose, (1 ml column, Pharmacia), using a salt gradient of 0.0-0.4 M NaCl in 20 mM Tris pH 7.5 over 40 ml at 1 ml/min. p67<sup>phox</sup> eluted at ~0.2 M NaCl in fractions which were >90% pure. Aliquots, with 10% (v/v) glycerol, were stored at -20°C.

<table>
<thead>
<tr>
<th>Terrific Broth</th>
<th><em>E. coli</em> Lysis Buffer</th>
<th>Thrombin Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>17 mM KH&lt;sub&gt;2&lt;/sub&gt;PO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>150 mM NaCl</td>
<td>150 mM NaCl</td>
</tr>
<tr>
<td>72 mM K&lt;sub&gt;2&lt;/sub&gt;HPO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>1 mM EDTA</td>
<td>5 mM MgCl&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>1.2 % (w/v) tryptone</td>
<td>0.5% (v/v) Nonidet p40</td>
<td>2.5 mM CaCl&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>2.4 % (w/v) yeast extract</td>
<td>50 mM Tris, pH 8.0</td>
<td>1 mM DTT</td>
</tr>
<tr>
<td>0.4 % (w/v) glycerol</td>
<td>50 mM Tris, pH 8.0</td>
<td></td>
</tr>
</tbody>
</table>
Regeneration of GST-beads
Number of separate washes for each solution shown in the order of use.
All solutions contain 1 mM DTT.

1x: 3M NaCl/ 10 mM GSH (reduced-form glutathione)
2x: 0.1 M Tris/ 0.5 M NaCl/ 10 mM GSH/ pH 8.5
1x: 0.1 M Na acetate/ 0.5 M NaCl/ 10 mM GSH/ pH 4.5
1x: 70% ethanol
2x: 50 mM Tris/ 0.5 M NaCl/ pH 7.5

Insect cell p67phox: p67phox had previously been cloned into a baculovirus vector for generation of recombinant p67phox in insect cells by Dr J.D. Lambeth, Emory University Medical School, Atlanta, Georgia (Uhlinger et al., 1992). Briefly, the baculovirus expression vector is a nuclear polyhedrosis virus from Autographa californica ("alphalpa looper") AcNPV, which replicates in SF9 (or SF21) insect ovary cell lines from Spodoptera frugiperda ("fall armyworm") (O'Reilly et al., 1994). The gene of interest is cloned in place of the polyhedron gene for high expression of the protein of interest. Protein synthesis starts 20 hr post-infection and after at least 72 hr, the virus is secreted from the cells into the media.

The p67phox baculovirus provided by Dr Lambeth served as the original stock of inoculum and was amplified as follows. The cells were grown to confluency in flat bottom flasks at 27°C (~1x10^8 cells for 150 cm^2 flask, 30 ml Insect Culture Media), to which 1-2 mls p67phox baculovirus inoculum was added and allowed to incubate for 6 days. The supernatant media was collected and spun twice at 2500 rpm (1000 g), 5 min, 4°C, then stored at 4°C as inoculum stock.

For p67phox protein expression, SF9 cells were grown in a shaken suspension. The optimal conditions such that the cell population doubles between 24-48 hr, are 4x10^5 - 4x10^6 cells/ml at 27°C, 110 rpm on an orbital shaker. Cell suspensions were diluted at least every 3 days to provide fresh media and always on the day of infection. The inoculum stock (2 ml) was added to the cell suspension (200 ml) at 2x10^6 cells/ml,
then incubated for 3 days, 27°C, 110 rpm orbital shaker. Cells were harvested at 1000 rpm (150 g), 5 min and washed with PBS, and resuspended on ice in PIPES Break Buffer with protease inhibitors (see 2.7) to 5 ml/200 ml culture. The cells were lysed by sonication, two 5 secs bursts, then spun 3200 rpm (1500 g), 10 min, 4°C, to remove nuclear material and unbroken cells. The PNS was centrifuged at 40k rpm (100 000 g), 60 min, 4°C, then filtered through 1.2 μM cellulose acetate and diluted 2.5× with Buffer A of the first chromatography step in the purification of p67 phox. The first chromatography step should be performed on the same day as sonication, then fractions may be kept on ice until the second step.

The first chromatography step was anion exchange on a 20 ml Q Sepharose column. p67 phox eluted at 0.2-0.3 M NaCl with a gradient of 0.0-0.4 M NaCl in 20 mM Tris pH 7.5 over 100 ml at 1 ml/min. The p67 phox-containing fraction pool was made up to 1 M (NH₄)₂SO₄ before applying to second FPLC step of hydrophobic interaction. The sample was loaded on a 25 ml Phenyl Sepharose column in 1M ammonium sulphate/ 20 mM Tris pH 7.0 and eluted by a linear gradient to 20 mM Tris pH 7.0 over 100 ml, 2 ml/min. p67 phox eluted in fractions <0.1 M (NH₄)₂SO₄, which were concentrated by centrifugation at 6k rpm (1500 g) in a Centricon filter, Mr 30 kDa cut off (Amicon). Aliquots were stored as for GST-cleaved p67 phox.

Insect cell culture media

500 ml IPL-41 medium; pH 6.2, Na Phosphate buffered, (Gibco-BRL)
50 ml Insect grade foetal bovine serum, (heat inactivated at 50°C, 30 min), 10% (v/v)
5 ml amphotericin B, (final 2.5 μg/ml)
0.5 ml gentamicin, (final 10 μg/ml)
5 ml penicillin/streptomycin, (final 100 μg/ml)
For shaken suspensions of ≥200 ml, Pluronic F-68, 0.2% (v/v), was added.

PIPES Break Buffer

50 mM KCl
3 mM NaCl
2 mM MgCl₂
5 mM PIPES
pH 7.5
2.11 *In vitro* phosphorylation assays

Several kinase preparations were prepared as follows:

- Cytosol and membrane fractions were prepared from neutrophils as described (see 2.7).
- Membrane-extracted kinase activity, prepared by Dr A. Lal, in Professor Segal’s laboratory, was the material usually applied to heparin agarose FPLC in the following purification procedure for p47^phox^ kinases: membranes were prepared from PMA-stimulated neutrophils and mixed with 0.5 M NaCl, and S/N material was applied to consecutive FPLC steps of Q sepharose, S sepharose and finally heparin agarose.
- MAP kinase; recombinant ERK 2 (extracellular-regulated kinase, 42 kDa), New England Biolabs.
- PKC; rat brain isolate of mixed isoforms from Dr S.J. Moss, MRC Laboratory for Molecular Cell Biology, London (Woodgett & Hunter, 1987).

25 µl reactions were set up on ice consisting of 2.5 or 5 µl 10× or 5×kinase buffer (see below), 5 µl 0.5 mM ATP at approximately 1000 cpm/pmol, 0.5-5 µg recombinant protein substrate (p67^phox^ or myelin basic protein (Sigma)) and kinase in the form of neutrophil cytosol (1-5 ×10^6^ cell eq), neutrophil membranes (1-3 ×10^6^ cell eq), membranes extract (an arbitrary volume), MAP kinase (5 units), PAK (0.7 µg) or PKC (5 ng). Following an incubation of 30°C, 20 min, the reactions were stopped by addition of 3 µl 10×Laemmli Sample Buffer, then boiled and subjected to SDS/PAGE. Each source of kinase had accompanying buffers of slightly different constitution. Phosphorylations in cytosol were carried out using either the MAP kinase or PKC buffers.
2.12 Cyanogen bromide digestion of p67<sup>phox</sup>

A 6×2 mm gel slice of immunoprecipitated p67<sup>phox</sup> was ground-up using eppendorf and pestle, and incubated with 66 μl 300 mg/ml cyanogen bromide (CNBr, stored as aliquots in 70% formic acid, -70°C) plus 133 μl fresh 70% formic acid, on ice, overnight. The reaction was dried using a Speedvac, resuspended and dried twice more in 50 μl water, then boiled in Laemmli sample buffer and subjected to tricine gel electrophoresis (see 2.3). The gel-loading of these viscous samples was aided by the use of MultiFlex flat pipette tips (Anachem).

2.13 HPLC-MS of trypsin digests

Tryptic digests were performed as above on unstained or Coomassie-stained gel slices containing phosphorylated p67<sup>phox</sup>. The resultant peptide mix was dried in a Speedvac and applied in 50 μl water to a Hewlett Packard 1090 HPLC with buffers A (0.06% (v/v) trifluoroacetic acid (TFA)) and B (0.052% (v/v) TFA/ 80% (v/v) acetonitrile). HPLC-grade solvents were purchased from BDH or Rathburn. A gradient of 0-60% B, 0.15 ml/min, over 60 min was used to elute the peptides from a 220 mm×2.1 mm Brownlee Aquapore OD-300, 7 micron, C<sub>18</sub> column (with AX-300 and C<sub>18</sub> precolumns) and 1 min fractions were collected. 15 μl of each fraction were added to 2 ml Canberra Packard Flo-Scint A and counted in a Canberra Packard Tri-Carb 4000 scintillation counter. Selected fractions were dried in a Speedvac for MALDI (matrix-
assisted laser-desorption ionisation) mass spectrometry (MS) performed on PerSeptive Biosystems Voyager Elite XL reflectron time-of-flight (TOF) apparatus equipped with a nitrogen laser (337 nm, 3 ns pulse) and delayed extraction.

2.14 Mutagenesis of p67<sub>phox</sub>

A polymerase chain reaction (PCR) overlap extension method (Ho <i>et al.</i>, 1989) was used to introduce a point mutation in wildtype p67<sub>phox</sub> such that amino acid 233 was changed from threonine to alanine. The location of the PCR primers within p67<sub>phox</sub> is indicated on Figure 4.2.3, with the following nucleotide sequences:

primer F: CTATATGAGCCAGTGCTCC
primer R: CTACCGGTGACTTGTAGTCACC
primer M1: AGACCGAAGCCCGAGATCTTCAG
primer M2: AAGATCTCTGGGGCTTTCGGTCTGG

Briefly, the method involved two PCR reactions (primer pairs F/M2, R/M1) performed on wildtype p67<sub>phox</sub> DNA, to generate DNA fragments with a point mutation at nucleotide 764 (A to G) introduced by the PCR mutagenic primers M1 and M2. These DNA fragments have overlapping ends, and a third PCR reaction (using primers F/R) was carried out on a mixture of the two fragments to anneal them together producing wildtype sequence except for the point mutation. The resultant PCR product was then digested by restriction enzymes at sites flanking the point mutation (AvrII, Bsu36I), and finally cloned into wildtype p67<sub>phox</sub> DNA. This cloning step was performed in the vector pSelect (Promega) which was necessarily resistant to the AvrII/Bsu36I enzyme digestion, and then the mutated p67<sub>phox</sub> was cloned into pGEX-2T (Pharmacia) using the StuI/EcoRI restriction sites (see Figure 4.2.3).

The PCR steps were performed with the standard parameters of ~25 ng DNA template, 100 pmol each primer, 0.2 mM dNTPs (2'-deoxyribonucleoside-5'-
triphosphates) and 2.5 units DNA polymerase. Vent polymerase (New England Biolabs) was chosen for its proof-reading exonuclease activity, and titration experiments showed that 2 mM MgCl\textsubscript{2} was optimal. Hotstart conditions were applied (McPherson et al., 1995) with 25 cycles used for each PCR step. Standard molecular biology protocols were applied for agarose gel electrophoresis, plasmid purification and cloning techniques (Sambrook et al., 1989). The DH5\textalpha{} strain of E. coli was used for competent cell procedures prepared with the use of RbCl (Sambrook et al., 1989). Oligonucleotides were synthesised by Genosys, restriction enzymes were from Ibi Fermentas or New England Biolabs, and the Qiaqex II DNA extraction kit (Qiagen) was used for DNA fragment purification. The full-length DNA sequence was checked using the Perkin Elmer DyeDeoxy Terminator kit and an Applied Biosystems 373A DNA sequencer. This was achieved by five separate sequencing reactions which together span the entire open reading frame of p67\textsuperscript{phox}. The N-terminal GST-fusion protein of the Ala-233 mutant was expressed and purified as for wildtype p67\textsuperscript{phox} (see 2.10).

2.15 NADPH oxidase activity assays

Superoxide release was measured by the superoxide dismutase-inhibitable reduction of cytochrome c (Garcia et al., 1986) using a Shimadzu UV-3000 dual beam spectrophotometer, with constant stirring in the cuvettes. Neutrophils were resuspended in PBS/glucose or HBS/glucose and incubated at 37°C. Aliquots of 1-2 ×10\textsuperscript{6} cells were added to the cuvettes maintained at 37°C and containing PBS/glucose with 0.5 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2} and 100 µM cytochrome c (from horse heart, Sigma). The absorbance was measured at 550 nm from the time the stimulus was added to both cuvettes, and reported as the difference between the sample cuvette and the reference cuvette (which contained 50 µg/ml superoxide dismutase). The rate of superoxide
production was determined as the rate of cytochrome c reduction using the absorption coefficient $21.1 \text{mM}^{-1}\text{cm}^{-1}$ (Van Gelder & Slater, 1962).

### 2.16 Application of inhibitors

The concentration and incubation time for the inhibitors used are given below. All experiments were on cells at $5\times10^7$/ml in HBS/glucose or PBS/glucose, at 37°C.

- PD098059 (Research Biochemicals International) was stored in aliquots at 50 mM in DMSO, -70°C. Neutrophils were incubated with 10-100 μM PD098059, 1 hr.
- Staurosporine (Sigma) was stored at 1 mM in DMSO, -20°C, and used with cells at 100 nM for 5 min preincubation.
- Okadaic acid (Sigma) was stored in dimethylformamide at 1 mM, -20°C, and used with cells at 1 μM for 30 min.
Chapter 3

Characterisation of p67phox phosphorylation

3.1 Method development for cell labelling

3.1 AIM:

The aim of this work was to investigate methods for optimising the labelling of p67phox for phosphorylation studies. Three approaches were taken in the attempt to gain the most efficient level of radiolabel incorporation into cells:

- glucose starvation of the cells by 2-deoxyglucose treatment to try to increase the specific activity of ATP,
- permeabilisation of cells to allow influx of radiolabelled ATP,
- metabolic labelling of B cells as compared with neutrophils.

3.1 INTRODUCTION:

The major practical limitation of in vivo protein phosphorylation studies is the level of radiolabel incorporation by metabolic labelling procedures. In order to label the intracellular stores of ATP, cells are incubated with $^{32}$P$_i$ (orthophosphate) which is taken into the inorganic phosphate pool by facilitated diffusion across the cell membrane (Vestergaard-Bogind, 1963). Only approximately 10% of radiolabelled orthophosphate externally applied to cells is taken up into the cell. Incubation times need to be sufficiently long such that the incorporation of $^{32}$P$_i$ into $^{32}$P-$\gamma$-ATP has reached an equilibrium. Although metabolic labelling allows cellular phosphorylation events to be studied in the most natural circumstances, it is an inefficient process.
p67\textsuperscript{phox} is not a heavily phosphorylated protein relative to other phosphoproteins in activated neutrophils. For example p67\textsuperscript{phox} is not identifiable as a distinct band by autoradiography of SDS/PAGE-resolved cytosol. Purification such as by immunoprecipitation is required for visualising the p67\textsuperscript{phox} phosphoprotein. Compared with p47\textsuperscript{phox}, the magnitude of phosphorylation is several times lower, and the cytosolic concentration is approximately half. The sensitivity of detection therefore, becomes a governing factor in designing the experiments for investigating the phosphorylation of p67\textsuperscript{phox}.

One approach that is sometimes taken, is to enhance the \textit{in vivo} phosphorylation of a given protein by using phosphatase inhibitors. By inhibiting dephosphorylation events, the degree of phosphorylation may be held at a maximum. There are problems with this technique however, since such interference may crucially divert the natural signalling route either at, or upstream of, the phosphorylation of interest. This manipulation can enhance phosphorylation events which would normally be “silent” or transient in the overall signalling process, as well as those which truly affect the protein function (Coligan \textit{et al.}, 1995). This is the reason that phosphatase inhibitors were not applied here to enhance phosphorylation. It is an approach best adopted if some knowledge is held of the exact signalling pathways involved. Equally, it may be a useful technique for gaining such information, by looking for the effect of inhibiting some factors on the native phosphorylation of p67\textsuperscript{phox} (see Chapter 5).

This section reports the approaches that were taken to optimise the radiolabelling of cells for the study of p67\textsuperscript{phox} phosphorylation.
3.1 RESULTS AND DISCUSSION:

3.1a Manipulation of ATP specific activity by 2-deoxyglucose

Cellular ATP turnover is continuous and rapid in a normally respiring cell. The process of glycolysis both requires ATP in the first stages and produces ATP in the later stages, with the net result of a gain in ATP (Alberts et al., 1983) (Figure 3.1.1). Inhibition of glycolysis therefore, causes a decrease in cellular ATP levels. 2-deoxyglucose (2-DG) inhibits glycolysis by acting as a competitive substrate for hexokinase because 2-DG-6-phosphate cannot be further metabolised (Wick et al., 1957; Kuo & Lampen, 1972).

Figure 3.1.1 The reactions of glycolysis.

Overall reaction for glycolysis:

\[ \text{glucose} + 2 \text{NAD}^+ + 2 \text{ADP} + 2 \text{P}_i \rightarrow 2 \text{pyruvate} + 2 \text{NADH} + 2 \text{H}^+ + 2 \text{ATP} + 2 \text{H}_2\text{O} \]
Treatment of cells with 2-DG has been shown to cause a 40% decrease in cellular ATP in guinea pig neutrophils (Boxer et al., 1977), and the reversibility of such ATP depletion has been demonstrated in macrophages by replacing the 2-DG with glucose (Michl et al., 1976). Glycolytic inhibitors such as 2-DG do not affect the uptake of $^{32}$P, only its intracellular distribution (Tenenhouse & Scriver, 1975).

Experiments were carried out to investigate whether simultaneous $^{32}$P loading and regeneration of depleted ATP levels would affect the efficiency of radiolabelling ATP. HPLC was employed for quantitation of ATP (Anderson & Murphy, 1976). Good resolution of nucleotides was obtained with the exception that GMP co-eluted with ATP (Figure 3.1.2A). The influence of GMP was thought to be insignificant in this study because cellular levels are much lower than those of ATP (Henderson & Paterson, 1973). A standard curve was constructed to ensure linearity in ATP quantitation. Titrations were carried out to determine the appropriate amount of cells to be prepared for analysis and $5 \times 10^7$ cells was found to be optimal.

Cellular nucleotides were extracted by aqueous/lipid phase fractionation of neutrophils. The trace from $5 \times 10^7$ cell equivalents showed a similar pattern to the nucleotide standards, with matching retention times, as well as a few additional peaks (Figure 3.1.2B). Free phosphate ($^{32}$P) from radiolabelled cells eluted with the solvent front, well resolved from the $^{32}$P-ATP signal. When compared with alternative ion exchange HPLC nucleotide separations, this isocratic reverse phase HPLC method had the advantage of short run times of approximately 15 minutes.

Figure 3.1.3 shows the effect of 2-DG on the HPLC traces. There was a significant drop in the ATP peak after incubation in 2-DG-containing medium, and the trace pattern was partially restored in terms of relative peak heights after replacing the 2-DG with glucose. The ATP levels were determined by the area under the ATP peak, normalised for the number of cells (total UV trace area) used per measurement. These
Figure 3.1.2  RP-HPLC for resolution of nucleotides.  A: Resolution of adenine and guanine nucleotides from an equimolar mix, each peak identified by individual application.  B: UV trace (260 nm) of the aqueous (aq) nucleotide-containing fraction from 5×10⁷ cells.  C: On-line detection of the radioactivity eluting in the HPLC run depicted in B, prepared from ³²P-labelled neutrophils.
Figure 3.1.3 Changes in neutrophil nucleotide composition during incubations. RP-HPLC UV absorbance at 260 nm, of nucleotide-containing fractions from 7.5×10^7 cells after different times and conditions of incubation. X-axis units: minutes, Y-axis units: mV×100. All traces are representative of triplicates, and came from the same experiment which was repeated. The total UV absorbance is a measure of the area under the curve and indicates the relative sample sizes.
calculated results are shown in Figure 3.1.4A. All timepoint measurements were normalised against the starting cells in which the ATP concentration was taken as 100%. Neutrophils incubated in glucose for 120 minutes showed a gradual decrease in ATP, but a rapid drop to 40% occurred after 30 minutes with 2-DG in place of glucose. This level was maintained over continued incubation in 2-DG, but increased to 70% of the starting level in cells that were returned to glucose medium.

It is emphasised here that there are major reservations over the accuracy of the method employed to determine the intracellular ATP level. Although the UV absorbance traces clearly show the reduction in ATP by 2-DG treatment, the apparent subsequent ATP regeneration (by an increase in the ratio of the ATP peak to the total UV trace) is not reflected in a corresponding increase in the ATP peak height. The trace changes pattern during the incubation in glucose after 2-DG treatment, but mostly in the peaks with retention times longer than ATP. Therefore the method of taking the ATP peak area (Cockcroft, 1984) normalised for the variable cell amount is misleading, as it does not control for the other changes in nucleotide content. If peak height were taken and normalised for the total cellular amount, the data would not indicate effective ATP regeneration beyond approximately 55% of the original ATP level. As mentioned, the influence of GMP’s contribution to the ATP peak presents another complication.

Despite these shortcomings of the analysis, the results of experiments to determine the changes in the specific activity of ATP in 2-DG-treated cells are reported.

The glucose-containing media used to replace the 2-DG media was spiked with $^{32}$P$_{i}$ to determine whether higher specific radioactivity of ATP was obtained in cells after starvation in the presence of 2-DG. Figure 3.1.4B compares the cells that were preincubated in 2-DG with control cells from glucose medium. Specific activity was measured as the ratio of the radioactive ATP signal to the UV absorbance signal, and shows equilibrium is reached after approximately 90 minutes, as previously estimated
Figure 3.1.4 Changes in the calculated cellular ATP levels. A: Timecourse of cellular ATP measurements during incubation of neutrophils in glucose, 2-DG, or 30 min in 2-DG followed by incubation in glucose. B: Timecourse of the specific activity of $^{32}$P-ATP in neutrophils after 30 min preincubation in either glucose or 2-DG. At time zero, cells were transferred to fresh medium with glucose and $^{32}$P. Each timepoint measurement is the average of triplicate samples (except duplicate, singlet) and error bars show ± standard error.
(Cockcroft, 1984). The glucose-starved cells lagged behind in their uptake of $^{32}$P into the ATP pool, and did not reach higher specific activity or a steeper uptake rate over the 120 minute incubation, as compared with the control cells. The uptake of $^{32}$P did not plateau over the time studied, but incubations of greater than 120 minutes after the 30 minute preincubation would not be suitable for neutrophils which can become stimulated over extended incubation times. For subsequent experiments on $^{32}$P$_7$-loaded resting neutrophils, minimal incubation times are required. Therefore it was concluded that glucose starvation gave no enhancement of the uptake of $^{32}$P, into the intracellular ATP pool.

The studies which claim complete regeneration of ATP after glucose starvation used enzymatic methods for ATP measurement; firefly-luciferase (Michl et al., 1976) and phosphoglycerate kinase (Boxer et al., 1977). These results were not reproduced here, where the disadvantages of the chosen HPLC method supersede the advantages. For a complete analysis of specific activity it would be necessary to determine the proportion of the ATP which was $^{32}$P-labelled in the $\gamma$-position. Another consideration to be made is the effect of glucose starvation on subsequent oxidase activation. 2-DG inhibits the superoxide production of neutrophils (Clark et al., 1990), presumably by blocking glucose utilisation which is essential for the production of NADPH. The reversibility of this inhibitory effect would need to be assessed if 2-DG treatment were to be used. Finally, the results indicate the limited regeneration of ATP achieved after 2-DG treatment does not confer any advantage to $^{32}$P$_7$-loading for in vivo phosphorylation experiments.

3.1b Cell permeabilisation for $^{32}$P-$\gamma$-ATP-loading

Another approach taken to try to increase the radiolabel uptake, was permeabilisation of cells by electroporation or with streptolysin-O. The introduction of
$^{32}\text{P-}\gamma\text{-ATP}$ into the cell is potentially more efficient than $^{32}\text{P}_i$ loading for achieving a labelled intracellular ATP pool. The nucleotide influx is fast relative to protein efflux so depending on the timecourse of subsequent manipulation, it is a good approximation of the whole cell system. The cell suspension medium needs to be made up with intracellular concentrations of salts, glucose and nucleotides such as NADPH.

Electroporation of neutrophils in the presence of $^{32}\text{P-}\gamma\text{-ATP}$ has been successfully applied in studies of gross phosphorylation changes (Nasmith et al., 1989; Lu et al., 1992; Fialkow et al., 1993; Heyworth et al., 1996), but there are no reports of application to immunoprecipitation studies. Even though permeabilisation techniques place restrictions on the number of cells used (up to one tenth the concentration of cells used in metabolic labelling), it was hoped that the gain in labelling efficiency would result in an increase of phosphoprotein yields.

Trials were undertaken to compare electroporation and streptolysin-O techniques. Both methods showed a change in phosphorylation pattern upon stimulation with PMA or fMLP (PMA data shown, Figure 3.1.5). A suitable concentration of cells was found to be $1\times10^7$/ml, as higher concentrations did not capture the radiolabel as effectively. The amount of $^{32}\text{P-}\gamma\text{-ATP}$ activity added to the medium was approximately 10 $\mu$Ci. Streptolysin-O showed more reproducible differences upon stimulation, so this system was optimised further. Timecourse experiments showed that 30 seconds was sufficient to let the labelled ATP diffuse into the cells from the suspension medium post addition of streptolysin-O, and 10 minutes of subsequent incubation with PMA gave maximal enhancement of phosphorylation of $p67^{\text{phox}}$ which was isolated by immunoprecipitation. The phosphorimage of immunoprecipitated streptolysin-O permeabilised cells (Figure 3.1.5) shows the unsatisfactory results obtained for purification of phosphorylated $p67^{\text{phox}}$.  

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Figure 3.1.5 Cell permeabilisation methods for labelling with $^{32}$P-γ-ATP. Phosphorimages of A: TCA precipitates of $1 \times 10^6$ electroporated or streptolysin-O treated neutrophils which were unstimulated or PMA-stimulated (1 μg/ml, 3 min) after the permeabilisation procedure. B: Immunoprecipitation (ip) of $p67^{phox}$ from $2.5 \times 10^6$ neutrophils following streptolysin-O permeabilisation and PMA stimulation (1 μg/ml, 10 min). The gels were exposed to phosphorimage screens for four days, and the position of molecular weight markers are shown on the left.

Attempts were made to scale-up the procedure for preparation of $p67^{phox}$ for phosphopeptide analysis, but the results from this were poor. Higher levels of radiolabel gave higher background without significant increases in $p67^{phox}$ labelling. The addition of recombinant $p67^{phox}$ to the permeabilisation medium was tried, but it was subject to proteolysis, thereby not increasing the yield of phosphorylated $p67^{phox}$. Protease inhibitors could be added to the system to overcome this problem, but may introduce artificial effects rendering a poor model of the native whole cell environment. It was considered therefore, that this system was unlikely to provide satisfactory improvement over other methods of labelled phosphorylation.

3.1c Metabolic labelling of B cells

Metabolic labelling is better suited to cultured cells than primary cells, because the cultured cells can remain in an almost unchanged environment and can withstand
longer incubation periods. The promyeloid cell line HL60, has recently been used for metabolic labelling experiments to characterise the phosphorylation of p40phox (Fuchs et al., 1997). EBV-transformed B cells have also been applied to the study of p47phox phosphorylation (El Benna et al., 1996). This is because B cells of CGD patients are incapable of superoxide production (Volkman et al., 1984), which is due to manifestation of the same genetic defect as in the neutrophil NADPH oxidase (Morel et al., 1993). Therefore, EBV-transformed B cells are recognised as an informative research tool and were also applied in this course of study.

A comparison was made between the radiolabelling of primary neutrophils and cultured B cells. Neutrophils were incubated with 200 µCi/ml 32P, for one hour at 30°C in phosphate-free HBS, and B cells were incubated with 200 µCi/ml 32P, for four hours at 37°C in phosphate-free RPMI medium. Cells were activated, and p67phox was immunoprecipitated from the cytosol fraction. Figure 3.1.6 shows that a higher background is obtained with B cell labelling but also a significantly higher level of the radiolabel is incorporated into phosphorylated p67phox. Also, the B cell immunoprecipitation was from one fifth of the number of cells used for the neutrophil immunoprecipitation. No Coomassie band is visible for the B cell immunoprecipitation, which is consistent with immunoblotting experiments which have shown that B cells contain as little as half the amount of p67phox as neutrophils (personal observations, Chetty et al., 1995). These results indicate that 32P,loading of B cells gives a much better labelling of phosphorylated p67phox than neutrophils.

A series of experiments were carried out to optimise the protocol for radiolabelling neutrophils, which included lowering the cell concentration as much as possible. A limiting factor in the metabolic labelling of neutrophils is that the handling must be kept to a minimum, otherwise stimulation occurs without the addition of
agonist. Therefore minimal agitation, and shortened incubation times at a temperature lower than 37°C, reduce the efficiency of labelling achieved.

Figure 3.1.6 Comparison of immunoprecipitated p67phox from metabolically labelled neutrophils and B cells. A: Coomassie-stained gel and B: corresponding phosphorimage (PI) after 36 hr exposure. Neutrophil: immunoprecipitate from 5x10^7 normal neutrophils labelled with 200 μCi ^32P, 1 ml, 1 hr, followed by 8 min stimulation with 1 mg/ml S.O.Zym. B cell: immunoprecipitate from 1x10^7 normal EBV-B cells labelled with 200 μCi ^32P, 1 ml, 4 hr, followed by 10 min stimulation with 1 μg/ml PMA.
3.2 **In vivo phosphorylation of p67phox**

3.2 AIM:

The main aim of this project was the determination of the phosphorylation sites on p67phox, and the investigation of the phosphoprotein’s role in the activation of the NADPH oxidase. The experiments reported in this section were carried out to establish the magnitude and nature of the phosphorylation of p67phox, thereby forming the basis for further characterisation.

3.2 INTRODUCTION:

It was first shown in 1993 that p67phox is phosphorylated upon activation of the NADPH oxidase (Dusi & Rossi, 1993; Dusi et al., 1993), an event that had been first observed for p47phox in 1985 (Segal et al., 1985). The cDNA encoding p47phox was not actually cloned until 1989 (Lomax et al., 1989; Volpp et al., 1989), but the original observation of the absence of this major phosphoprotein in the neutrophil cytosol of some CGD patients, illustrates the massive change in phosphorylation state of p47phox upon stimulation. The PMA-induced phosphorylation sites on p47phox have been identified (El Benna et al., 1994a), and site-directed mutagenesis was recently performed to determine the significance of the different sites on NADPH oxidase activation. The mutant proteins were transfected into p47phox-deficient B cell lines, which demonstrated that replacement of the phosphorylated serines with alanine rendered p47phox inactive (Faust et al., 1995), and that PKC was responsible for phosphorylation of most of the sites (El Benna et al., 1996).

At the time of commencing this study, other than two papers reporting an overall increase in the phosphorylation of p67phox in cytosol and membranes after activation of neutrophils (Dusi & Rossi, 1993; Dusi et al., 1993), there had been no further studies characterising this reaction. During the course of this study, a paper was published...
which questioned whether $p_{67}^{phos}$ is phosphorylated at all (Heyworth et al., 1996). The authors claim that no phosphoprotein was immunoprecipitated by antibodies against $p_{67}^{phos}$. They suggest that other previous observations to the contrary have mistakenly focused on some other, unidentified, 67 kDa proteins which they showed to be significantly phosphorylated. In an earlier study where phosphorylated $p_{47}^{phos}$ was detected in cell lysate, there was no obvious phosphorylation of native or added recombinant $p_{67}^{phos}$ (Nauseef et al., 1990). Therefore, it was of primary interest to determine whether $p_{67}^{phos}$ became phosphorylated in the intact cell, and whether this was regulated by cell stimulation.

3.2 RESULTS AND DISCUSSION:

Several tests were carried out to ensure the specificity of the antibody used to identify the $p_{67}^{phos}$ phosphoprotein. It was a polyclonal antibody raised in rabbits against recombinant GST-$p_{67}^{phos}$ and then affinity purified against recombinant (GST-cleaved or insect cell-derived) $p_{67}^{phos}$. The recombinant proteins were either synthesised in *E. coli* (bacterial) or *Spodoptera frugiperda* (insect cell) systems from fully sequenced cDNA clones of human $p_{67}^{phos}$. The antibody was tested against Western blots of TCA precipitates of whole neutrophils from a CGD patient deficient in $p_{67}^{phos}$ and normal control neutrophils (Figure 3.2.1A). Both neutrophil samples gave positive signals with antibodies to $p_{47}^{phos}$ and $p_{40}^{phos}$ (also affinity purified antibodies raised in rabbits against the recombinant proteins), but no signal was obtained in the $p_{67}^{phos}$-deficient CGD neutrophils using the $p_{67}^{phos}$ antibody. In order to check the antibody specificity under immunoprecipitation conditions, normal and $p_{67}^{phos}$-deficient CGD EBV-transformed B cells were radiolabelled with $^{32}$P, and subjected to immunoprecipitation with rabbit antibodies. The immunoprecipitates were blotted and probed with an affinity purified antibody raised in goats against recombinant $p_{67}^{phos}$. $p_{67}^{phos}$ was detected in the normal
Figure 3.2.1 Immunoblots and autoradiographs of p67\textsuperscript{phox} from whole cells and immunoprecipitates from cytosol.  

A: TCA precipitates of 1×10\textsuperscript{6} neutrophils from a normal subject and from a CGD patient lacking p67\textsuperscript{phox} were applied to SDS/PAGE, blotted onto nitrocellulose and probed with affinity purified antibodies to p67\textsuperscript{phox}, p47\textsuperscript{phox} and p40\textsuperscript{phox}. The bands were detected with the ECL system. 

B: EBV-B cells were radiolabelled with \textsuperscript{32}P, and the cytosol immunoprecipitated with the rabbit antibody against p67\textsuperscript{phox}. The immunoblot was carried out with a goat antibody to p67\textsuperscript{phox} and developed by ECL. 

C: The radiolabelled immunoprecipitates were exposed to film for 16 hr.

cells only (Figure 3.2.1B). The corresponding autorad signal at 67 kDa was present in the normal cells whereas no 67 kDa phosphoprotein was immunoprecipitated from the p67\textsuperscript{phox}-deficient cells (Figure 3.2.1C). This immunoprecipitated 67 kDa phosphoprotein was also recognised by two other antibodies raised against p67\textsuperscript{phox} peptides (see Chapter 4.1; cyanogen bromide digestion). In summary, these tests all verify the antibody specificity and confirm the identity of the 67 kDa phosphoprotein as p67\textsuperscript{phox}. 

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To study the changes in p67$^{phox}$ phosphorylation upon cell stimulation, immunoprecipitations were made of cytosol from metabolically-labelled neutrophils which had been stimulated prior to lysis (Figure 3.2.2). The results of repeated experiments showed a consistent increase in the incorporation of radiolabel on p67$^{phox}$ upon stimulation, although the cells not treated by a stimulus also showed a low level of incorporation. PMA was used as the stimulant of phosphorylation for B cells because they do not respond to fMLP (Kalunta et al., 1992), or phagocytose particles (Furukawa et al., 1992). Again some basal phosphorylation of p67$^{phox}$ was evident (Figure 3.2.3). p67$^{phox}$ phosphorylation has not been previously described in B cells.

![Coomassie-stained gels and autorads](image)

**Figure 3.2.2** Phosphorylation of p67$^{phox}$ upon stimulation of neutrophils. $^{32}P_i$-labelled neutrophils were treated in four ways (a, resting; b, S.O.Zym.; c, fMLP; d, PMA), homogenised, and then cytosol was prepared. Coomassie-stained gels and autorads are shown for A: 2×10$^5$ cell eq cytosol. B: p67$^{phox}$ immunoprecipitated from 5×10$^7$ cell eq cytosol. The gels were exposed to autorad film for 5 days at -70°C.
Figure 3.2.3 Stimulation of p67$^{\text{phox}}$ phosphorylation in B cells. $^{32}$P$_{i}$-labelled B cells were unstimulated or stimulated with 1 µg/ml PMA, 20 min. Autorads are shown of cytosol (2×10$^6$ cell eq), 5 hr exposure, and p67$^{\text{phox}}$ immunoprecipitates (from 1×10$^7$ cell eq cytosol), 16 hr exposure.

There are no reports in the literature of p67$^{\text{phox}}$ phosphorylation studies using a stimulant of phagocytosis. As the best approximation of physiological activation of the NADPH oxidase, zymosan (particles of Saccharomyces cerevisiae yeast) was opsonised with human serum to be used as an agent for phagocytosis. The opsonised zymosan, but not unopsonised zymosan, stimulated the NADPH oxidase of neutrophils as demonstrated by superoxide dismutase inhibitable cytochrome c reduction using spectrophotometric detection. A timecourse study of p67$^{\text{phox}}$ phosphorylation after phagocytic stimulation was carried out by taking aliquots of radiolabelled neutrophils over a 20 minute period (Figure 3.2.4). Since the amount of immunoprecipitated p67$^{\text{phox}}$ was not uniform, the $^{32}$P signal was normalised with respect to the protein (densitometric analysis). After five to ten minutes of stimulation the level of p67$^{\text{phox}}$ phosphorylation had more than doubled.
It is interesting to note that the phosphorylation returned to the basal level after 20 minutes of zymosan stimulation. Kinetic studies of neutrophils stimulated by opsonised latex, showed that the timecourse of superoxide production closely paralleled that of particle uptake (Segal & Coade, 1978). Phosphorylation of p47^{phox} stimulated by opsonised latex or fMLP is transient, whereas it is more persistent with PMA stimulation (Heyworth & Segal, 1986). It has been suggested that continuous phosphorylation of p67^{phox} and p47^{phox} is required to maintain NADPH oxidase activation stimulated by PMA or fMLP (Dusi et al., 1993), and that dephosphorylation of p47^{phox} precedes the decrease in the rate of oxygen consumption (Heyworth & Segal, 1986). The results here also support a role for phosphatases in deactivation of the oxidase.

There are no reports in the literature regarding quantification of p67^{phox} phosphorylation or comparison with p47^{phox} phosphorylation. Figure 3.2.5A shows the contrast in the magnitude of the phosphorylation of p67^{phox} and p47^{phox} upon activation and is representative of several experiments. The quantitation data (Figure 3.2.5B) shows that stimulation by serum-opsonised zymosan (S.O.Zym.) or fMLP yields a two-
Figure 3.2.5 Comparison of p67phox and p47phox phosphorylation in neutrophils. A: Immunoprecipitates of p67phox or p47phox from 4.5x10^7 cell eq cytosol of radiolabelled neutrophils were subjected to SDS/PAGE, then Coomassie-stained (gels), and exposed to film for 2 weeks (autorads). Neutrophils were either unstimulated or stimulated with 1 mg/ml S.O.Zym for 5 min, or 1 µg/ml PMA for 6 min, prior to lysis and immunoprecipitation. This is an example of repeated experiments used to generate the data in B. Phosphorylated p40phox is visible as an extra band in the stimulated p67phox immunoprecipitations (see Figure 5.1.4 for immunoblotting). Stimulation with fMLP was also performed (not shown here, but see Figure 5.1.3), using a 5 min preincubation with 5 µg/ml cytochalasin B followed by 1 µM fMLP for 2 min. B: Tabulation of the level of increase in phosphorylation from multiple experiments (n value in brackets), ± standard deviation for n >2. Values derived from band densitometry and normalised against 1.00 for phosphorylation in unstimulated cells.
fold increase in p47phox phosphorylation compared with the increase in p67phox phosphorylation. The increase in phosphorylation is highest when the agonist is PMA, and is particularly high for p47phox. The phosphorylation of p47phox is characterised on multiple sites, resulting in a phosphoprotein population of several isoforms (Rotrosen & Leto, 1990; El Benna et al., 1994a). The number of sites for p67phox has not been previously studied.

For investigation of the number and nature of the p67phox phosphorylation sites, 2-dimensional phosphopeptide mapping was used. Considerable time was spent on optimising the preparation of phosphorylated p67phox immunoprecipitates and the use of a Hunter Thin Layer Electrophoresis apparatus. Passing radiolabelled cytosol through an anti-p67phox column was tried as a larger scale alternative to protein-A sepharose immunoprecipitation, but it proved a cumbersome process with no significant gain in yield. Instead, better results were obtained by ensuring the ratio of cytosol to antibody and protein-A was optimised for complete precipitation of p67phox, and including steps for reducing nonspecific binding so that there was resolution of a pure p67phox band by SDS/PAGE.

In order to determine whether the increase upon stimulation was caused by the phosphorylation of multiple sites on p67phox, tryptic phosphopeptide maps of unstimulated and stimulated cells were compared (Figure 3.2.6). The phosphopeptide pattern was the same for resting and stimulated cells, suggesting that no new sites are phosphorylated. Neutrophil and B cell p67phox phosphopeptide maps were very similar, both with one major tryptic peptide signal. Phosphopeptide mapping of neutrophil p67phox was performed several times and showed the same pattern for the three stimuli used; PMA, S.O.Zym, or fMLP. Analysis of the phosphoamino acid content showed that p67phox was phosphorylated on threonine and serine in neutrophils and B cells (Figure 3.2.6 insets).
Figure 3.2.6 Phosphoamino acid and phosphopeptide maps of p67\textsuperscript{phox} before and after stimulation. p67\textsuperscript{phox} was immunoprecipitated from \(^{32}\)P\textsubscript{3}-labelled cells. A: neutrophils before stimulation, B: neutrophils after stimulation with S.O.Zym., C: unstimulated EBV-transformed B cells, and D: PMA-stimulated EBV-B cells. The phosphorimages of 2D phosphopeptide maps are shown with insets a-d of phosphoamino acid analyses. The standard resolution of phospho-serine (S), -threonine (T) and -tyrosine (Y) is indicated on inset a.
3.3 *In vitro* models of p67\(^{phox}\) phosphorylation

3.3 AIM:

To investigate methods for *in vitro* phosphorylation of p67\(^{phox}\) in order to mimic *in vivo* phosphorylation.

3.3 INTRODUCTION:

The purpose of this work was two-fold; firstly as an indicator of possible kinases in the *in vivo* phosphorylation of p67\(^{phox}\) and secondly, to provide a convenient experimental method for the efficient phosphorylation of p67\(^{phox}\). The approach entailed exposing recombinant p67\(^{phox}\) to various sources of kinase, then comparing the phosphorylation with the intact cell phosphorylation. This work is divided in two main sections:

- the application of neutrophil fractions (membranes, membrane extract, and cytosol),
- the use of specific kinase preparations (βPAK, PKC and MAP kinase).

Also included here are some observations of anomalies found with recombinant p67\(^{phox}\) derived from the insect cell expression system.

3.3 RESULTS AND DISCUSSION:

3.3a p67\(^{phox}\) phosphorylation by neutrophil fractions

Phosphorylation of recombinant p67\(^{phox}\) by isolated membrane and cytosol fractions of neutrophils could potentially provide insight to the localisation of the kinase responsible for phosphorylation in the intact cell. Membrane and cytosol fractions were prepared from sonicated lysates by centrifugation through a discontinuous sucrose gradient.
In vitro phosphorylation by membranes was highly reproducible. Phosphopeptide mapping was carried out and comparisons were made with in vivo phosphorylation by coapplication of p67\(^{\text{phox}}\) purified from both systems (Figure 3.3.1). This confirmed that the phosphopeptides produced by membrane phosphorylation and intact cell phosphorylation were different because the images do not coincide. The phosphoamino acid map showed that p67\(^{\text{phox}}\) was phosphorylated on a tyrosine residue(s), whereas threonine (and minor serine) phosphorylation takes place in the whole cell. To be sure that no co-eluting membrane protein was responsible for the phosphotyrosine signal in the in vitro system, p67\(^{\text{phox}}\) was immunoprecipitated from the phosphorylation reaction prior to running on SDS/PAGE. The p67\(^{\text{phox}}\) was immunoprecipitated from the cytosol of intact cells, as this was the only means of purifying sufficient protein for phosphopeptide mapping of p67\(^{\text{phox}}\) phosphorylated in vivo. It is interesting to note that the p67\(^{\text{phox}}\) phosphorylated in intact cells (Figure 3.3.1B) gave a weak signal, marked (i), corresponding to the membranes-derived signal (i). Although the appearance of weaker signals such as (i) and (iii) varied between experiments, the appearance of the signal in position (i) was not seen again in intact cell phosphorylation. Whether there is indeed some weak tyrosine phosphorylation in vivo accompanying the threonine-serine phosphorylation is an issue raised in Chapter 5.

Although the phosphorylation of p67\(^{\text{phox}}\) by the isolated membranes fraction did not mimic the whole cell system, the membranes were investigated further as a source of kinase. This was because the p67\(^{\text{phox}}\) kinase may be membrane-associated but in very low concentration therefore requiring separation from the crude membrane preparation which would contain multiple protein kinases. Dr A. Lal, another graduate student in Professor Segal’s laboratory, had developed a method of extracting kinase activity from membranes for the study of p47\(^{\text{phox}}\) phosphorylation. Kinase activity was partially purified by a series of extraction and chromatography steps from membranes of
Figure 3.3.1 Phosphopeptide maps comparing isolated membrane and intact cell phosphorylation of p67<sup>p60</sup>. 2D phosphopeptide analysis of A: <em>in vitro</em> phosphorylation of recombinant p67<sup>p60</sup> by unstimulated neutrophil membranes, B: neutrophil p67<sup>p60</sup> immunoprecipitated from cytosol of PMA-stimulated cells, and C: coapplication of duplicate samples as applied to A and B. Insets (a,b) are phosphoamino acid analyses.
neutrophils after stimulation with PMA. The fully purified chromatography fractions were selected for their ability to phosphorylate p47phox, but the semi-purified material also phosphorylated p67phox (Figure 3.3.2). In two experiments, phosphorylation by this membrane extract gave a different phosphopeptide map from the membrane fraction, with serine phosphorylation of p67phox (Figure 3.3.3). The 2D phosphopeptide map of p67phox also differed from that obtained from intact cells, therefore this purification of kinase activity from neutrophils did not provide a physiological in vitro system for phosphorylation of p67phox.

The results from the membranes work suggested that either the p67phox kinase is located in the cytosol, or there is some involvement of a cytosolic factor that is integral to the phosphorylation process. In vitro phosphorylation of p67phox by cytosol proved more difficult to establish than the membranes system. The stoichiometry of phosphorylation was very low, as illustrated in a comparison with the phosphorylation of recombinant p47phox (Figure 3.3.4). This is discussed further in the next section (3.3b), and experiments that were carried out to investigate in vitro conditions for achieving optimal p67phox phosphorylation are reported here.

The activation state of the neutrophils prior to sonication did not significantly affect p67phox phosphorylation by cytosol. Results of one such experiment are shown (Figure 3.3.5) indicating a slight increase in phosphorylation by PMA and fMLP stimulated cytosol preparations, but the results of several experiments failed to produce a consensus on this point. The same lack of gross effect of cell stimulation was observed for phosphorylation of p67phox by the membranes. Various additions were made to the cytosol to determine the sensitivity of the in vitro reactions to other factors. The addition of PMA caused some enhancement of phosphorylation, whereas the non-hydrolysable nucleotide GTP-γ-S did not (Figure 3.3.5). Recombinant Rac1, (in both
Figure 3.3.2 In vitro phosphorylation of p67\textsuperscript{phox} by neutrophil membranes and membrane-extracted kinase activity. Autorads of in vitro phosphorylation reactions applied to SDS/PAGE with the position of p67\textsuperscript{phox} indicated by an arrow. A: unstimulated membranes with and without added recombinant p67\textsuperscript{phox}. B: kinase-active fraction of a semipurified, salt wash of PMA-stimulated membranes.

Figure 3.3.3 Phosphopeptide analysis of membrane-extracted kinase phosphorylation of p67\textsuperscript{phox}. 2D phosphopeptide map and phosphoamino acid analysis (inset, indicating phosphoserine (S)) of recombinant p67\textsuperscript{phox} phosphorylated in vitro by membrane-extracted kinase-active fraction.
Figure 3.3.4 Comparison of in vitro phosphorylation of p67phox and p47phox by neutrophil cytosol. Coomassie-stained gel and autorad of in vitro phosphorylation reactions of recombinant p67phox and p47phox by unstimulated neutrophil cytosol. Background phosphorylation is shown as cytosol alone.

Figure 3.3.5 Variations of in vitro phosphorylation reactions of p67phox with cytosol. Coomassie-stained gels and autorads of recombinant p67phox with (A) buffer only, unstimulated cytosol with additions of PMA and GTP-γ-S, and (B) comparison of reactions with cytosol from and PMA- or fMLP-stimulated neutrophils.
the active GTP-bound form, and the constitutively active form Val-12 Rac1) and p40phox, were also without an effect.

The addition of phosphatidic acid has been reported to increase the level of phosphorylation in a similar in vitro system (particularly for native p47phox) using cytosol and membrane fractions together (McPhail et al., 1995). This was investigated for p67phox, where recombinant p67phox was added to the system described in the paper. Although enhancement of total phosphorylation was observed, no increase in p67phox phosphorylation was found. Therefore this was not a useful method to achieve enhanced labelling of p67phox.

The results of phosphopeptide mapping of p67phox phosphorylated in vitro by cytosol reproducibly showed a match with the in vivo phosphorylation in whole cells (Figure 3.3.6A,B). For comparison of similar maps, it was important to carry out 2D mapping in parallel due to slight variations in the resolution between experiments. For example, the major phosphopeptide signal (Figure 3.2.6) sometimes appeared as two closely resolved spots (Figure 3.3.6A,B), which is probably due to partial tryptic digestion. This in vitro phosphorylation however, provided a mimic of the native p67phox phosphorylation.

3.3b p67phox phosphorylation by βPAK, PKC, and MAP kinase

Recombinant p67phox protein was also phosphorylated in vitro with the recombinant kinases, MAP kinase and PAK, and a mixed isoform brain isolate of PKC. The phosphopeptide maps show that only MAP kinase mimicked the in vivo phosphorylation (Figure 3.3.6C-E). The PAK and PKC phosphorylations were markedly different and occurred on serine residues only. These particular kinases were chosen for study as each has been implicated in the signal transduction network for activation of the NADPH oxidase.
Figure 3.3.6 Phosphopeptide analysis of the in vitro phosphorylation of p67phox. The 2D phosphopeptide map of (A:) p67phox immunoprecipitated from S.O.Zym-stimulated neutrophils, is compared with phosphopeptide maps of in vitro phosphorylated recombinant p67phox using different sources of kinase (B: neutrophil cytosol, C: recombinant MAP kinase, D: recombinant βPAK, E: rat brain PKC mixed isoforms). Insets a-e show phosphoamino acid analysis of the same samples.
Recent findings raised the possibility of an association between PAK and p67\textsuperscript{phox}, since both PAK and p67\textsuperscript{phox} interact with Rac. A 68 kDa protein, closely related to brain βPAK p65, was purified from neutrophil cytosol and shown to react with GTP-bound p21 proteins including Rac1 (Prigmore et al., 1995). It is established that p67\textsuperscript{phox} is an effector molecule for Rac in the activation of neutrophils (Diekmann et al., 1994, 1995; Dorseuil et al., 1996), but an interaction between PAK and p67\textsuperscript{phox} has yet to be proven. A constitutively active, (therefore not requiring activation by Rac-GTP binding) GST-fusion recombinant βPAK p65 (Manser et al., 1995) (provided by Ms E. Prigmore at the Institute of Neurology, London), was used for these in vitro phosphorylation studies. This rat brain βPAK p65 shares 73% amino acid identity with neutrophil PAK, hPAK65 (Martin et al., 1995), which has ubiquitous mRNA but with particularly high expression in neutrophils and HL60 cells. There remains the possibility that the PAK phosphorylation of p67\textsuperscript{phox} did not mimic in vivo phosphorylation of p67\textsuperscript{phox} because βPAK was used and not neutrophil PAK. A suitable source of neutrophil PAK was not available for testing.

The phosphorylation of p47\textsuperscript{phox} by βPAK was also carried out. Figure 3.3.7 shows that the maps from PMA-stimulated neutrophils and βPAK phosphorylation are not identical, but that some of the p47\textsuperscript{phox} phosphopeptides may be the same. Solely phosphoserine was detected for both PMA and βPAK phosphorylation of p47\textsuperscript{phox}. After these experiments were performed, a study was published showing two neutrophil proteins p65 and p68 (isolated by immunoaffinity to brain PAK antibodies) were able to phosphorylate p47\textsuperscript{phox} (Knaus et al., 1995). These PAK preparations did not phosphorylate recombinant p67\textsuperscript{phox} under the same conditions. These findings support the conclusions drawn from the work with recombinant βPAK, in that p67\textsuperscript{phox}
Figure 3.3.7 p47
 phosphorylation by βPAK. Phosphopeptide maps of (upper:) p47
 immunoprecipitated from PMA-stimulated neutrophils, and (lower:) recombinant p47
 phosphorylated by βPAK.

Phosphorylation appears not to involve PAKs, whereas p47
 could be a PAK substrate in neutrophils.

PKC was an obvious candidate kinase to undertake the phosphorylation of p67
. This is because PMA, a direct agonist of PKC, activates p67
 phosphorylation in whole cells and was shown to give the same phosphopeptide map of p67
 as the more physiological stimuli, fMLP and zymosan. However, the phosphopeptide map obtained with phosphorylation of p67
 by PKC did not match in vivo phosphorylation, and was of purely phosphoserine content. A very recent publication, which was the first report of p67
 phosphopeptide mapping, claims that PKC phosphorylated p67
 on the same site as for phosphorylation in intact cells (El Benna et al., 1997). They did not identify the phosphorylation site, but showed only a phosphoserine signal, with no phosphothreonine. These findings do not agree with the experiments reported here despite the use of similar analytical methods.
In summary, the kinase preparations of PKC, βPAK and neutrophil membranes, phosphorylated recombinant p67<sub>phox</sub> under the same conditions that they phosphorylated p47<sub>phox</sub> (Figure 3.3.8). Phosphopeptide mapping showed that the pattern of phosphorylation obtained with these kinases in vitro did not match the pattern of p67<sub>phox</sub> phosphorylation in vivo. Therefore these components appear to have no direct physiological role in the phosphorylation of neutrophil p67<sub>phox</sub>. Both cytosol and MAP kinase, however, do mimic the phosphorylation obtained in vivo by producing the same major tryptic phosphopeptide. These in vitro systems were taken further in the identification of the phosphorylation site (Chapter 4).

Calculations of the stoichiometry of p67<sub>phox</sub> phosphorylation were carried out for the cytosol and MAP kinase phosphorylations by measuring the amount of incorporated radioactivity. The values were consistently in the range of 1 mol phosphate to 100-200 mol p67<sub>phox</sub>, corresponding to 0.5-1.0% of p67<sub>phox</sub> molecules becoming phosphorylated. The concentration of ATP and kinase were not limiting in the experiments, as demonstrated by the phosphorylation of p47<sub>phox</sub> which was several times stronger. The low stoichiometry of p67<sub>phox</sub> is in stark contrast to p47<sub>phox</sub> which has been shown to be 1-8 mol phosphate/mol p47<sub>phox</sub> in some in vitro reactions (Park, J.-W. et al., 1997). This difference in p67<sub>phox</sub> and p47<sub>phox</sub> as substrates of in vitro phosphorylation probably reflects the number of phosphorylation sites per molecule, but may also indicate that the optimal (or physiological) conditions for p67<sub>phox</sub> phosphorylation were not met in the cytosol and MAP kinase reactions.

Although the in vivo phosphorylation of p47<sub>phox</sub> was also greater than for p67<sub>phox</sub> (Figure 3.2.5), the difference between the phosphorylation of p47<sub>phox</sub> and p67<sub>phox</sub> by cytosol in vitro seemed even more pronounced (Figure 3.3.4). As described earlier, attempts to manipulate the conditions of the in vitro phosphorylation of p67<sub>phox</sub> by cytosol, did not result in significant improvements to the stoichiometry of
**Figure 3.3.8** Comparison of *in vitro* phosphorylation of p67<sup>phox</sup> and p47<sup>phox</sup>. Coomassie-stained gels and autorads of *in vitro* phosphorylation reactions with PKC, βPAK, and membrane-extracted kinase activity. Background phosphorylation without protein substrate shown by buffer-only reactions (A), and with added recombinant p47<sup>phox</sup> (B) or p67<sup>phox</sup> (C). Film was exposed to the gels for 24 hr., -70°C.
phosphorylation. An explanation of the apparently limited phosphorylation of p67\textsuperscript{phox} in vitro could be that in the cytosol of neutrophils, native p67\textsuperscript{phox} is accompanied by a co-factor which is not present, or too dilute, in the MAP kinase and cytosol in vitro systems. Recombinant p67\textsuperscript{phox} undergoes the same phosphorylation by isolated cytosol or MAP kinase as native p67\textsuperscript{phox} in the intact cell (shown by 2D mapping), but it seems likely that other specific intermolecular interactions would play a role in the dynamics of p67\textsuperscript{phox} phosphorylation in vivo.

3.3c Phosphorylation of insect cell recombinant p67\textsuperscript{phox}

Some interesting findings were made in the course of in vitro phosphorylation work regarding recombinant p67\textsuperscript{phox} expressed in the insect cell system. The data shown above are from experiments using p67\textsuperscript{phox} cleaved from a GST-fusion protein synthesised by bacteria. Initially the insect cell p67\textsuperscript{phox} was used because it could be easily produced in bulk and purified to approximately 95% purity. However it was observed that in negative controls, where \textsuperscript{32}P-\gamma-ATP, kinase buffer and the substrate p67\textsuperscript{phox} were incubated in the absence of any kinase, insect cell p67\textsuperscript{phox} became phosphorylated. This phenomenon was not observed for GST-fusion recombinant p67\textsuperscript{phox} or immunoprecipitated neutrophil p67\textsuperscript{phox}. When the specific activity of the reactions was increased, the autorad of such phosphorylated p67\textsuperscript{phox} showed two extra bands on either side of insect cell p67\textsuperscript{phox} (Figure 3.3.9A). Even at very high loading of p67\textsuperscript{phox}, these bands could not be seen by Coomassie staining.

The purification of p67\textsuperscript{phox} from the insect cell expression system involved anion exchange chromatography followed by hydrophobic interaction chromatography (Chapter 4.3), therefore it seemed unlikely that these phosphorylating bands could be bound in a complex with p67\textsuperscript{phox}. To test whether any insect cell proteins could show high affinity binding to p67\textsuperscript{phox}, a lysate of uninfected insect cells was passed down a
p67\textsuperscript{phox} affinity column prepared with GST-cleaved, bacterially expressed, p67\textsuperscript{phox}. No bands were detected in the eluant fractions by staining, or by autoradiography following incubation with \textsuperscript{32}P-\gamma-ATP. This suggested that the bands co-purifying with insect cell expressed p67\textsuperscript{phox} were not strongly bound proteins. Also, immunoprecipitation of p67\textsuperscript{phox} from the \textsuperscript{32}P-\gamma-ATP incubation mixture, showed that these two co-purifying bands could be dissociated from insect cell p67\textsuperscript{phox} (Figure 3.3.9B).

To determine whether the phosphorylation of insect cell p67\textsuperscript{phox} was due to the presence of the copurifying factors, the p67\textsuperscript{phox} was immunoprecipitated before it was incubated with \textsuperscript{32}P-\gamma-ATP (Figure 3.3.9C). Even without the other bands, p67\textsuperscript{phox} was phosphorylated. Although the two visible bands were removed by immunoprecipitation, this experiment does not rule out the possibility of a different factor, or kinase, remaining with insect cell p67\textsuperscript{phox}. A previous batch of insect cell p67\textsuperscript{phox} was tested and although the effect was less pronounced, it showed similar results.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.3.9.png}
\caption{Phosphorylated bands of insect cell p67\textsuperscript{phox}. Autorads of insect cell expressed p67\textsuperscript{phox} after incubation with \textsuperscript{32}P-\gamma-ATP. A: p67\textsuperscript{phox} incubated with \textsuperscript{32}P-\gamma-ATP in the absence and presence of neutrophil cytosol. B: Unbound (UB) and immunoprecipitate (ip) fractions of p67\textsuperscript{phox} which had been incubated with \textsuperscript{32}P-\gamma-ATP prior to immunoprecipitation procedure using antibodies to p67\textsuperscript{phox}. C: p67\textsuperscript{phox} was immunoprecipitated first, then the PAS beads were incubated with \textsuperscript{32}P-\gamma-ATP.}
\end{figure}
A motif search using the GCG package (Genetics Computer Group, version 8) identified amino acids 145-152 as a consensus sequence for an ATP/GTP-binding motif, except for one mismatched amino acid (see Figure 4.2.3). The question of whether p67\textsuperscript{phox} is a nucleotide-binding protein has been raised before in relation to the binding of NADPH (Smith, R.M. et al., 1996). However, earlier reports claim that p67\textsuperscript{phox} does not bind ATP or GTP (Leto et al., 1991; Iyer, S.S. et al., 1994). The fact that insect cell p67\textsuperscript{phox} was radiolabelled after denaturing-SDS/PAGE, indicated that the observation was due to covalent phosphorylation and not ATP-binding. It is unlikely that the p67\textsuperscript{phox} is autophosphorylated as there are no matches in the amino acid sequence of p67\textsuperscript{phox} for protein kinase ATP-binding or catalytic domain motifs. Therefore the most probable explanation is the presence of an active kinase which copurifies with p67\textsuperscript{phox} from insect cell cytosol.

Tryptic phosphopeptide mapping was carried out on insect cell p67\textsuperscript{phox}. The protein was incubated in \textsuperscript{32}P-\gamma-ATP without added kinase, subjected to SDS/PAGE (an 8% acrylamide gel run overnight for good resolution) and the p67\textsuperscript{phox} band was excised to isolate it from the other two phosphorylating components. The phosphopeptide map showed one phosphopeptide, and both phosphoserine and phosphothreonine content (Figure 3.3.10). The phosphopeptide is in a similar position to the major phosphopeptide of neutrophil p67\textsuperscript{phox} maps, but the comparison was not taken further. The use of insect cell recombinant p67\textsuperscript{phox} for \textit{in vitro} phosphorylation studies was not continued, because of possible interference of this background phosphorylation.

These observations of the insect cell recombinant protein may be useful for future work in designing cell-free systems using p67\textsuperscript{phox}. Two papers have drawn comparisons between the activities of recombinant p67\textsuperscript{phox} and p47\textsuperscript{phox} from bacteria and insect cell systems in amphiphile-activated cell-free oxidase assays (Leto \textit{et al}., 1991; Abo \textit{et al}., 1992). In the former report the authors claimed that the recombinant proteins produced
by insect cells were much more effective in reconstituting CGD cytosol activity, and the latter report showed the bacteria-expressed proteins were more active in a different cell-free system. Therefore it is difficult to postulate on the nature or the significance of differences in protein expression and processing by these prokaryotic and eukaryotic systems. However, the possibility of inherent differences in recombinant protein production should be taken into account when interpreting conflicting data such as that relating to protein-protein interactions.

Figure 3.3.10 Phosphopeptide analysis of insect cell p67\(^{plus}\). Insect cell expressed p67\(^{plus}\) was incubated with \(^{32}\)P-\(\gamma\)-ATP, subjected to SDS/PAGE and the 67 kDa phosphoprotein was then excised for tryptic digestion and 2D phosphopeptide mapping. The phosphoamino acid analysis of the same sample is shown in the inset.
SUMMARY OF CHAPTER 3:

Characterisation of p67\textsuperscript{phox} phosphorylation

The low level of p67\textsuperscript{phox} phosphorylation in neutrophils can incur detection difficulties. The results presented here confirm the previous findings that p47\textsuperscript{phox} phosphorylation is more easily detected. There is debate in the literature regarding whether p67\textsuperscript{phox} is indeed phosphorylated. Therefore, the initial aim was to optimise the labelling methods, and determine if p67\textsuperscript{phox} is phosphorylated or not. Efforts to enhance the specific activity of ATP in neutrophils by glucose starvation and permeabilisation failed to produce improved labelling methods. The conditions of standard metabolic labelling of neutrophils and B cells were optimised, as was the immunoprecipitation protocol.

In vivo phosphorylation experiments showed that p67\textsuperscript{phox} is phosphorylated in neutrophils and B cells. There is low basal phosphorylation which is increased up to three-fold upon stimulation. No new sites are phosphorylated upon stimulation, but the number of phosphorylated molecules of p67\textsuperscript{phox} increases. This was shown by reproducible phosphopeptide mapping, which indicated one major tryptic phosphopeptide for basal and stimulated phosphorylation. The same phosphopeptide was produced by phorbol ester or membrane receptor mediated agonists alike. Amino acid analysis revealed threonine and serine phosphorylation, with the predominant signal as phosphothreonine.

In vitro phosphorylation models for p67\textsuperscript{phox} were pursued to provide a practical method for identifying the phosphorylation site, and to obtain insight into the cellular mechanisms involved. Phosphopeptide mapping served to eliminate membranes, βPAK, and PKC as possible kinases for p67\textsuperscript{phox}. The major phosphopeptide, and threonine-serine phosphorylation, was mimicked by cytosol or MAP kinase. Further information on the regulation of the reaction was not gained by manipulation of cytosol
fractions. Also reported here were observations of differing behaviour between insect cell expressed and bacterially expressed recombinant p67phox.

The results from this chapter formed the basic characterisation of p67phox phosphorylation, to be extended in determination of the phosphorylation site (Chapter 4), and in studies of the regulation of phosphorylation (Chapter 5).
Chapter 4

Identification of the major phosphorylation site of p67phox

4.1 Localisation of the p67phox phosphorylation site

4.1 AIM:

To determine the position of the phosphorylation site on p67phox.

4.1 INTRODUCTION:

In order to broadly locate the part of the p67phox protein that is phosphorylated, cyanide bromide digestion was used. HPLC-mass spectrometry (MS) was then applied to analyse tryptic peptides of in vitro phosphorylated p67phox. This allowed an assumption to be made as to the site of phosphorylation. This site was modified by mutagenesis and shown not to be phosphorylated in the mutant protein.

4.1 RESULTS AND DISCUSSION:

p67phox contains 13 methionines, so cleavage by cyanogen bromide could theoretically give rise to 14 peptides ranging in size from one to 116 amino acids in length (Figure 4.1.1). Radiolabelled p67phox was immunoprecipitated and cleaved by cyanogen bromide, then the resultant peptides were separated on a high percentage tricine gel. Although no peptide bands were detected by colloidal Coomassie staining, the phosphoimages clearly show the position of the phosphorylated peptide (Figure 4.1.2). This is a peptide of approximately 13 kDa for the in vivo phosphorylations, the larger fainter bands are suspected to be incomplete cleavage products of the same region. The same pattern is seen from unstimulated and stimulated neutrophils and B cells alike.
Figure 4.1.1  Diagram of the theoretical cleavage of p67\textsuperscript{phox} by cyanogen bromide.  
A: The amino acid position of cyanogen bromide cleavage sites (methionine residues) are marked on a schematic diagram of p67\textsuperscript{phox}.  B: Representation of the theoretical fragments from complete digestion of p67\textsuperscript{phox}, fragment size in kDa.

Figure 4.1.2  Cyanogen bromide digestions of phosphorylated p67\textsuperscript{phox}. p67\textsuperscript{phox} was \textsuperscript{12}P-phosphorylated, isolated by SDS/PAGE and digested by cyanogen bromide. Phosphorimages are shown of the tricine gels from three experiments. Tricine gel electrophoresis resolved the peptides obtained from p67\textsuperscript{phox} immunoprecipitated from unstimulated, S.O.Zym.- and PMA-stimulated neutrophils (N), unstimulated and PMA-stimulated B cells (B), and recombinant p67\textsuperscript{phox} phosphorylated \textit{in vitro} by βPAK and neutrophil membranes.
PMA and opsonised zymosan stimulation of neutrophils give the same phosphopeptide. These results are consistent with the 2D phosphopeptide mapping findings reported in Chapter 3.

The in vitro phosphorylations of recombinant p67\textsuperscript{phox}, showed that membranes phosphorylated the same region as in vivo phosphorylated p67\textsuperscript{phox}, but βPAK gave a different profile. As indicated by 2D phosphopeptide mapping (Chapter 3) both membranes and βPAK in vitro systems phosphorylated p67\textsuperscript{phox} at different sites from intact cell phosphorylation. However, since the tyrosine phosphorylation by membranes occurs in the same cyanogen bromide fragment as the threonine/serine phosphorylation in intact cells, it was considered worthwhile to try to identify the region of phosphorylation using the membranes in vitro system. At this stage of experimental work, neither the cytosol nor MAP kinase in vitro methods had been established, otherwise they would have been used as phosphorylation models.

Various recombinant p67\textsuperscript{phox} constructs made by Dr F. Wientjes, Professor Segal’s laboratory, were applied to the same in vitro phosphorylation assay as full length p67\textsuperscript{phox} (Figure 4.1.3). The results implicated an N-terminal region containing the site for phosphorylation. Neither N-58 nor N-131 fragments were phosphorylated, and both the N-192 and N-238 fragments were strongly phosphorylated, consistent with a site between 131-192aa, but this was contradicted by the 192-238aa fragment also being phosphorylated. A single site for phosphorylation by membranes would be expected based on the tryptic phosphopeptide map. However, the limitations of this experimental approach must be emphasised. The use of protein fragments could give misleading information with respect to the full length molecule because the tertiary structure of the isolated part may well vary from the way the region folds when in the whole protein. Also, differing net electrostatic charges of polypeptides would affect their performance as phosphorylation substrates. The obvious differences in the stoichiometry of labelling
Figure 4.1.3 *In vitro* phosphorylation of recombinant fragments and mutants of p67<sup>Phox</sup> by neutrophil membranes. Coomassie-stained gels and autorads of *in vitro* phosphorylation reactions carried out with unstimulated membranes as the source of kinase. Recombinant substrates are described as GST-(fusion proteins), number of amino acids indicating length or position in p67<sup>Phox</sup>, Δ (deletion) and SH3 (N- or C-terminal SH3 domains). Phosphorylation of substrate denoted as ✓ or ✗, or ? for unclear result.
of different fragments from the same protein, seen by autoradiography (Figure 4.1.4),
serve as reminders that in vitro phosphorylation is fundamentally an artificial system.
This method was not pursued for locating the phosphorylation site, although the results
do support the general location of phosphorylation as being towards the N-terminus.

Immunoblots were carried out on cyanogen bromide digestions of \( p67^{phox} \)
phosphorylated in intact neutrophils, using antibodies raised against polypeptides
contained in \( p67^{phox} \). These are against the N-terminal 192 amino acids and the last 20
amino acids of the C-terminus. The 13 kDa phosphopeptide is recognised by the \( \alpha N-192 aa \) antibody, whereas the \( \alpha C-20 aa \) antibody detects a different larger peptide (Figure 4.1.4). The specific epitopes recognised by these polyclonal antibodies are not known. The size of the major fragments recognised by the antibodies indicates that the digestion
by cyanogen bromide was only partial. The C-terminus antibody recognised a large
fragment of approximately 35 kDa, also the size of a common breakdown product of
\( p67^{phox} \). Possible partial digestion products that may have reacted with the N- and C-
terminal antibodies are portrayed in Figure 4.1.5. The approximate positions of all
threonines and serines are also shown. The fact that the phosphopeptide is recognised by
the N-terminal antibody, eliminated the threonines and serines situated in the C-terminal
half of the protein as phosphorylation sites.

Identification of candidate phosphorylation sites was made by mass spectrometry
(MS). Since phosphorylation by cytosol and by MAP kinase had been shown by 2D
phosphopeptide mapping to provide good models for the intact cell phosphorylation,
these in vitro methods were applied to generate \( ^{32}P \)-phosphorylated \( p67^{phox} \). Tryptic
digests of such radio-labelled \( p67^{phox} \) were applied to reverse phase HPLC to separate the
peptides. Scintillation counting of the HPLC fractions allowed detection of the
radio-labelled peptide. MALDI-TOF MS was used to determine the masses of the
peptides in the fraction. The MS facility was located in the Ludwig Institute for Cancer
Figure 4.1.4 Localisation of p67phox phosphorylation by immunoblotting. Cyanogen bromide digestions of immunoprecipitated p67phox were subjected to tricine gel electrophoresis (gel, colloidal stain), and immunoblotted with peptide antibodies against the N-terminal 192 amino acids (αN), and C-terminal 20 amino acids (αC). The ECL system was used for detection. p67phox immunoprecipitated from 32P labelled, PMA-stimulated neutrophils, was digested by cyanogen bromide and exposed in a phosphorimager (PI).

![Image of gel and immunoblots]

Figure 4.1.5 Position of p67phox threonine and serine residues. The 21 threonines and 28 serines shown in relation to the cyanogen bromide cleavage sites (▼) and position of the N- and C-terminus antibody recognition sites (N192, C20). Possible partial digestion fragments detected by the N192 antibody (15.1, 12.7, 13.3 kDa) and C20 antibody (29.9 kDa). Threonines 233 and 366, and serine 373 are depicted in bold type.

![Diagram of threonine and serine residues]
Research, London, and was operated by Ms O. Truong. **Figure 4.1.6** shows a representative spectrum for the analysis, with a summary of the assignment of signals. The strongest signals of 762 and 1245 Da were subjected to further analysis using Post Source Decay spectra, to determine the amino acid sequences. The 842 Da signal was originally thought to be the phosphopeptide of 762 (ie: 762 + 80 PO₄), but was later sequenced from a different spectrum as a common contaminating peptide of trypsin from the tryptic digestion.

The major components of the fraction were the two p67phox peptides, amino acids 233-238 and 366-376. HPLC-MS was applied to repeated experiments with the same results. No phosphorylated peptide signal was detected, despite the detection of a phosphopeptide by scintillation counting. This was thought to be due to low stoichiometry of the phosphorylation reaction, or could be due to dephosphorylation during sample handling after scintillation counting. An alternative matrix (2,6-dihydroxyacetophenone/di-ammonium hydrogen citrate (DHAP/DAHC) often used for phosphopeptide analyses, was also unable to allow detection of any phosphorylated peptides. The relatively steep HPLC gradient made it possible that the phosphorylated and non-phosphorylated forms of the same peptide co-eluted in the same fraction.

Attempts were made to recover peptides off the cellulose plates after 2D mapping (Boyle *et al.*, 1991). Particulate matter proved difficult to remove during this process, and the MS spectra indicated high background of unknown contaminants. As this approach was unsuccessful, direct analysis of resolved phosphopeptides was not possible.

Both of the sequenced peptides contained threonine or serine residues; Thr-233, Thr-366 and Ser-373. These residues are marked in bold on the diagram of p67phox in **Figure 4.1.5**. The information from the cyanogen bromide experiments and the MS data were combined to deduce the most likely phosphorylation site. The peptide 366-373 could not be contained within a cyanogen bromide digestion fragment that would be
Figure 4.1.6  MS analysis of radiolabelled RP-HPLC fraction from tryptic digest of cytosol-phosphorylated p67phox. Recombinant p67phox was phosphorylated in vitro by neutrophil cytosol, gel purified, and digested by trypsin. The resultant peptide mix was applied to RP-HPLC, and the fractions analysed by scintillation counting to determine the elution of $^{32}$P. The radiolabelled fraction was applied to MALDI-TOF MS, and sequence analysis was carried out on the two most abundant peptide signals (762.365 and 1245.62 Da) using post source decay spectra. The solved amino acid sequence is shown for these two peptides. The signal at 842.462 Da was later identified as a peptide of trypsin.
detected by the N-terminal antibody as it is from the C-terminal half of p67phox. However, the residue Thr-233 could be contained in the cyanogen bromide 13 kDa phosphopeptide. So an assumption was made that Thr-233 was the most likely candidate phosphorylation site, and mutagenesis was carried out to confirm this.
4.2 Mutagenesis of threonine 233

4.2 AIM:

To test the deduction that threonine 233 was a phosphorylation site, mutagenesis of p67\textsuperscript{phox} was performed to introduce a single amino acid substitution in which threonine 233 was replaced by alanine. It would then be possible to determine if the phosphorylation of p67\textsuperscript{phox} was prevented by this mutation.

4.2 INTRODUCTION:

From the 2D phosphopeptide mapping, cyanogen bromide digestion and HPLC-MS results, Thr-233 was implicated as the major phosphorylation site on p67\textsuperscript{phox}. This section of work describes the site-directed mutagenesis of this residue, and the use of the resultant recombinant protein in the \textit{in vitro} phosphorylation systems for p67\textsuperscript{phox}. Experiments to examine the role of Thr-233 phosphorylation in the regulation of the NADPH oxidase were also attempted. The introduction of mutant or wild type p67\textsuperscript{phox} to p67\textsuperscript{phox}-deficient cells, will be the key system for evaluating the importance of this phosphorylation site \textit{in vivo}. Viral delivery of p67\textsuperscript{phox} constructs to EBV-transformed B cell lines of p67\textsuperscript{phox}-deficient CGD patients is the system currently being developed (Thrasher \textit{et al.}, 1992; Weil \textit{et al.}, 1997) but as it has not yet been successful, no results can be included here.

4.2 RESULTS AND DISCUSSION:

The method chosen for mutagenesis of p67\textsuperscript{phox} was a PCR technique known as primer overlap extension. In brief, two consecutive PCR reactions using primers with single point mutations were used to produce a double stranded mutagenic cassette which was then cloned by restriction digestion into wildtype cDNA. The final construct was a GST-fusion protein with the point mutation of threonine to alanine at amino acid 233 of
Complete DNA sequencing of the final DNA clone was performed to ensure that the construct was otherwise identical to wildtype, and the recombinant protein was expressed and purified as for wildtype p67\textsuperscript{phox}. The mutant p67\textsuperscript{phox} behaved in exactly the same way as wildtype p67\textsuperscript{phox} during the entire expression and purification procedure.

The ability for the Ala-233 mutant to act as a substrate for \textit{in vitro} phosphorylation was examined in parallel with wildtype. The Ala-233 mutant and p67\textsuperscript{phox} wildtype were phosphorylated to an equal extent by PKC (data not shown). However, the mutation of threonine 233 resulted in almost complete loss of phosphorylation of p67\textsuperscript{phox} exposed to cytosol or MAP kinase \textit{in vitro} (Figure 4.2.1A). Phosphopeptide mapping of the p67\textsuperscript{phox} Ala-233 mutant as substrate in both of these \textit{in vitro} models of phosphorylation resulted in the disappearance of the major phosphopeptide, leaving the background (noise) signals only (data for cytosol assay shown, Figure 4.2.1B). The major phosphorylation signal is therefore due to the phosphorylation of threonine 233. Phosphoamino acid analysis was attempted on the Ala-233 mutant phosphorylations to test for phosphoserine, but the signals were undetectable.

The Ala-233 mutant p67\textsuperscript{phox} was also subjected to HPLC-MS. The HPLC UV traces of the tryptic digests of mutant and wildtype p67\textsuperscript{phox} were almost identical. Fraction 20 was analysed from both mutant and wildtype phosphorylation reactions using cytosol or MAP kinase as the source of kinase. Figure 4.2.2 only shows the data from the MAP kinase \textit{in vitro} system, but the same results were obtained for recombinant p67\textsuperscript{phox} phosphorylated by cytosol. The MS signal at 1245 Da (peptide 366-376) as seen in Figure 4.1.6, was present in fraction 20 of the mutant and wildtype experiments alike, just as the rest of the spectra were very similar. The only significant difference was the 732 Da signal for the mutant experiment instead of the 762 Da signal of wildtype. The
Figure 4.2.1  Effect on *in vitro* phosphorylation of Thr-233 to Ala substitution in p67phox.  

**A:** Coomassie-stained gel and autorad showing p67phox*wt* (wt) or Ala-233 mutant p67phox*mut* (mut), with either no kinase (buffer), neutrophil cytosol (Cytosol) or MAP kinase (MAPK). (Mr: albumin molecular weight standard for 67 kDa, C: cytosol alone control, M: MAP kinase alone control).  

**B:** 2D phosphopeptide maps of p67phox*wt* wildtype background phosphorylation without added kinase (wt bg), p67phox*wt* wildtype phosphorylated by cytosol (cyt/wt) and p67phox*mut* Ala-233 mutant phosphorylated by cytosol (cyt/mut).
Figure 4.2.2 Peptide sequence analysis of HPLC fractions from in vitro phosphorylated p67\textsuperscript{phos}. A: one tenth of each fraction collected from HPLC resolution of tryptic peptides of MAP kinase phosphorylated p67\textsuperscript{phos} was subjected to scintillation counting. Fraction 20 contained radiolabel for p67\textsuperscript{phos} wildtype. B: fractions 20 of p67\textsuperscript{phos} wildtype and Ala-233 mutant experiments were analysed by MALDI-TOF MS using an α-cyano-4-hydroxycinnamic acid (CHCA) matrix for molecular weight measurements. The peptide sequences were confirmed by post source decay mass spectra.
732 Da peak was sequenced as the mutant 233-238 peptide. This result confirmed that the lack of phosphorylation of the mutant p67phos was due to the absence of Thr-233.

The results presented here disagree with a very recent publication reporting that p67phos is only phosphorylated on serine residues (El Benna et al., 1997). Although some phosphoserine was also detected, the majority of experiments in this project showed the stronger signal was for phosphothreonine. It may be possible that the weaker phosphoserine signal can be enhanced by subtle differences in cell handling or phosphopeptide mapping procedures. Phosphoamino acid analysis by partial acid hydrolysis does not necessarily provide an exact representation of the relative abundance of phosphorylated amino acids (Kamps, 1991), so there is a limit to the confidence with which these data can be interpreted.

There are some variable, weaker signals in addition to the main phosphopeptide signal on the 2D maps of intact cell and cytosol-phosphorylated p67phos (Figures 3.2.6 and 3.3.6). However, for MAP kinase phosphorylation, which also indicated phosphoserine content, there is just the single phosphopeptide spot which disappeared after mutation of Thr-233. Therefore the implication is that the phosphoserine signal is produced by many serines phosphorylated at low frequency. This would account for phosphoserine detection in the absence of distinct signals corresponding to specific serine phosphopeptides.

The identified Thr-233 phosphorylation site is in a consensus sequence for the proline-directed MAP kinases (PXS/TP) (Erickson, A.K. et al., 1990; Clark-Lewis et al., 1991) as it is situated in a proline-rich region (Figure 4.2.3). Searches for other consensus sequences were carried out using the GCG package (Genetics Computer Group, version 8) and the ExPASy Molecular Biology Server, University of Geneva. Ten PKC phosphorylation sites (Woodgett et al., 1986), eleven casein kinase II sites (Pinna, 1990), and one cAMP/cGMP-dependent protein kinase site (Framisco et al.,
**Key to Figure 4.2.3:**

- Threonine 233
- Src homology 3 (SH3) domain (Mayer et al., 1988)
- Polyproline region (Ren et al., 1993)
- Tetratricopeptide repeat (TPR) (Ponting, 1996)
- Exon boundary (Kenney et al., 1993)

**Motifs / phosphorylation sites:**

- **MAPK** mitogen-activated protein kinase phosphorylation site, PX(S/T)P, (Clark-Lewis et al., 1991)
- **PKC** protein kinase C phosphorylation site, S/TKR/K, (Woodgett et al., 1986)
- **CKII** casein kinase II phosphorylation site, S/TXXD/E, (Pinna, 1990)
- **cAMP/GMP** cyclic AMP/GMP-dependent protein kinase phosphorylation site, R/KXXS/T, (Framisco et al., 1980)
- **A/GTP** ATP/GTP binding site motif, with one major mismatch at Met-150, (A/G)\_4GK(S/T), (Walker et al., 1982)

**Information relating to mutagenesis procedure:**
(see Chapter 2 for details)

- Polymerase chain reaction (PCR) primer
- Restriction enzyme recognition site
Figure 4.2.3  cDNA and amino acid sequence of p67\textsuperscript{phox}. p67\textsuperscript{phox} cDNA was cloned by screening an expression library derived from HL60 cells (Leto et al., 1990). The gene was mapped to chromosome 1q25 (MIM number 233710) and named neutrophil cytosolic factor 2 (NCF 2) (Francke et al., 1990). The 526 amino acid sequence has been assigned the database accession numbers P19878 (SwissProt) and M32011 (EMBL). See facing page for key.
1980), were identified on $p67^{phox}$. It may be that the phosphoserine signal observed by phosphoamino acid mapping represents the activity of one of these kinases. The assignment of the major signal of tryptic phosphopeptide mapping as Thr-233, as mimicked by recombinant MAP kinase phosphorylation, suggests that MAP kinase could be the candidate kinase. This issue is pursued in Chapter 5.

In order to examine the requirement of $p67^{phox}$ phosphorylation for translocation from cytosol to the membranes, an experiment was designed to test for a change in affinity of $p67^{phox}$ for the membranes after phosphorylation. Wildtype $p67^{phox}$ and mutant Ala-233 $p67^{phox}$ were subjected to in vitro phosphorylation by neutrophil cytosol. Therefore the mutant $p67^{phox}$ was in effect an unphosphorylated form of $p67^{phox}$ to be compared with wildtype phosphorylated $p67^{phox}$. After 20 minutes at 30°C, the phosphorylation reactions were incubated with neutrophil whole cell lysate, containing phosphatase inhibitors, for 5 minutes at 37°C. The whole cell lysate was prepared from unstimulated or PMA-stimulated neutrophils. The phosphorylation reaction/cell lysate mixture was then centrifuged through a sucrose gradient for the isolation of neutrophil plasma membranes. A comparison was made between the amounts of $p67^{phox}$ that were recovered with the membranes. However, this experiment failed to show any effect of phosphorylation on the interaction between $p67^{phox}$ and membranes, for two reasons. Firstly, both recombinant proteins fractionated with stimulated membranes even without undergoing the phosphorylation reaction (Figure 4.2.4A), indicating that the activation state of the membranes had a significant effect on the affinity that was independent of $p67^{phox}$ phosphorylation. Secondly, there was no discernible increase in the amount of $p67^{phox}$ in stimulated membrane fractions for either recombinant protein compared to the control with no added $p67^{phox}$ (Figure 4.2.4B). That is, the translocation of native $p67^{phox}$ (from the whole cell lysate) to the membranes upon stimulation saturated the $p67^{phox}$ binding sites. Unfortunately, $p67^{phox}$-deficient neutrophils from CGD patients
were not available for this experiment, and the low amount of cytochrome in EBV-B cells (Morel et al., 1993; Chetty et al., 1995) renders it a poor model for such studies.

![Figure 4.2.4 Membrane affinity experiment comparing wildtype and Ala-233 mutant p67\textsuperscript{phox}](image)

As a means for studying the \textit{in vivo} phosphorylation of p67\textsuperscript{phox} and to demonstrate the effect of substituting alanine for threonine 233, attempts were made to introduce p67\textsuperscript{phox} to cells. The first attempt used cells of a p67\textsuperscript{phox}-deficient EBV-B cell line, which were electroporated in the presence of recombinant p67\textsuperscript{phox} protein. The only other similar experiment reported in the study of the NADPH oxidase is the delivery of a peptide of p47\textsuperscript{phox} to intact cells using an osmotic shock technique (Hendey et al., 1992). Electroporation is a proven method for delivery of proteins into cultured cells (Verkhoef et al., 1993). The electroporated cells were washed thoroughly to remove any extracellular p67\textsuperscript{phox}, and then homogenised. The homogenate was centrifuged to obtain crude cytosol which was analysed by immunoblotting for the presence of p67\textsuperscript{phox}. 

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Although p67\textsuperscript{phox} was detected in the supernatant fraction in approximately the same quantity as for normal, untreated cells, suggesting successful internalisation of the introduced protein, there was an excess of p67\textsuperscript{phox} in the pellet (Figure 4.2.5). By nature, these pellets contain the cell membranes and other insoluble matter. This suggested that recombinant p67\textsuperscript{phox} protein added to the electroporation medium either bound to the cell membranes to an artificially excessive extent, or the p67\textsuperscript{phox} formed some other insoluble complex. The delivery of p67\textsuperscript{phox} protein to cells by electroporation therefore, did not succeed in producing a whole cell model for studying the Ala-233 mutant.

An alternative approach is to deliver the DNA of p67\textsuperscript{phox} for expression of the protein within the cells. Wildtype and mutant Ala-233 plasmid-cDNA expression constructs have been prepared for delivery of p67\textsuperscript{phox} DNA to EBV-B cells. Two...
vectors have been used; pEGFP-N1 expression vector (Clontech), and pBabe Neo for retroviral transfer (Thrasher et al., 1992). Electroporation and a commercial liposome-based kit (SuperFect, Qiagen) were tested as transfection methods using the p67phox DNA clones in the former vector. These methods have been reported as successful in similar studies for restoring the superoxide production of CGD B cells (Chanock et al., 1992; Volpp & Lin, 1993). Transfection efficiencies were determined by fluorescence microscopy for detection of a green fluorescent protein (GFP) control. For both methods of delivery, the transient transfection of EBV-B cells was very poor at only 1-5% of cells expressing GFP. No p67phox expression could be detected by immunoblotting. It was hoped that transient transfection would be sufficient for metabolic studies of reconstituted B cells, but this level of transfection is unsatisfactory. Interestingly, there is a report in the literature of partial reconstitution (10-20%) of oxidase activity in B cells by transfection of p67phox which was undetectable by immunoblotting (Chanock et al., 1996). However, as superoxide production in normal B cells is very low, this represents a rather compromised system.

Most of the successful NADPH oxidase reconstitution studies using CGD cell lines involve the preparation of stable transfectants, using either retroviral (Thrasher et al., 1992; Sekhsaria et al., 1993; Porter et al., 1993; Li et al., 1994; Ding, C. et al., 1996; Weil et al., 1997) or adenoviral (Thrasher et al., 1995a,b,c) transduction systems. These studies have focused on the identification of the genetic lesions of CGD and the development of reconstitution systems for correction of the protein deficiency, with the long term goal of gene therapy for these patients. The first clinical trial of gene therapy recently reported the functional correction of peripheral blood granulocytes for up to six months in p47phox-deficient CGD patients, using ex vivo retroviral transduction of p47phox (Malech et al., 1997). Three other studies have applied the methods of reconstituting CGD B cells to examining the role of p47phox phosphorylation sites (Faust et al., 1995; as
discussed in Chapter 1, 1.7) and the requirement of regions within p67<sup>phox</sup> (de Mendez et al., 1994; Chanock et al., 1996; see 1.8). Work is ongoing in the development of a reconstituted EBV-B cell model, using the retroviral system, for assessment of the p67<sup>phox</sup> Ala-233 mutation.
4.3 Crystallisation trials for p67<sup>phox</sup>

4.3 AIM:

To crystallise recombinant p67<sup>phox</sup> for structure analysis, to lead to studies of structure-function associations within p67<sup>phox</sup>.

4.3 INTRODUCTION:

Identification of the phosphorylation site Thr-233, raises many questions relating to the possible structure and function aspects of the phosphorylation reaction. Is it significant that Thr-233 is located in the polyproline domain of p67<sup>phox</sup>? Is this region of the molecule exposed to the surface? Are the polyproline region and the C-terminal SH3 domain usually closely aligned? Does phosphorylation at Thr-233 cause a change in conformation? What are the relative positions of the binding sites of the other NADPH oxidase components? Solving the crystal structure of p67<sup>phox</sup> would help to find answers to these questions.

None of the NADPH oxidase components have been crystallised, except for Rac (Hirshberg et al., 1997) and RhoGDI (Keep et al., 1997). Nuclear magnetic resonance (NMR) technology has also been successfully applied to solve the structure of RhoGDI (Gosser et al., 1997; Keep et al., 1997). Otherwise, the only structural information available is from modelling, especially relating to conserved domains such as the β sheets of SH3 domains (Cohen et al., 1995). SH3 domain structure has been solved by crystallography (Mussachio et al., 1992) and by NMR (Yu et al., 1992) for many proteins now (review, Najmudin et al., 1997) enabling good modelling for SH3 domains in unsolved proteins. Molecular modelling of p67<sup>phox</sup> has suggested that p47<sup>phox</sup> can bind the C-terminal SH3 domain in either of two orientations and that certain amino acids are central to this interaction (Finan et al., 1996).
Crystallography requires good individual crystal quality which in turn depends upon high purity and concentration of protein. Crystallisation trials are carried out initially as a screening process covering a range of ionic strength and composition, pH, precipitant and temperature variants for initiation of nucleation. Conditions may then be refined for optimal crystal growth if some crystallisation is observed. There are several approaches that can be adopted to help promote stabilisation of the protein to increase the tendency to nucleate, such as including ligands.

Reported here is the purification of insect cell expressed p67phox and subsequent crystallisation trials. Despite preparing p67phox of high purity, no crystals were obtained. Therefore no conclusions regarding the structural relevance of the mid-protein position of the Thr-233 phosphorylation site could be drawn.

4.3 RESULTS AND DISCUSSION:

The insect cell expression system has been employed to overcome insolubility problems encountered with the *E. coli* GST-fusion expression system for generation of p67phox. It also removed the need for thrombin cleavage to generate full-length protein of p67phox. Yields were higher with the insect cell system, which may also be due to lower proteolytic damage than in the bacterial system. p67phox, both of recombinant and native neutrophil sources, is an unstable protein, prone to forming breakdown products, the major one at approximately 35 kDa. It must be handled on ice where possible and it was also noted that significant loss of yield occurred by spontaneous precipitation if the insect cell lysate was frozen prior to purification.

Good purity of recombinant p67phox was achieved via a two step chromatography method; anion exchange on Q-sepharose followed by hydrophobic interaction on phenyl sepharose (Figure 4.3). Of the total protein in the lysate, approximately 80 mg from a 200 ml culture, approximately 20% was collected in p67phox-containing fractions from the
first step, and the second step gave approximately 5 mg resulting in a final yield of 6.4%.
This was reproducible in at least two insect cell preparations. Minor contaminant bands
were observed after this purification process, and were kept to a minimum by including a
full complement of protease inhibitors in all buffers during the purification.

Figure 4.3  Purification of recombinant p67<sub>phox</sub> for crystallisation trials.
Coomassie-stained gel of insect cell cytosol after induction of p67<sub>phox</sub> expression
(cytosol). This was the start material applied to Q-sepharose FPLC, from which fractions
were pooled for phenyl sepharose FPLC (p. sepharose start), which yielded purified
p67<sub>phox</sub> (final).

Several attempts were made to further purify p67<sub>phox</sub> from the minor protein
contaminants still present after the two chromatography steps, but no improvement was
found. These included Mono-P (chromatofocusing), heparin agarose (affinity/cation
exchange), Q-sepharose at higher pH (pH 8.5) and Superose-12 gel filtration. Other
methods to ensure the highest purity could include affinity chromatography employing
SH3 binding properties such as a polyproline column, or a protein binding partner such
as Rac.

A fifty solution crystallisation screen was applied to the purified proteins using
sitting drop and hanging drop techniques (Ducruix & Giege, 1992). The set of solutions
was prepared in conjunction with the Biochemistry Department, UCL (Drs. L. Pearl and S. O’Hara), following a protocol based on the original Jancarik and Kim sparse matrix (Jancarik & Kim, 1991). Briefly, 1µl of 10 mg/ml p67phos and 1µl crystallisation solution were mixed and trays of sitting drops were incubated at room temperature and 4°C, and viewed using a spectroscopic microscope two or three times a week. To control for spontaneous screen solution precipitation or crystallisation, blank screens were monitored using buffer only.

If signs of nucleation were observed, titrations of the salt and precipitant for that particular solution were set up as hanging drop assays. This was carried out for p67phos in the case of (NH₄)₂SO₄/polyethylene glycol 4000, where both were titrated against each other. The results were negative, with only precipitation or salt formation. No convincing observations of crystallisation were made, even over several weeks. The stability of p67phos may not be high enough for extended time periods, so screens were set up with and without protease inhibitors, since such additives may interfere with the nucleation process. A lower concentration of p67phos (3 mg/ml) was also used to avoid precipitation, but no advantage was evident. The observations made from monitoring the crystallisation screens were analysed for any patterns of insolubility with relation to the solution constituents. No patterns were found, therefore providing no direction as to which other conditions to try.

It is generally considered that if proteins have the tendency to crystallise well, and certain size and quality requirements for X-ray analysis are to be met, this will be evident by sparse matrix screening. However, various exhaustive screens can be applied, as can the use of binding partners such as other proteins, antibodies and chemical ligands to help stabilise a soluble protein into a matrix-forming conformation (Ducruix & Giege, 1992). Other approaches can also be taken, such as truncating the full-length protein or making domain constructs, to remove possible disordered amino acid chains. This can
sometimes allow a more folded core protein to pack well. However, it is a compromising measure, as the more altered the protein, the less authentic the structural data will be. Crystallisation studies for the *phox* proteins are still being pursued in Professor Segal's laboratory.
SUMMARY OF CHAPTER 4:

**Identification of the major phosphorylation site of p67\textsuperscript{phox}**

The site of phosphorylation on p67\textsuperscript{phox} was localised to the N-terminal half of the protein by cyanogen bromide digestion and the use of antibodies to the N- and C-termini. An HPLC-MS application was established for isolation and analysis of the phosphopeptide by trypsin digestion. Although this system did not provide definitive sequencing of a phosphopeptide, it lead to the deduction of the most likely candidate threonine contained in the N-terminal half of p67\textsuperscript{phox}. To test the theory that Thr-233 was phosphorylated, mutagenesis of p67\textsuperscript{phox} was performed to introduce a single amino acid substitution to alanine 233. Only traces of phosphorylation of recombinant p67\textsuperscript{phox} by cytosol or MAP kinase were observed after the mutation of Thr-233. The major phosphorylation signal is therefore due to the phosphorylation of Thr-233. The observation of serine phosphorylation has not been accounted for. The results of 2D phosphopeptide mapping however, would be consistent with many phosphorylated serines throughout the molecule, but none phosphorylated to the extent of Thr-233.

The relevance of phosphorylation of Thr-233 to the molecular structure and interactions of p67\textsuperscript{phox}, remains to be determined. Attempts to crystallise p67\textsuperscript{phox} for structure determination were unsuccessful. For proper assessment of the function of p67\textsuperscript{phox} phosphorylation in the intact cell, a mechanism must be established to introduce the proteins into cells. DNA constructs of wildtype and Ala-233 mutant p67\textsuperscript{phox} have been made, and it is planned to use them in a reconstitution assay for evaluating the possible role of p67\textsuperscript{phox} phosphorylation in signal transduction.
Chapter 5

Studies on the mechanism of $p67^{phox}$ phosphorylation

5.1 Application of signal transduction inhibitors

5.1 AIM:

To use the inhibitor PD098059 to examine the role of MAP kinase in $p67^{phox}$ phosphorylation. Secondly, to determine whether $p67^{phox}$ phosphorylation was affected by the inhibitors staurosporine and okadaic acid which cause gross changes in neutrophil phosphorylation and activity.

5.1 INTRODUCTION:

MAP kinase (ERK 2) and cytosol in vitro phosphorylations of $p67^{phox}$ occur on the same site as in vivo phosphorylated $p67^{phox}$ (Chapter 3.3). The ERK group of MAP kinases are present in neutrophil cytosol (Torres et al., 1993). Activation of this MAP kinase pathway has been associated with the activation of the NADPH oxidase by phorbol ester (Worthen et al., 1994; Downey et al., 1996) and chemotactic stimuli (Grinstein & Furuya, 1992; Buhl et al., 1994; Dusi et al., 1994; Downey et al., 1996; Kuroki & O'Flaherty, 1997). However, one study proved that MAP kinase and NADPH oxidase activation can be dissociated in an environment of elevated cAMP suggesting that MAP kinase is not always activated (Yu et al., 1995). The upstream activator of MAP kinase is MEK (MAP/ERK kinase) which requires phosphorylation for the MAP kinase cascade to be activated. Unphosphorylated MEK can be inhibited by the synthetic compound PD098059, thereby blocking the downstream events (Alessi et al., 1995; Dudley et al., 1995; Pang et al., 1995). PD098059 has been used to study MAP kinase
involvement in regulation of the NADPH oxidase, with conflicting results. One study found that the application of PD098059 caused significant reduction in fMLP-induced oxidase activity (Avdi et al., 1996). Another study in which a range of chemotactic activators were used, including fMLP, showed no effect on oxidase activity, but chemotaxis was blocked (Kuroki & O'Flaherty, 1997). Experiments are reported in this chapter in which PD098059 was used to investigate a role for MAP kinase in p67phox phosphorylation.

As a more general approach to the study of the cellular significance of p67phox phosphorylation, experiments were also performed using staurosporine and okadaic acid. These compounds are relatively nonspecific inhibitors, of PKC (Tamaoki, 1991) and phosphatase types 1 and 2A (Hardie et al., 1991), respectively. Staurosporine has been used to explore the participation of PKC in the activation of the NADPH oxidase. It is established that staurosporine markedly inhibits stimulation of the oxidase by PMA (Badway et al., 1989; Robinson et al., 1990; Watson et al., 1991). With fMLP or phagocytic stimuli, the oxidase activity is reduced to a lesser extent (Robinson et al., 1990; Watson et al., 1991; Combadiere et al., 1993), indicative of alternative, PKC-independent, signalling. All these studies report a correlation between the inhibition of the oxidase and reduction in the phosphorylation of p47phox. There is one previous report of reduced p67phox phosphorylation in the presence of staurosporine (Dusi et al., 1993), but the cytosolic 67 kDa protein was not proven to be p67phox. Therefore the effect of staurosporine on the phosphorylation of p67phox remained to be evaluated.

The inhibition of phosphatase activity by okadaic acid results in a massive increase in phosphorylation of many neutrophil proteins, including p47phox (Ding, J. & Badway, 1992; Garcia et al., 1992; Yamaguchi et al., 1993; Curnutte et al., 1994; Bengis-Garber & Gruener, 1995; Suzuki et al., 1995). The observation that okadaic acid has different effects on the oxidase activity of neutrophils when treated with
different stimuli has been the focus of several studies (Garcia et al., 1992; Lu et al., 1992; Yamaguchi et al., 1993; Harbecke et al., 1996). The consensus is that PMA or phagocytic stimulation is abolished whereas the superoxide release stimulated by fMLP is prolonged and increased. There are contradicting reports however, that in certain conditions (different concentrations of stimulus and okadaic acid), PMA and opsonised zymosan stimulation is enhanced (Ding, J. & Badway, 1992; Suzuki et al., 1995). One explanation that has been suggested for the differing effect of okadaic acid on PMA and fMLP activation is the associated changes in intracellular calcium levels (Garcia et al., 1992). These findings illustrate two points; firstly, that the use of okadaic acid reveals heterogeneity in the signal transduction pathways controlling NADPH oxidase activation, and secondly, that deactivation of an fMLP-stimulated NADPH oxidase involves dephosphorylation.

The chosen approach was to determine whether changes in p67phox phosphorylation in response to these inhibitors followed the same pattern as p47phox phosphorylation in relation to the oxidase activity. If p67phox phosphorylation trends were different, it would provide significant insight into the regulation of p67phox phosphorylation.

5.1 RESULTS AND DISCUSSION:

In order to test whether MAP kinase is involved in p67phox phosphorylation by isolated cytosol or in intact cells, the MEK inhibitor PD098059 (Dudley et al., 1995) was applied to the in vitro and in vivo phosphorylation experiments. Neutrophils were incubated with 100 µM PD098059 (or the equivalent volume of DMSO) for one hour at 37°C prior to treatment with cytochalasin B and fMLP, after which cytosol was prepared for use as the source of kinase in in vitro phosphorylation experiments. Quantitation of the autorad signals in two experiments showed that partial inhibition by 27% or 52% of
the phosphorylation of recombinant p67\textsuperscript{phox} was achieved. In control experiments, the phosphorylation of myelin basic protein (MBP) was used to determine the efficacy of the inhibitor (Pang \textit{et al.}, 1995). For MBP, only a partial reduction (20\%, 22\%) in phosphorylation was also observed. The inhibition of MEK therefore, appeared to affect the phosphorylation of p67\textsuperscript{phox} in the \textit{in vitro} model to a similar level.

\textbf{Table 5.1.1} shows the results of preincubation of cells with PD098059 before stimulation, which was followed by immunoprecipitation of p67\textsuperscript{phox}. After pretreatment with PD098059, p67\textsuperscript{phox} phosphorylation was still increased upon stimulation by S.O.Zym, fMLP or PMA. The degree of elevation remained approximately the same as in untreated cells for S.O.Zym and fMLP, but in two experiments with PMA there was a >30\% reduction in the level of phosphorylation. The effect of PD098059 on the NADPH oxidase activity was also tested to determine whether there was any correlation with the p67\textsuperscript{phox} phosphorylation findings. No effect was observed for S.O.Zym or PMA stimulation including the cells from experiments with >30\% reduction of p67\textsuperscript{phox} phosphorylation. A significant reduction in the oxidase activity of fMLP stimulated cells occurred in all four experiments with PD098059 (\textbf{Table 5.1.1}), and even at the concentration of 10 \(\mu\)M (data not shown), but this occurred without a significant change in p67\textsuperscript{phox} phosphorylation. In summary, no correlation was observed between the effect of PD098059 on p67\textsuperscript{phox} phosphorylation and NADPH oxidase activity.

Although these results do not prove a role for MAP kinase \textit{in vivo}, they do not eliminate it either. PD098059 is known to inhibit the upstream activator of MAP kinase, MEK, but not MAP kinase directly (Dudley \textit{et al.}, 1995), therefore any preactivated MAP kinase will not be inhibited. The extent of the effect of PD098059 has also been demonstrated to depend on the conditions in which cells are activated (Alessi \textit{et al.}, 1995). These may be the reasons why only partial inhibition of either MBP or p67\textsuperscript{phox} phosphorylation was achieved by cytosol prepared from PD098059-treated, stimulated
neutrophils. Such partial inhibition has been reported elsewhere for fMLP stimulated neutrophils (Kuroki & O'Flaherty, 1997). The incomplete inhibition of in vivo phosphorylation (maximum observed was 38% reduction), may explain the lack of correlation between p67phox phosphorylation and NADPH oxidase activity. Alternatively, p67phox phosphorylation is not a rate-limiting requirement for activation.

<table>
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<tr>
<th>Stimulation</th>
<th>p67phox phosphorylation</th>
<th>NADPH oxidase activity</th>
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<td>+ PD</td>
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<td>resting</td>
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<td>S.O.Zym</td>
<td>2.1 ± 0.7</td>
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<td>PMA</td>
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Table 5.1.1 The effect of PD098059 inhibitor on phosphorylation of p67phox in intact cells and on NADPH oxidase activity. p67phox was immunoprecipitated in parallel from 32P-labelled neutrophils that had been either untreated, or pretreated with PD098059 (+PD), prior to stimulation. Phosphorylation of p67phox was quantified as the ratio of the Coomassie-stained band to the intensity of the autorad signal. The change in phosphorylation is normalised to that in untreated cells. Effect of PD098059 is expressed as the percentage of the phosphorylation measured in untreated cells (+PD (% control)). NADPH oxidase activity was measured as maximal rates of cytochrome c reduction for untreated and pretreated cells. The number of experiments is in brackets [ ], and the standard deviation is reported for those experiments repeated more than twice.

The use of PD098059 has not given conclusive results, which reflects the lack of agreement between previous studies using this inhibitor (Avdi et al., 1996; Kuroki & O'Flaherty, 1997). As an alternative way to study MAP kinase and p67phox phosphorylation, attempts were made to deplete cytosol of MAP kinase by immunoprecipitation. Only partial depletion was achieved using a commercial, polyclonal antibody (ERK 2 (C-14), which cross-reacts with ERK 1 and 2, Santa Cruz Biotechnology), so this approach was not pursued. Therefore the possible interactions
between MAP kinase activation and the NADPH oxidase, together with p67phox phosphorylation, remain to be investigated further.

Experiments were conducted with the inhibitors staurosporine and okadaic acid to determine if p67phox phosphorylation correlated with NADPH oxidase activation. Figure 5.1.2 shows spectra portraying the superoxide production of neutrophils stimulated by different agonists, and the effect of pretreating the cells with staurosporine or okadaic acid. Staurosporine caused a diminution of the oxidase activity upon PMA stimulation, but almost no change with the phagocytic stimulus, serum opsonised zymosan. Although the reduction in the PMA response is not as pronounced as some reports, these data are consistent with previous studies. The issue to be addressed was whether this apparent difference in the extent of PKC-dependent activation, was reflected in p67phox phosphorylation.

The okadaic acid responses also confirmed the established effects using different stimuli. PMA activation was completely blocked by okadaic acid, the opsonised zymosan response was diminished, and the initial rate of fMLP stimulated superoxide production was reduced but a prolonged response continued. The interest for studying the phosphorylation of p67phox in this context was to determine if the divergent effects on the oxidase activity corresponded to a similar pattern in p67phox phosphorylation.

The magnitude of phosphorylation was measured by densitometric analysis of immunoprecipitates from the cytosol of 32P-labelled neutrophils (Figure 5.1.3). As an example, the autorads are shown of the effect of okadaic acid on neutrophil cytosol and immunoprecipitates (Figure 5.1.3A). The phosphorylation of p47phox was monitored as a source of comparison for the changes in p67phox phosphorylation (Figure 5.1.3B). Staurosporine had the effect of reducing the magnitude of basal and stimulated phosphorylation of p67phox and p47phox. Okadaic acid caused significant enhancement of phosphorylation throughout the cytosol, including p67phox and p47phox phosphorylation.
Figure 5.1.2 Effects of staurosporine and okadaic acid on superoxide production of neutrophils. Spectrophotometric analysis of the superoxide dismutase-inhibitable reduction of ferricytochrome c. X-axis: time, units ×100 seconds, Y-axis: 550 nm absorbance, arbitrary units. A: Neutrophils were preincubated at 37°C for 5 min with (dotted line) or without (solid line) 100 nM staurosporine then stimulated at time 0 by 0.5 mg/ml S.O.Zym or 0.5 μg/ml PMA. B: Preincubations in 1 μM okadaic acid were carried out at 37°C for 30 min. The 0.5 μM fMLP stimulation followed a 5 min preincubation with 5 μg/ml cytochalasin B. Maximum rates of superoxide production are shown, nmol O₂⁻/min/10⁷ cells.
Figure 5.1.3 Effect of staurosporine and okadaic acid on phosphorylation of \( p_{67}^{\text{phox}} \) and \( p_{47}^{\text{phox}} \) in intact cells. Neutrophils were radiolabelled, stimulated, and \( p_{67}^{\text{phox}} \) and \( p_{47}^{\text{phox}} \) were immunoprecipitated from the cytosol fraction. A: Illustration of the raw results from which phosphorylation was quantified. The autorads are shown of (cytosol:) \( 5 \times 10^5 \) cell eq cytosol from unstimulated, fMLP-stimulated cells, and cells which were pretreated with okadaic acid (OA) then fMLP-stimulated, 16 hr exposure. (Immunoprecipitates:) \( p_{67}^{\text{phox}} \) and \( p_{47}^{\text{phox}} \) bands from immunoprecipitates of 3.3 and 1.7\( \times 10^5 \) cell eq cytosol respectively, 6 day exposure. B: Quantitation of changes in phosphorylation of \( p_{67}^{\text{phox}} \) and \( p_{47}^{\text{phox}} \). Values are derived from band densitometry and normalised against 1.00 for phosphorylation in neutrophils not treated with an inhibitor or stimulus. The incubations with staurosporine and okadaic acid, and the stimulation conditions are as described above.
These results show that p67phox and p47phox phosphorylation are influenced in the same way as each other by staurosporine and okadaic acid. p67phox phosphorylation did not account for the different effect of okadaic acid on PMA and fMLP stimulation. Therefore, no information was gained relating to a special role that p67phox might have in regulating the NADPH oxidase.

An interesting observation was made in these experiments. Okadaic acid caused hyperphosphorylation of p67phox, and its immunoprecipitation from stimulated cells resulted in a phosphorylated doublet at 67 kDa (Figure 5.1.3B). It was initially thought that this might be a co-precipitating protein with affinity for phosphorylated p67phox. Immunoblotting revealed that both bands of the doublet were p67phox (Figure 5.1.4). The co-precipitation of p47phox and p40phox is also shown by immunoblotting. It seems that the additional band of p67phox is due to the slower migration of p67phox that is hyperphosphorylated in the presence of okadaic acid, because of the inhibition of phosphatase activity. This is consistent with the upper band being almost invisible by Coomassie staining but with a disproportionally strong phosphorylation signal.

Phosphopeptide mapping revealed extra spots supporting the conclusion that okadaic acid induces additional phosphorylation of p67phox on different sites (Figure 5.1.5). In this particular experiment, the phosphopeptide map had stronger “minor” spots than usual. These were variable in intensity between experiments, and this map shows them at their strongest. However, both of these maps were prepared in the same experiment and their comparison clearly shows the appearance of new spots, and tyrosine phosphorylation, in the okadaic acid treated cells. p47phox also becomes phosphorylated on novel sites (compared with PMA stimulation) by inhibition of phosphatases 1 and 2A (Yamaguchi et al., 1996). It is assumed that phosphorylation of these additional sites is caused by an imbalance in the kinase and phosphatase activities due to okadaic acid. A less likely scenario is that okadaic acid treatment preserves
Figure 5.1.4 Immunoprecipitation of p67<sub>phox</sub> from okadaic acid-treated cells.

Coomassie-stained gel and immunoblots of p67<sub>phox</sub> immunoprecipitations from neutrophil cytosol. The migration of p67<sub>phox</sub>, p47<sub>phox</sub> and p40<sub>phox</sub> are indicated by the stained molecular weight markers (Mr) and recombinant protein standards (stds), and the immunoblot against all three proteins. The gel shows very weak staining of an extra band of p67<sub>phox</sub> with okadaic acid (OA) treatment, which is detected by the immunoblot. An anti-phosphotyrosine (αPY) immunoblot of an equivalent immunoprecipitation of p67<sub>phox</sub> from stimulated neutrophils, shows a weakly visible doublet of bands at 67 kDa.
Figure 5.1.5 Phosphopeptide and phosphoamino acid analysis of $p67^{\text{phos}}$ from okadaic acid-treated cells. $p67^{\text{phos}}$ was immunoprecipitated from neutrophils which were stimulated in the absence (upper panel) or presence (lower panel) of okadaic acid (OA). $p67^{\text{phos}}$ was gel purified, and subjected to 2D tryptic mapping for phosphopeptide analysis, and acid hydrolysis for amino acid analysis (insets). Immunoprecipitated $p67^{\text{phos}}$ resolved as a doublet of bands from the okadaic acid-treated cells which were processed together.
multiple-site phosphorylation that occurs transiently during activation of the NADPH oxidase. This issue is addressed in the next section.
5.2 Investigation of p67phox tyrosine phosphorylation

5.2 AIM:

To investigate the possibility of tyrosine phosphorylation of p67phox.

5.2 INTRODUCTION:

Phosphoamino acid analysis has shown that phosphorylated p67phox immunoprecipitated from neutrophil cytosol, is phosphorylated on threonine and serine residues. The method of acid hydrolysis is known to be less sensitive for the detection of phosphotyrosine as phosphotyrosine is labile under these conditions (Kozma et al., 1991). Phosphotyrosine was detected on p67phox using this method after the p67phox was phosphorylated in vitro by membranes (section 3.3), and in p67phox separated from intact cells after incubation in okadaic acid (section 5.1). This raised the possibility of undetected tyrosine phosphorylation occurring in the cell. p67phox may undergo tyrosine phosphorylation after translocation to the membrane, or cytosolic p67phox may be phosphorylated on tyrosine at much lower stoichiometry than for threonine (or serine).

Tyrosine phosphorylation has been implicated as playing an important role in signal transduction in the neutrophil. This has been demonstrated for phorbol ester and chemoattractant stimuli alike (Green & Phillips, 1994; Rollet et al., 1994). Most interest has been directed towards the prominent tyrosine phosphorylation of MAP kinases (Grinstein & Furuya, 1992; Torres et al., 1993; Dusi et al., 1994), but few other targets have been identified. To determine whether tyrosine phosphorylation of p67phox occurs naturally in vivo, two approaches were taken in this study; the isolation of p67phox from the cellular membrane fraction, and immunodetection with a specific phosphotyrosine antibody.
5.2 RESULTS AND DISCUSSION:

Neutrophils were $^{32}\text{P}_i$-labelled, stimulated and fractionated by centrifugation through a discontinuous sucrose gradient. The membrane fraction was subjected to immunoprecipitation as for cytosol, with the inclusion of deoxycholate being vital. As only a small proportion of total neutrophil p67$^{phox}$ translocates to the membrane, at least $3\times10^8$ cells is required for immunoprecipitation of detectable amounts of p67$^{phox}$. Phosphorylation of membrane p67$^{phox}$ was only detectable by phosphorimaging with long exposure times (Figure 5.2.1). These yields were too low to use for phosphopeptide mapping. As an alternative approach, immunoprecipitation was attempted on whole cell homogenates, without fractionation. However, no p67$^{phox}$ was recovered by immunoprecipitation, presumably due to proteolysis of p67$^{phox}$ when whole cells are exposed to detergent, despite the addition of protease inhibitors.

Figure 5.2.1 Immunoprecipitation of p67$^{phox}$ from neutrophil membranes. A: Coomassie-stained gel of p67$^{phox}$ immunoprecipitated from the membrane fraction of $7.5\times10^6$ neutrophils prelabelled with $^{32}\text{P}_i$. B: Phosphorimage after 6 days exposure of p67$^{phox}$ immunoprecipitations from the membrane fractions of $7.5\times10^6$ cell neutrophils either unstimulated, fMLP- or PMA-stimulated. Different molecular weight markers were used in A and B.
Two dimensional electrophoresis, involving isoelectric focusing followed by SDS/PAGE, was also tried. This was used to test for multiple isoforms of phosphorylated p67\(^{phox}\), but the resolution obtained was not adequate. In summary, it was not possible to derive a comparison between the overall phosphorylation states of p67\(^{phox}\) located at the membrane and in the cytosol. The availability of highly specific anti-phosphotyrosine antibodies however, makes it possible to study very low levels of tyrosine phosphorylation.

To test for the occurrence of tyrosine phosphorylation of p67\(^{phox}\) \textit{in vivo}, immunoblotting was carried out on cytosol and membrane fractions (Figure 5.2.2). A recombinant phosphotyrosine antibody detected more proteins in stimulated cytosol and membranes than in unstimulated fractions, with the more prominent effect caused by PMA compared to fMLP. The translocation of p67\(^{phox}\) from cytosol to membranes was also more pronounced for PMA than for fMLP. This observation of lower translocation by activation with fMLP has been made before (Clark \textit{et al.}, 1990; Quinn & Bokoch, 1995).

Immunoprecipitation showed a weak phosphotyrosine signal for p67\(^{phox}\) located in the cytosol and membranes (Figure 5.2.2B,C). In order to measure any changes in tyrosine phosphorylation of p67\(^{phox}\) upon stimulation, the p67\(^{phox}\) and phosphotyrosine signals were quantified and compared to unstimulated p67\(^{phox}\) (Figure 5.2.2D). The pattern of relative tyrosine phosphorylation was the same in three separate experiments. There was an increase in cytosolic p67\(^{phox}\) phosphotyrosine by PMA stimulation only. This probably reflects the documented effect of PMA lowering phosphotyrosine phosphatase activity (Kansha \textit{et al.}, 1993).

In membranes, the p67\(^{phox}\) phosphotyrosine signal was decreased for PMA and fMLP stimulations alike. Since the amount of p67\(^{phox}\) at the membrane increases upon stimulation, this result must mean that the translocating p67\(^{phox}\) is not tyrosine
Figure 5.2.2 Phosphotyrosine analysis of p67^{phox} immunoprecipitated from neutrophils. A: Anti-phosphotyrosine immunoblots of 7.5×10^6 cell eq cytosol and 4.5×10^8 cell eq membranes from unstimulated, PMA- or fMLP-stimulated neutrophils. The blots were developed by the ECL method. B: Anti-phosphotyrosine immunoblots of p67^{phox} immunoprecipitates from 7.5×10^7 cell eq cytosol and 7.5×10^8 cell eq membranes, corresponding to the samples in A. C: Immunoblots of the same immunoprecipitations, using a p67^{phox} antibody. D: Quantitation of the amount of tyrosine phosphorylation of p67^{phox} by densitometric analysis of the immunoblots. The ratio of the phosphotyrosine signal to the p67^{phox} signal is normalised against that of unstimulated cells which was assigned 1.00. The entire experiment was performed three times and the value reported is the average ± standard deviation.
phosphorylated. The conclusion is that the weak tyrosine phosphorylation of p67phox in intact cells, which was only detectable by this sensitive method, is not a requirement for assembly or activation of the NADPH oxidase. Therefore the tyrosine phosphorylations detected in \textit{in vitro} phosphorylation by membranes, and by okadaic acid treatment, are unlikely to represent a physiological phenomenon.
5.3 Phosphorylation in chronic granulomatous disease cells

5.3 AIM:

To examine whether the phosphorylation of p67$^{phox}$ in vivo depends on the presence of p47$^{phox}$, and vice versa.

5.3 INTRODUCTION:

p67$^{phox}$, p47$^{phox}$, and p40$^{phox}$ exist in the cytosol of resting neutrophils in a ~240 kDa complex, and upon activation the proteins translocate to the plasma membrane (Park, J.-W. et al., 1992, 1994; Iyer, S.S. et al., 1994). p67$^{phox}$ can bind to p47$^{phox}$ and to p40$^{phox}$, with a higher affinity for p40$^{phox}$ (Someya et al., 1993). It has been proposed that p40$^{phox}$ performs a regulatory role by competing with p67$^{phox}$ for interaction with p47$^{phox}$ (Wientjes et al., 1993; Ito et al., 1996). This could be the mechanism enabling p40$^{phox}$ to down-regulate the activity of an intact cell NADPH oxidase system (Sathyamoorthy et al., 1997). The interaction between p67$^{phox}$ and p47$^{phox}$ appears to be central to the activation of the NADPH oxidase. p67$^{phox}$ translocation to the membrane requires p47$^{phox}$ (Heyworth et al., 1991), suggesting either an association at the membrane or a modification to p67$^{phox}$ that depends on p47$^{phox}$ binding.

Using various binding techniques, three sites of interaction have been identified between p67$^{phox}$ and p47$^{phox}$ (see Figure 1.4). Two of these appear to be interactions between SH3 and proline-rich domains, and the other involves a region on p47$^{phox}$ (amino acids 323-332) which has been shown to bind the cytochrome. This third interaction is reported to be activation dependent in cell free studies (De Leo et al., 1996). It was suggested that p67$^{phox}$ and p47$^{phox}$ bind together via this site after activation in order to translocate to the membrane, where the interaction is disrupted allowing p47$^{phox}$ to use the site to bind the cytochrome (De Leo et al., 1996).
Phosphorylation of p67\textsuperscript{phox} and p47\textsuperscript{phox} may be crucial to the control of these events, and may require association with each other for phosphorylation to occur.

Neutrophils, or EBV-B cell lines, of p47\textsuperscript{phox}-deficient or p67\textsuperscript{phox}-deficient CGD patients provide ideal tools to test whether p67\textsuperscript{phox} and p47\textsuperscript{phox} undergo normal phosphorylation in the absence of the other. It is known that p47\textsuperscript{phox} does become phosphorylated in p67\textsuperscript{phox}-deficient neutrophils (Nunoi & Malech, 1988) but this may not necessarily be to completion. There are three publications where this issue has been approached. Two studies showed that the same array of spots representing different isoforms of phosphorylated p47\textsuperscript{phox} was observed by 2D electrophoresis of normal and p67\textsuperscript{phox}-deficient CGD neutrophil cytosol and membranes (Okamura et al., 1990; Rotrosen & Leto, 1990). Another reported that the same magnitude of p47\textsuperscript{phox} phosphorylation was achieved in normal and p67\textsuperscript{phox}-deficient CGD neutrophils (Dusi & Rossi, 1993). Analysis of p47\textsuperscript{phox} phosphorylation by 2D phosphopeptide mapping has not been carried out. Neither are there any studies of the effect of p47\textsuperscript{phox} deficiency on p67\textsuperscript{phox} phosphorylation.

5.3 RESULTS AND DISCUSSION:

p67\textsuperscript{phox} deficiency is a rare phenotype of CGD, representing approximately 5% of all patients. Hence, the opportunity to work with such neutrophils is restricted. Once during this course of study, a p67\textsuperscript{phox}-deficient patient donated blood for an experiment to determine whether p47\textsuperscript{phox} was normally phosphorylated.

The CGD neutrophils were completely deficient of p67\textsuperscript{phox}, and had a significantly reduced amount of p40\textsuperscript{phox} compared with normal neutrophils (Figure 5.3.1A). This is a characteristic feature of p67\textsuperscript{phox}-deficient neutrophils (Wientjes et al., 1993; Tsunawaki et al., 1994; Dusi et al., 1996). An increase in phosphorylation upon PMA stimulation was observed in the CGD neutrophils as for normal neutrophils by immunoprecipitation of
Figure 5.3.1 Phosphorylation of p47phox in p67phox-deficient neutrophils. A: TCA precipitates of 1×10⁶ neutrophils from a normal subject and p67phox-deficient CGD patient were immunoblotted for p67phox, p47phox, and p40phox. Detection was by the ECL method. B: Autoradiogram of p47phox immunoprecipitates of 5×10⁵ cell eq cytosol from ³²P-labelled neutrophils, either unstimulated or PMA-stimulated. C: 2D phosphopeptide maps (with phosphoamino acid analysis in insets) of phosphorylated p47phox from normal (upper) and p67phox-deficient (lower) neutrophils, either unstimulated (left) or PMA-stimulated (right).
cytosolic p47<sup>phox</sup> (Figure 5.3.1B). p47<sup>phox</sup> phosphopeptide mapping showed almost identical changes upon stimulation of normal and p67<sup>phox</sup>-deficient neutrophils, with solely serine phosphorylation (Figure 5.3.1C). One possible deviation from normal is the presence of an extra phosphopeptide as marked by an arrow on the p67<sup>phox</sup>-deficient PMA map. However, this experiment seemed to confirm previous reports that p47<sup>phox</sup> phosphorylation is not dependent on p67<sup>phox</sup>.

To determine whether the phosphorylation of p67<sup>phox</sup> was equally independent of p47<sup>phox</sup>, p47<sup>phox</sup>-deficient EBV-B cells were used. It had already been established that the phosphorylation of p67<sup>phox</sup> in B cells was the same as in neutrophils (section 3.2). Normal neutrophils were included here as an extra control for comparison between phosphopeptide maps. p67<sup>phox</sup> was immunoprecipitated from one normal, and two p47<sup>phox</sup>-deficient EBV-B cell lines (Figure 5.3.2A). The phosphopeptide maps of p67<sup>phox</sup> from normal and p47<sup>phox</sup>-deficient cells were identical (Figure 5.3.2B). Therefore the phosphorylation of p67<sup>phox</sup> occurs normally in the absence of p47<sup>phox</sup>.
Figure 5.3.2  Phosphorylation of p67<sup>phox</sup> in p47<sup>phox</sup>-deficient EBV-B cells. A: ECL-developed immunoblots for p67<sup>phox</sup> and p47<sup>phox</sup> in EBV-B cells of one normal subject and two p47<sup>phox</sup>-deficient CGD patients; (1),(2). B: 2D phosphopeptide maps of p67<sup>phox</sup> immunoprecipitated from radiolabelled normal neutrophils stimulated with fMLP, and PMA-stimulated EBV-B cells of the normal and CGD subjects characterised in A.
SUMMARY OF CHAPTER 5:

Studies on the mechanism of p67\textsuperscript{phox} phosphorylation

The aim of this chapter was to investigate some of the factors which may play a role in the phosphorylation of p67\textsuperscript{phox}. MAP kinase was implicated as a candidate kinase for p67\textsuperscript{phox} by phosphopeptide analysis. The \textit{in vitro} phosphorylation of p67\textsuperscript{phox} by cytosol was reduced by inhibiting MEK activation with PD098059. However, the intact cell experiments carried out using PD098059 did not clearly provide evidence for MAP kinase involvement in native phosphorylation of p67\textsuperscript{phox}.

The inhibitors staurosporine and okadaic acid were used to determine if there was close correlation between p67\textsuperscript{phox} phosphorylation and NADPH oxidase activation. No deviations from the documented effects on p47\textsuperscript{phox} were observed. That is, phosphorylation of p67\textsuperscript{phox} was decreased by staurosporine and increased by okadaic acid. The same broad conclusions can be drawn for p67\textsuperscript{phox} phosphorylation as have been drawn previously for p47\textsuperscript{phox}; phosphorylation occurs under conditions of NADPH oxidase activity, and dephosphorylation may play an important role in deactivation of the NADPH oxidase.

The possibility of native tyrosine phosphorylation of p67\textsuperscript{phox} was investigated because it had been observed by \textit{in vitro} phosphorylation with membranes and by phosphatase inhibition using okadaic acid. Insufficient yields of membrane-localised p67\textsuperscript{phox} prevented phosphopeptide analysis, but detection was successful using a phosphotyrosine antibody. Both cytosolic and membrane-bound p67\textsuperscript{phox} contained some phosphotyrosine. The level of the phosphotyrosine signal on membrane p67\textsuperscript{phox} decreased upon activation, hence the translocation of p67\textsuperscript{phox} effectively dilutes rather than increases the tyrosine phosphorylation. The conclusion from this work is that tyrosine phosphorylation occurs very weakly and is not associated with the formation of an active NADPH oxidase.
That \textit{p67}^{phox} phosphorylation does not involve the membrane was also implied by the normal phosphorylation of \textit{p67}^{phox} in \textit{p47}^{phox}-deficient cells. Translocation of \textit{p67}^{phox} to the membrane is dependent on \textit{p47}^{phox}, so neither translocation nor cytosolic interaction with \textit{p47}^{phox} can play a role in \textit{p67}^{phox} phosphorylation. The phosphorylation of \textit{p47}^{phox} did not appear to be affected by \textit{p67}^{phox} deficiency either. Therefore, cytosolic phosphorylation events taking place on \textit{p67}^{phox} and \textit{p47}^{phox} occur independently of each other.

Further elucidation of the mechanism of \textit{p67}^{phox} phosphorylation will be facilitated by the development of an intact cell system comparing wildtype and Ala-233 mutant \textit{p67}^{phox}. Such a system will allow assessment of the intertwined roles of phosphorylation, intermolecular interaction and translocation in the regulation of the NADPH oxidase.
The phosphorylation of p67\textsuperscript{phox} occurs upon activation of the NADPH oxidase in neutrophils. It appears to be a reaction localised in the cytosol, since isolated cytosol can mimic phosphorylation in the intact cell. The tyrosine phosphorylation of p67\textsuperscript{phox} by isolated membranes is not strongly evident upon translocation in activated cells. Therefore it does not seem likely that additional phosphorylation occurs at the membrane. Neither does it appear that the phosphorylation involves transient docking at the membrane because p67\textsuperscript{phox} is equally phosphorylated in the absence of p47\textsuperscript{phox}, which is required for p67\textsuperscript{phox} membrane binding.

p67\textsuperscript{phox} phosphorylation is stimulated by the soluble stimulus PMA, and by the receptor-mediated stimuli fMLP and opsonised zymosan alike, implicating a single kinase which is activated downstream of various divergent signalling pathways. MAP kinase is a candidate kinase for p67\textsuperscript{phox} phosphorylation because it can also mimic the intact cell phosphorylation, and is activated upon neutrophil stimulation. A role for MAP kinase in the \textit{in vivo} phosphorylation of p67\textsuperscript{phox} remains to be proven. The level of p67\textsuperscript{phox} phosphorylation is reduced by staurosporine, and increased by okadaic acid with additional sites becoming phosphorylated. These changes correlate with those observed for the phosphorylation of p47\textsuperscript{phox}, suggesting similar roles for the phosphorylation of both of these cytosolic components in the regulation of the NADPH oxidase.

The stoichiometry of phosphorylation of p67\textsuperscript{phox} is low, but then the amount of p67\textsuperscript{phox} which translocates to the membrane can be as low as 2\% (DeLeo & Quinn, 1996). A similar number of molecules of p47\textsuperscript{phox} and p40\textsuperscript{phox} probably become phosphorylated upon stimulation as for p67\textsuperscript{phox}, but the incorporation of 32P is
significantly higher because multiple sites are phosphorylated on single molecules of p47$^{phox}$ and p40$^{phox}$, as evident by multiple isoforms separated by isoelectric focusing (Rotrosen & Leto, 1990; Fuchs et al., 1997). The observation of basal phosphorylation of the cytosolic phox components in unstimulated cells suggests that there is a threshold level of phosphorylation which must be reached, and/or other signalling events are co-requirements for activation of the oxidase. This is supported by certain kinase inhibitor studies where phosphorylation is reduced, but not totally eliminated, with little effect on oxidase activity (Robinson et al., 1990; Dusi et al., 1993). In the case of p67$^{phox}$, the same phosphorylation profile by 2D mapping occurs in basal and stimulated phosphorylation, including the major phosphopeptide signal from Thr-233. Therefore it is suggested that the amount of p67$^{phox}$ phosphorylated on this site increases at the same time as other modifying events to bring about translocation. The phosphorylation of p67$^{phox}$ on serines is less defined, so the significance of this remains to be evaluated.

The identification of threonine 233 as the major phosphorylation site of p67$^{phox}$ may provide important structure-function information. The proline-rich region surrounding Thr-233 is a putative binding site for SH3 domains. It is tempting to hypothesise that phosphorylation in this region induces a conformational change in p67$^{phox}$ and alters the resting state protein interactions. However, despite all the binding studies of the components of the NADPH oxidase, the polyproline region of p67$^{phox}$ has yet to be specifically assigned a binding partner. The C-terminal SH3 domain of p47$^{phox}$ interacts with the N-terminal 246 amino acid region of p67$^{phox}$ (de Mendez et al., 1997), but this has not been localised to the polyproline region.

Phosphorylation with proline-rich regions has been identified on other molecules, particularly substrates of the proline-directed MAP kinases, but a literature search failed to identify examples of such phosphorylation disrupting SH3-polyproline interactions. However, the activation of some tyrosine kinases apparently involves phosphorylation of
SH3 domains disrupting intramolecular binding (Najmudin et al., 1997). Interestingly, 
the phosphorylation sites of p47phox are also located very closely to a polyproline region, 
which has been shown to bind to the C-terminal SH3 domain of p67phox, the N-terminal 
SH3 domain of p47phox and the only SH3 domain of p40phox (Leto et al., 1994; Fuchs et 
al., 1996). It is feasible therefore that phosphorylation events in the NADPH oxidase 
modulate the intra- and inter-molecular interactions ultimately leading to an active 
electron transport chain.

Other studies lend support to the idea that a structural change in p67phox may 
occur in activation of the NADPH oxidase. Kinetic studies indicate that p67phox and 
p47phox are kinetically independent components (Okamura et al., 1990), and the 
inactivation of the NADPH oxidase correlates with inactivation of p67phox (Fujimoto et 
al., 1989). Therefore, the structural changes and/or signal transduction initiated by 
p47phox phosphorylation are probably under separate control from p67phox modifications. 
Interaction between p67phox and p47phox is not a prerequisite for phosphorylation as 
normal phosphorylation of one can occur in the absence of the other. The requirement 
of activation-dependent phosphorylation other than on p47phox is proven by only low 
level activity induced by phosphorylated p47phox when other kinase activity is blocked 
(Park, J.-W. et al., 1997). A conformational change in p67phox is implicated in the 
molecular rearrangements that occur upon stimulation. Observations of changes in the 
binding affinity between p67phox and p47phox during activation have been made with the 
amphiphile-induced cell free oxidase system (Sumimoto et al., 1994; DeLeo et al., 
1996).

The residue Thr-233 is flanked by the C-terminal Rac-binding region (Diekmann 
et al., 1994) and the inter-SH3 region that binds p40phox (Fuchs et al., 1996; 
Sathyamoorthy et al., 1997). These nearby associations may be regulated by 
phosphorylation at Thr-233 on p67phox. Rac binds to the N-terminal 199 amino acid
region of $p67^{phox}$ (Diekmann et al., 1994), an interaction which is essential for activation of the NADPH oxidase (Diekmann et al., 1994; Freeman et al., 1996). The kinetics and cell localisation of this interaction are not fully resolved. The affinity between $p67^{phox}$ and $p40^{phox}$ is very high, apparently involving non-SH3 type interactions (Fuchs et al., 1996; Wientjes et al., 1996). It is not known whether this interaction is modified upon activation, but demonstration of a negative effect of $p40^{phox}$ on oxidase activity (Sathyamoorthy et al., 1997) has suggested a role in inhibiting hyperactivation of the NADPH oxidase. The low levels of $p40^{phox}$ in $p67^{phox}$-deficient cells (Wientjes et al., 1993; Tsunawaki et al., 1994; Dusi et al., 1996) suggests that the interaction with $p67^{phox}$ is important for $p40^{phox}$ stability. $p40^{phox}$ also becomes phosphorylated upon stimulation, so it is feasible that their interaction is somehow modified.

**Figure 6** outlines two models for the role of $p67^{phox}$ phosphorylation. The first model (**Figure 6B**) is that phosphorylation at Thr-233 causes a change in $p67^{phox}$ resting state conformation, enabling Rac to bind. It is proposed that there is an internal SH3-polyproline interaction which is disrupted by the addition of a phosphate group to the polyproline region. The binding between Rac and $p67^{phox}$ may mutually facilitate binding to the membrane activating the required signal transduction events at the cytochrome to bring about electron transfer.

The second model includes the association of $p47^{phox}$ (**Figure 6C**). This is based on the evidence that $p47^{phox}$ is required for $p67^{phox}$ to bind to the membrane (Heyworth et al., 1991), and that there are activation-dependent changes in the interactions of $p67^{phox}$ and $p47^{phox}$. Cell-free studies have shown that two changes occur upon addition of amphiphiles to resting state cytosol. The intramolecular SH3-polyproline binding of $p47^{phox}$ is broken (Sumimoto et al., 1994), and a new interaction between $p67^{phox}$ and $p47^{phox}$ occurs via $p47^{phox}$ 323-332 (De Leo et al., 1996). It has been assumed that the simultaneous phosphorylation of $p67^{phox}$ and $p47^{phox}$ replaces the effect of amphiphiles in
Figure 6    Amino acid Thr-233 is in a proline-rich region of p67$^{phox}$ and might regulate intramolecular or intermolecular binding. **A:** Schematic diagram of p67$^{phox}$ showing the positions of the two SH3 domains (amino acids 245-295 and 462-512), the polyproline region (227-234), and the phosphorylation site Thr-233.  **B:** It is proposed that the N-terminal region (1-99) of p67$^{phox}$ is made available for binding Rac by a conformational change caused by the phosphorylation of Thr-233.  **C:** It is proposed that the resting state interactions between p67$^{phox}$ and p47$^{phox}$, (p40$^{phox}$ is omitted for simplicity) are altered upon stimulation by the phosphorylation of p67$^{phox}$ (P), and binding of Rac. The p47$^{phox}$ region 323-332 (in black) is released by p67$^{phox}$ and then binds to the flavocytochrome. In the active state, Rac is GTP-bound and p47$^{phox}$ is heavily phosphorylated (P').
whole cells, causing an arrangement of p67phox and p47phox which is appropriate for cytochrome binding. The region of p47phox 323-332 has been shown by peptide competition in intact cells to be required for PMA-stimulated p47phox phosphorylation, translocation and oxidase activation (Labadia et al., 1996). Binding of the region p47phox 323-332 to p67phox occurs after stimulation but appears to be under competition by the cytochrome (DeLeo et al., 1996). It was proposed that the interaction of Rac with p67phox may be the event which disrupts this p47phox-p67phox interaction and allow transfer to the cytochrome.

These models require substantiation by experimental studies. The coordination of phosphorylation with changes in the interaction between these proteins, and other signal transduction events, is the subject of future study. A key element to be investigated further is the involvement of p67phox phosphorylation. The application of experimental intact cell systems, such as the reconstitution of p67phox-deficient CGD cells, will allow the assessment of p67phox phosphorylation in the activation of the multicomponent NADPH oxidase.
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