

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

by

Louisa Vivien Forbes

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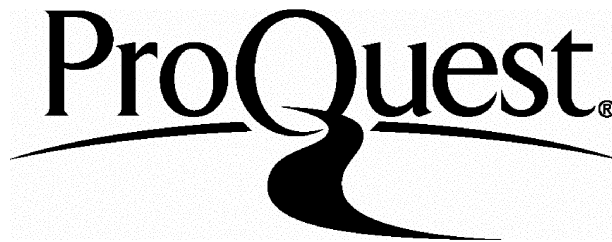
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Abstract

p67^{phox} is a component of the NADPH oxidase, the multicomponent enzyme that causes the production of superoxide in phagocytes. The phosphorylation of p67^{phox} upon cellular activation was studied in neutrophils and B lymphocytes. p67^{phox} showed low basal phosphorylation which increased up to three-fold upon stimulation. Phosphopeptide mapping revealed one major tryptic peptide, for p67^{phox} 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 p67^{phox} 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 p67^{phox} 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 p67^{phox} phosphorylation were performed. Although a MAP kinase pathway inhibitor (PD098059) reduced the *in vitro* phosphorylation of p67^{phox} by cytosol, intact cell experiments did not clearly provide evidence for MAP kinase in native p67^{phox} phosphorylation. The inhibitors staurosporine and okadaic acid gave results similar to those for p47^{phox}, in that phosphorylation of p67^{phox} correlates with activity, and that dephosphorylation may be involved in deactivation of the NADPH oxidase.

Experiments using p47^{phox}-deficient cells showed that p67^{phox} phosphorylation is independent of p47^{phox} 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 p67^{phox}. 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 p67^{phox} phosphorylation within the NADPH oxidase.

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Abbreviations

aa	amino acid
A, C, G, T	adenine, cytosine, guanine, thymine
ADP	adenosine 5'-diphosphate
Ala	alanine
AMP	adenosine 5'-monophosphate
Asp	aspartic acid
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
°C	degrees Celsius
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
cell eq	cell equivalents
CGD	chronic granulomatous disease
Ci	Curie
CNBr	cyanogen bromide
cpm	counts per minute
C-terminal	carboxy terminal
2D	two dimensional
Da	Daltons
2-DG	2-deoxy glucose
DMSO	dimethyl sulphoxide
DTT	dithiothreitol
EBV	Epstein Barr virus
ECL	enhanced chemiluminescence
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediamine tetra-acetic acid
ERK	extracellular-regulated kinase
FAD	flavin adenine dinucleotide
fMLP	N-formyl-methionyl-leucyl-phenylalanine
FPLC	fast protein liquid chromatography
g	gram
<i>g</i>	relative centrifugal force
GAP	GTPase activating protein
GDI	GDP-dissociation inhibitor
GDP	guanosine 5'-diphosphate
GEF	GDP/GTP exchange factor
Glu	glutamic acid
Gly	glycine
GMP	guanosine 5'-monophosphate
GST	glutathione S-transferase
GTP- γ -S	guanosine 5'-(γ -thio)triphosphate
HBS	HEPES -buffered saline
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethane-sulphonic acid)
HPLC	high pressure liquid chromatography
hr	hours
IgG	immunoglobulin G

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
<i>phox</i>	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	microFarad
UV	ultra violet
Val	valine
v/v	volume to volume ratio
w/v	weight to volume ratio

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Publications

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Forbes LV, Truong O, Moss SJ, Segal AW The major phosphorylation site of the NADPH oxidase component p67^{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 "φαγο", 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 margined 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 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 B₁₂-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.

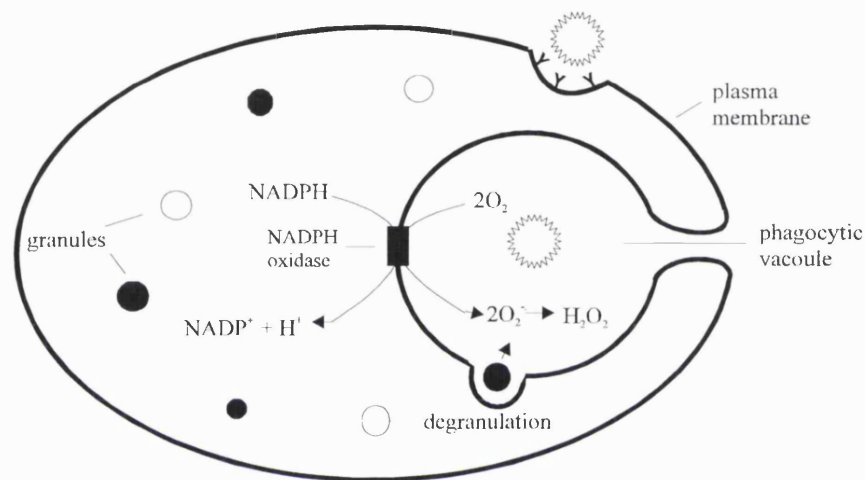


Figure 1.2 Schematic representation of the phagocytosis of a microbe.

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 micro-organisms. 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 (Baldrige & 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).

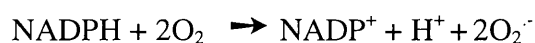
Major advances in the understanding of the biochemistry of phagocytosis then came from the discovery of a novel b-type cytochrome system in the vacuoles of phagocytes (Segal & Jones, 1978). The cytochrome is localised in the plasma membrane and in the membrane of the secondary granules, and upon stimulation 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 b₅₅₈

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 b₂₄₅ and cytochrome b₅₅₈. 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:



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 p47^{phox} (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 p47^{phox} (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 p67^{phox}-deficiency.

It was observed that p47^{phox} and p67^{phox} 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 p67^{phox} revealed a third protein of 40 kDa in this complex (Someya *et al.*, 1993; Wientjes *et al.*, 1993) which was cloned and named p40^{phox} (Wientjes *et al.*, 1993). A proportion of these three proteins, p47^{phox}, p67^{phox} and p40^{phox} 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^{phox} and p67^{phox} and a Rac-containing fraction were all present (Abo *et al.*, 1991). Rac (also known as p21rac) 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^{phox}, p67^{phox}, 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 $p67^{phox}$, $p47^{phox}$, $p40^{phox}$ 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 *et al.*, 1990; Quinn & Bokoch, 1995).

1.8 Priming

Superoxide production for the activation of the NADPH oxidase occurs after a delay following neutrophil stimulation (Rossi *et al.*, 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 *et al.*, 1983; McPhail *et al.*, 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 *et al.*, 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 *et al.*, 1986). The process is believed to be partly due to increases in intracellular

Ca^{2+} 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 p67^{phox} (Dusi *et al.*, 1993), p47^{phox} (Segal *et al.*, 1985), p40^{phox} (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 electroporabilised cells is dependent on ATP and Mg^{2+} (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 p47^{phox} 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 C γ 2 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 C γ 2 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 Ca²⁺ 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 Ca²⁺-

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 α 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

p47^{phox} and p67^{phox} have been identified. These are between the C-terminal SH3 domain of p67^{phox} and the C-terminal p47^{phox} 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 p67^{phox} with the C-terminal-, or both, SH3 domains of p47^{phox} (de Mendez *et al.*, 1996, 1997), and a third interaction involving amino acids 323-322 of p47^{phox} (De Leo *et al.*, 1996). The binding of p47^{phox} to p40^{phox} takes place between the p40^{phox} SH3 domain and the polyproline domain of p47^{phox} (Fuchs *et al.*, 1995; Ito *et al.*, 1996; Sathyamoorthy *et al.*, 1997). This region of p47^{phox} can also bind p67^{phox} 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 p47^{phox} binding of p67^{phox} (Sumimoto *et al.*, 1994). That is, the p47^{phox} SH3 domains only become available to interact with p67^{phox} 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 p47^{phox} is disrupted, unshielding the SH3 domains for intermolecular binding. In another study, binding between p67^{phox} and the 323-332 amino acid region of p47^{phox} 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^{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^{phox}, and indicated that the N-terminal SH3 domain binds to the α subunit and the C-terminal one binds to p67^{phox} (de Mendez *et al.*, 1997). Interestingly, the translocation of the N-terminal half of p67^{phox}, via interaction with this SH3 domain of p47^{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^{phox} corresponds with the kinetics of activation (Heyworth & Segal, 1986). It has been shown that while the NADPH oxidase is active, p47^{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^{phox} seem to be related processes (see also section 1.7). p47^{phox} phosphorylation starts in the cytosol (Heyworth *et al.*, 1989) but it becomes more heavily phosphorylated at the membrane (Okamura *et al.*, 1988; Rotrosen & Leto, 1990). This seems to be dependent on binding to the β subunit of the cytochrome because lack of the cytochrome (Ding, J. *et al.*, 1993) or disruption of the interaction between p47^{phox} and the β subunit (Rotrosen *et al.*, 1990) causes p47^{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^{phox} and p67^{phox} remains at the membrane (Dusi *et al.*, 1993), and the interaction between p47^{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^{phox} in opsonised zymosan-stimulated neutrophils, but with 5% of total cellular p47^{phox} remaining at the membrane after activation (Sergeant & McPhail, 1997).

1.11 Phosphorylation of p47^{phox}

After stimulation of neutrophils, there are at least six p47^{phox} 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^{phox} 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^{phox}-deficient Epstein 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^{phox} and oxidant production of the transfected B cells. Also, PKC-phosphorylated Ala-379 p47^{phox} 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^{phox} 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^{phox} 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 p47^{phox}, 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 p47^{phox} SH3 domains (Sumimoto *et al.*, 1994; DeLeo *et al.*, 1996). Amphiphiles cause p47^{phox} 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 p47^{phox} preceded activity. The superoxide production was 70% inhibited by staurosporine or GF109203X PKC inhibitors, with a concomitant decrease in the phosphorylation of p47^{phox} and other proteins. Recombinant p47^{phox} 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 p47^{phox} 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 $p47^{phox}$ phosphorylation. It is of interest that if the cytosol of the cell-free system were ATP-depleted before the addition of pre-phosphorylated $p47^{phox}$, 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 $p47^{phox}$.

Many studies have tried to define the relationship between $p47^{phox}$ phosphorylation, translocation and oxidase activation. Inhibition of PMA-induced $p47^{phox}$ 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 Ca^{2+} 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 $p47^{phox}$ phosphorylation and translocation for oxidase activity. Two studies report the inhibition

of p47^{phox} phosphorylation, translocation and oxidase activity by the introduction of peptides to the whole cell. One study used a peptide of the β subunit (Rotrosen *et al.*, 1990) and the other used a peptide of p47^{phox} (Labadia *et al.*, 1996). This amounts to circumstantial evidence that phosphorylation of p47^{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 Ca²⁺ depletion) significantly inhibits the phosphorylation of p67^{phox} and p47^{phox} but translocation and oxidase activity proceed almost unaffected (Robinson *et al.*, 1990; Dusi *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^{phox} (Badway *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^{phox} was sufficient to obtain maximal translocation and oxidase activity (Levy *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^{phox}. In one study, comparisons were made between the phosphopeptide maps from PMA-stimulated EBV-B cell p47^{phox}, and p47^{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 p67^{phox}

p67^{phox} 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 p47^{phox}- and cytochrome-positive (Leto *et al.*, 1990). p67^{phox}-deficiency is a rare phenotype of CGD, amounting to only 5% of all cases, compared with about 25% for p47^{phox}-deficiency (Clark *et al.*, 1989; Casimir *et al.*, 1992). The expression of p67^{phox} appears to be restricted to the same cell lineages as for p47^{phox} (see section 1.6), but the gene is located on a different chromosome, 1q25 (Francke *et al.*, 1990; Kenney *et al.*, 1993).

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

This phenotype of CGD was originally described as total lack of p67^{phox} protein, but with normal levels of mRNA (Leto *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 p67^{phox} protein have been reported to be detected, with inability to translocate

to the membrane (Leusen *et al.*, 1996). Recombinant p67^{phox} 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^{phox} 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^{phox}, 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^{phox}-deficient CGD.

(reference)	mRNA	genetic defect	predicted effect
case 1 ^a	normal level	homozygous G233 to A in exon 3	Gly 78 to Glu
case 2 ^b	normal level	heterozygous; in exon 5 A279 to T and A 481 to G	Lys 160 to Glu from one allele, Asp 161 to Val from other allele
case 3 ^c	normal level	homozygous AG insertion 399 in exon 5	frameshift causing stop codon at 433 in exon 5 (amino acid 118)
case 4 ^d	reduced amount	homozygous T to C in splice site intron 3	exon 3 deletion in mRNA causes unstable mRNA
case 5 ^e	smaller size	homozygous G to A in splice site intron 9	deletion of exons 8,9 coding for a polyproline and SH3 region
case 6 ^f	normal level	heterozygous, GAA deletion and 11-13 kb deletion	Lys 58 deletion from one allele, unstable mRNA from other allele

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

There is less p67^{phox} than p47^{phox} 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^{phox} is an NADPH binding component of the oxidase (Smith, R.M. *et al.*, 1996). This was developed from an observation that a 66 kDa protein in the oxidase bound labelled NADPH (Umei *et al.*, 1986; DeLeo & Quinn, 1996). p67^{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. *et al.*, 1996). p67^{phox} does not bind flavin, GTP or ATP (Leto *et al.*, 1991; Iyer, S.S. *et al.*, 1994). As discussed, there is strong evidence that the β subunit is the significant NADPH binding factor of the oxidase. Therefore, the data suggest that p67^{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^{phox}. Unlike for p47^{phox} phosphorylation, lack of a 67 kDa phosphoprotein was not noticeable in p67^{phox}-deficient CGD. In fact, one publication challenged whether there is any phosphorylation of p67^{phox} (Heyworth *et al.*, 1996). Another study showed, by immunoprecipitation, that phosphorylated p67^{phox} was found in the cytosol and membrane fractions of PMA-stimulated neutrophils (Dusi & Rossi, 1993). PMA- or fMLP-stimulated phosphorylation of p67^{phox} (although not purified by immunoprecipitation) was shown to be inhibited by staurosporine (Dusi *et al.*, 1993). Conversely, a recent paper reported that p67^{phox} phosphorylation was inhibited by a PKC inhibitor (GF109203X) in PMA but not fMLP stimulation, suggesting PKC-dependent and -independent regulation (El Benna *et al.*, 1997). This phosphorylation was detected as serine phosphorylation, and it was suggested that it could be mimicked by PKC *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^{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 p67^{phox} (Fujimoto *et al.*, 1989). This conclusion is based on the similarity in the rates of oxidase activation and p67^{phox} inactivation. Such an alteration of p67^{phox} could result from a change in binding or a chemical modification such as phosphorylation. Various findings indicate that the structure of p67^{phox} is critical to its stability. These include the fact that single amino acid substitutions coded by the mRNA can be the cause of p67^{phox} deficiency in CGD. In addition to p67^{phox} 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 p67^{phox} 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 p47^{phox}. 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). p67^{phox} 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 p67^{phox} is not known, except the genetic defect of a p67^{phox}-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^{phox} (Ponting, 1996).

There is evidence for two SH3-polyproline interactions between p67^{phox} and p47^{phox}, plus another interaction independent of SH3 domains, as discussed for p47^{phox}. However, no specific binding partners have been appointed to the N-terminal SH3 domain of p67^{phox} or to the polyproline domain, although the latter region may be involved in p47^{phox} binding to the N-terminal half of p67^{phox}. Also, the intramolecular SH3-polyproline binding that has been demonstrated within p47^{phox} (Leto *et al.*, 1994; de Mendez *et al.*, 1997), has not been shown for p67^{phox}. Deletion studies in transfected whole cell (p67^{phox}-deficient B cells) experiments have provided some important structure-function information for p67^{phox}. Both SH3 domains of p67^{phox} 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^{phox}, and Southern blotting was used for detection of p67^{phox} transfection as the level of expression was below detection by immunoblotting.

It must be emphasised that the intact cell requirements for p67^{phox} differ from those for cell-free oxidase activity. N-terminal p67^{phox} (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^{phox}, amino acids 1-212 (Hata *et al.*, 1997). This truncated p67^{phox} supported higher activity than full-length p67^{phox} if the polyproline region of p47^{phox} were simultaneously deleted. The authors claim that this implies a regulatory role for the p67^{phox} SH3 to p47^{phox} polyproline interaction. It is difficult to extrapolate these results to the cellular

oxidase (without μM amphiphiles) where the C-terminal half of p67^{phox} is a proven requirement (de Mendez *et al.*, 1994, 1997).

There are two other binding partners of p67^{phox} ; p40^{phox} and Rac. It has been observed that there is a particular affinity between p67^{phox} and p40^{phox} (Wientjes *et al.*, 1996). The two proteins remain tightly bound during purification of the p67^{phox} - p47^{phox} - p40^{phox} 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 p67^{phox} (Fuchs *et al.*, 1996; Sathyamoorthy *et al.*, 1997). There is also one report of high affinity binding between the N-terminus of p67^{phox} and p40^{phox} (Wientjes *et al.*, 1996). The N-terminal 199 amino acids of p67^{phox} 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 p67^{phox} with p40^{phox} and Rac is examined in more detail in the following sections.

1.13 p40^{phox}

p40^{phox} is an 339 amino acid protein with one SH3 domain and a high degree of homology with p47^{phox} (Wientjes *et al.*, 1993). The N-termini of p40^{phox} and p47^{phox} 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). p40^{phox} 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 ι/λ and ζ , and possesses four conserved acidic residues suggesting the ability to bind divalent cations such as 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 $p40^{phox}$ and $p67^{phox}$ is almost unbreakable, one immunoprecipitation study showed that arachidonic acid treatment at 20°C of the $p47^{phox}$ - $p67^{phox}$ - $p40^{phox}$ complex caused dissociation of $p40^{phox}$ (Someya *et al.*, 1996). Whether this indicates that separation of $p40^{phox}$ and $p67^{phox}$ occurs during *in vivo* activation is not clear.

The polyproline region on $p47^{phox}$ that binds the C-terminal SH3 of $p67^{phox}$ can also bind the $p40^{phox}$ SH3 domain (Ito *et al.*, 1996). This suggests that competition in the intermolecular interactions may play a regulatory role in activation. The concept that $p40^{phox}$ and $p47^{phox}$ have related roles in the regulation of $p67^{phox}$ action is consistent with $p40^{phox}$ and $p47^{phox}$ 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 $p40^{phox}$, antibodies against $p40^{phox}$ which disrupt the binding between $p40^{phox}$ and $p67^{phox}$, can significantly inhibit the cell-free SDS-activated oxidase of cytosol plus membranes (Tsunawaki *et al.*, 1996). A C-terminal peptide of $p40^{phox}$ 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, $p67^{phox}$ and $p47^{phox}$ is sufficient to generate an NADPH oxidase (de Mendez & Leto, 1995). If $p40^{phox}$, or even just the SH3 domain which binds to $p47^{phox}$, is cotransfected in these cells, the oxidase activity decreases by at least 40% (Sathyamoorthy *et al.*, 1997). This may indicate that $p40^{phox}$ plays a moderating role in controlling superoxide production. These results from different studies suggest that $p40^{phox}$ is a key element of oxidase activation, probably by

regulating the interactions between p47^{phox}, p67^{phox} and the cytochrome. It has now been postulated that phosphorylation plays a role in p40^{phox} modulation (Fuchs *et al.*, 1997).

The phosphorylation of p40^{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^{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^{phox}, p47^{phox} and p67^{phox} did not change upon stimulation, but the incorporation of radiolabelled phosphate increased three-fold for p40^{phox} and p47^{phox}, and only by 25% for p67^{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 Rac1s, or Rac1s containing nonhydrolysable GTP- γ -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^{phox} and p47^{phox} to the membrane. Despite the discrepancies in the literature, the most convincing data suggests Rac2 is the physiological binding partner of

p67^{phox} (Dorseuil *et al.*, 1996), and they translocate independently to the membrane (Dusi *et al.*, 1995, 1996). Once at the membrane, p67^{phox}, p47^{phox} and Rac appear to be present in equimolar amounts (Quinn *et al.*, 1993; Nisimoto *et al.*, 1997).

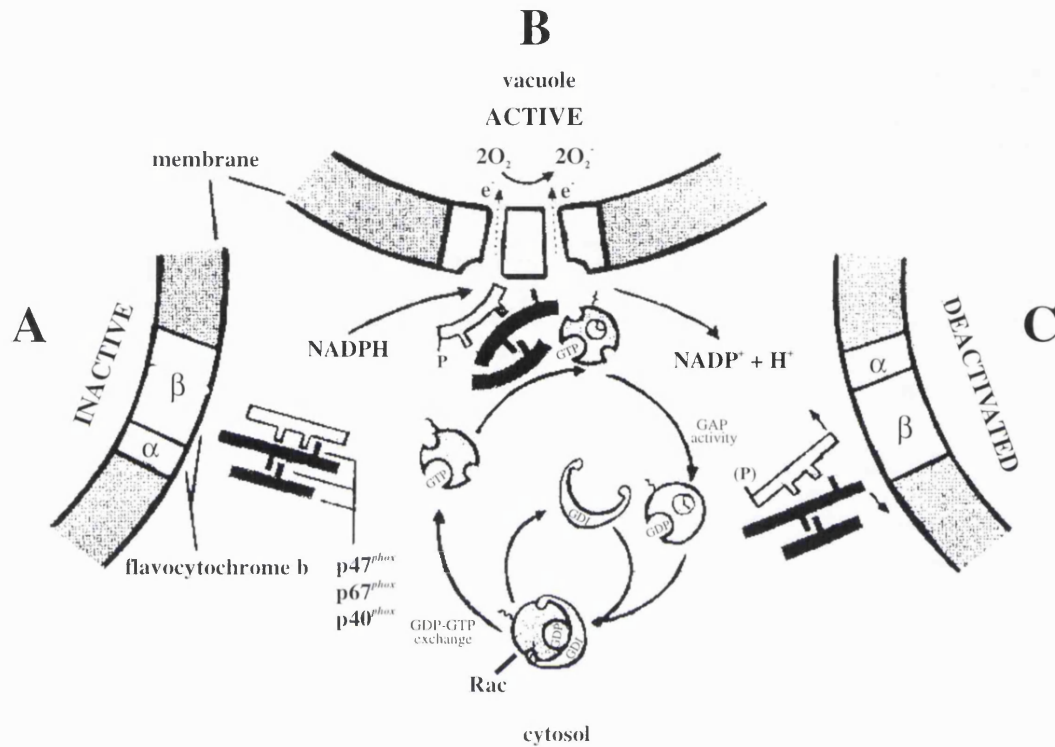


Figure 1.4 Model of the activation and deactivation process of the NADPH oxidase. The formation of the multimolecular complex at the flavocytochrome in phagocyte membranes is shown in the A: inactive, B: active and C: deactivated states. Before activation, p47^{phox}, p67^{phox} and p40^{phox} are in 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 *et al.*, 1996), although nonisoprenylated Racs preloaded with GTP- γ -S can support oxidase activity under some cell-free conditions (Heyworth *et al.*, 1993b). Isoprenylation does not appear to be a requirement for p67^{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 p67^{phox} (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 p47^{phox} *in vitro*, the relevance of this has yet to be determined *in vivo* (Knaus *et al.*, 1995).

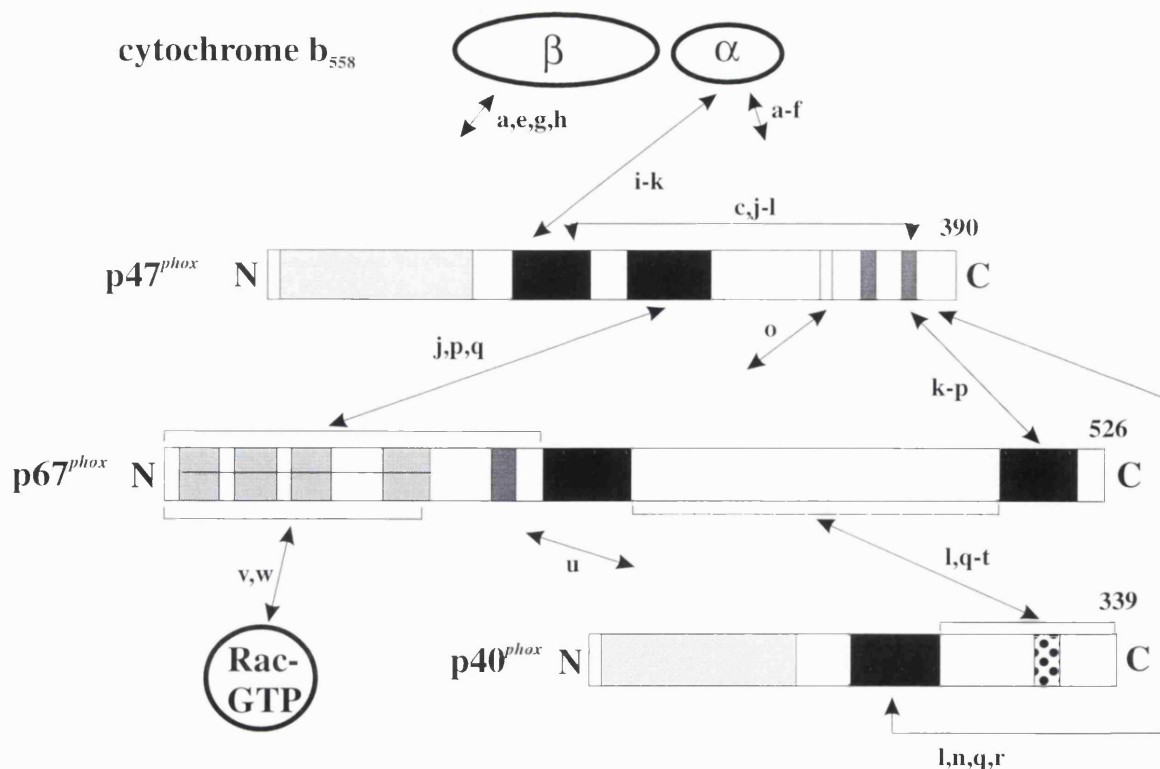
1.15 Summary of protein-protein interactions

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



Key to the domains:



PX (phox) : p47^{phox} 20-125
p40^{phox} 37-140



SH3 (Src homology 3) : p47^{phox} 151-214, 227-284
p67^{phox} 245-295, 458-517
p40^{phox} 175-226



polyproline : p47^{phox} 341-349, 361-369
p67^{phox} 219/227-234



p47^{phox} 323-332



TPR (tetratricopeptide) : p67^{phox} 6-154



OPR (octicosapeptide) : p40^{phox} 283-310

Figure 1.5 Diagram of p67^{phox}, p47^{phox} and p40^{phox} depicting the documented sites of protein-protein interactions. p67^{phox} has 526 amino acids with 2 SH3 domains (Leto *et al.*, 1990), p47^{phox} has 390 amino acids with 2 SH3 domains (Volpp *et al.*, 1989), and p40^{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). p67^{phox} 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 p67^{phox}, and it can also bind one of p67^{phox}'s binding partners, C-terminal p40^{phox}. 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, p67^{phox} is an essential, labile, component of the NADPH oxidase. It is known to bind p47^{phox} and p40^{phox}, 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 p67^{phox}. 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 *in vivo*) and recombinant systems (*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 p67^{phox}, 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 haemocytometer.

HEPES-buffered saline (HBS) phosphate-free buffer

137 mM NaCl

2.7 mM KCl

20 mM HEPES (N-(2-hydroxyethyl)piperazine-N'-(2-ethane-sulphonic acid))

(continued from over:)

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 5×10^7 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_i, 10 mCi/ml aqueous solution, Amersham), and incubated for a further 2 hr. Aliquots of 7.5×10^7 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 μ F to cells at 10^7 /ml in ATP-Electroporation Buffer. Cells were put on ice and used immediately after 32 P- 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 μ l with 5-25 μ Ci 32 P- 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.

<u>Streptolysin-O Buffer:</u>	20 mM PIPES*	+ 100 μ M ATP
	137 mM NaCl	+ 2 mM NADPH
	2.7 mM KCl	+ 1 mM MgCl ₂
	pH 6.8	+ 100 nM Ca ²⁺ / 3 mM EGTA*

<u>ATP-Electroporation Buffer :</u>	140 mM KCl	+ 100 μ M ATP
	10 mM HEPES	+ 2 mM NADPH
	20 mM glucose	+ 1 mM MgCl ₂
	pH 7.0	+ 100 nM Ca ²⁺ / 3 mM EGTA*

*PIPES; 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 ³²P_i

Neutrophils: ³²P_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 ³²P_i incorporation increased with decreased cell concentration and increased ³²P_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 ³²P_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 5×10^7 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 2×10^8 /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 5× 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)

140 mM NaCl
10 mM KCl
10 mM NaH₂PO₄
to pH 7.3 with NaOH

Lamberth's break buffer

10 mM KCl
3 mM NaCl
4 mM MgCl₂
10 mM PIPES, pH 7.0

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 1 µM 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 vanadate* (stock: 0.2 M, -20°C)

*Na vanadate preparation: 200 mM in water, adjust to pH 10, boil to dissolve, readjust to pH 10.

2.8 Immunoprecipitation of p67^{phox}

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 5×10^7 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^{phox} antibody raised in rabbits against full-length glutathione S-transferase (GST)-p67^{phox} 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^{phox}. 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^{phox} immunoprecipitate. Membrane preparations of 7.5×10⁸ cell eq/500 µl were found to be suitable.

Relaxation Buffer

100 mM KCl
3 mM NaCl
3.5 mM MgCl₂
1.25 mM EGTA
10 mM PIPES, pH 7.3

2× Solubilisation Buffer

50 mM Tris, pH 7.5
2% (v/v) Triton X-100*
300 mM NaCl
2% (w/v) Na deoxycholate
0.2% (w/v) SDS

*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^{phox} bands excised from unstained gels were carried out in one of two ways: first method; elution of p67^{phox} 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.

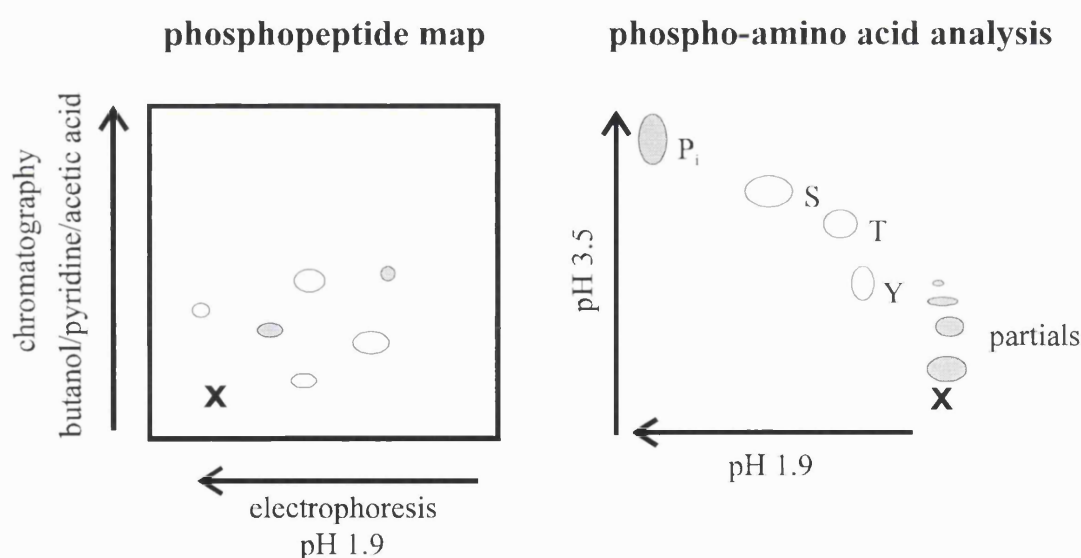


Figure 2.1 2D phosphopeptide and phosphoamino acid analyses. The direction of the two steps applied to the cellulose-coated glass plate is indicated. The sample origin is at position X for both procedures, and the relative migration of free phosphate (P_i) and phospho-serine (S), -threonine (T) and -tyrosine (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. p67^{phox} 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 p47^{phox} and truncated p67^{phox} 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 p67^{phox}: Full-length p67^{phox} cDNA had been previously cloned plasmid in Professor Segal's laboratory from an induced HL60 λ gt10 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 p67^{phox} using this system was as follows: 500 ml Terrific Broth (with 100 μ g/ml ampicillin) was inoculated with an overnight culture of pGEX-p67^{phox} from a glycerol stock, such that absorbance at 600 nm was 0.05. This culture was grown in a 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 p67^{phox} 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^{phox}-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 through this procedure twice again, and the S/Ns combined after checking for p67^{phox} by SDS/PAGE. Glutathione beads can be regenerated for repeated use as detailed below.

Purification of p67^{phox} 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^{phox} eluted at ~0.2 M NaCl in fractions which were >90% pure. Aliquots, with 10% (v/v) glycerol, were stored at -20°C.

<u>Terrific Broth</u>	<u><i>E. coli</i> Lysis Buffer</u>	<u>Thrombin Buffer</u>
17 mM KH ₂ PO ₄	150 mM NaCl	150 mM NaCl
72 mM K ₂ HPO ₄	1 mM EDTA	5 mM MgCl ₂
1.2 % (w/v) tryptone	0.5% (v/v) Nonidet p40	2.5 mM CaCl ₂
2.4 % (w/v) yeast extract	50 mM Tris, pH 8.0	1 mM DTT
0.4 % (w/v) glycerol		50 mM Tris, pH 8.0

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 p67^{phox} : p67^{phox} had previously been cloned into a baculovirus vector for generation of recombinant p67^{phox} 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 p67^{phox} 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 (~1×10⁸ cells for 150 cm² flask, 30 ml Insect Culture Media), to which 1-2 mls p67^{phox} 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 p67^{phox} protein expression, SF9 cells were grown in a shaken suspension. The optimal conditions such that the cell population doubles between 24-48 hr, are 4×10⁵ - 4×10⁶ 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 2×10⁶ 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.
- PAK; GST-fusion protein of rat brain β PAK 65 kDa from Ms E. Prigmore, Institute of Neurology, London (Prigmore *et al.*, 1995).
- PKC; rat brain isolate of mixed isoforms from Dr S.J. Moss, MRC Laboratory for Molecular Cell Biology, London (Woodgett & Hunter, 1987).

25 μ l reactions were set up on ice consisting of 2.5 or 5 μ l 10 \times or 5 \times kinase buffer (see below), 5 μ l 0.5 mM ATP at approximately 1000 cpm/pmol, 0.5-5 μ g recombinant protein substrate (p67^{phox} or myelin basic protein (Sigma)) and kinase in the form of neutrophil cytosol ($1-5 \times 10^4$ cell eq), neutrophil membranes ($1-3 \times 10^6$ cell eq), membranes extract (an arbitrary volume), MAP kinase (5 units), PAK (0.7 μ g) or PKC (5 ng). Following an incubation of 30°C, 20 min, the reactions were stopped by addition of 3 μ l 10 \times 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.

<u>10×MAP kinase buffer</u>	<u>5×PAK buffer</u>	<u>5×PKC buffer</u>
100 mM MgCl ₂	25 mM MgCl ₂	50 mM MgCl ₂
10 mM EGTA	25 mM MnCl ₂	2.5 mM CaCl ₂
20 mM DTT	5 mM DTT	100 mM HEPES, pH 7.4
0.1% (v/v) Brij 35	0.25% (v/v) Triton-X 100	
500 mM Tris, pH 7.5	250 mM HEPES, pH 7.0	

2.12 Cyanogen bromide digestion of p67^{phox}

A 6×2 mm gel slice of immunoprecipitated p67^{phox} 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^{phox}. 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, C₁₈ column (with AX-300 and C₁₈ 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^{phox}

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

primer F: CTATATGAGCCAGTGGTGATCC

primer R: CTACCGTGTACTTGTAGTGCACC

primer M1: AGACCGAAAGCCCCAGAGATCTTCAG

primer M2: AAGATCTCTGGGGCTTTCGGTCTGG

Briefly, the method involved two PCR reactions (primer pairs F/M2, R/M1) performed on wildtype p67^{phox} 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^{phox} 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^{phox} 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 MgCl₂ 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 α 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 Qiagex 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^{phox}. The N-terminal GST-fusion protein of the Ala-233 mutant was expressed and purified as for wildtype p67^{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 $1-2 \times 10^6$ cells were added to the cuvettes maintained at 37°C and containing PBS/glucose with 0.5 mM CaCl₂, 1 mM MgCl₂ and 100 μ M 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 μ g/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 $5 \times 10^7/\text{ml}$ 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 p67^{phox} 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 p67^{phox} 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 ³²P_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 ³²P_i into ³²P-γ-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^{phox} is not a heavily phosphorylated protein relative to other phosphoproteins in activated neutrophils. For example p67^{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^{phox} phosphoprotein. Compared with p47^{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^{phox}.

One approach that is sometimes taken, is to enhance the *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 *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^{phox} (see Chapter 5).

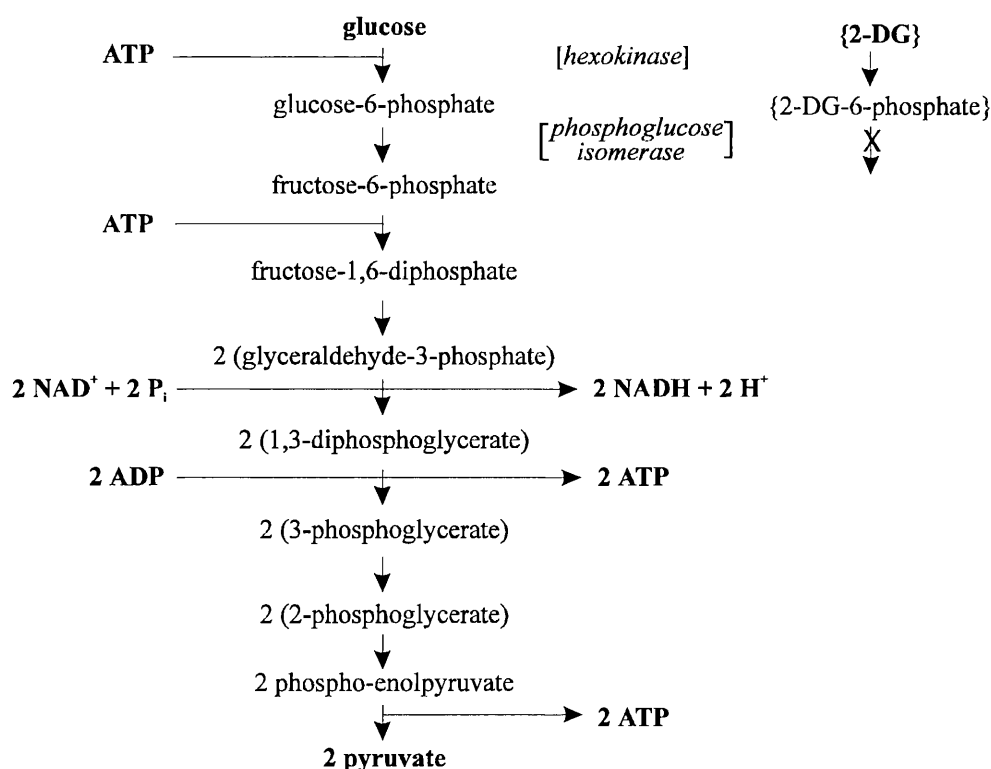
This section reports the approaches that were taken to optimise the radiolabelling of cells for the study of p67^{phox} phosphorylation.

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 can not be further metabolised (Wick *et al.*, 1957; Kuo & Lampen, 1972).

Figure 3.1.1 The reactions of glycolysis.



Overall reaction for glycolysis:



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 $^{32}\text{P}_i$, only its intracellular distribution (Tenenhouse & Sriver, 1975).

Experiments were carried out to investigate whether simultaneous $^{32}\text{P}_i$ 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 5×10^7 cells was found to be optimal.

Cellular nucleotides were extracted by aqueous/lipid phase fractionation of neutrophils. The trace from 5×10^7 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 ($^{32}\text{P}_i$) 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

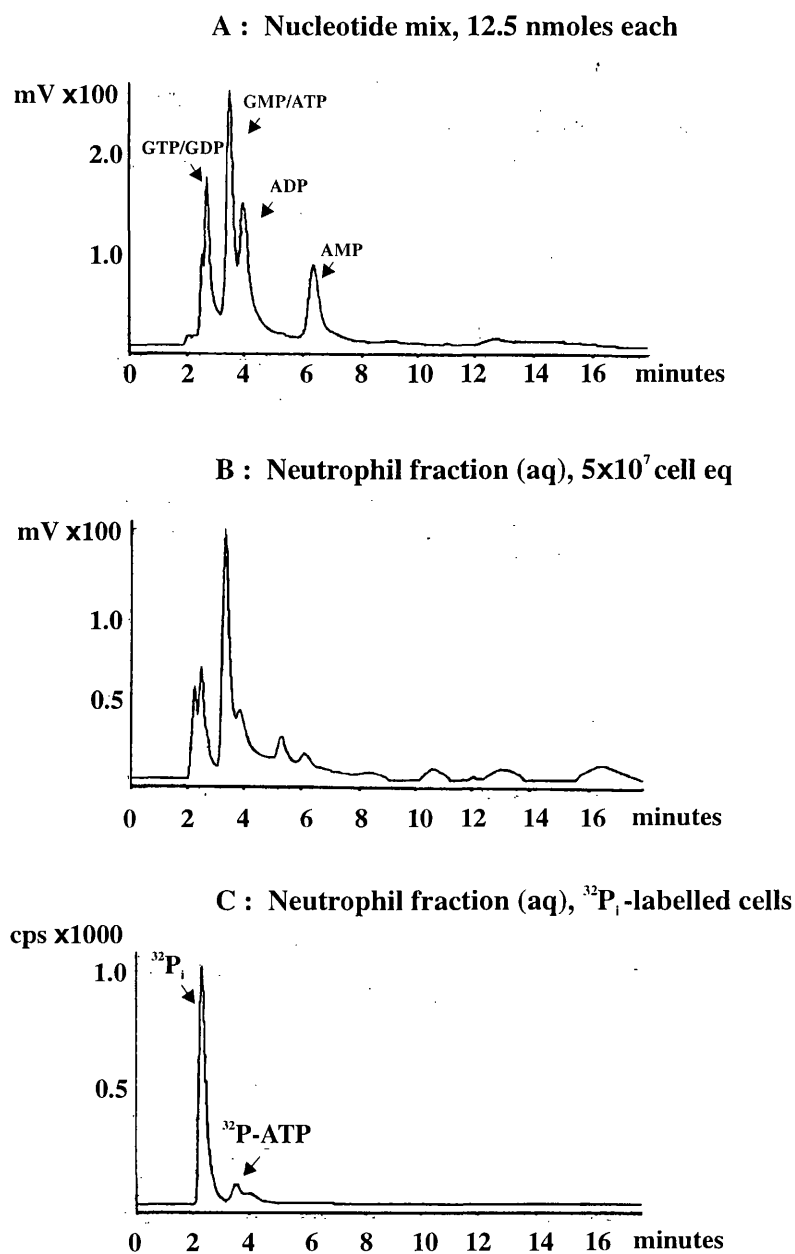


Figure 3.1.2 RP-HPLC for resolution of nucleotides. **A:** Resolution of adenine and guanine nucleotides from an equimolar mix, each peak identified by individual application. **B:** UV trace (260 nm) of the aqueous (aq) nucleotide-containing fraction from 5×10^7 cells. **C:** On-line detection of the radioactivity eluting in the HPLC run depicted in **B**, prepared from $^{32}\text{P}_i$ -labelled neutrophils.

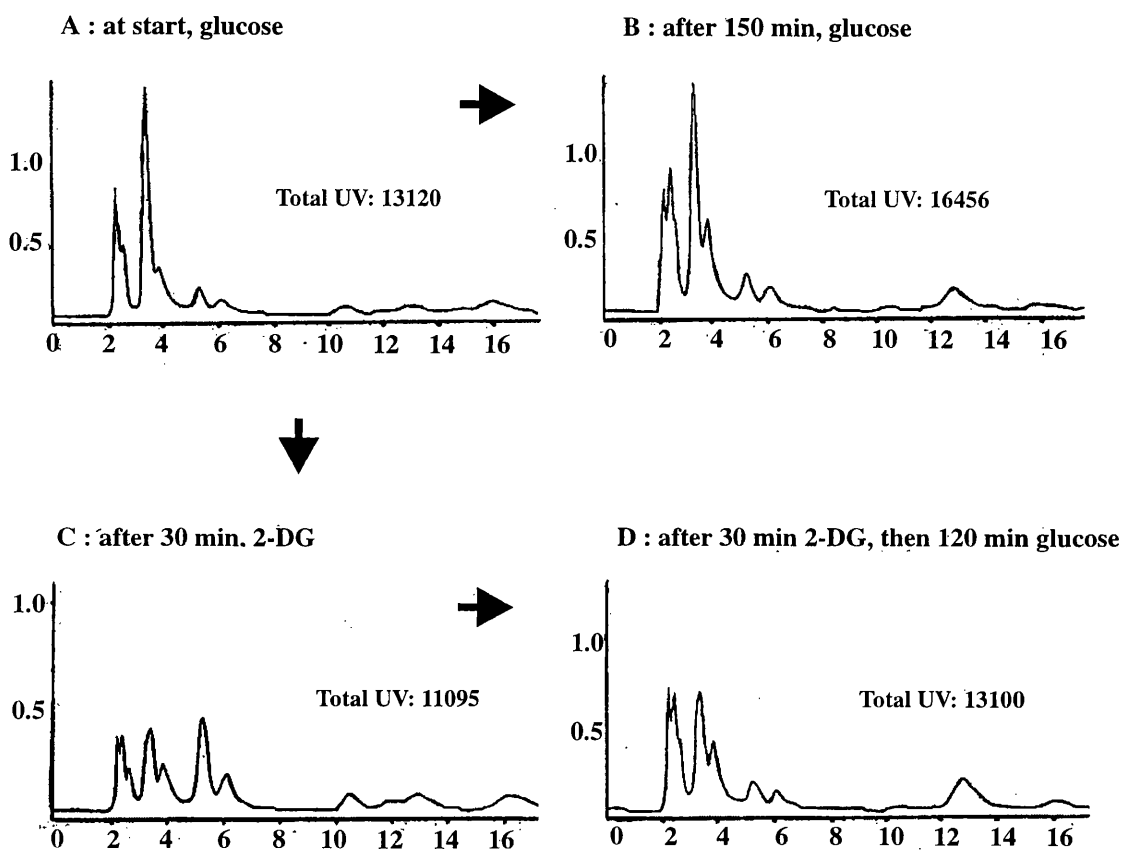


Figure 3.1.3 Changes in neutrophil nucleotide composition during incubations. RP-HPLC UV absorbance at 260 nm, of nucleotide-containing fractions from 7.5×10^7 cells after different times and conditions of incubation. X-axis units: minutes, Y-axis units: mV $\times 100$. All traces are representative 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 $^{32}\text{P}_i$ 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

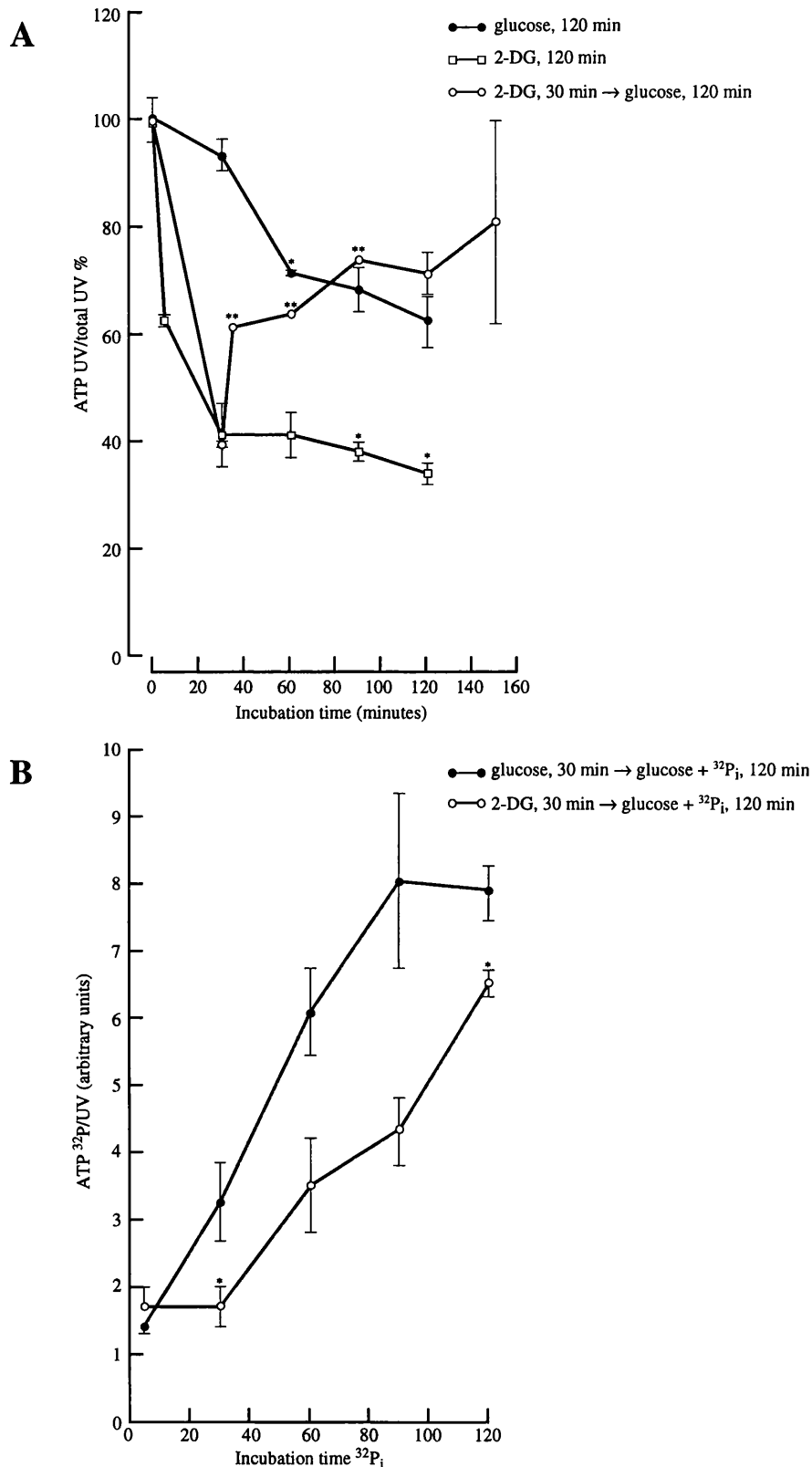


Figure 3.1.4 Changes in the calculated cellular ATP levels. **A:** Timecourse of cellular ATP measurements during incubation of neutrophils in glucose, 2-DG, or 30 min in 2-DG followed by incubation in glucose. **B:** Timecourse of the specific activity of ³²P-ATP in neutrophils after 30 min preincubation in either glucose or 2-DG. At time zero, cells were transferred to fresh medium with glucose and ³²P_i. 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 $^{32}\text{P}_i$ -loaded resting neutrophils, minimal incubation times are required. Therefore it was concluded that glucose starvation gave no enhancement of the uptake of $^{32}\text{P}_i$ 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 γ -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 $^{32}\text{P}_i$ -loading for *in vivo* phosphorylation experiments.

3.1b Cell permeabilisation for ^{32}P - γ -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}P - γ -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}P - γ -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 $1 \times 10^7/\text{ml}$, as higher concentrations did not capture the radiolabel as effectively. The amount of ^{32}P - γ -ATP activity added to the medium was approximately 10 μCi . 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^{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^{phox} .

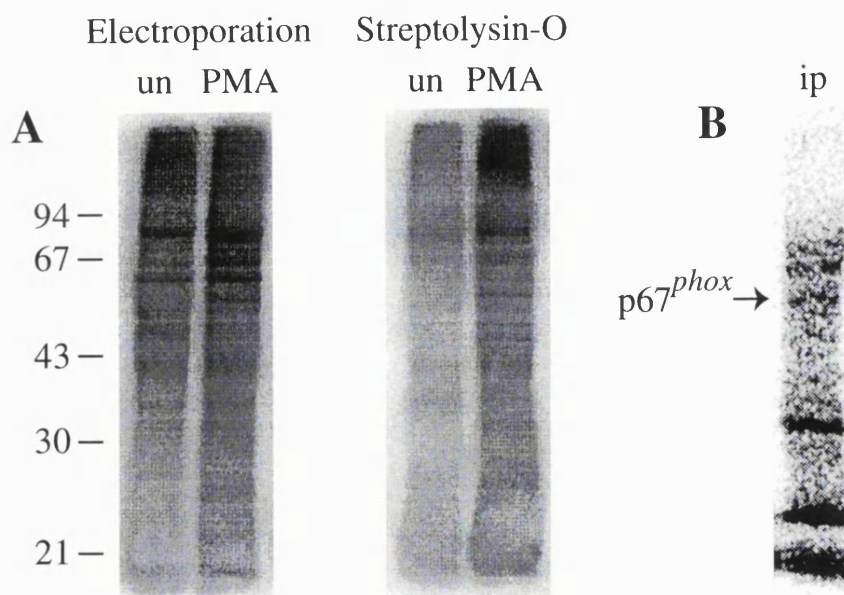


Figure 3.1.5 Cell permeabilisation methods for labelling with ^{32}P - γ -ATP. Phosphorimages of **A**: TCA precipitates of 1×10^6 electroporated or streptolysin-O treated neutrophils which were unstimulated or PMA-stimulated ($1 \mu\text{g}/\text{ml}$, 3 min) after the permeabilisation procedure. **B**: Immunoprecipitation (ip) of p67^{phox} from 2.5×10^6 neutrophils following streptolysin-O permeabilisation and PMA stimulation ($1 \mu\text{g}/\text{ml}$, 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 p40^{phox} (Fuchs *et al.*, 1997). EBV-transformed B cells have also been applied to the study of p47^{phox} 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 $\mu\text{Ci/ml}$ $^{32}\text{P}_i$ for one hour at 30°C in phosphate-free HBS, and B cells were incubated with 200 $\mu\text{Ci/ml}$ $^{32}\text{P}_i$ for four hours at 37°C in phosphate-free RPMI medium. Cells were activated, and p67^{phox} 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 p67^{phox}. 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 p67^{phox} as neutrophils (personal observations, (Chetty *et al.*, 1995)). These results indicate that $^{32}\text{P}_i$ -loading of B cells gives a much better labelling of phosphorylated p67^{phox} 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.

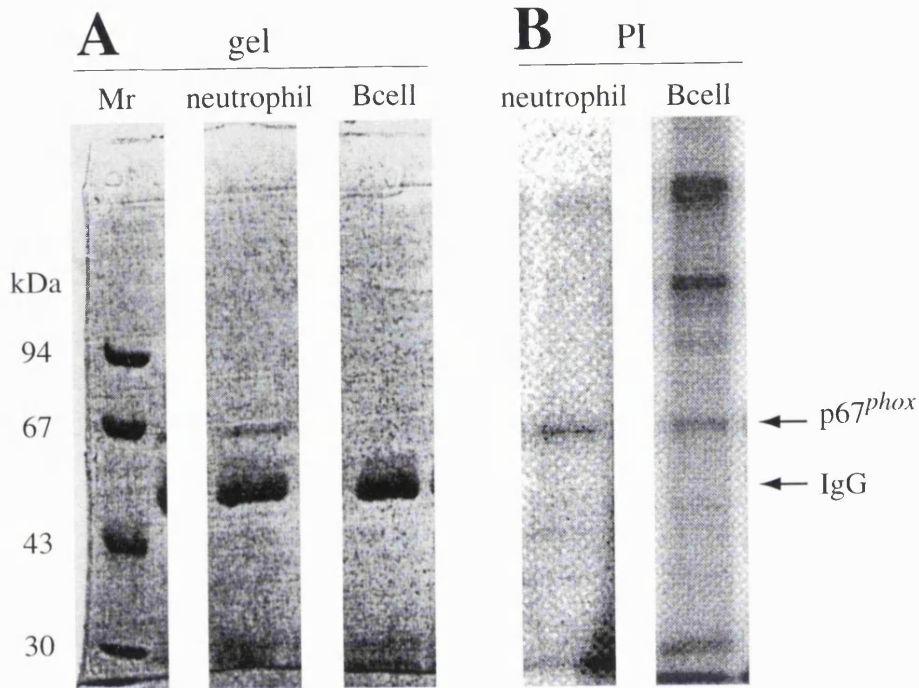


Figure 3.1.6 Comparison of immunoprecipitated p67^{phox} from metabolically labelled neutrophils and B cells. **A:** Coomassie-stained gel and **B:** corresponding phosphorimage (PI) after 36 hr exposure. **Neutrophil:** immunoprecipitate from 5×10^7 normal neutrophils labelled with 200 μCi $^{32}\text{P}_i$, 1 ml, 1 hr, followed by 8 min stimulation with 1 mg/ml S.O.Zym. **B cell:** immunoprecipitate from 1×10^7 normal EBV-B cells labelled with 200 μCi $^{32}\text{P}_i$, 1 ml, 4 hr, followed by 10 min stimulation with 1 $\mu\text{g}/\text{ml}$ PMA.

3.2 *In vivo* phosphorylation of p67^{phox}

3.2 AIM :

The main aim of this project was the determination of the phosphorylation sites on p67^{phox}, 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 p67^{phox}, thereby forming the basis for further characterisation.

3.2 INTRODUCTION :

It was first shown in 1993 that p67^{phox} is phosphorylated upon activation of the NADPH oxidase (Dusi & Rossi, 1993; Dusi *et al.*, 1993), an event that had been first observed for p47^{phox} in 1985 (Segal *et al.*, 1985). The cDNA encoding p47^{phox} 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 p47^{phox} upon stimulation. The PMA-induced phosphorylation sites on p47^{phox} 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 p47^{phox}-deficient B cell lines, which demonstrated that replacement of the phosphorylated serines with alanine rendered p47^{phox} 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 p67^{phox} 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 p67^{phox} is phosphorylated at all (Heyworth *et al.*, 1996). The authors claim that no phosphoprotein was immunoprecipitated by antibodies against p67^{phox}. 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 p47^{phox} was detected in cell lysate, there was no obvious phosphorylation of native or added recombinant p67^{phox} (Nauseef *et al.*, 1990). Therefore, it was of primary interest to determine whether p67^{phox} 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 p67^{phox} phosphoprotein. It was a polyclonal antibody raised in rabbits against recombinant GST-p67^{phox} and then affinity purified against recombinant (GST-cleaved or insect cell-derived) p67^{phox}. The recombinant proteins were either synthesised in *E. coli* (bacterial) or *Spodoptera frugiperda* (insect cell) systems from fully sequenced cDNA clones of human p67^{phox}. The antibody was tested against Western blots of TCA precipitates of whole neutrophils from a CGD patient deficient in p67^{phox} and normal control neutrophils (**Figure 3.2.1A**). Both neutrophil samples gave positive signals with antibodies to p47^{phox} and p40^{phox} (also affinity purified antibodies raised in rabbits against the recombinant proteins), but no signal was obtained in the p67^{phox}-deficient CGD neutrophils using the p67^{phox} antibody. In order to check the antibody specificity under immunoprecipitation conditions, normal and p67^{phox}-deficient CGD EBV-transformed B cells were radiolabelled with ³²P_i and subjected to immunoprecipitation with rabbit antibodies. The immunoprecipitates were blotted and probed with an affinity purified antibody raised in goats against recombinant p67^{phox}. p67^{phox} was detected in the normal

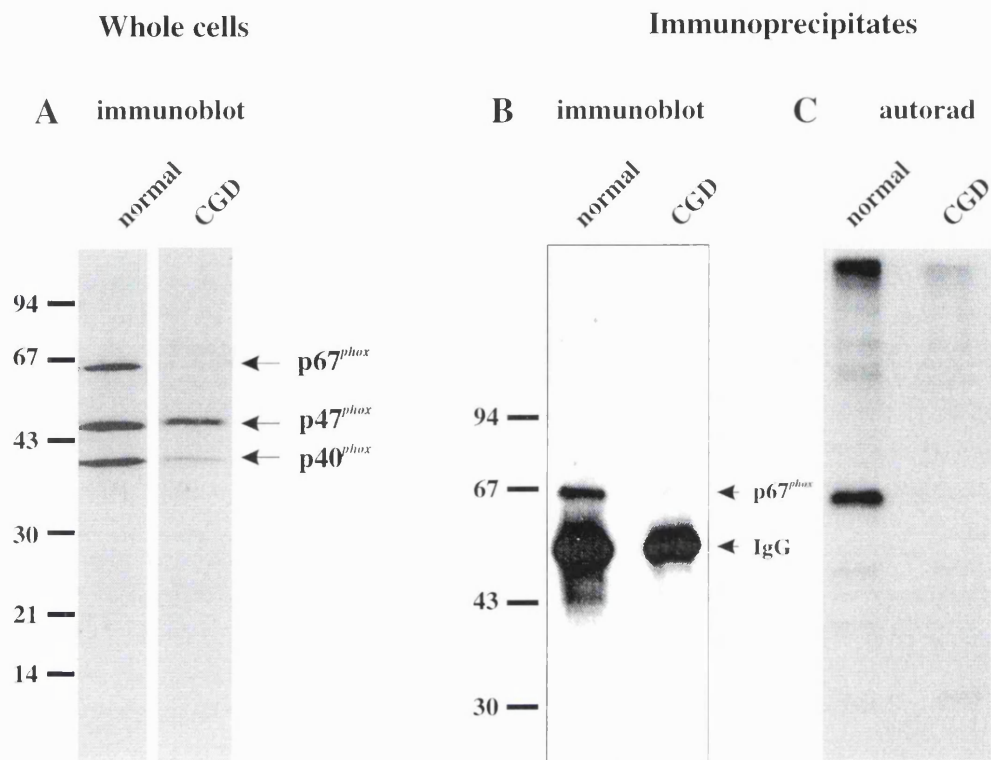


Figure 3.2.1 Immunoblots and autoradiographs of p67^{phox} from whole cells and immunoprecipitates from cytosol. **A:** TCA precipitates of 1×10^6 neutrophils from a normal subject and from a CGD patient lacking p67^{phox} were applied to SDS/PAGE, blotted onto nitrocellulose and probed with affinity purified antibodies to p67^{phox}, p47^{phox} and p40^{phox}. The bands were detected with the ECL system. **B:** EBV-B cells were radiolabelled with $^{32}\text{P}_i$ and the cytosol immunoprecipitated with the rabbit antibody against p67^{phox}. The immunoblot was carried out with a goat antibody to p67^{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^{phox}-deficient cells (**Figure 3.2.1C**). This immunoprecipitated 67 kDa phosphoprotein was also recognised by two other antibodies raised against p67^{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^{phox}.

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.

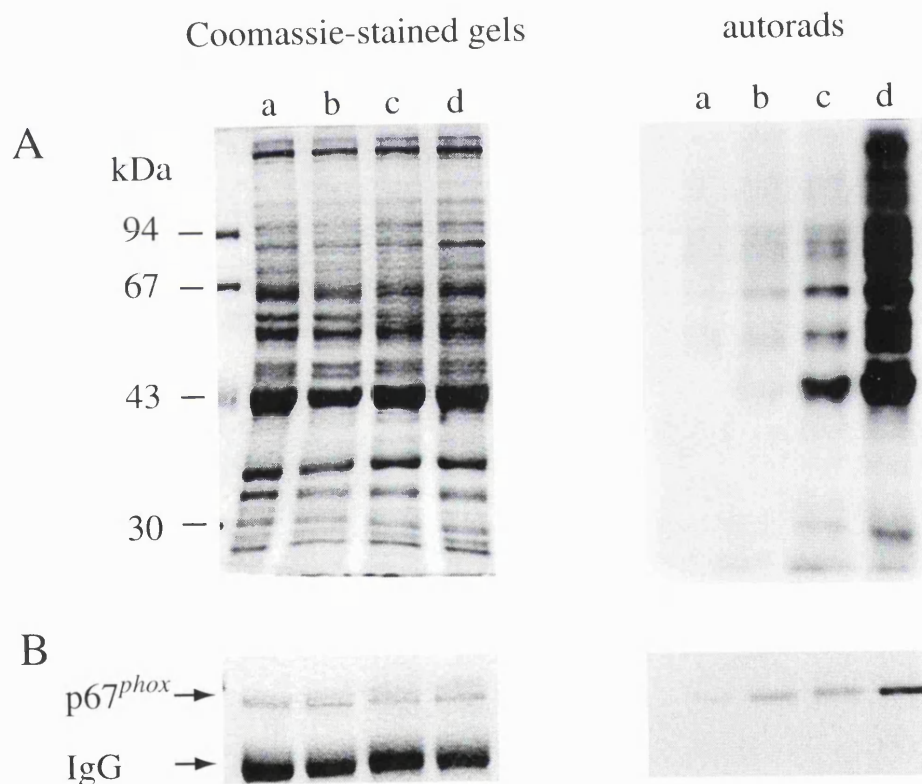


Figure 3.2.2 Phosphorylation of $p67^{phox}$ upon stimulation of neutrophils. $^{32}\text{P}_i$ -labelled neutrophils were treated in four ways (a, resting; b, S.O.Zym.; c, fMLP; d, PMA), homogenised, and then cytosol was prepared. Coomassie-stained gels and autorads are shown for **A**: 2×10^5 cell 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 .

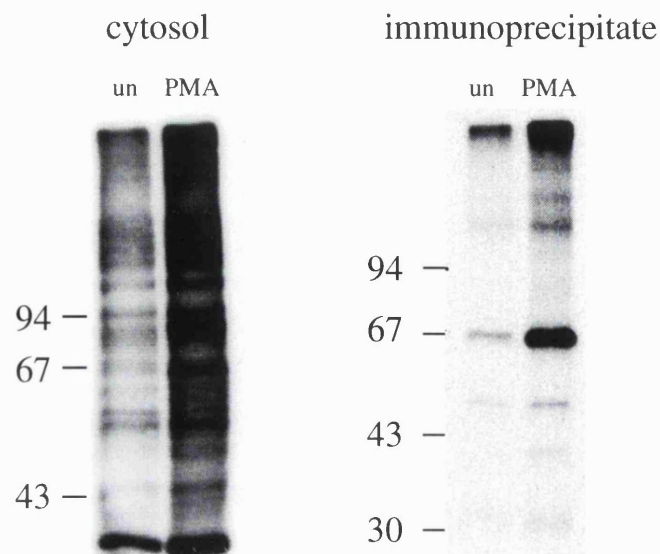


Figure 3.2.3 Stimulation of p67^{phox} phosphorylation in B cells. ³²P_i-labelled B cells were unstimulated or stimulated with 1 µg/ml PMA, 20 min. Autorads are shown of cytosol (2×10⁶ cell eq), 5 hr exposure, and p67^{phox} immunoprecipitates (from 1×10⁷ cell eq cytosol), 16 hr exposure.

There are no reports in the literature of p67^{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^{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^{phox} was not uniform, the ³²P signal was normalised with respect to the protein (densitometric analysis). After five to ten minutes of stimulation the level of p67^{phox} phosphorylation had more than doubled.

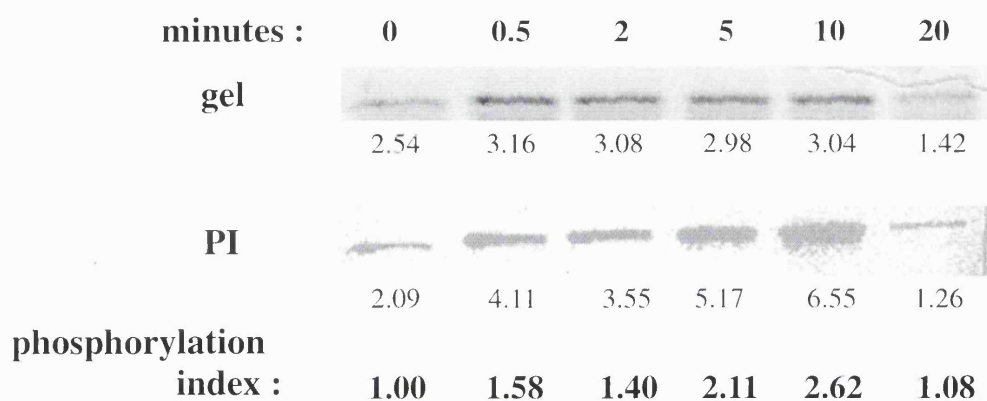
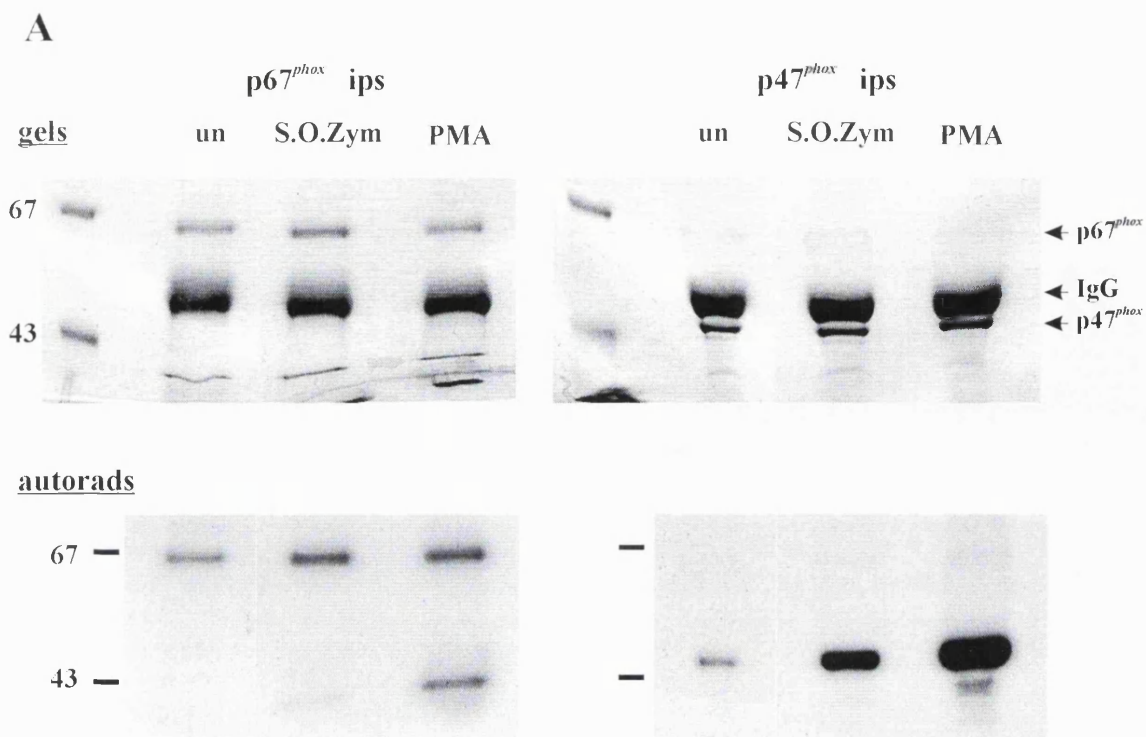


Figure 3.2.4 Timecourse of p67^{phox} phosphorylation by S.O.Zym stimulation. Neutrophils were radiolabelled with ³²P_i and then incubated with 1 mg/ml S.O.Zym for 20 min. Aliquots of cells were removed and lysed at timepoints for immunoprecipitation of p67^{phox} from the cytosol. Coomassie-stained gel and corresponding phosphorimage bands (PI) were quantified by band densitometry and the ratio of these values gave a level of phosphorylation. The phosphorylation is normalised against timepoint 0 (resting cells) to give the phosphorylation index.

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-



B

	S.O.Zym	PMA	fMLP
p67 ^{phox}	2.17 ± 0.52 (5)	3.24 ± 1.14 (4)	1.43 ± 0.17 (3)
p47 ^{phox}	4.97 ± 2.50 (5)	26.77 ± 10.12 (4)	2.82 (2)

Figure 3.2.5 Comparison of p67^{phox} and p47^{phox} phosphorylation in neutrophils. A: Immunoprecipitates of p67^{phox} or p47^{phox} from 4.5×10^7 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 μ g/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 p40^{phox} is visible as an extra band in the stimulated p67^{phox} 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 μ g/ml cytochalasin B followed by 1 μ M fMLP for 2 min. **B:** Tabulation of the level of increase in phosphorylation from multiple experiments (n value in brackets), \pm standard deviation for n > 2. Values derived from band densitometry and normalised against 1.00 for phosphorylation in unstimulated cells.

fold increase in p47^{phox} phosphorylation compared with the increase in p67^{phox} phosphorylation. The increase in phosphorylation is highest when the agonist is PMA, and is particularly high for p47^{phox}. The phosphorylation of p47^{phox} 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 p67^{phox} has not been previously studied.

For investigation of the number and nature of the p67^{phox} phosphorylation sites, 2-dimensional phosphopeptide mapping was used. Considerable time was spent on optimising the preparation of phosphorylated p67^{phox} immunoprecipitates and the use of a Hunter Thin Layer Electrophoresis apparatus. Passing radiolabelled cytosol through an anti-p67^{phox} 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 p67^{phox}, and including steps for reducing nonspecific binding so that there was resolution of a pure p67^{phox} band by SDS/PAGE.

In order to determine whether the increase upon stimulation was caused by the phosphorylation of multiple sites on p67^{phox}, 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 p67^{phox} phosphopeptide maps were very similar, both with one major tryptic peptide signal. Phosphopeptide mapping of neutrophil p67^{phox} 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 p67^{phox} was phosphorylated on threonine and serine in neutrophils and B cells (**Figure 3.2.6 insets**).

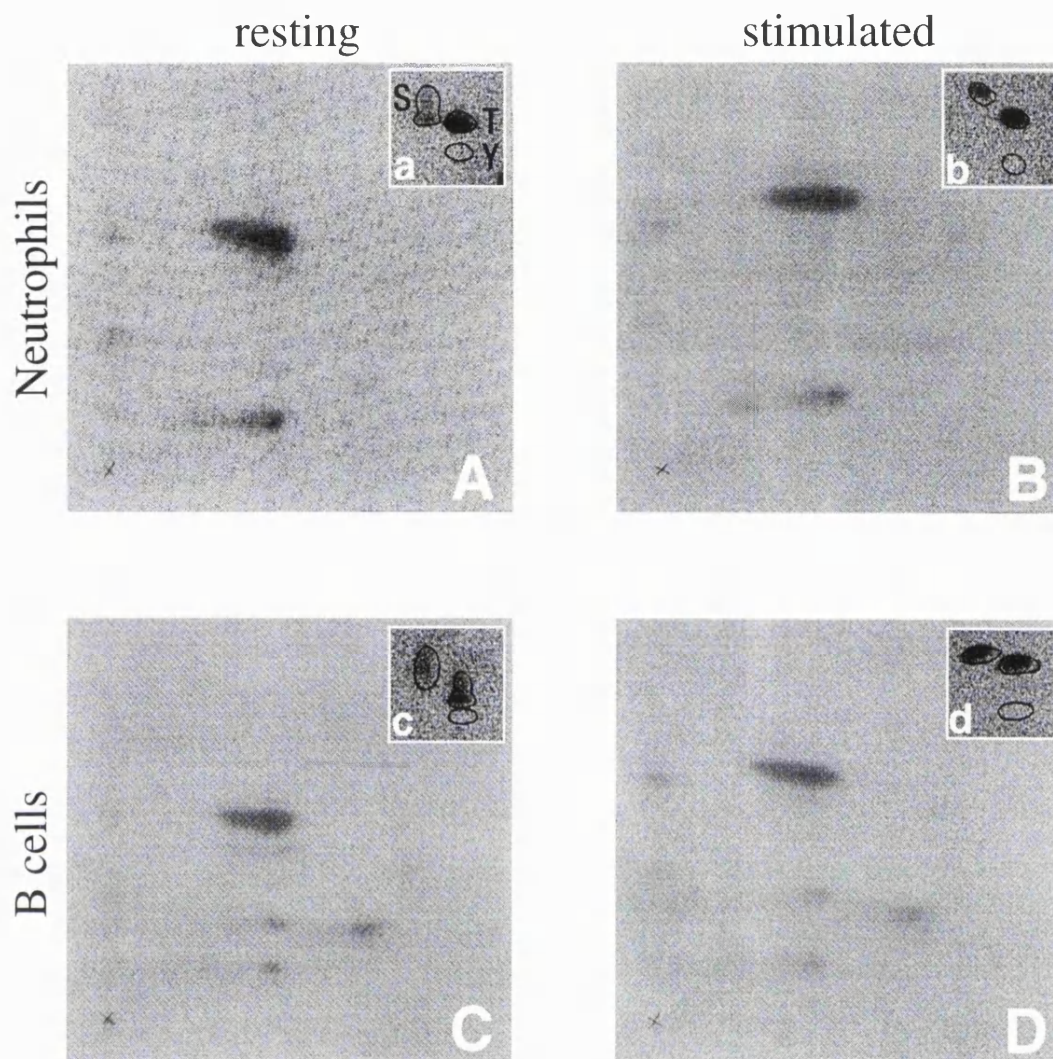


Figure 3.2.6 Phosphoamino acid and phosphopeptide maps of p67^{phox} before and after stimulation. p67^{phox} was immunoprecipitated from ³²P_i-labelled cells. **A:** neutrophils before stimulation, **B:** neutrophils after stimulation with S.O.Zym., **C:** unstimulated EBV-transformed B cells, and **D:** 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 (β PAK, 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^{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^{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^{phox} was immunoprecipitated from the phosphorylation reaction prior to running on SDS/PAGE. The p67^{phox} was immunoprecipitated from the cytosol of intact cells, as this was the only means of purifying sufficient protein for phosphopeptide mapping of p67^{phox} phosphorylated *in vivo*. It is interesting to note that the p67^{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^{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^{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^{phox} phosphorylation. Kinase activity was partially purified by a series of extraction and chromatography steps from membranes of

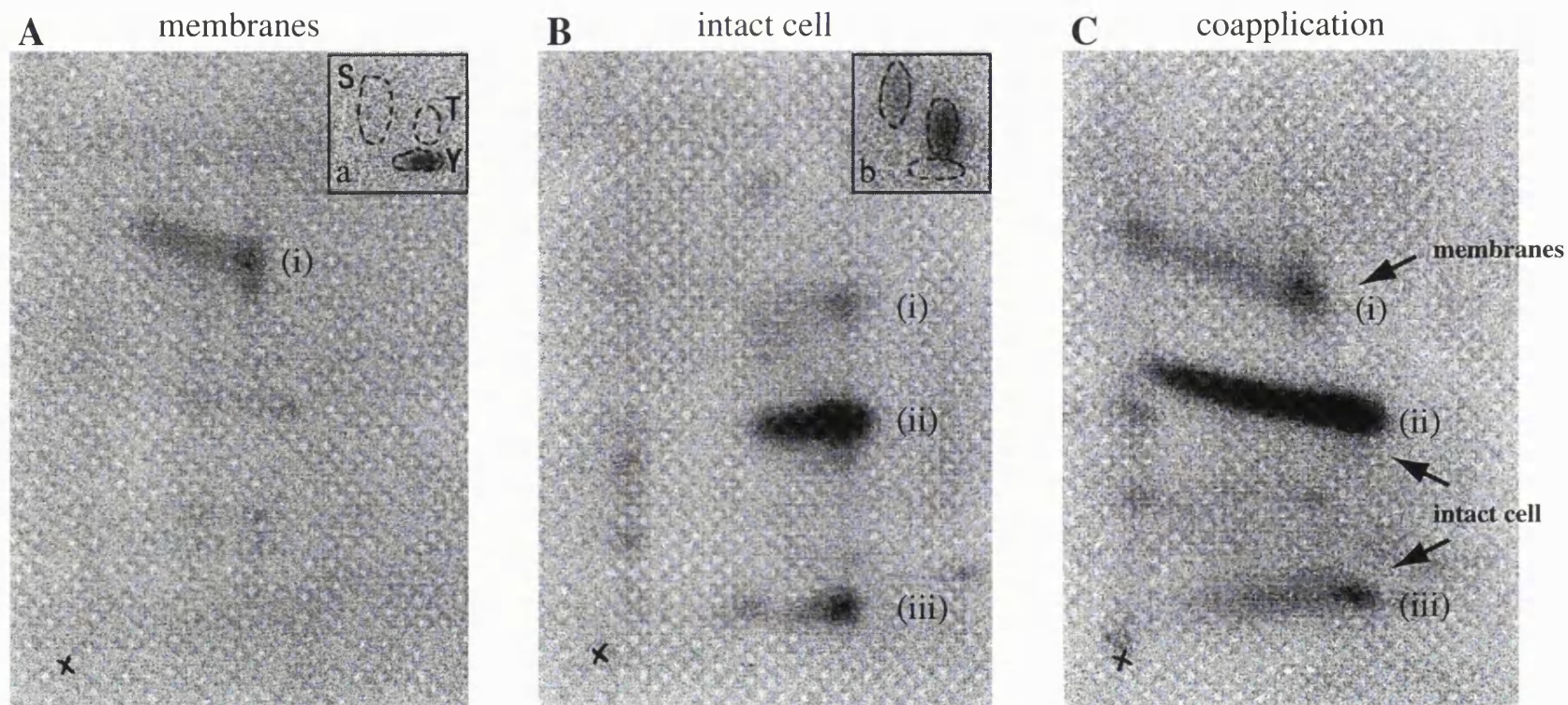


Figure 3.3.1 Phosphopeptide maps comparing isolated membrane and intact cell phosphorylation of $p67^{phox}$. 2D phosphopeptide analysis of **A**: *in vitro* phosphorylation of recombinant $p67^{phox}$ by unstimulated neutrophil membranes, **B**: neutrophil $p67^{phox}$ immunoprecipitated from cytosol 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 $p47^{phox}$, but the semi-purified material also phosphorylated $p67^{phox}$ (**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 $p67^{phox}$ (**Figure 3.3.3**). The 2D phosphopeptide map of $p67^{phox}$ 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 $p67^{phox}$.

The results from the membranes work suggested that either the $p67^{phox}$ 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 $p67^{phox}$ 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 $p47^{phox}$ (**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 $p67^{phox}$ phosphorylation are reported here.

The activation state of the neutrophils prior to sonication did not significantly affect $p67^{phox}$ 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 $p67^{phox}$ 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

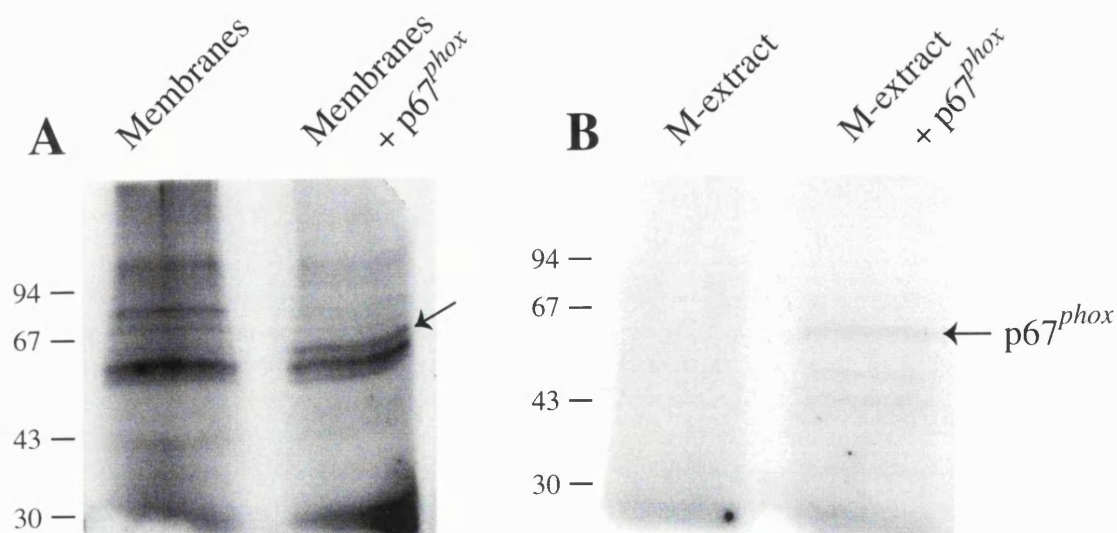


Figure 3.3.2 *In vitro* phosphorylation of p67^{phox} by neutrophil membranes and membrane-extracted kinase activity. Autorads of *in vitro* phosphorylation reactions applied to SDS/PAGE with the position of p67^{phox} indicated by an arrow. **A:** unstimulated membranes with and without added recombinant p67^{phox}. **B:** kinase-active fraction of a semipurified, salt wash of PMA-stimulated membranes.

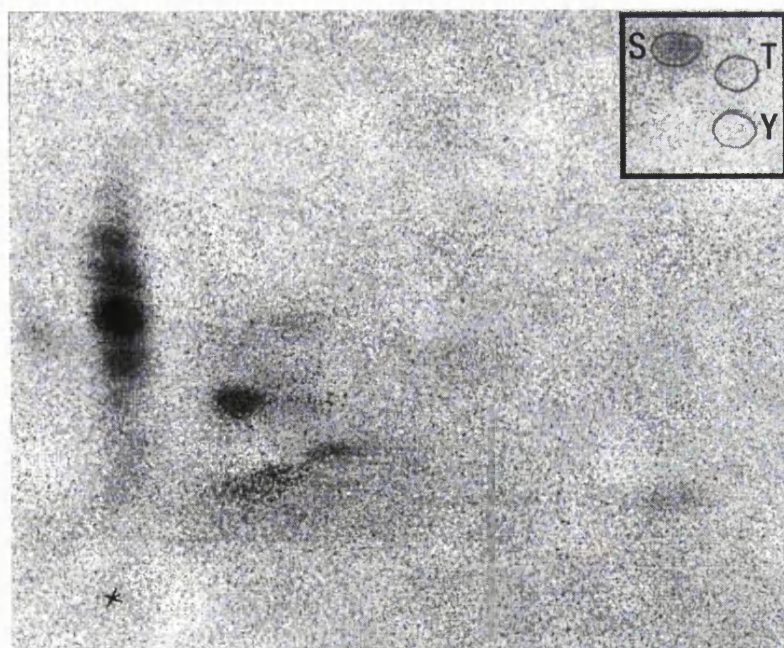


Figure 3.3.3 Phosphopeptide analysis of membrane-extracted kinase phosphorylation of p67^{phox}. 2D phosphopeptide map and phosphoamino acid analysis (inset, indicating phosphoserine (S)) of recombinant p67^{phox} phosphorylated *in vitro* by membrane-extracted kinase-active fraction.

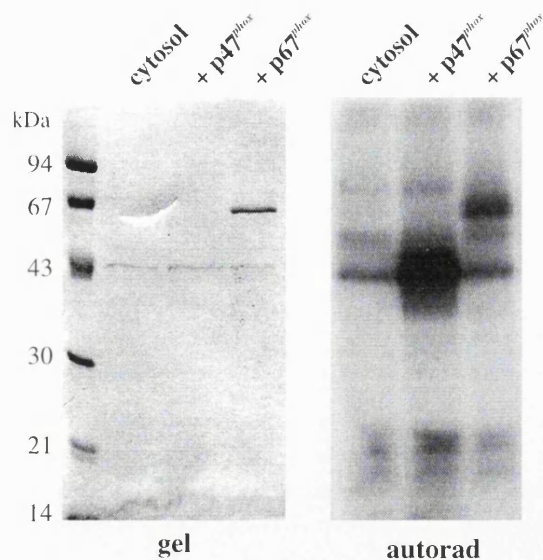


Figure 3.3.4 Comparison of *in vitro* phosphorylation of p67^{phox} and p47^{phox} by neutrophil cytosol. Coomassie-stained gel and autorad of *in vitro* phosphorylation reactions of recombinant p67^{phox} and p47^{phox} by unstimulated neutrophil cytosol. Background phosphorylation is shown as cytosol alone.

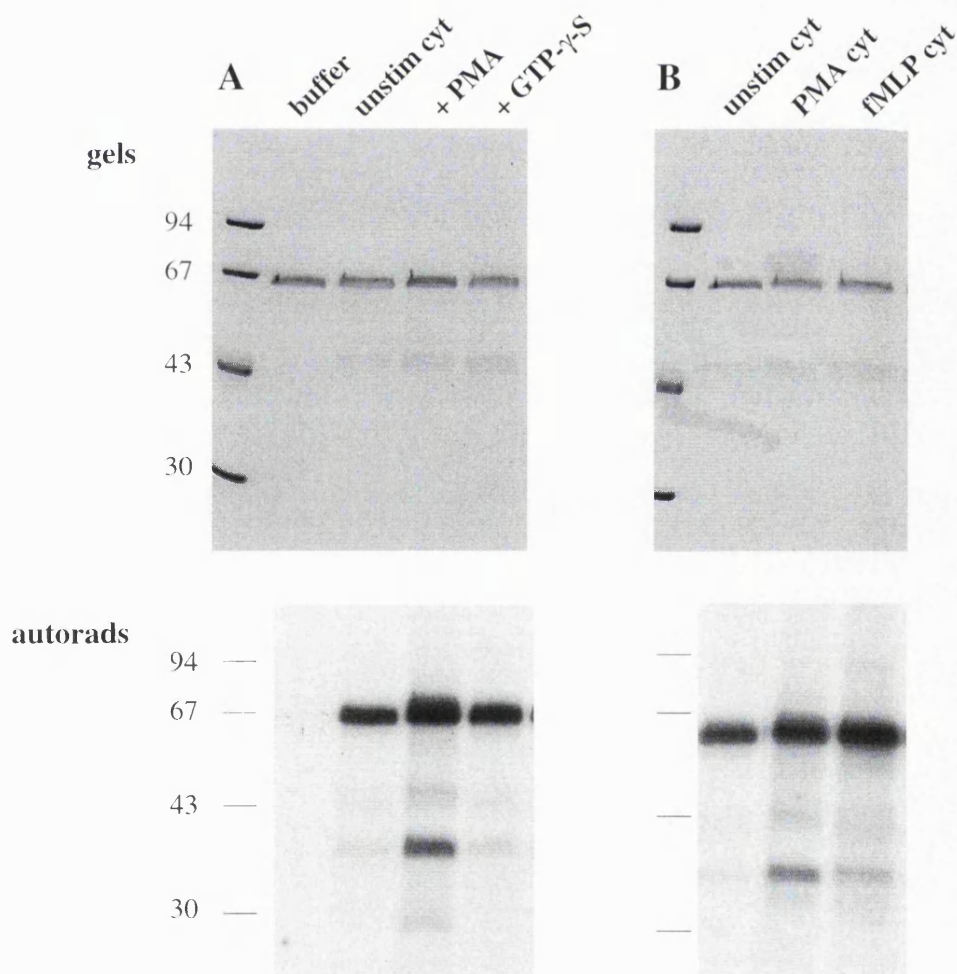


Figure 3.3.5 Variations of *in vitro* phosphorylation reactions of p67^{phox} with cytosol. Coomassie-stained gels and autorads of recombinant p67^{phox} with (A) buffer only, unstimulated cytosol with additions of PMA and GTP- γ -S, and (B) comparison of reactions with cytosol from and PMA- or fMLP-stimulated neutrophils.

the active GTP-bound form, and the constitutively active form Val-12 Rac1) and p40^{phox}, were also without an effect.

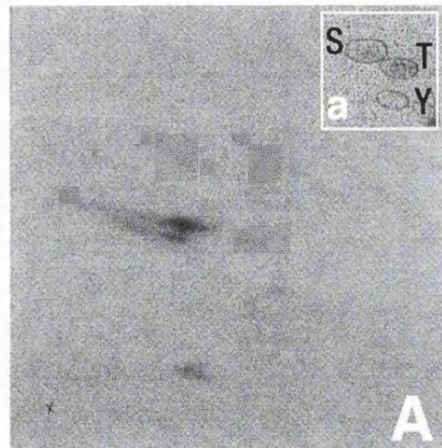
The addition of phosphatidic acid has been reported to increase the level of phosphorylation in a similar *in vitro* system (particularly for native p47^{phox}) using cytosol and membrane fractions together (McPhail *et al.*, 1995). This was investigated for p67^{phox}, where recombinant p67^{phox} was added to the system described in the paper. Although enhancement of total phosphorylation was observed, no increase in p67^{phox} phosphorylation was found. Therefore this was not a useful method to achieve enhanced labelling of p67^{phox}.

The results of phosphopeptide mapping of p67^{phox} 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 p67^{phox} phosphorylation.

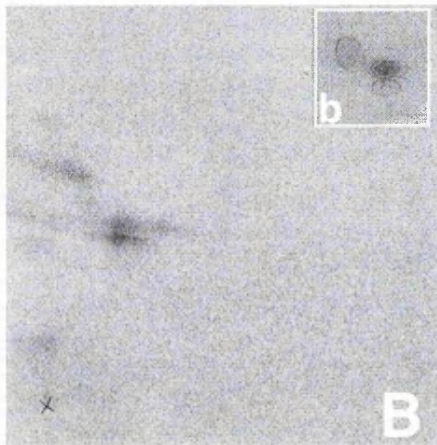
3.3b p67^{phox} phosphorylation by β PAK, PKC, and MAP kinase

Recombinant p67^{phox} 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.

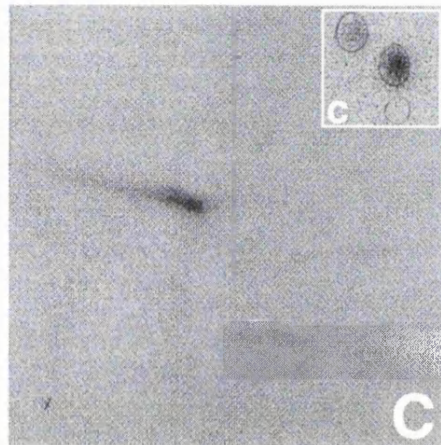
Neutrophils



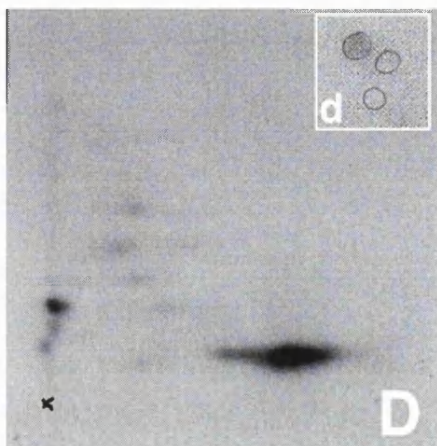
Cytosol



MAPK



PAK



PKC

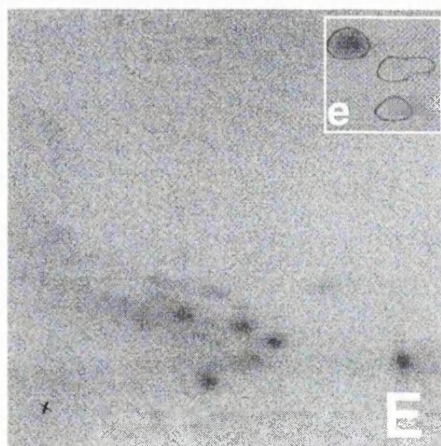
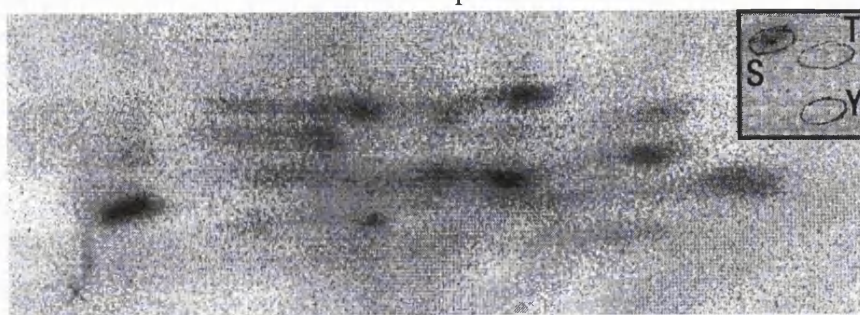


Figure 3.3.6 Phosphopeptide analysis of the *in vitro* phosphorylation of p67^{phox}. The 2D phosphopeptide map of (A:) p67^{phox} immunoprecipitated from S.O.Zym-stimulated neutrophils, is compared with phosphopeptide maps of *in vitro* phosphorylated recombinant p67^{phox} using different sources of kinase (B: neutrophil cytosol, C: recombinant MAP kinase, D: recombinant β PAK, 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^{phox}, since both PAK and p67^{phox} interact with Rac. A 68 kDa protein, closely related to brain β PAK 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^{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^{phox} has yet to be proven. A constitutively active, (therefore not requiring activation by Rac-GTP binding) GST-fusion recombinant β PAK 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 β PAK 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^{phox} did not mimic *in vivo* phosphorylation of p67^{phox} because β PAK was used and not neutrophil PAK. A suitable source of neutrophil PAK was not available for testing.

The phosphorylation of p47^{phox} by β PAK was also carried out. **Figure 3.3.7** shows that the maps from PMA-stimulated neutrophils and β PAK phosphorylation are not identical, but that some of the p47^{phox} phosphopeptides may be the same. Solely phosphoserine was detected for both PMA and β PAK phosphorylation of p47^{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^{phox} (Knaus *et al.*, 1995). These PAK preparations did not phosphorylate recombinant p67^{phox} under the same conditions. These findings support the conclusions drawn from the work with recombinant β PAK, in that p67^{phox}

Neutrophils



β PAK *in vitro*



Figure 3.3.7 p47^{phox} phosphorylation by β PAK. Phosphopeptide maps of (upper:) p47^{phox} immunoprecipitated from PMA-stimulated neutrophils, and (lower:) recombinant p47^{phox} phosphorylated by β PAK.

phosphorylation appears not to involve PAKs, whereas p47^{phox} could be a PAK substrate in neutrophils.

PKC was an obvious candidate kinase to undertake the phosphorylation of p67^{phox}. This is because PMA, a direct agonist of PKC, activates p67^{phox} phosphorylation in whole cells and was shown to give the same phosphopeptide map of p67^{phox} as the more physiological stimuli, fMLP and zymosan. However, the phosphopeptide map obtained with phosphorylation of p67^{phox} 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^{phox} phosphopeptide mapping, claims that PKC phosphorylated p67^{phox} 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, β PAK and neutrophil membranes, phosphorylated recombinant $p67^{phox}$ under the same conditions that they phosphorylated $p47^{phox}$ (**Figure 3.3.8**). Phosphopeptide mapping showed that the pattern of phosphorylation obtained with these kinases *in vitro* did not match the pattern of $p67^{phox}$ phosphorylation *in vivo*. Therefore these components appear to have no direct physiological role in the phosphorylation of neutrophil $p67^{phox}$. 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 $p67^{phox}$ 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 $p67^{phox}$, corresponding to 0.5-1.0% of $p67^{phox}$ molecules becoming phosphorylated. The concentration of ATP and kinase were not limiting in the experiments, as demonstrated by the phosphorylation of $p47^{phox}$ which was several times stronger. The low stoichiometry of $p67^{phox}$ is in stark contrast to $p47^{phox}$ which has been shown to be 1-8 mol phosphate/mol $p47^{phox}$ in some *in vitro* reactions (Park, J.-W. *et al.*, 1997). This difference in $p67^{phox}$ and $p47^{phox}$ 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 $p67^{phox}$ phosphorylation were not met in the cytosol and MAP kinase reactions.

Although the *in vivo* phosphorylation of $p47^{phox}$ was also greater than for $p67^{phox}$ (**Figure 3.2.5**), the difference between the phosphorylation of $p47^{phox}$ and $p67^{phox}$ 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 $p67^{phox}$ by cytosol, did not result in significant improvements to the stoichiometry of

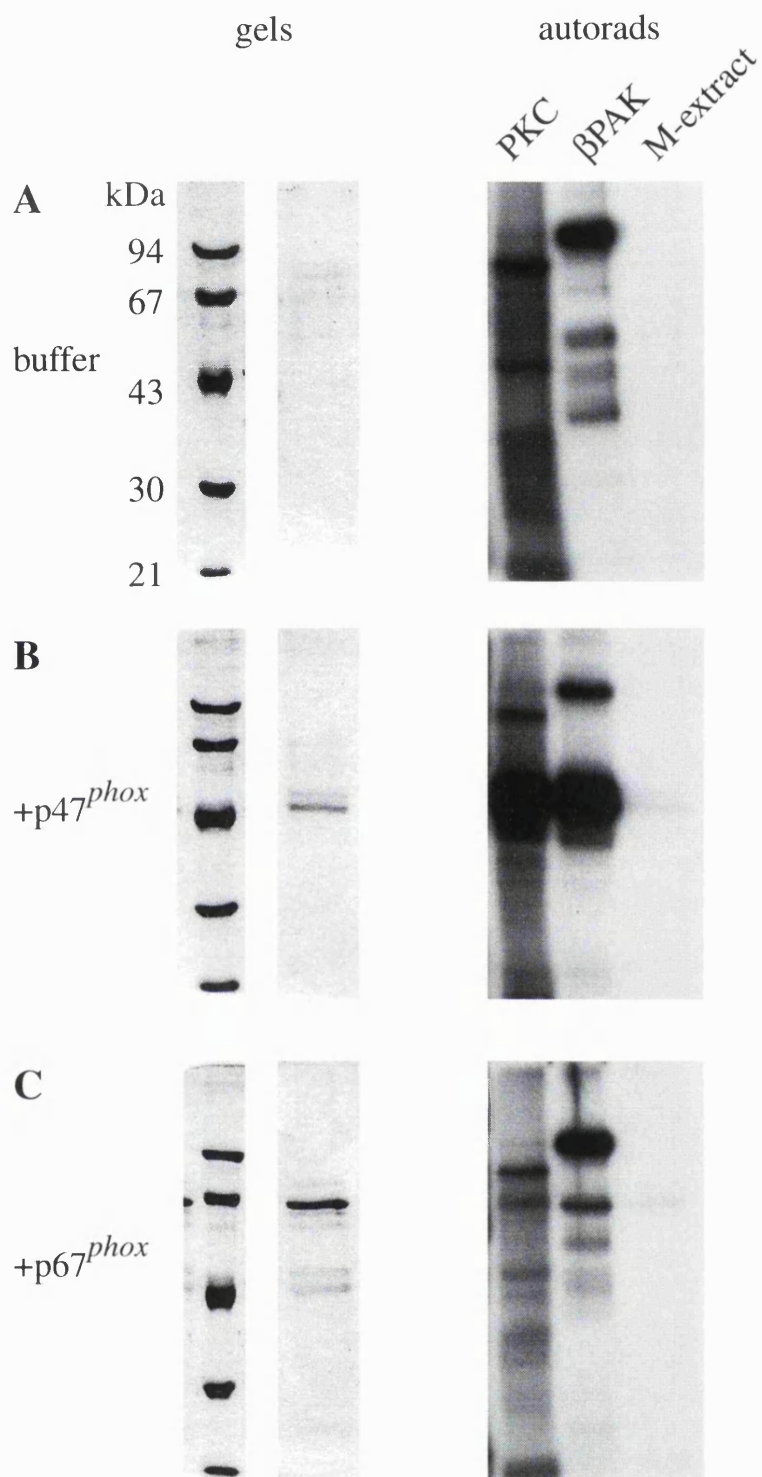


Figure 3.3.8 Comparison of *in vitro* phosphorylation of p67^{phox} and p47^{phox}. Coomassie-stained gels and autorads of *in vitro* phosphorylation reactions with PKC, βPAK, and membrane-extracted kinase activity. Background phosphorylation without protein substrate shown by buffer-only reactions (A), and with added recombinant p47^{phox} (B) or p67^{phox} (C). Film was exposed to the gels for 24 hr, -70°C.

phosphorylation. An explanation of the apparently limited phosphorylation of p67^{phox} *in vitro* could be that in the cytosol of neutrophils, native p67^{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^{phox} undergoes the same phosphorylation by isolated cytosol or MAP kinase as native p67^{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^{phox} phosphorylation *in vivo*.

3.3c Phosphorylation of insect cell recombinant p67^{phox}

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

The purification of p67^{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^{phox}. To test whether any insect cell proteins could show high affinity binding to p67^{phox}, a lysate of uninfected insect cells was passed down a

p67^{phox} affinity column prepared with GST-cleaved, bacterially expressed, p67^{phox}. No bands were detected in the eluant fractions by staining, or by autoradiography following incubation with ³²P-γ-ATP. This suggested that the bands co-purifying with insect cell expressed p67^{phox} were not strongly bound proteins. Also, immunoprecipitation of p67^{phox} from the ³²P-γ-ATP incubation mixture, showed that these two co-purifying bands could be dissociated from insect cell p67^{phox} (**Figure 3.3.9B**).

To determine whether the phosphorylation of insect cell p67^{phox} was due to the presence of the copurifying factors, the p67^{phox} was immunoprecipitated before it was incubated with ³²P-γ-ATP (**Figure 3.3.9C**). Even without the other bands, p67^{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^{phox}. A previous batch of insect cell p67^{phox} was tested and although the effect was less pronounced, it showed similar results.

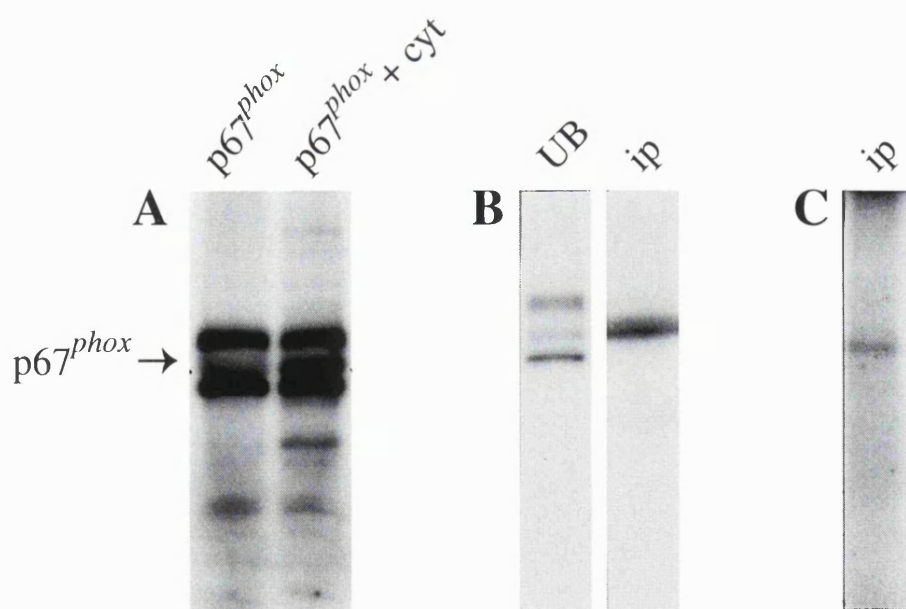


Figure 3.3.9 Phosphorylated bands of insect cell p67^{phox}. Autorads of insect cell expressed p67^{phox} after incubation with ³²P-γ-ATP. **A:** p67^{phox} incubated with ³²P-γ-ATP in the absence and presence of neutrophil cytosol. **B:** Unbound (UB) and immunoprecipitate (ip) fractions of p67^{phox} which had been incubated with ³²P-γ-ATP prior to immunoprecipitation procedure using antibodies to p67^{phox}. **C:** p67^{phox} was immunoprecipitated first, then the PAS beads were incubated with ³²P-γ-ATP.

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^{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^{phox} does not bind ATP or GTP (Leto *et al.*, 1991; Iyer, S.S. *et al.*, 1994). The fact that insect cell p67^{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^{phox} is autophosphorylated as there are no matches in the amino acid sequence of p67^{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^{phox} from insect cell cytosol.

Tryptic phosphopeptide mapping was carried out on insect cell p67^{phox}. The protein was incubated in ³²P-γ-ATP without added kinase, subjected to SDS/PAGE (an 8% acrylamide gel run overnight for good resolution) and the p67^{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^{phox} maps, but the comparison was not taken further. The use of insect cell recombinant p67^{phox} for *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^{phox}. Two papers have drawn comparisons between the activities of recombinant p67^{phox} and p47^{phox} from bacteria and insect cell systems in amphiphile-activated cell-free oxidase assays (Leto *et al.*, 1991; Abo *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.

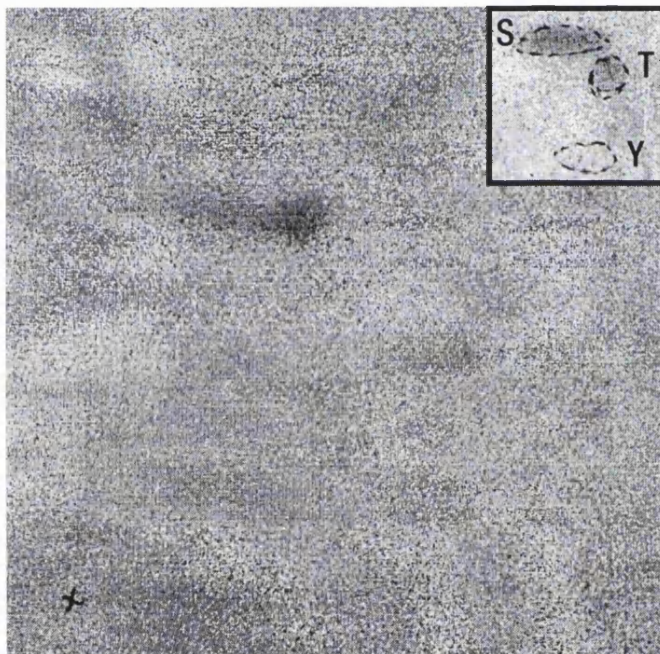


Figure 3.3.10 Phosphopeptide analysis of insect cell p67^{phox}. Insect cell expressed p67^{phox} was incubated with ³²P- γ -ATP, subjected to SDS/PAGE and the 67 kDa phosphoprotein was then excised for tryptic digestion and 2D phosphopeptide mapping. The phosphoamino acid analysis of the same sample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

SUMMARY OF CHAPTER 3 :

Characterisation of p67^{phox} phosphorylation

The low level of p67^{phox} phosphorylation in neutrophils can incur detection difficulties. The results presented here confirm the previous findings that p47^{phox} phosphorylation is more easily detected. There is debate in the literature regarding whether p67^{phox} is indeed phosphorylated. Therefore, the initial aim was to optimise the labelling methods, and determine if p67^{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^{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^{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^{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, β PAK, and PKC as possible kinases for p67^{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 p67^{phox}.

The results from this chapter formed the basic characterisation of p67^{phox} 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 p67^{phox}

4.1 Localisation of the p67^{phox} phosphorylation site

4.1 AIM :

To determine the position of the phosphorylation site on p67^{phox}.

4.1 INTRODUCTION :

In order to broadly locate the part of the p67^{phox} protein that is phosphorylated, cyanide bromide digestion was used. HPLC-mass spectrometry (MS) was then applied to analyse tryptic peptides of *in vitro* phosphorylated p67^{phox}. 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 :

p67^{phox} 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 p67^{phox} 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.

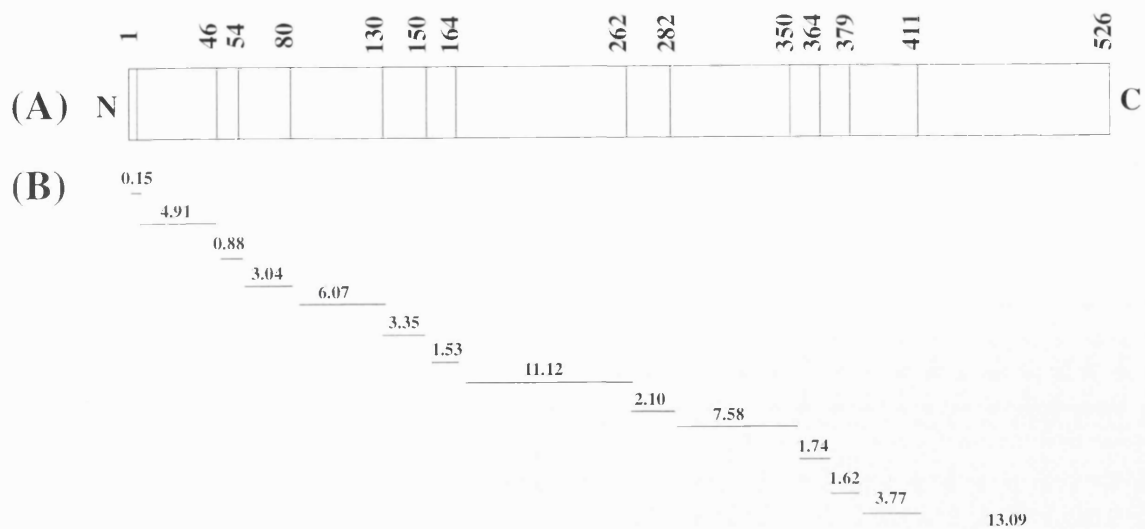


Figure 4.1.1 Diagram of the theoretical cleavage of $p67^{phox}$ by cyanogen bromide. **A:** The amino acid position of cyanogen bromide cleavage sites (methionine residues) are marked on a schematic diagram of $p67^{phox}$. **B:** Representation of the theoretical fragments from complete digestion of $p67^{phox}$, fragment size in kDa.

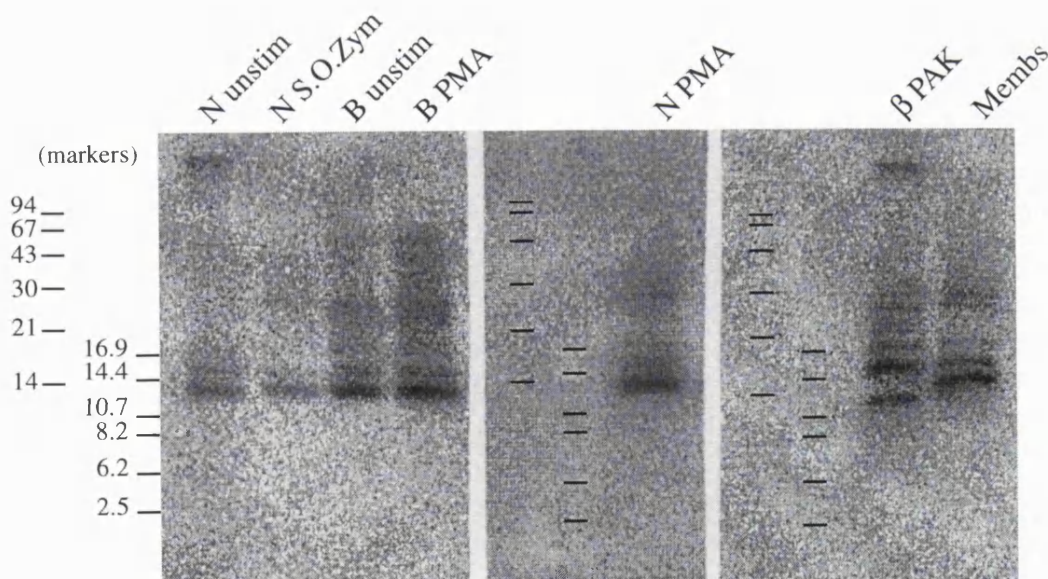


Figure 4.1.2 Cyanogen bromide digestions of phosphorylated $p67^{phox}$. $p67^{phox}$ was ^{32}P -phosphorylated, isolated by SDS/PAGE and digested by cyanogen bromide. Phosphorimages are shown of the tricine gels from three experiments. Tricine gel electrophoresis resolved the peptides obtained from $p67^{phox}$ immunoprecipitated from unstimulated, S.O.Zym.- and PMA-stimulated neutrophils (**N**), unstimulated and PMA-stimulated B cells (**B**), and recombinant $p67^{phox}$ phosphorylated *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^{phox}, showed that membranes phosphorylated the same region as *in vivo* phosphorylated p67^{phox}, but β PAK gave a different profile. As indicated by 2D phosphopeptide mapping (Chapter 3) both membranes and β PAK *in vitro* systems phosphorylated p67^{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^{phox} constructs made by Dr F. Wientjes, Professor Segal's laboratory, were applied to the same *in vitro* phosphorylation assay as full length p67^{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

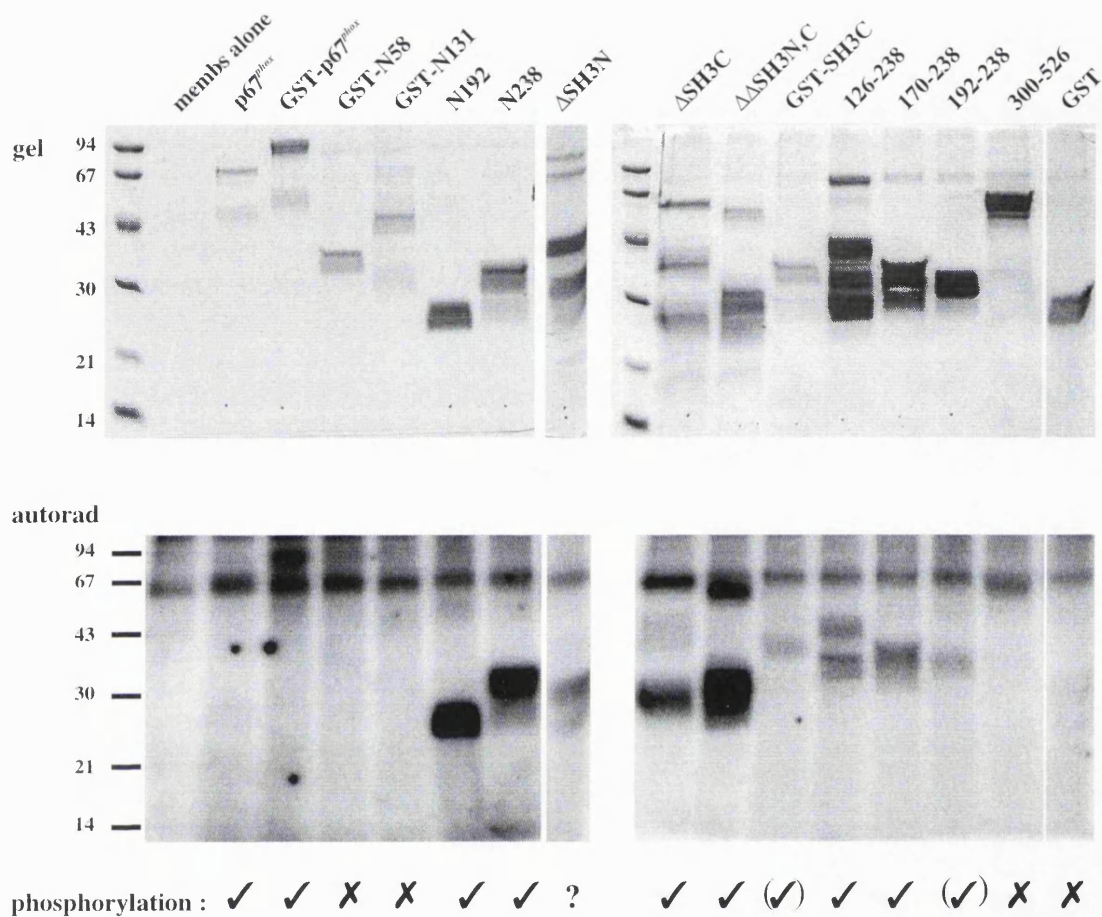


Figure 4.1.3 *In vitro* phosphorylation of recombinant fragments and mutants of p67^{phox} by neutrophil membranes. Coomassie-stained gels and autorads of *in vitro* phosphorylation reactions carried out with unstimulated membranes as the source of kinase. Recombinant substrates are described as GST-(fusion proteins), number of amino acids indicating length or position in p67^{phox}, 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 α N-192aa antibody, whereas the α C-20aa 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 ³²P-phosphorylated p67^{phox}. Tryptic digests of such radiolabelled p67^{phox} were applied to reverse phase HPLC to separate the peptides. Scintillation counting of the HPLC fractions allowed detection of the radiolabelled 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

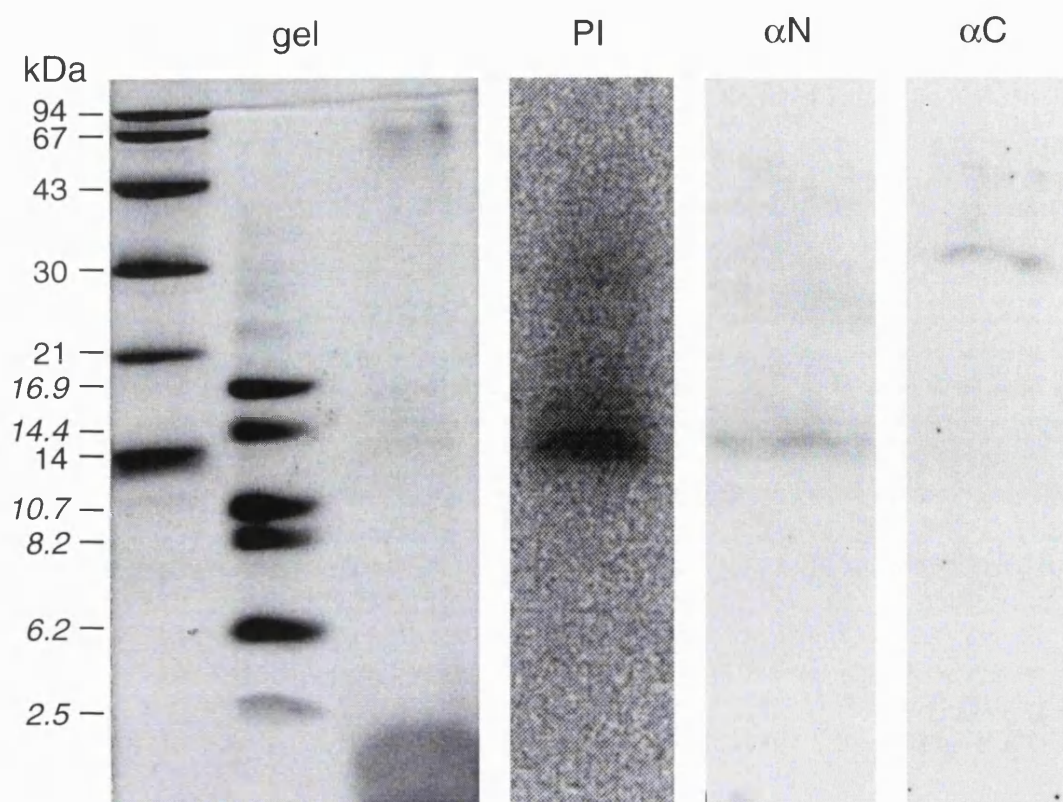


Figure 4.1.4 Localisation of p67^{phox} phosphorylation by immunoblotting. Cyanogen bromide digestions of immunoprecipitated p67^{phox} were subjected to tricine gel electrophoresis (gel, colloidal stain), and immunoblotted with peptide antibodies against the N-terminal 192 amino acids (α N), and C-terminal 20 amino acids (α C). The ECL system was used for detection. p67^{phox} immunoprecipitated from ³²P_i-labelled, PMA-stimulated neutrophils, was digested by cyanogen bromide and exposed in a phosphorimager (PI).

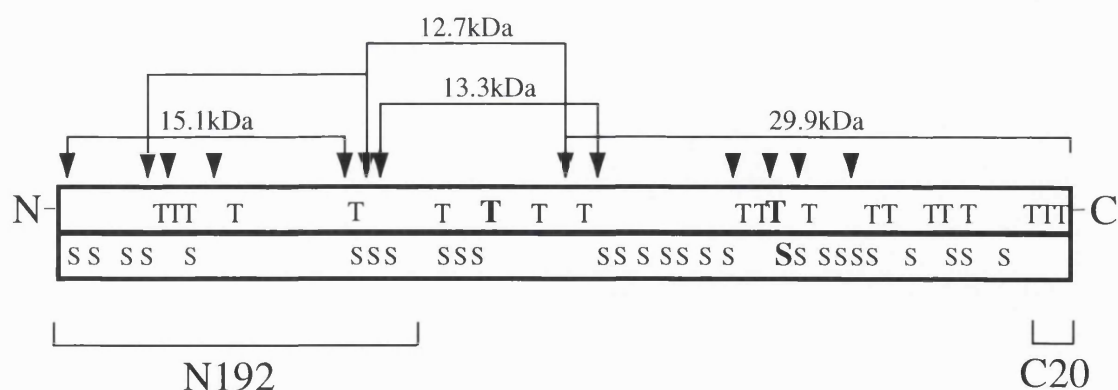


Figure 4.1.5 Position of p67^{phox} threonine and serine residues. The 21 threonines and 28 serines shown in relation to the cyanogen bromide cleavage sites (▼) and position of the N- and C-terminus antibody recognition sites (N192, C20). Possible partial digestion fragments detected by the N192 antibody (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.

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: $762 + 80 \text{ PO}_4^-$), 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 p67^{phox} 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 p67^{phox} 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

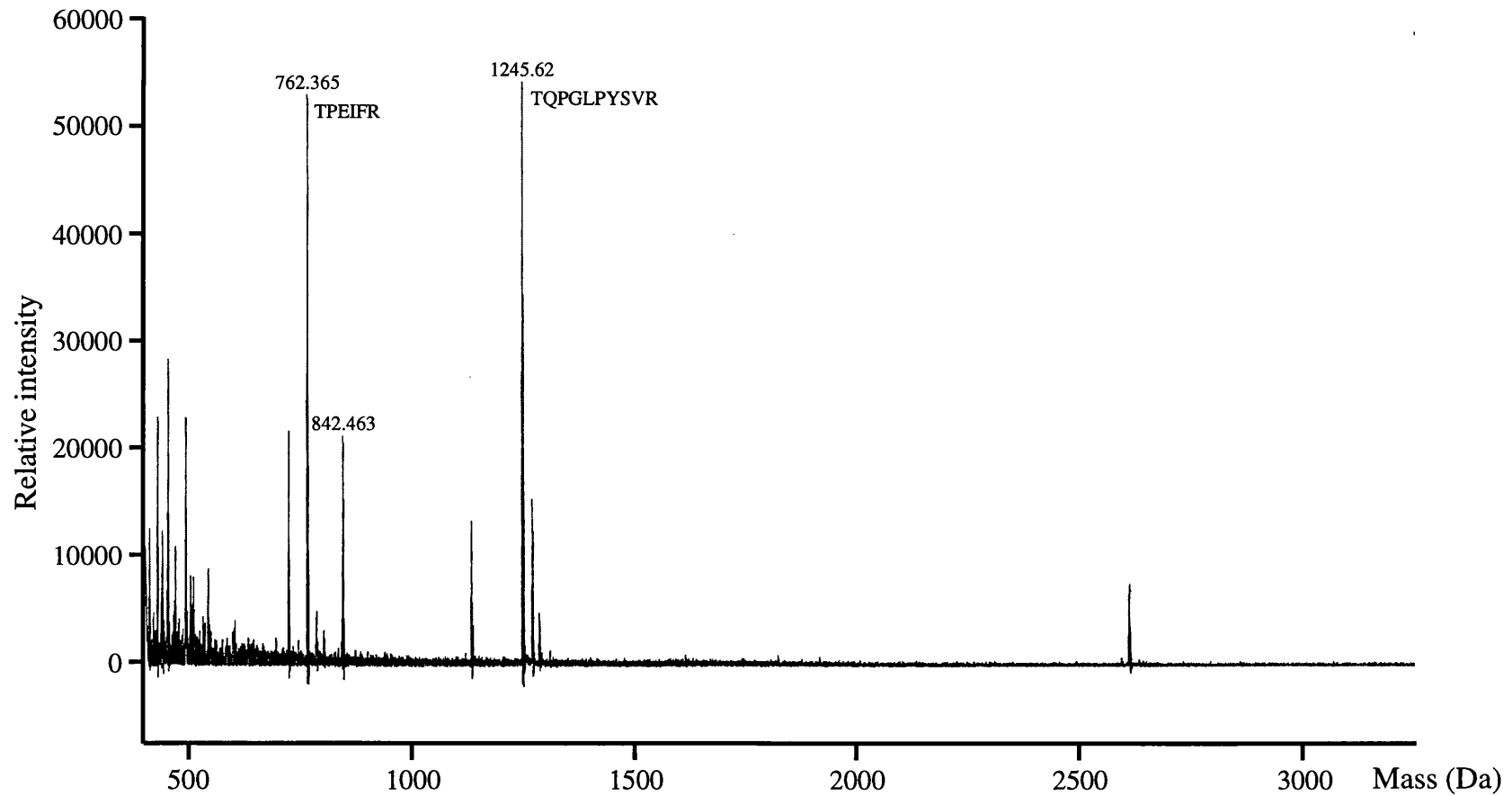


Figure 4.1.6 MS analysis of radiolabelled RP-HPLC fraction from tryptic digest of cytosol-phosphorylated p67^{phox}. Recombinant p67^{phox} was phosphorylated *in vitro* by neutrophil cytosol, gel purified, and digested by trypsin. The resultant peptide mix was applied to RP-HPLC, and the fractions analysed by scintillation counting to determine the elution of ³²P. 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 p67^{phox}. 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^{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^{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^{phox}. This section of work describes the site-directed mutagenesis of this residue, and the use of the resultant recombinant protein in the *in vitro* phosphorylation systems for p67^{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^{phox} to p67^{phox}-deficient cells, will be the key system for evaluating the importance of this phosphorylation site *in vivo*. Viral delivery of p67^{phox} constructs to EBV-transformed B cell lines of p67^{phox}-deficient CGD patients is the system currently being developed (Thrasher *et al.*, 1992; Weil *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^{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

p67^{phox}. 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^{phox}. The mutant p67^{phox} behaved in exactly the same way as wildtype p67^{phox} during the entire expression and purification procedure.

The ability for the Ala-233 mutant to act as a substrate for *in vitro* phosphorylation was examined in parallel with wildtype. The Ala-233 mutant and p67^{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^{phox} exposed to cytosol or MAP kinase *in vitro* (**Figure 4.2.1A**). Phosphopeptide mapping of the p67^{phox} Ala-233 mutant as substrate in both of these *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^{phox} was also subjected to HPLC-MS. The HPLC UV traces of the tryptic digests of mutant and wildtype p67^{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 *in vitro* system, but the same results were obtained for recombinant p67^{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

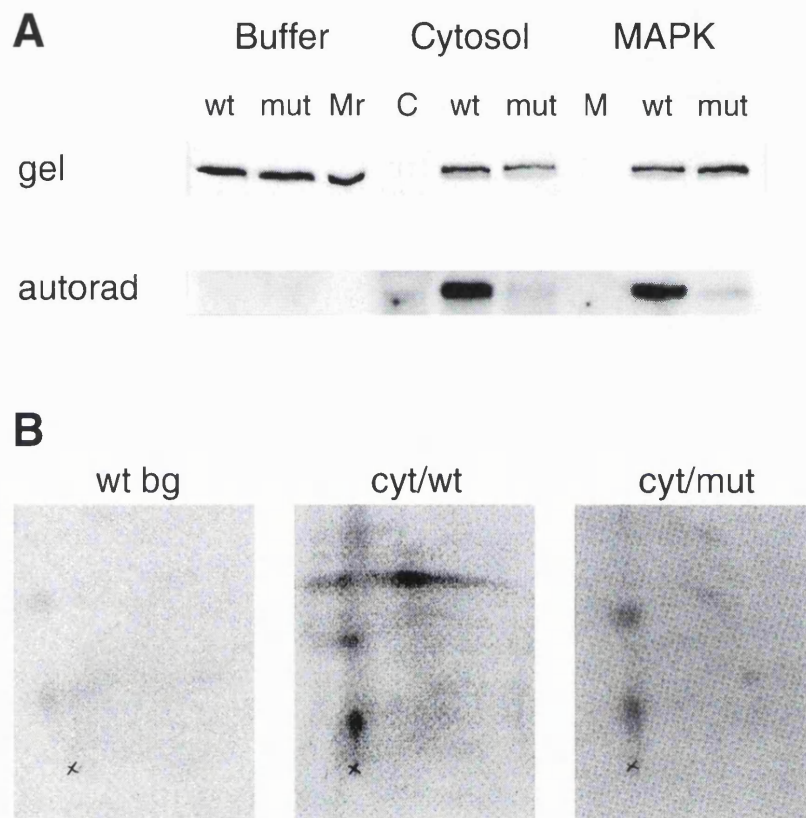


Figure 4.2.1 Effect on *in vitro* phosphorylation of Thr-233 to Ala substitution in p67^{phox}. **A:** Coomassie-stained gel and autorad showing p67^{phox} from *in vitro* phosphorylation reactions using wildtype p67^{phox} (wt) or Ala-233 mutant p67^{phox} (mut), with either no kinase (buffer), neutrophil 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 p67^{phox} wildtype background phosphorylation without added kinase (wt bg), p67^{phox} wildtype phosphorylated by cytosol (cyt/wt) and p67^{phox} Ala-233 mutant phosphorylated by cytosol (cyt/mut).

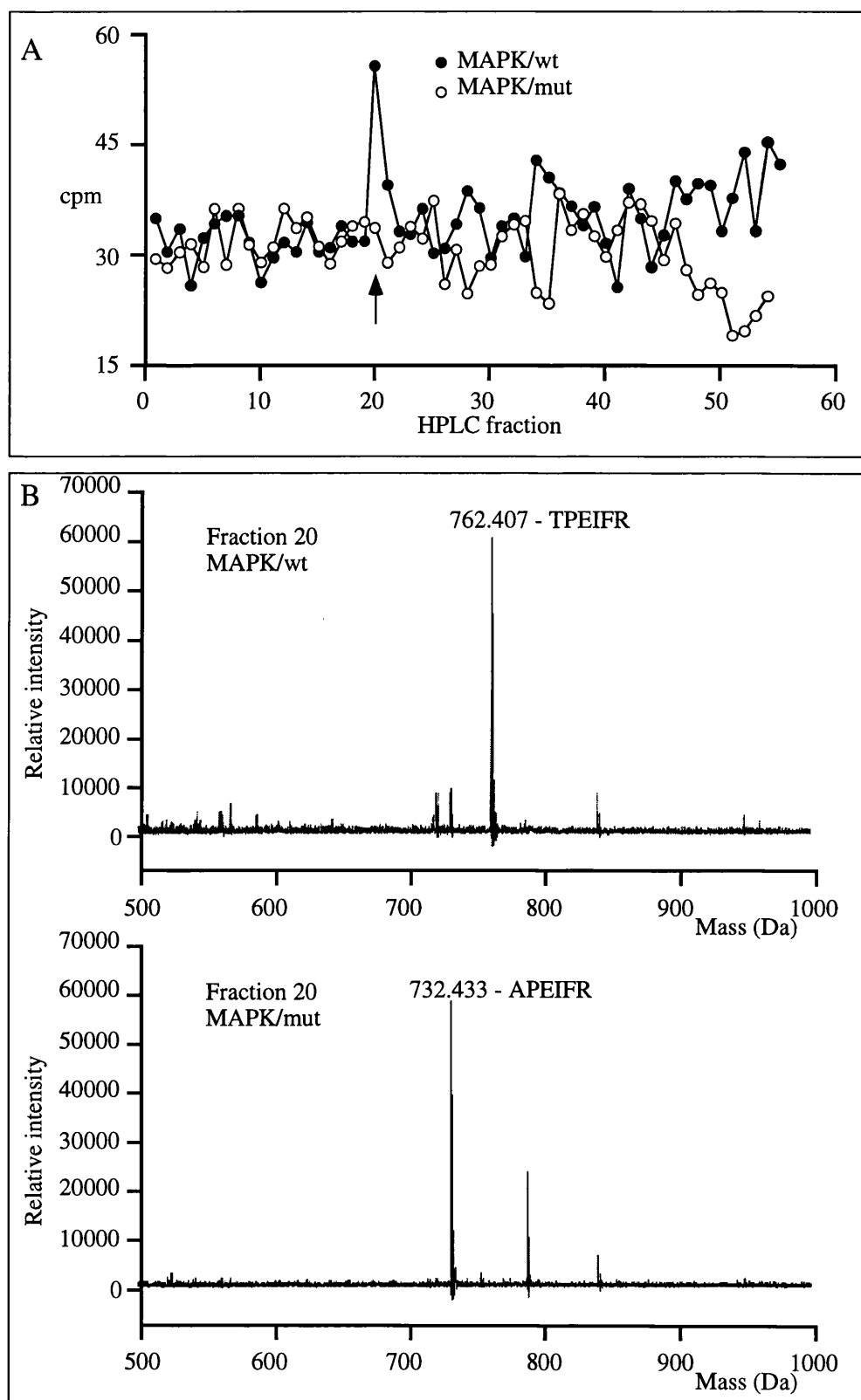


Figure 4.2.2 Peptide sequence analysis of HPLC fractions from *in vitro* phosphorylated p67^{phox}. **A:** one tenth of each fraction collected from HPLC resolution of tryptic peptides of MAP kinase phosphorylated p67^{phox} was subjected to scintillation counting. Fraction 20 contained radiolabel for p67^{phox} wildtype. **B:** fractions 20 of p67^{phox} 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 p67^{phox} was due to the absence of Thr-233.

The results presented here disagree with a very recent publication reporting that p67^{phox} 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 p67^{phox} (**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/TXR/K, (Woodgett *et al.*, 1986)
- CKII** casein kinase II phosphorylation site, S/TXXD/E, (Pinna, 1990)
- cA/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

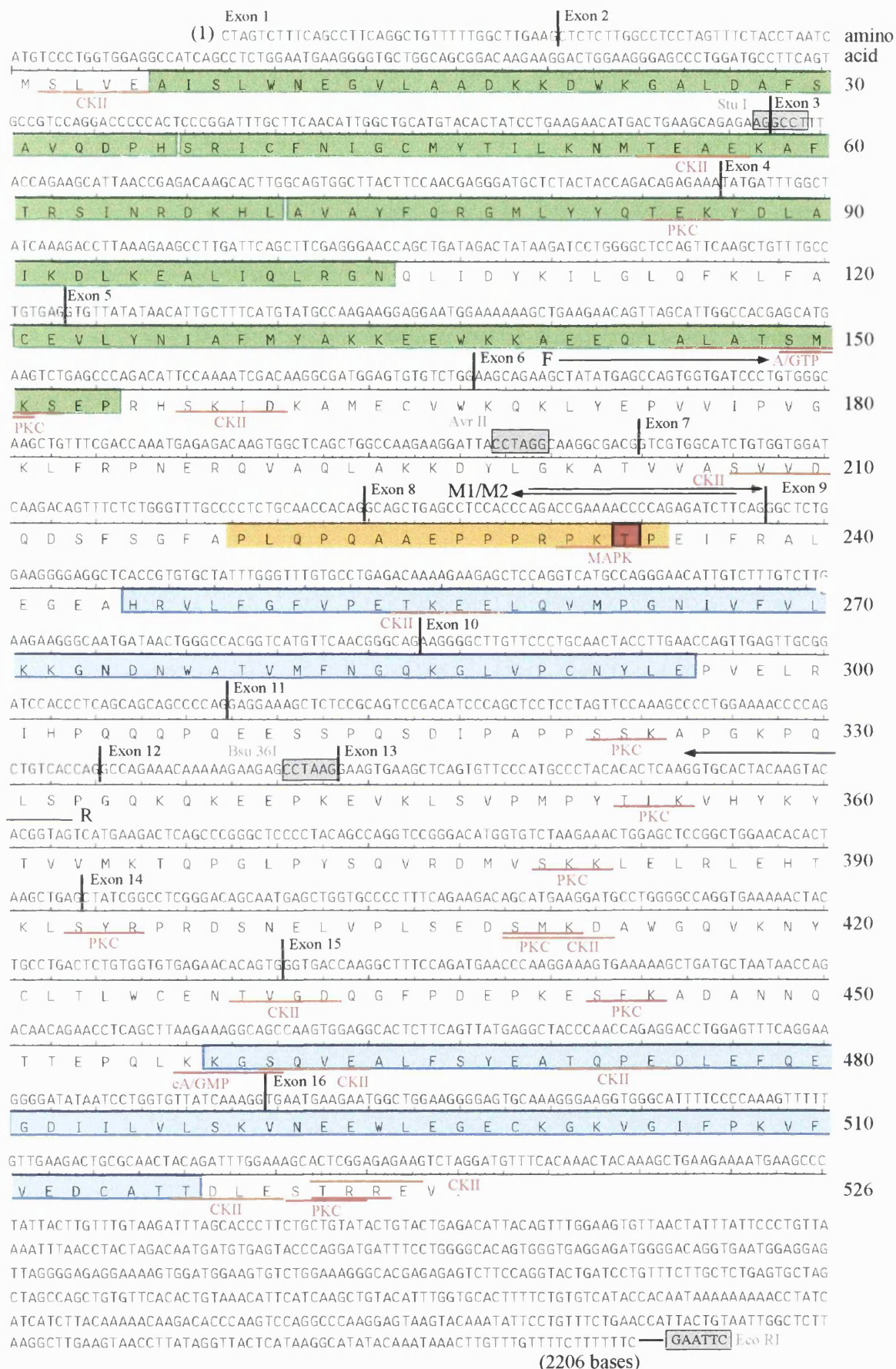


Figure 4.2.3 cDNA and amino acid sequence of p67^{phox}. p67^{phox} cDNA was cloned by screening an expression library derived from HL60 cells (Leto *et al.*, 1990). The gene was mapped to chromosome 1q25 (MIM number 233710) and named neutrophil cytosolic factor 2 (NCF 2) (Francke *et al.*, 1990). The 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.

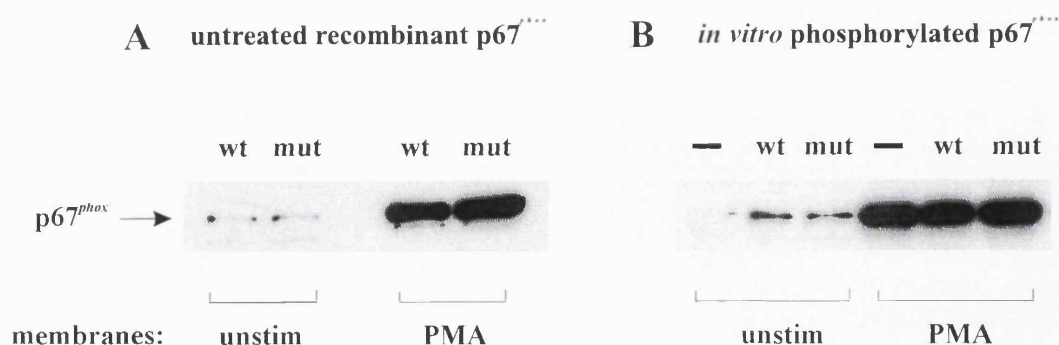


Figure 4.2.4 Membrane affinity experiment comparing wildtype and Ala-233 mutant p67^{phox}. Immunoblots of p67^{phox} bound to membranes of fractionated cell lysate from unstimulated and PMA-stimulated neutrophils. Wildtype (wt) and Ala-233 (mut) recombinant p67^{phox} were either **A**: added to neutrophil whole cell lysate without pretreatment, or **B**: subjected to *in vitro* phosphorylation by neutrophil cytosol and then added (- indicates no added recombinant p67^{phox}). Fractionation of the lysate with the added p67^{phox} was carried out by centrifugation through a sucrose gradient, and the membrane fraction was washed with 250 mM KCl prior to applying the samples to SDS/PAGE.

As a means for studying the *in vivo* phosphorylation of p67^{phox} and to demonstrate the effect of substituting alanine for threonine 233, attempts were made to introduce p67^{phox} to cells. The first attempt used cells of a p67^{phox}-deficient EBV-B cell line, which were electroporated in the presence of recombinant p67^{phox} protein. The only other similar experiment reported in the study of the NADPH oxidase is the delivery of a peptide of p47^{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^{phox}, and then homogenised. The homogenate was centrifuged to obtain crude cytosol which was analysed by immunoblotting for the presence of p67^{phox}.

Although $p67^{phox}$ 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 $p67^{phox}$ in the pellet (**Figure 4.2.5**). By nature, these pellets contain the cell membranes and other insoluble matter. This suggested that recombinant $p67^{phox}$ protein added to the electroporation medium either bound to the cell membranes to an artificially excessive extent, or the $p67^{phox}$ formed some other insoluble complex. The delivery of $p67^{phox}$ protein to cells by electroporation therefore, did not succeed in producing a whole cell model for studying the Ala-233 mutant.

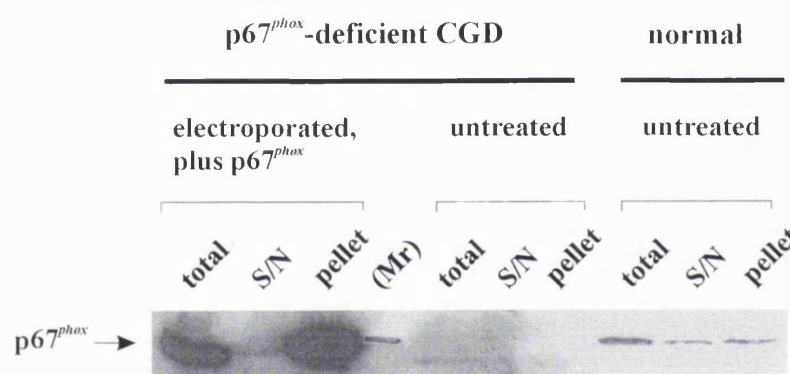


Figure 4.2.5 Electroporation of recombinant $p67^{phox}$ protein into $p67^{phox}$ -deficient B cells. Immunoblot for $p67^{phox}$ in untreated normal and $p67^{phox}$ -deficient EBV-B cells, and cells which had been subjected to electroporation in the presence of recombinant $p67^{phox}$ (plus $p67^{phox}$). Cells were sonicated and centrifuged at 50k rpm, 12 min, for cytosol supernatant (S/N) fraction and membrane-containing pellet. The loading of whole cell lysate (total) and S/N was 1×10^6 cell eq, and 5×10^6 cell eq of resuspended pellet. Detection was by ECL. The position of the 67 kDa molecular weight marker is shown (Mr).

An alternative approach is to deliver the DNA of $p67^{phox}$ for expression of the protein within the cells. Wildtype and mutant Ala-233 plasmid-cDNA expression constructs have been prepared for delivery of $p67^{phox}$ 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 p67^{phox} 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 p67^{phox} 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 p67^{phox} 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 p47^{phox}-deficient CGD patients, using *ex vivo* retroviral transduction of p47^{phox} (Malech *et al.*, 1997). Three other studies have applied the methods of reconstituting CGD B cells to examining the role of p47^{phox} phosphorylation sites (Faust *et al.*, 1995; as

discussed in Chapter 1, 1.7) and the requirement of regions within p67^{phox} (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^{phox} Ala-233 mutation.

4.3 Crystallisation trials for p67^{phox}

4.3 AIM :

To crystallise recombinant p67^{phox} for structure analysis, to lead to studies of structure-function associations within p67^{phox}.

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^{phox}? 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^{phox} 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^{phox} has suggested that p47^{phox} 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 p67^{phox} and subsequent crystallisation trials. Despite preparing p67^{phox} 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 p67^{phox}. It also removed the need for thrombin cleavage to generate full-length protein of p67^{phox}. Yields were higher with the insect cell system, which may also be due to lower proteolytic damage than in the bacterial system. p67^{phox}, 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 p67^{phox} 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 p67^{phox}-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.

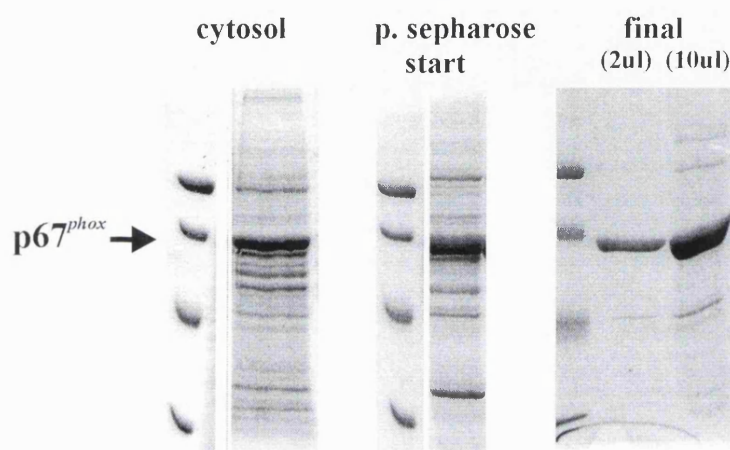


Figure 4.3 Purification of recombinant p67^{phox} for crystallisation trials. Coomassie-stained gel of insect cell cytosol after induction of p67^{phox} expression (cytosol). This was the start material applied to Q-sepharose FPLC, from which fractions were pooled for phenyl sepharose FPLC (p. sepharose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

Several attempts were made to further purify p67^{phox} 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 p67^{phox} 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 p67^{phox} 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 p67^{phox} 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 p67^{phox} (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^{phox}

The site of phosphorylation on p67^{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 led to the deduction of the most likely candidate threonine contained in the N-terminal half of p67^{phox}. To test the theory that Thr-233 was phosphorylated, mutagenesis of p67^{phox} was performed to introduce a single amino acid substitution to alanine 233. Only traces of phosphorylation of recombinant p67^{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^{phox}, remains to be determined. Attempts to crystallise p67^{phox} for structure determination were unsuccessful. For proper assessment of the function of p67^{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^{phox} have been made, and it is planned to use them in a reconstitution assay for evaluating the possible role of p67^{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 p67^{phox} phosphorylation.

As a more general approach to the study of the cellular significance of p67^{phox} 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 p47^{phox}. There is one previous report of reduced p67^{phox} phosphorylation in the presence of staurosporine (Dusi *et al.*, 1993), but the cytosolic 67 kDa protein was not proven to be p67^{phox}. Therefore the effect of staurosporine on the phosphorylation of p67^{phox} remained to be evaluated.

The inhibition of phosphatase activity by okadaic acid results in a massive increase in phosphorylation of many neutrophil proteins, including p47^{phox} (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 p67^{phox} phosphorylation in response to these inhibitors followed the same pattern as p47^{phox} phosphorylation in relation to the oxidase activity. If p67^{phox} phosphorylation trends were different, it would provide significant insight into the regulation of p67^{phox} phosphorylation.

5.1 RESULTS AND DISCUSSION :

In order to test whether MAP kinase is involved in p67^{phox} 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 μ M 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^{phox} was achieved. In control experiments, the phosphorylation of myelin basic protein (MBP) was used to determine the efficacy of the inhibitor (Pang *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^{phox} in the *in vitro* model to a similar level.

Table 5.1.1 shows the results of preincubation of cells with PD098059 before stimulation, which was followed by immunoprecipitation of p67^{phox}. After pretreatment with PD098059, p67^{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^{phox} phosphorylation findings. No effect was observed for S.O.Zym or PMA stimulation including the cells from experiments with >30% reduction of p67^{phox} phosphorylation. A significant reduction in the oxidase activity of fMLP stimulated cells occurred in all four experiments with PD098059 (**Table 5.1.1**), and even at the concentration of 10 μ M (data not shown), but this occurred without a significant change in p67^{phox} phosphorylation. In summary, no correlation was observed between the effect of PD098059 on p67^{phox} phosphorylation and NADPH oxidase activity.

Although these results do not prove a role for MAP kinase *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 *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 *et al.*, 1995). These may be the reasons why only partial inhibition of either MBP or p67^{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 p67^{phox} phosphorylation and NADPH oxidase activity. Alternatively, p67^{phox} phosphorylation is not a rate-limiting requirement for activation.

Stimulation	<u>p67^{phox} phosphorylation</u>				<u>NADPH oxidase activity</u>	
	+ PD	+PD (% control)			+PD (% control)	
resting	1.0	1.0	-		-	
S.O.Zym	2.1 ± 0.7	1.9 ± 0.4	97 ± 18	[3]	109	[2]
fMLP	1.4	1.5	108	[2]	51 ± 13	[4]
PMA	3.4 ± 1.3	2.4 ± 0.5	76 ± 22	[3]	89	[2]

Table 5.1.1 The effect of PD098059 inhibitor on phosphorylation of p67^{phox} in intact cells and on NADPH oxidase activity. p67^{phox} was immunoprecipitated in parallel from ³²P_i-labelled neutrophils that had been either untreated, or pretreated with PD098059 (+PD), prior to stimulation. Phosphorylation of p67^{phox} 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 p67^{phox} 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 $p67^{phox}$ phosphorylation, remain to be investigated further.

Experiments were conducted with the inhibitors staurosporine and okadaic acid to determine if $p67^{phox}$ 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 $p67^{phox}$ 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 $p67^{phox}$ in this context was to determine if the divergent effects on the oxidase activity corresponded to a similar pattern in $p67^{phox}$ phosphorylation.

The magnitude of phosphorylation was measured by densitometric analysis of immunoprecipitates from the cytosol of ^{32}P -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 $p47^{phox}$ was monitored as a source of comparison for the changes in $p67^{phox}$ phosphorylation (**Figure 5.1.3B**). Staurosporine had the effect of reducing the magnitude of basal and stimulated phosphorylation of $p67^{phox}$ and $p47^{phox}$. Okadaic acid caused significant enhancement of phosphorylation throughout the cytosol, including $p67^{phox}$ and $p47^{phox}$ phosphorylation.

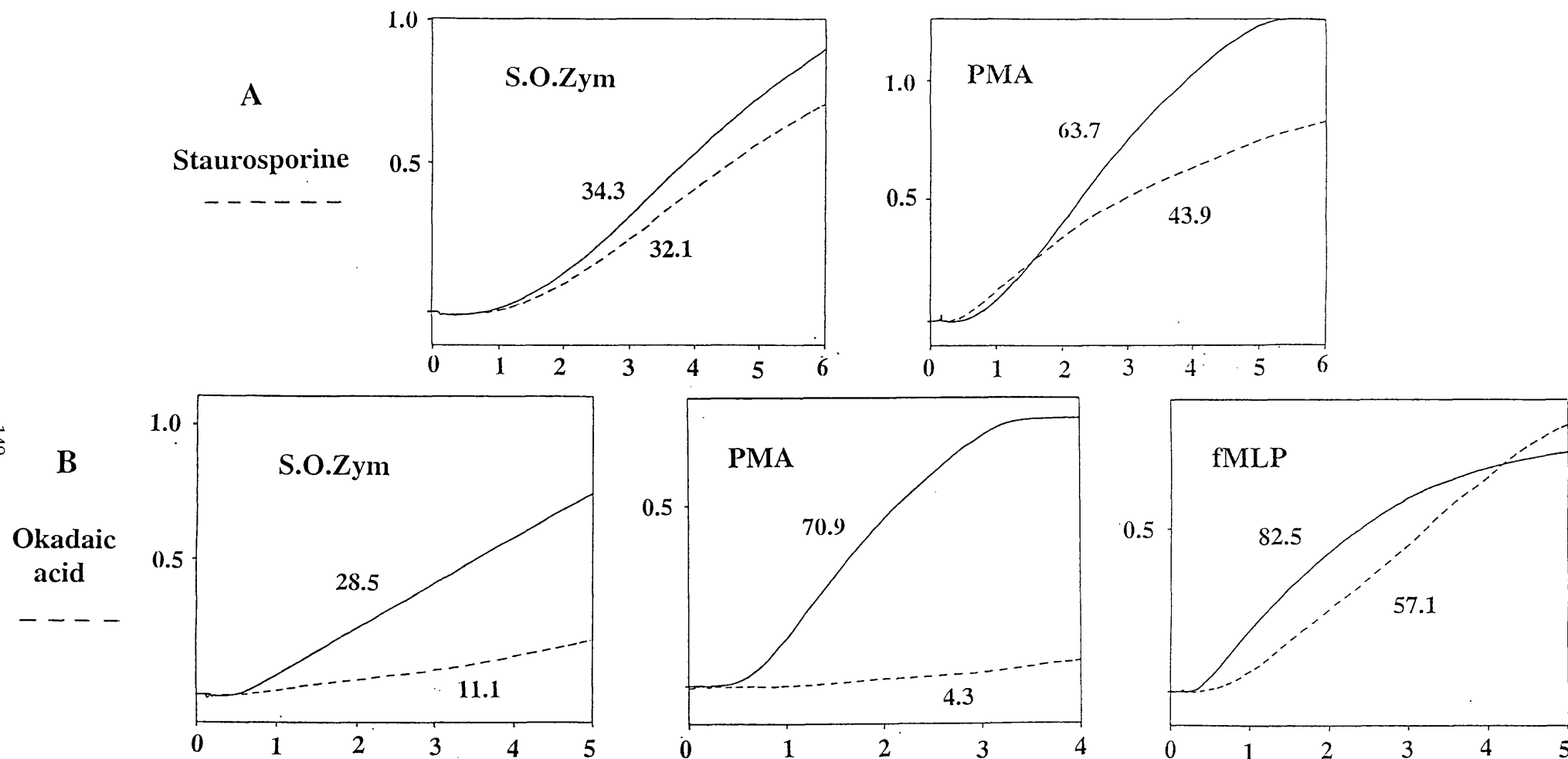
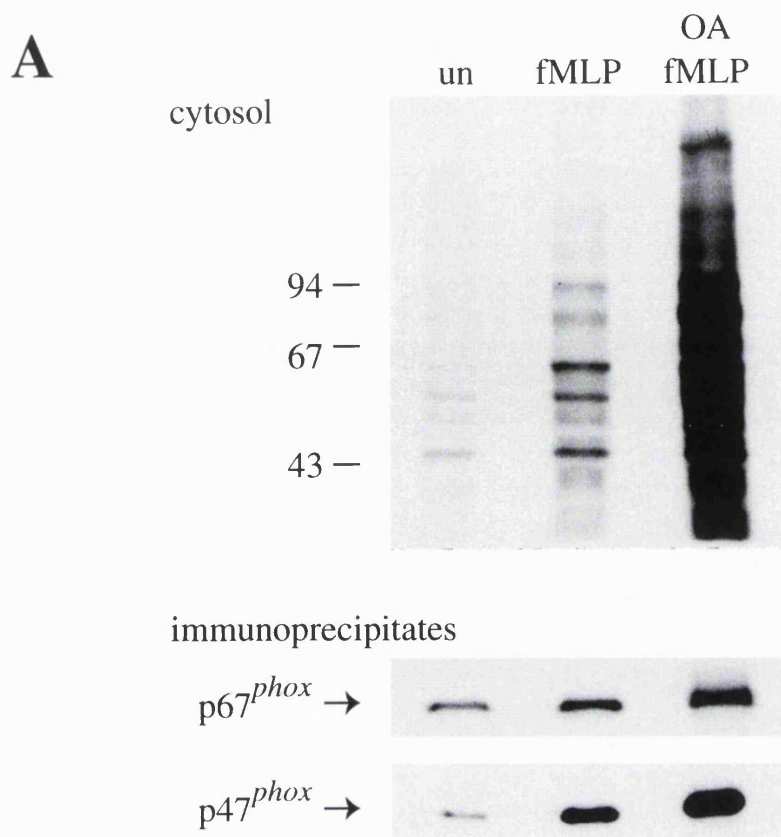


Figure 5.1.2 Effects of staurosporine and okadaic acid on superoxide production of neutrophils. Spectrophotometric analysis of the superoxide dismutase-inhibitable reduction of ferricytochrome c. X-axis: time, units $\times 100$ seconds, Y-axis: 550 nm absorbance, arbitrary units. **A:** Neutrophils 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 μ g/ml PMA. **B:** Preincubations in 1 μ M okadaic acid were carried out at 37°C for 30 min. The 0.5 μ M fMLP stimulation followed a 5 min preincubation with 5 μ g/ml cytochalasin B. Maximum rates of superoxide production are shown, nmol O₂⁻/min/10⁷ cells.



B

	without inhibitor				+ Staurosporine			+ Okadaic acid			
	un	fMLP	S.O.Z	PMA	un	S.O.Z	PMA	un	fMLP	S.O.Z	PMA
p67 ^{phox}	1.00	1.60	2.63	4.00	0.76	1.78	3.03	1.33	3.36	6.31	8.70
p47 ^{phox}	1.00	3.98	5.50	19.3	0.71	2.92	10.56	6.22	6.41	18.85	30.74

Figure 5.1.3 Effect of staurosporine and okadaic acid on phosphorylation of p67^{phox} and p47^{phox} in intact cells. Neutrophils were radiolabelled, stimulated, and p67^{phox} and p47^{phox} were immunoprecipitated from the cytosol fraction. **A:** Illustration of the raw results from which phosphorylation was quantified. The autorads are shown of (cytosol:) 5×10⁵ cell eq cytosol from unstimulated, fMLP-stimulated cells, and cells which were pretreated with okadaic acid (OA) then fMLP-stimulated, 16 hr exposure. (Immunoprecipitates:) p67^{phox} and p47^{phox} bands from immunoprecipitates of 3.3 and 1.7×10⁷ cell eq cytosol respectively, 6 day exposure. **B:** Quantitation of changes in phosphorylation of p67^{phox} and p47^{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 $p67^{phox}$ and $p47^{phox}$ phosphorylation are influenced in the same way as each other by staurosporine and okadaic acid. $p67^{phox}$ 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 $p67^{phox}$ might have in regulating the NADPH oxidase.

An interesting observation was made in these experiments. Okadaic acid caused hyperphosphorylation of $p67^{phox}$, 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 $p67^{phox}$. Immunoblotting revealed that both bands of the doublet were $p67^{phox}$ (**Figure 5.1.4**). The co-precipitation of $p47^{phox}$ and $p40^{phox}$ is also shown by immunoblotting. It seems that the additional band of $p67^{phox}$ is due to the slower migration of $p67^{phox}$ 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 $p67^{phox}$ 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. $p47^{phox}$ 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

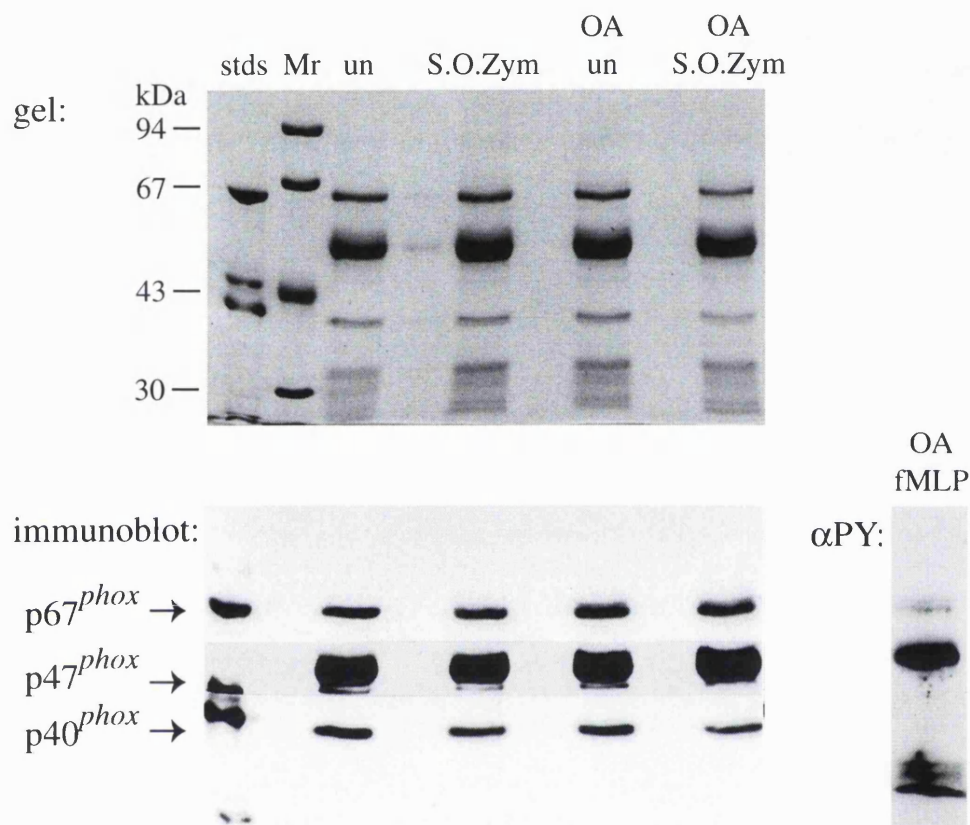
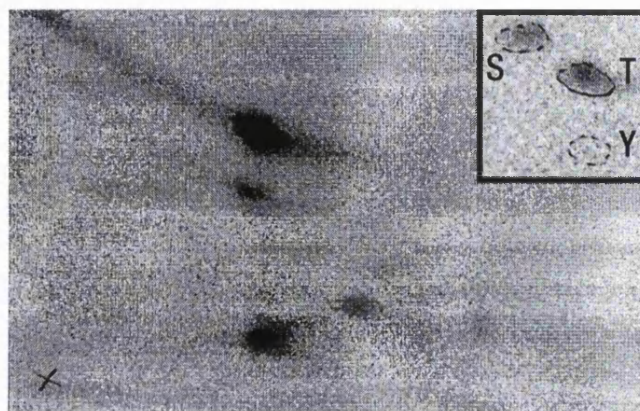


Figure 5.1.4 Immunoprecipitation of p67^{phox} from okadaic acid-treated cells. Coomassie-stained gel and immunoblots of p67^{phox} immunoprecipitations from neutrophil cytosol. The migration of p67^{phox}, p47^{phox} and p40^{phox} are indicated by the stained molecular weight markers (Mr) and recombinant protein standards (stds), and the immunoblot against all three proteins. The gel shows very weak staining of an extra band of p67^{phox} with okadaic acid (OA) treatment, which is detected by the immunoblot. An anti-phosphotyrosine (αPY) immunoblot of an equivalent immunoprecipitation of p67^{phox} from stimulated neutrophils, shows a weakly visible doublet of bands at 67 kDa.

stimulated neutrophils



OA-treated, stimulated neutrophils

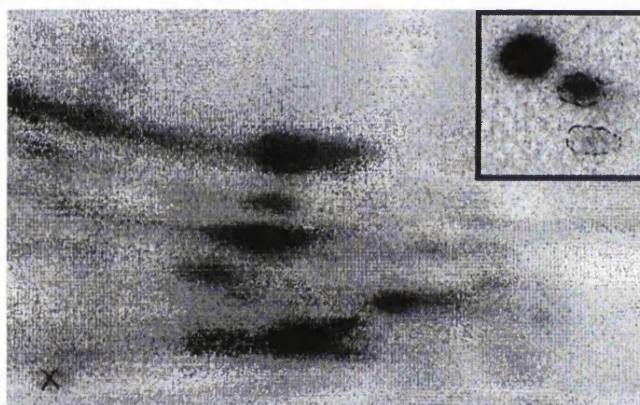


Figure 5.1.5 Phosphopeptide and phosphoamino acid analysis of p67^{phox} from okadaic acid-treated cells. p67^{phox} was immunoprecipitated from neutrophils which were stimulated in the absence (upper panel) or presence (lower panel) of okadaic acid (OA). p67^{phox} was gel purified, and subjected to 2D tryptic mapping for phosphopeptide analysis, and acid hydrolysis for amino acid analysis (insets). Immunoprecipitated p67^{phox} 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 p67^{phox} tyrosine phosphorylation

5.2 AIM :

To investigate the possibility of tyrosine phosphorylation of p67^{phox}.

5.2 INTRODUCTION :

Phosphoamino acid analysis has shown that phosphorylated p67^{phox} 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 p67^{phox} using this method after the p67^{phox} was phosphorylated *in vitro* by membranes (section 3.3), and in p67^{phox} separated from intact cells after incubation in okadaic acid (section 5.1). This raised the possibility of undetected tyrosine phosphorylation occurring in the cell. p67^{phox} may undergo tyrosine phosphorylation after translocation to the membrane, or cytosolic p67^{phox} 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 p67^{phox} occurs naturally *in vivo*, two approaches were taken in this study; the isolation of p67^{phox} 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 3×10^8 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.

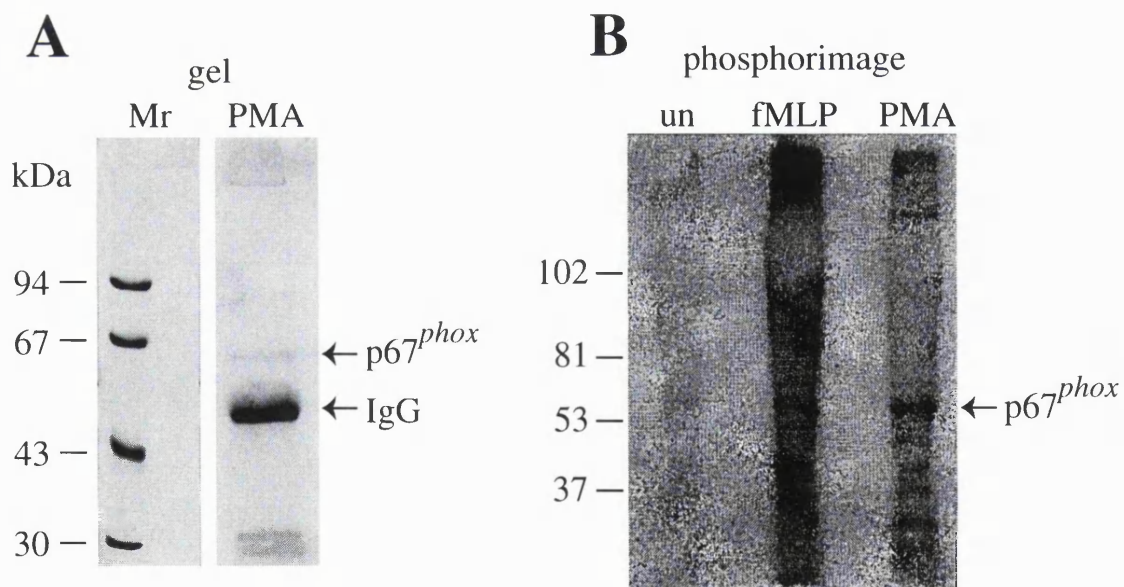


Figure 5.2.1 Immunoprecipitation of p67^{phox} from neutrophil membranes. **A:** Coomassie-stained gel of p67^{phox} immunoprecipitated from the membrane fraction of 7.5×10^8 neutrophils prelabelled with $^{32}\text{P}_i$. **B:** Phosphorimage after 6 days exposure of p67^{phox} immunoprecipitations from the membrane fractions of 7.5×10^8 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} *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 *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 *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

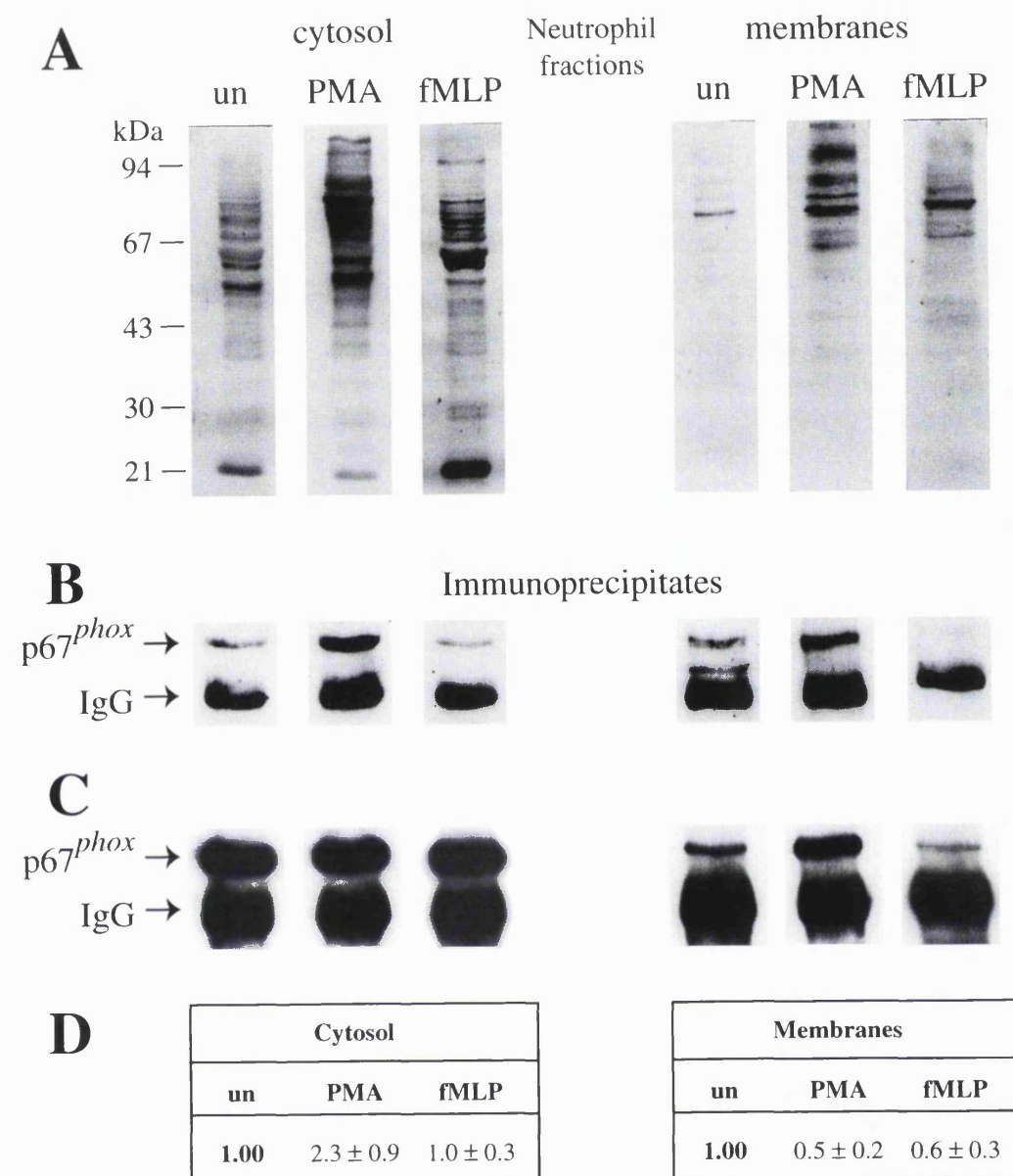


Figure 5.2.2 Phosphotyrosine analysis of p67^{phox} immunoprecipitated from neutrophils. **A:** Anti-phosphotyrosine immunoblots of 7.5×10^5 cell eq cytosol and 4.5×10^7 cell eq membranes from unstimulated, PMA- or fMLP-stimulated neutrophils. The blots were developed by the ECL method. **B:** Anti-phosphotyrosine 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 \pm standard deviation.

phosphorylated. The conclusion is that the weak tyrosine phosphorylation of p67^{phox} 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 *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 $p67^{phox}$ and $p47^{phox}$ 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 $p47^{phox}$ -deficient or $p67^{phox}$ -deficient CGD patients provide ideal tools to test whether $p67^{phox}$ and $p47^{phox}$ undergo normal phosphorylation in the absence of the other. It is known that $p47^{phox}$ does become phosphorylated in $p67^{phox}$ -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 $p47^{phox}$ was observed by 2D electrophoresis of normal and $p67^{phox}$ -deficient CGD neutrophil cytosol and membranes (Okamura *et al.*, 1990; Rotrosen & Leto, 1990). Another reported that the same magnitude of $p47^{phox}$ phosphorylation was achieved in normal and $p67^{phox}$ -deficient CGD neutrophils (Dusi & Rossi, 1993). Analysis of $p47^{phox}$ phosphorylation by 2D phosphopeptide mapping has not been carried out. Neither are there any studies of the effect of $p47^{phox}$ deficiency on $p67^{phox}$ phosphorylation.

5.3 RESULTS AND DISCUSSION :

$p67^{phox}$ 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 $p67^{phox}$ -deficient patient donated blood for an experiment to determine whether $p47^{phox}$ was normally phosphorylated.

The CGD neutrophils were completely deficient of $p67^{phox}$, and had a significantly reduced amount of $p40^{phox}$ compared with normal neutrophils (**Figure 5.3.1A**). This is a characteristic feature of $p67^{phox}$ -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

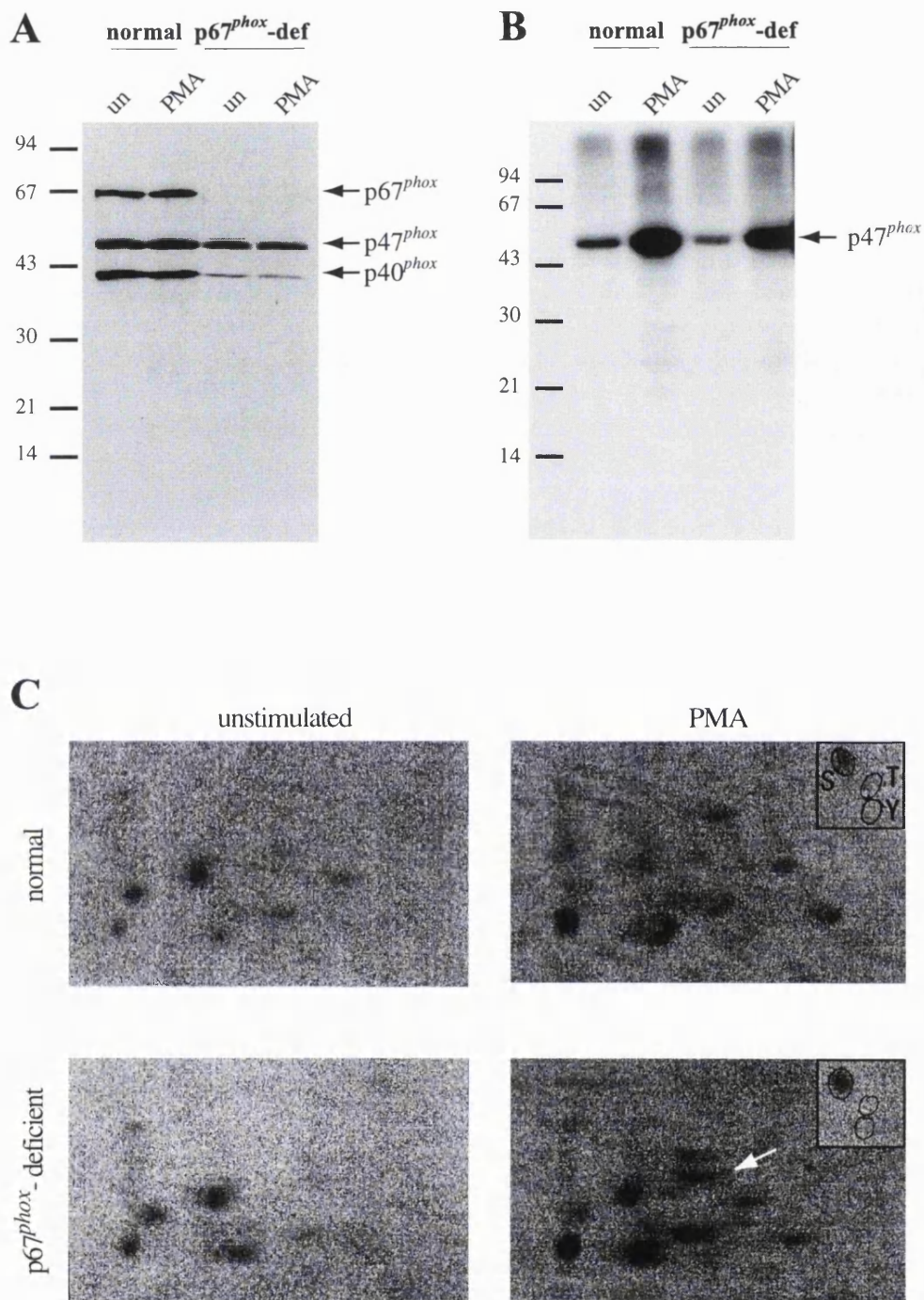


Figure 5.3.1 Phosphorylation of $p47^{phox}$ in $p67^{phox}$ -deficient neutrophils. **A:** TCA precipitates of 1×10^6 neutrophils from a normal subject and $p67^{phox}$ -deficient CGD patient were immunoblotted for $p67^{phox}$, $p47^{phox}$ and $p40^{phox}$. Detection was by the ECL method. **B:** Autorad of $p47^{phox}$ immunoprecipitates of 5×10^7 cell eq cytosol from $^{32}P_i$ -labelled neutrophils, either unstimulated or PMA-stimulated. **C:** 2D phosphopeptide maps (with phosphoamino acid analysis in insets) of phosphorylated $p47^{phox}$ from normal (upper) and $p67^{phox}$ -deficient (lower) neutrophils, either unstimulated (left) or PMA-stimulated (right).

cytosolic p47^{phox} (**Figure 5.3.1B**). p47^{phox} phosphopeptide mapping showed almost identical changes upon stimulation of normal and p67^{phox}-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^{phox}-deficient PMA map. However, this experiment seemed to confirm previous reports that p47^{phox} phosphorylation is not dependent on p67^{phox}.

To determine whether the phosphorylation of p67^{phox} was equally independent of p47^{phox}, p47^{phox}-deficient EBV-B cells were used. It had already been established that the phosphorylation of p67^{phox} 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^{phox} was immunoprecipitated from one normal, and two p47^{phox}-deficient EBV-B cell lines (**Figure 5.3.2A**). The phosphopeptide maps of p67^{phox} from normal and p47^{phox}-deficient cells were identical (**Figure 5.3.2B**). Therefore the phosphorylation of p67^{phox} occurs normally in the absence of p47^{phox}.

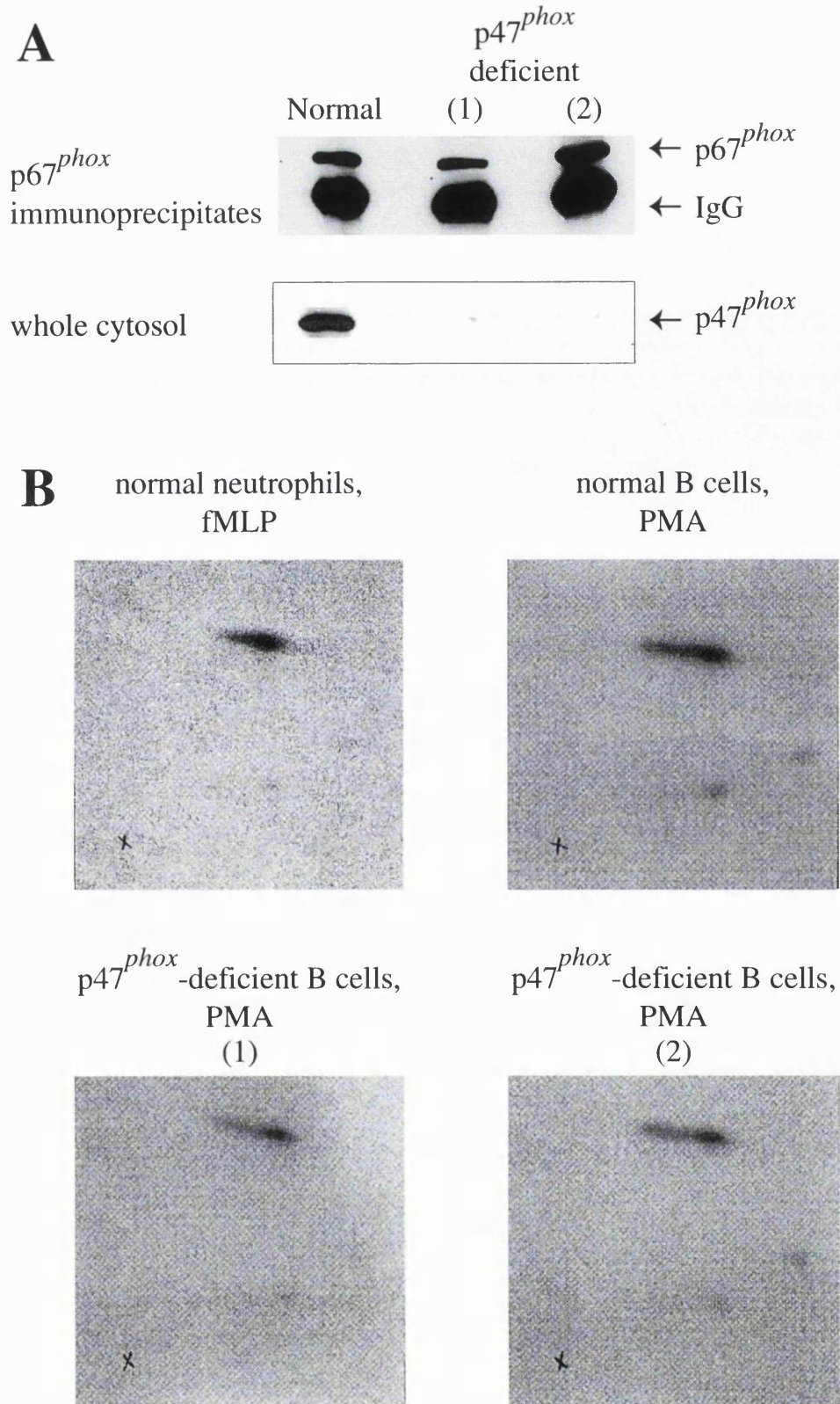


Figure 5.3.2 Phosphorylation of p67^{phox} in p47^{phox}-deficient EBV-B cells. **A:** ECL-developed immunoblots for p67^{phox} and p47^{phox} in EBV-B cells of one normal subject and two p47^{phox}-deficient CGD patients; (1),(2). **B:** 2D phosphopeptide maps of p67^{phox} immunoprecipitated from radiolabelled 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 $p67^{phox}$ phosphorylation

The aim of this chapter was to investigate some of the factors which may play a role in the phosphorylation of $p67^{phox}$. MAP kinase was implicated as a candidate kinase for $p67^{phox}$ by phosphopeptide analysis. The *in vitro* phosphorylation of $p67^{phox}$ 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 $p67^{phox}$.

The inhibitors staurosporine and okadaic acid were used to determine if there was close correlation between $p67^{phox}$ phosphorylation and NADPH oxidase activation. No deviations from the documented effects on $p47^{phox}$ were observed. That is, phosphorylation of $p67^{phox}$ was decreased by staurosporine and increased by okadaic acid. The same broad conclusions can be drawn for $p67^{phox}$ phosphorylation as have been drawn previously for $p47^{phox}$; 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 $p67^{phox}$ was investigated because it had been observed by *in vitro* phosphorylation with membranes and by phosphatase inhibition using okadaic acid. Insufficient yields of membrane-localised $p67^{phox}$ prevented phosphopeptide analysis, but detection was successful using a phosphotyrosine antibody. Both cytosolic and membrane-bound $p67^{phox}$ contained some phosphotyrosine. The level of the phosphotyrosine signal on membrane $p67^{phox}$ decreased upon activation, hence the translocation of $p67^{phox}$ 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 p67^{phox} phosphorylation does not involve the membrane was also implied by the normal phosphorylation of p67^{phox} in p47^{phox}-deficient cells. Translocation of p67^{phox} to the membrane is dependent on p47^{phox}, so neither translocation nor cytosolic interaction with p47^{phox} can play a role in p67^{phox} phosphorylation. The phosphorylation of p47^{phox} did not appear to be affected by p67^{phox} deficiency either. Therefore, cytosolic phosphorylation events taking place on p67^{phox} and p47^{phox} occur independently of each other.

Further elucidation of the mechanism of p67^{phox} phosphorylation will be facilitated by the development of an intact cell system comparing wildtype and Ala-233 mutant 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.

Chapter 6

General discussion

The phosphorylation of $p67^{phox}$ 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 $p67^{phox}$ 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 $p67^{phox}$ is equally phosphorylated in the absence of $p47^{phox}$, which is required for $p67^{phox}$ membrane binding.

$p67^{phox}$ 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 $p67^{phox}$ phosphorylation because it can also mimic the intact cell phosphorylation, and is activated upon neutrophil stimulation. A role for MAP kinase in the *in vivo* phosphorylation of $p67^{phox}$ remains to be proven. The level of $p67^{phox}$ 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 $p47^{phox}$, suggesting similar roles for the phosphorylation of both of these cytosolic components in the regulation of the NADPH oxidase.

The stoichiometry of phosphorylation of $p67^{phox}$ is low, but then the amount of $p67^{phox}$ which translocates to the membrane can be as low as 2% (DeLeo & Quinn, 1996). A similar number of molecules of $p47^{phox}$ and $p40^{phox}$ probably become phosphorylated upon stimulation as for $p67^{phox}$, but the incorporation of ^{32}P 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 p47^{phox} are also located very closely to a polyproline region, which has been shown to bind to the C-terminal SH3 domain of p67^{phox}, the N-terminal SH3 domain of p47^{phox} and the only SH3 domain of p40^{phox} (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 p67^{phox} may occur in activation of the NADPH oxidase. Kinetic studies indicate that p67^{phox} and p47^{phox} are kinetically independent components (Okamura *et al.*, 1990), and the inactivation of the NADPH oxidase correlates with inactivation of p67^{phox} (Fujimoto *et al.*, 1989). Therefore, the structural changes and/or signal transduction initiated by p47^{phox} phosphorylation are probably under separate control from p67^{phox} modifications. Interaction between p67^{phox} and p47^{phox} 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 p47^{phox} is proven by only low level activity induced by phosphorylated p47^{phox} when other kinase activity is blocked (Park, J.-W. *et al.*, 1997). A conformational change in p67^{phox} is implicated in the molecular rearrangements that occur upon stimulation. Observations of changes in the binding affinity between p67^{phox} and p47^{phox} 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 p40^{phox} (Fuchs *et al.*, 1996; Sathymoorthy *et al.*, 1997). These nearby associations may be regulated by phosphorylation at Thr-233 on p67^{phox}. 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 (Sathymoorthy *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

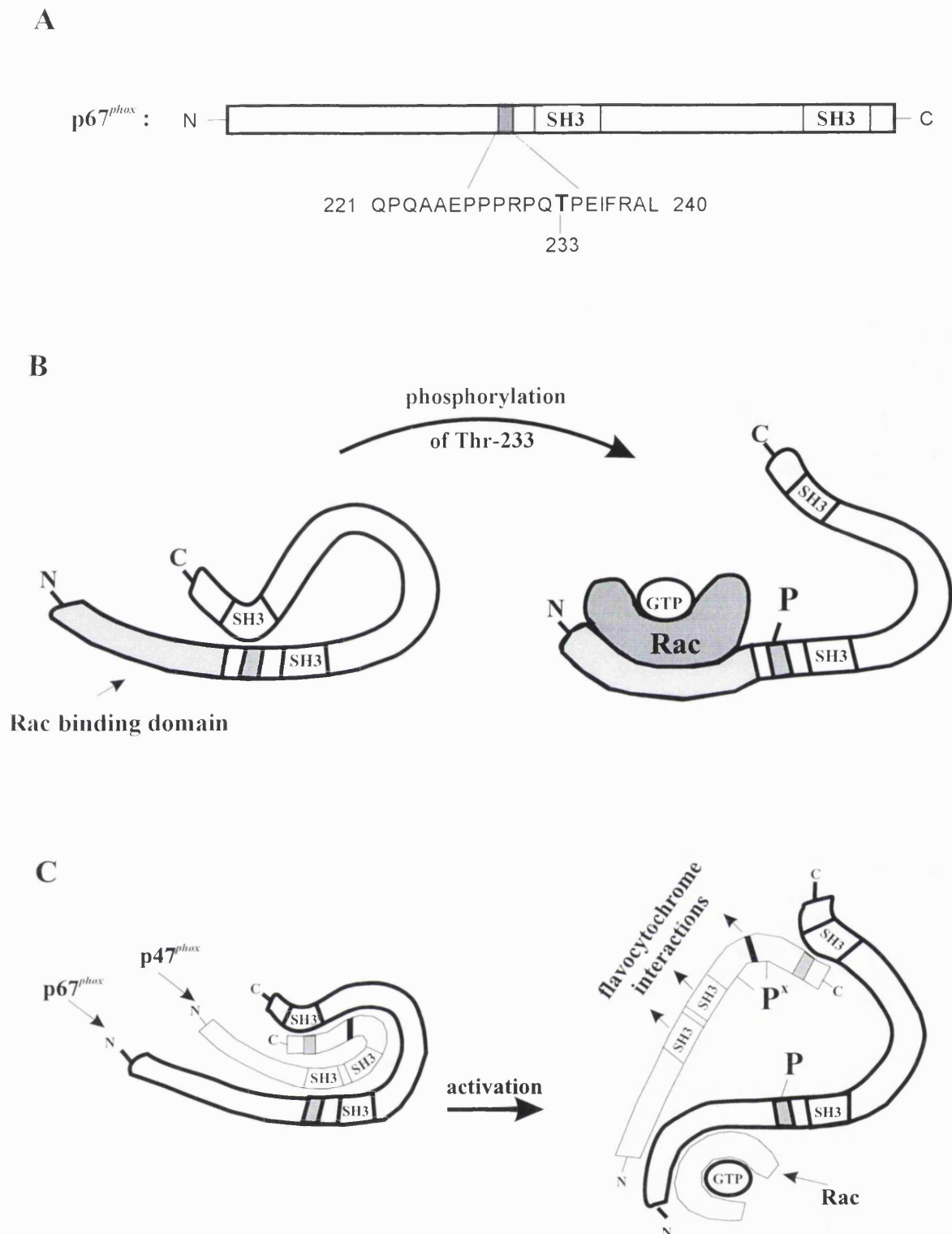


Figure 6 Amino acid Thr-233 is in a proline-rich region of p67^{phox} and might regulate intramolecular or intermolecular binding. **A:** Schematic diagram of p67^{phox} showing the positions of the two SH3 domains (amino acids 245-295 and 462-512), the polyproline region (227-234), and the phosphorylation 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^v).

whole cells, causing an arrangement of p67^{phox} and p47^{phox} which is appropriate for cytochrome binding. The region of p47^{phox} 323-332 has been shown by peptide competition in intact cells to be required for PMA-stimulated p47^{phox} phosphorylation, translocation and oxidase activation (Labadia *et al.*, 1996). Binding of the region p47^{phox} 323-332 to p67^{phox} 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 p67^{phox} may be the event which disrupts this p47^{phox}-p67^{phox} 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 p67^{phox} phosphorylation. The application of experimental intact cell systems, such as the reconstitution of p67^{phox}-deficient CGD cells, will allow the assessment of p67^{phox} phosphorylation in the activation of the multicomponent NADPH oxidase.

References

- Abo A, Segal AW (1995) Reconstitution of cell-free NADPH oxidase activity by purified components. *Methods Enzymol* 256:268-278
- Abo A, Pick E, Hall A, Totty N, Teahan CG, Segal AW (1991) Activation of the NADPH oxidase involves the small GTP-binding protein p21rac1. *Nature* 353:668-670
- Abo A, Boyhan A, West I, Thrasher AJ, Segal AW (1992) Reconstitution of neutrophil NADPH oxidase activity in the cell-free system by four components: p67^{phox}, p47^{phox}, p21rac1, and cytochrome b₂₄₅. *J Biol Chem* 267:16767-16770
- Adams ER, Diatz EA, Gizachew D, DeLeo FR, Yu L, Volpp BD, Vlases M, Jesaitis AJ, Quinn MT (1997) Interaction of human neutrophil flavocytochrome b with cytosolic proteins: transferred-NOESY NMR studies of a gp91^{phox} C-terminal peptide bound to p47^{phox}. *Biochem J* 325:249-257
- Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD (eds) (1983) *Molecular biology of the cell*. Garland Publishing, New York
- Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR (1995) PD098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and *in vivo*. *J Biol Chem* 270:27489-27494
- Altman PL, Dittmer DS (eds) (1961) *Blood and other body fluids*. Federation of American Societies for Experimental Biology, Washington
- Anderson FS, Murphy RC (1976) Isocratic separation of some purine nucleotide, nucleoside, and base metabolites from biological extracts by high-performance liquid chromatography. *J Chromatogr* 121:251-262
- Aoshima M, Nunoi H, Shimazu M, Shimizu S, Tatsuzawa O, Kenney RT, Kanegasaki S (1996) Two-exon skipping due to a point mutation in p67^{phox} deficient chronic granulomatous disease. *Blood* 88(5):1841-1845
- Arnold RR, Russell JE, Champion WJ, Brewer M, Gauthier JT (1982) Bactericidal activity of human lactoferrin: differentiation from the stasis of iron deprivation. *Infect Immun* 35:792-799
- Athens J (1981) Leukocytes. In: Wintrobe M (ed) *Clinical Haematology*. Lex and Febiger, Philadelphia
- Avdi NJ, Winston BW, Russel M, Young SK, Johnson GL, Worthen GS (1996) Activation of MEKK by formyl-methionyl-leucyl-phenylalanine in human neutrophils: mapping pathways for mitogen-activated protein kinase activation. *J Biol Chem* 271:33598-33606

Babior BM, Kipnes RS, Curnutte JT (1973) The production by leukocytes of superoxide: a potential bactericidal agent. *J Clin Invest* 52:741-744

Babior BM, Curnutte JT, Kipnes RS (1975) Biological defence mechanisms. Evidence for the participation of superoxide in bacterial killing by xanthine oxidase. *J Lab Clin Med* 85:235-244

Badway JA, Robinson JM, Heyworth PG, Curnutte JT (1989) 1,2-di-octanoyl-*sn*-glycerol can stimulate neutrophils by different mechanisms: evidence for a pathway that does not involve phosphorylation of the 47-kDa protein. *J Biol Chem* 264:20676-20682

Baehner RL, Nathan CD (1967) Leukocyte oxidase: defective activity in chronic granulomatous disease. *Science* 155:835-836

Baehner RL, Kunkel LM, Monaco AP, Haines JL, Conneally PM, Palmer C, Heerema N, Orkin SH (1986) DNA linkage analysis of X chromosome-linked chronic granulomatous disease. *Proc Natl Acad Sci USA* 83:3398-3401

Baggiolini M, Wymann MP (1990) Turning on the respiratory burst. *Trends Biochem Sci* 15:69-72

Bainton DF, Farquhar MG (1966) Origin of granules in polymorphonuclear leukocytes: two types derived from opposite faces of the Golgi complex in developing granulocytes. *J Cell Biol* 28:277-310

Bainton DF, Ulliyot JL, Farquhar MG (1971) The development of neutrophilic polymorphonuclear leukocytes in human bone marrow. *J Exp Med* 134:907-934

Baldrige CW, Gerard RW (1933) The extra respiration of phagocytosis. *Am J Physiol* 103:235-236

Bass DA, Olbantz P, Szejda P, Seeds MC, McCall CE (1986) Subpopulations of neutrophils with increased oxidative product formation in blood of patients with infection. *J Immunol* 136:860-866

Bender JG, McPhail LC, Van Epps DE (1983) Exposure of human neutrophils to chemotactic factors potentiates activation of the respiratory burst enzyme. *J Immunol* 130:2316-2323

Bengis-Garber C, Gruener N (1995) Involvement of protein kinase C and of protein phosphatases 1 and/or 2A in p47^{phox} phosphorylation in formylmet-leu-phe stimulated neutrophils: studies with selective inhibitors RO 31-81220 and calyculin A. *Cell Signal* 7(7):721-732

Bengis-Garber C, Gruener N (1996) Protein kinase A downregulates the phosphorylation of p47^{phox} in human neutrophils: a possible pathway for inhibition of the respiratory burst. *Cell Signal* 8(4): 291-296

Bonizzato A, Russo MP, Donini M, Dusi S (1997) Identification of a double mutation (D160V-K161E) in the p67^{phox} gene of a chronic granulomatous disease patient. *Biochem Biophys Res Commun* 231:861-863

Borregaard N, Lollike K, Kjeldsen L, Sengelov H, Bastholm L, Neilson MH, Bainton DF (1993) Human neutrophil granules and secretory vesicles. *Eur J Haem* 51:187-198

Boxer LA, Baehner RL, Davis J (1977) The effect of 2-deoxyglucose on guinea pig polymorphonuclear leukocyte phagocytosis. *Cell Physiol* 91:89-102

Boxer LA, Axtell R, Suchard S (1990) The role of the neutrophil in inflammatory diseases of the lung. *Blood Cells* 16:25-42

Boyle WJ, van der Geer P, Hunter T (1991) Phosphopeptide mapping and phosphoamino acid analysis by two dimensional separation on thin layer cellulose plates. *Methods Enzymol* 199:110-149

Bromberg Y, Pick E (1985) Activation of NADPH-dependent superoxide production in a cell-free system by sodium dodecyl sulphate. *J Biol Chem* 260:13539-13545

Buhl AM, Avdi N, Worthen GS, Johnson GL (1994) Mapping of the C5a receptor signal transduction network in human neutrophils. *Proc Natl Acad Sci USA* 91:9190-9194

Casimir CM, Bu-Ghanim HN, Rodaway ARF, Bentley DL, Rowe P, Segal AW (1991) Autosomal recessive chronic granulomatous disease caused by deletion at a dinucleotide repeat. *Proc Natl Acad Sci USA* 88:2753-2757

Casimir C, Chetty M, Bohler M-C, Garcia R, Fischer A, Griscelli C, Johnson B, Segal AW (1992) Identification of the defective NADPH-oxidase component in chronic granulomatous disease: a study of 57 families. *Eur J Clin Invest* 22:403-406

Chanock SJ, Faust LRP, Barrett D, Bizali C, Maly FE, Newburger PE, Ruedi JM, Smith RM, Babior BM (1992) O₂⁻ production by B lymphocytes lacking the respiratory burst oxidase subunit p47^{phox} after transfection with an expression vector containing a p47^{phox} cDNA. *Proc Natl Acad Sci USA* 89:10174-10177

Chanock SJ, Faust LRP, Barrett D, Christensen B, Newburger PE, Babior BM (1996) Partial reconstitution of the respiratory burst oxidase in lymphoblastoid B cell lines lacking p67^{phox} after transfection with an expression vector containing wild type and mutant p67^{phox} cDNAs: Deletions of the carboxy and amino terminal residues of p67^{phox} are not required for activity. *Exp Hematol* 24:531-536

Chao W, Spragg RG, Smith RM (1995) Inhibitory effect of porcine surfactant on the respiratory burst oxidase in human neutrophils: attenuation of p47^{phox} and p67^{phox} membrane translocation as the mechanism. *J Clin Invest* 96:2654-2660

Chetty M, Thrasher AJ, Abo A, Casimir C (1995) Low NADPH oxidase activity in Epstein-Barr-virus immortalised B-lymphocytes is due to a post-transcriptional block in expression of cytochrome b₅₅₈. *Biochem J* 306:141-145

Clark RA, Malech HL, Gallin JL, Nunoi H, Volpp BD, Pearson DW, Nauseef WM, Curnutte JT (1989) Genetic variants of chronic granulomatous disease: prevalence of deficiencies of two cytosolic components of the NADPH oxidase system. *New Engl J Med* 321:647-652

Clark RA, Volpp BD, Leidal KG, Nauseef WM (1990) Two cytosolic components of the human neutrophil respiratory burst oxidase translocate to the plasma membrane during cell activation. *J Clin Invest* 85:714-721

Clark-Lewis I, Sanghera JS, Pelech SL (1991) Definition of a consensus sequence for peptide substrate recognition by p44^{mpk}, the meiosis-activated myelin basic protein kinase. *J Biol Chem* 266:15180-15184

Cockcroft S (1984) Ca²⁺-dependent conversion of phosphatidylinositol to phosphatidate in neutrophils stimulated with fMet-Leu-Phe or ionophore A23187. *Biochim Biophys Acta* 795:37-46

Cockcroft S, Thomas GMH, Cunningham E, Ball A (1994) Use of cytosol-depleted HL-60 cells for reconstitution studies of G-protein-regulated phosphoinositide-specific phospholipase C- β isozymes. *Methods Enzymol* 238:154-168

Cohen GB, Ren R, Baltimore D (1995) Modular binding domains in signal transduction proteins. *Cell* 80:237-248

Coligan JE, Dunn BM, Ploegh HL, Speicher DW, Wingfield PT (eds) (1995) *Current protocols in protein science*, vol 2. John Wiley and Sons, New York

Combadiere C, El Benna J, Pedruzzi E, Hakim J, Perianin A (1993) Stimulation of the human neutrophil respiratory burst by formyl peptides is primed by a protein kinase inhibitor, staurosporine. *Blood* 82(9):2890-2898

Cox JA, Jeng AY, Sharkey NA, Blumberg PM, Tauber AI (1985) Activation of the human neutrophil nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase by protein kinase C. *J Clin Invest* 76:1932-1938

Crawford N, Eggleton P (1991) Dynamic change in neutrophil cytoskeleton during priming and subsequent surface stimulant functions. *Biochemical Society Transactions* 19

Cronkite EP, Vincent PC (1969) Granulocytopoiesis. *Semin Hemat* 2:3-15

Cross AR, Curnutte JT (1995) The cytosolic activating factors p47^{phox} and p67^{phox} have distinct roles in the regulation of electron flow in NADPH oxidase. *J Biol Chem* 270:6543-6548

Cross AR, Jones OT, Harper AM, Segal AW (1981) Oxidation-reduction properties of the cytochrome b found in the plasma-membrane fractions of human neutrophils. *Biochem J* 194:599-606

Cross AR, Higson FK, Jones OT, Harper AM, Segal AW (1982) The enzymic reduction and kinetics of oxidation of cytochrome b₂₄₅ of neutrophils. *Biochem J* 204:479-485

Cross AR, Parkinson JF, Jones OT (1985) Mechanism of the superoxide-producing oxidase of neutrophils: O₂ is necessary for the fast reduction of cytochrome b₂₄₅ by NADPH. *Biochem J* 226:881-884

Cross AR, Rae J, Curnutte JT (1995) Cytochrome b₂₄₅ of the neutrophil superoxide-generating system contains two nonidentical haems. *J Biol Chem* 270:17075-17077

Curnutte JT, Berkow RL, Roberts RL, Shurin SB, Scott PJ (1988) Chronic granulomatous disease due to a defect in the cytosolic factor required for nicotinamide adenine dinucleotide phosphate oxidase activation. *J Clin Invest* 81:606-610

Curnutte JT, Erickson RW, Ding J, Badway JA (1994) Reciprocal interactions between protein kinase C and components of the NADPH oxidase complex may regulate superoxide production by neutrophils stimulated with a phorbol ester. *J Biol Chem* 269:10813-10819

de Boer M, Hilarius-Stokman PM, Hossle J-P, Verhoeven AJ, Graf N, Kenney RT, Seger R, Roos D (1994) Autosomal recessive chronic granulomatous disease with absence of the 67-kD cytosolic NADPH oxidase component: identification of mutation and detection of carriers. *Blood* 83(2):531-536

DeLeo FR, Quinn MT (1996) Assembly of the NADPH oxidase: molecular interaction of oxidase proteins. *J Leukoc Biol* 60:677-691

DeLeo FR, Nauseef WM, Jesaitis AJ, Burritt JB, Clark RA, Quinn MT (1995a) A domain of p47^{phox} that interacts with human neutrophil flavocytochrome b₅₅₈. *J Biol Chem* 270:26246-26251

DeLeo FR, Yu L, Burritt JB, Loetterle LR, Bond CW, Jesaitis AJ, Quinn MT (1995b) Mapping sites of interaction of p47^{phox} and flavocytochrome b with random-sequence peptide phage display libraries. *Proc Natl Acad Sci USA* 92:7110-7114

DeLeo FR, Ulman KV, Davis AR, Julita KL, Quinn MT (1996) Assembly of the human neutrophil NADPH oxidase involves binding of p67^{phox} and flavocytochrome b to a common functional domain in p47^{phox}. *J Biol Chem* 271:17013-17020

de Mendez I, Leto TL (1995) Functional reconstitution of the phagocyte NADPH oxidase by transfection of its multiple components in a heterologous system. *Blood* 85(4):1104-1110

de Mendez I, Garrett MC, Adams AG, Leto TL (1994) Role of p67^{phox} SH3 domains in assembly of the NADPH oxidase system. *J Biol Chem* 269:16326-16332

de Mendez I, Adams AG, Soholic RA, Malech HL, Leto TL (1996) Multiple SH3 domain interactions regulate NADPH oxidase assembly in whole cells. *EMBO J* 15(6):1211-1220

de Mendez I, Homayounpour N, Leto TL (1997) Specificity of p47^{phox} SH3 domain interactions in NADPH oxidase assembly and activation. *Mol Cell Biol* 17:2177-2185

Dewald B, Linker-Ludwig R, Bretz U, Baggiolini M (1975) Subcellular localisation and heterogeneity of neutral proteases in neutrophilic polymorphonuclear leukocytes. *J Exp Med* 141:709-723

Dewald B, Thelen M, Baggiolini M (1988) Two transduction sequences are necessary for neutrophil activation by receptor agonists. *J Biol Chem* 263:16179-16184

- Didsbury J, Weber RF, Bokoch GW, Evans T, Syderman RJ (1989) Rac, a novel Ras-related family of proteins that are botulinum toxin substrates. *J Biol Chem* 264:16378-16382
- Diekmann D, Abo A, Johnston C, Segal AW, Hall A (1994) Interaction of Rac with p67^{phox} and regulation of phagocytic NADPH oxidase activity. *Science* 265:531-532
- Diekmann D, Nobes CD, Burbelo PD, Abo A, Hall A (1995) Rac GTPase interacts with GAPs and target proteins through multiple effector sites. *EMBO J* 14(21):5297-5305
- Dinauer MC, Pierce EA, Bruns GAP, Curnutte JT, Orkin SH (1990) Human neutrophil cytochrome b light chain (p22^{phox}): gene structure, chromosomal location, and mutations in cytochrome-negative autosomal recessive chronic granulomatous disease. *J Clin Invest* 86:1729-1737
- Ding C, Kume A, Bjorgvinsdottir H, Hawley RG, Pech N, Dinauer MC (1996) High-level reconstitution of respiratory burst activity in a human X-linked chronic granulomatous disease (X-CGD) cell line and correction of murine X-CGD bone marrow cells by retroviral-mediated gene transfer of human gp91^{phox}. *Blood* 88(5):1834-1840
- Ding J, Badway JA (1992) Effects of antagonists of protein phosphatases on superoxide release by neutrophils. *J Biol Chem* 267:6442-6448
- Ding J, Badway JA (1993) Stimulation of neutrophils with a chemoattractant activates several novel protein kinases that can catalyse the phosphorylation of peptides derived from the 47-kDa protein component of the phagocyte oxidase and myristoylated alanine-rich C kinase substrate. *J Biol Chem* 268:17326-17333
- Ding J, Badway JA, Erickson RW, Balazovich KJ, Curnutte JT (1993) Protein kinases potentially capable of catalysing the phosphorylation of p47^{phox} in normal neutrophils and neutrophils of patients with chronic granulomatous disease. *Blood* 82(3):940-947
- Dorseuil O, Quinn MT, Bokoch GM (1995) Dissociation of Rac translocation from p47^{phox}/p67^{phox} movements in human neutrophils by tyrosine kinase inhibitors. *J Leukoc Biol* 58:108-113
- Dorseuil O, Reibel L, Bokoch GM, Camonis J, Gacon G (1996) The Rac target NADPH oxidase p67^{phox} interacts preferentially with Rac2 rather than Rac1. *J Biol Chem* 271:83-88
- Doussiere J, Brandolin G, Derrien V, Vignais PV (1993) Critical assessment of the presence of an NADPH binding site on neutrophil cytochrome b₅₅₈ by photoaffinity and immunochemical labelling. *Biochemistry* 32:8880-8887
- Downey GP, Butler JR, Brumell J, Borregaard N, Sue-A-Quan AK, Grinstein S (1996) Chemotactic peptide-induced activation of MEK-2, the predominant isoform in human neutrophils: inhibition by wortmannin. *J Biol Chem* 271:21005-21011
- Ducruix A, Giege R (eds) (1992) Crystallisation of nucleic acids and proteins: a practical approach. Oxford University Press, New York

Dudley DT, Pang L, Decker SJ, Bridges AJ, Saltiel AR (1995) A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc Natl Acad Sci USA* 92:7686-7689

Dusi S, Rossi F (1993) Activation of NADPH oxidase of human neutrophils involves the phosphorylation and the translocation of cytosolic p67^{phox}. *Biochem J* 296:367-371

Dusi S, Della Bianca V, Grzeskowiak M, Rossi F (1993) Relationship between phosphorylation and translocation to the plasma membrane of p47^{phox} and p67^{phox} and activation of the NADPH oxidase in normal and Ca²⁺-depleted human neutrophils. *Biochem J* 290:173-178

Dusi S, Donini M, Rossi F (1994) Tyrosine phosphorylation and activation of NADPH oxidase in human neutrophils: a possible role for MAP kinases and for a 75 kDa protein. *Biochem J* 304:243-250

Dusi S, Donini M, Rossi F (1995) Mechanisms of NADPH oxidase activation in human neutrophils: p67^{phox} is required for the translocation of Rac 1 but not of Rac 2 from cytosol to the membranes. *Biochem J* 308:991-994

Dusi S, Donini M, Rossi F (1996) Mechanisms of NADPH oxidase activation: translocation of p40^{phox}, Rac1 and Rac2 from the cytosol to the membranes in human neutrophils lacking p47^{phox} or p67^{phox}. *Biochem J* 314:409-412

El Benna J, Ruedi JM, Babior BM (1994a) Cytosolic guanine nucleotide-binding protein Rac2 operates *in vivo* as a component of the neutrophil respiratory burst oxidase: transfer of Rac2 and the cytosolic components p47^{phox} and p67^{phox} to the submembranous actin cytoskeleton during oxidase activation. *J Biol Chem* 269:6729-6734

El Benna J, Faust LP, Babior BM (1994b) The phosphorylation of the respiratory burst oxidase component p47^{phox} during neutrophil activation. *J Biol Chem* 269:23431-23436

El Benna J, Faust LRP, Johnson JL, Babior BM (1996) Phosphorylation of the respiratory burst oxidase subunit p47^{phox} as determined by two-dimensional phosphopeptide mapping. *J Biol Chem* 271:6374-6378

El Benna J, Dang PM-C, Gaudry M, Fay M, Morel F, Hakim J, Gourgerot-Pocidalo M-A (1997) Phosphorylation of the respiratory burst oxidase subunit p67^{phox} during human neutrophil activation: regulation by protein kinase C-dependent and independent pathways. *J Biol Chem* 272:17204-17208

Elsbach P, Weiss J (1992) Oxygen-independent antimicrobial systems of phagocytes. In: Gallin JI, Goldstein IM, Snyderman R (eds) *Inflammation: basic principles and clinical correlates*, 2nd edn. Raven Press, New York

Erickson AK, Payne DM, Martino PA, Rossomondo AJ, Shabanowitz J, Weber MJ, Hunt DF, Sturgill TW (1990) Identification by mass spectrometry of threonine 97 in bovine myelin basic protein as a specific phosphorylation site for mitogen-activated protein kinase. *J Biol Chem* 265:19728-19735

Erickson RW, Malawista SE, Garrett MC, Blaricom GV, Leto TL, Curnutte JT (1992) Identification of a thermolabile component of the human neutrophil NADPH oxidase: a

model for chronic granulomatous disease caused by deficiency of the p67^{phox} cytosolic component. J Clin Invest 89:1587-1595

Exton JH (1997) Cell signalling through guanine-nucleotide-binding regulatory proteins (G proteins) and phospholipases. Eur J Biochem 243:10-20

Faust LRP, El Benna J, Babior BM, Chanock SJ (1995) The phosphorylation targets of p47^{phox}, a subunit of the respiratory burst oxidase: functions of the individual target serines as evaluated by site-directed mutagenesis. J Clin Invest 96:1499-1505

Fialkow L, Chan CK, Grinstein S, Downey GP (1993) Regulation of tyrosine phosphorylation in neutrophils by the NADPH oxidase; role of reactive oxygen intermediates. J Biol Chem 268:17131-17137

Finan P, Shimizu Y, Gout I, Hsuan J, Truong O, Butcher C, Bennett P, Waterfield MD, Kellie S (1994) An SH3 domain and proline-rich sequence mediate an interaction between two components of the phagocyte NADPH oxidase complex. J Biol Chem 269:13752-13755

Finan P, Koga H, Zvelebil MJ, Waterfield MD, Kellie S (1996) The C-terminal domain of p67^{phox} binds its natural ligand in a reverse orientation. J Molec Biol 261:173-180

Framisco JR, Glass DB, Krebs EG (1980) Optimal spatial requirements for the location of basic residues in peptide substrates for the cyclic AMP-dependent protein kinase. J Biol Chem 255:4240-4245

Francke U, Hsieh C-L, Foellmer BE, Lomax KJ, Malech HL, Leto TL (1990) Genes for two autosomal recessive forms of chronic granulomatous disease assigned to 1q25 (NCF2) and 7q11.23 (NCF1). Am J Hum Genet 47:483-492

Franke U, Ochs HD, De Martinville B, Giacalone J, Lindgren V, Distech C, Pagon RA, Hofker MH, Van Ommen G-J B, Pearson PL, Wedgwood RJ (1985) Minor Xp21 chromosome deletion in a male associated with expression of Duchenne muscular dystrophy, chronic granulomatous disease, retinitis pigmentosa, and McLeod Syndrome. Am J Hum Genet 37:250-267

Freeman JL, Lambeth JD (1996) NADPH oxidase activity is independent of p47^{phox} *in vitro*. J Biol Chem 271:22578-22582

Freeman JL, Abo A, Lambeth JD (1996) Rac "insert region" is a novel effector region that is implicated in the activation of NADPH oxidase, but not PAK65. J Biol Chem 271:19794-19801

Fuchs A, Dagher M-C, Jouan A, Vignais PV (1994) Activation of the O₂⁻-generating NADPH oxidase in a semirecombinant cell-free system: assessment of the function of Rac in the activation process. Eur J Biochem 226:587-595

Fuchs A, Dagher M-C, Vignais PV (1995) Mapping the domains of interaction of p40^{phox} with both p47^{phox} and p67^{phox} of the neutrophil oxidase complex using the two-hybrid system. J Biol Chem 270:5695-5697

Fuchs A, Dagher M-C, Faure J, Vignais PV (1996) Topological organisation of the superoxide-generating NADPH-oxidase. Pinpointing the sites of interaction between p47^{phox}, p67^{phox} and p40^{phox} using the two-hybrid system. *Biochim Biophys Acta* 1312:39-47

Fuchs A, Bouin A-P, Rabilloud T, Vignais PV (1997) The 40-kDa component of the phagocyte NADPH oxidase (p40^{phox}) is phosphorylated during activation in differentiated HL60 cells. *Eur J Biochem* 249:531-539

Fujimoto S, Smith RM, Curnette JT, Babior BM (1989) Evidence that activation of the respiratory burst oxidase in a cell-free system from human neutrophils is accomplished in part through an alteration of the oxidase-related 67-kDa cytosolic protein. *J Biol Chem* 264:21629-21632

Furukawa K, Tengler R, Nakamura M, Urwyler A, De Weck AL, Kanegasaki S, Maly FE (1992) B lymphoblasts show oxidase activity in response to cross-linking of surface IgM and HLA-DR. *Scand J Immunol* 35:561-567

Gabay JE, Scott RW, Campanetti D, Griffith J, Wilde C, Marra M, Seega M, Nathan CF (1989) Antibiotic proteins of human polymorphonuclear leukocytes. *Proc Natl Acad Sci USA* 86:5610-5614

Garcia RC, Segal AW (1988) Phosphorylation of the subunits of cytochrome b₂₄₅ upon triggering of the respiratory burst of human neutrophils and macrophages. *Biochem J* 252:901-904

Garcia RC, Cross AR, Segal AW (1986) The development of cytochrome b₂₄₅ in maturing human macrophages. *Biochem J* 239:647-651

Garcia RC, Whitaker M, Heyworth PG, Segal AW (1992) Okadaic acid produces changes in phosphorylation and translocation of proteins and in intracellular calcium in human neutrophils: relationship with the activation of the NADPH oxidase by different stimuli. *Biochem J* 286:687-692

Gomperts BD, Tatham PER (1992) Regulated exocytotic secretion from permeabilised cells. *Methods Enzymol* 219:178-189

Gosser YQ, Nomanbhoy TK, Aghazadeh B, Manor D, Combs C, Cerione RA, Rosen MK (1997) C-terminal binding domain of RhoGDP-dissociation inhibitor directs N-terminal inhibitory peptide to GTPases. *Nature* 387:814-819

Grand RJA, Owen D (1991) The biochemistry of Ras p21. *Biochem J* 279:609-631

Green SP, Phillips WA (1994) Activation of the macrophage respiratory burst by phorbol myristate acetate: evidence for both tyrosine-kinase-dependent and -independent pathways. *Biochim Biophys Acta* 1222:241-248

Grinstein S, Furuya W (1992) Chemoattractant-induced tyrosine phosphorylation and activation of microtubule-associated protein kinase in human neutrophils. *J Biol Chem* 267:18122-18225

- Grinstein S, Hill M, Furuya W (1989) Activation of electropermeabilised neutrophils by adenosine 5'[\gamma-thio]triphosphate (ATP[S]). *Biochem J* 261:755-759
- Grogan A, Wientjes F, Reeves E, Segal AW (1996) p67^{phox}, p47^{phox} and p40^{phox} in the cytosol of neutrophils are predominantly not associated with the assembled cytoskeleton. *Eur J Clin Invest* 26:Supp 1 154
- Grogan A, Reeves E, Keep N, Wientjes F, Totty NF, Burlingame AL, Hsuan JJ, Segal AW (1997) Cytosolic phox proteins interact with and regulate the assembly of coronin in neutrophils. *J Cell Science* 110:3071-3081
- Grzeskowiak M, Della Bianca V, Cassatella MA, Rossi F (1986) Complete dissociation between the activation of phosphoinositide turnover and of NADPH oxidase by formyl-methionyl-leucyl-phenylalanine in human neutrophils depleted of Ca²⁺ and primed by subthreshold doses of phorbol 12-myristate 13-acetate. *Biochem Biophys Res Commun* 135:785-794
- Gullick WJ (1983) Production of antisera to synthetic peptides. In: Walker JM (ed) *Methods in molecular biology*, vol 3; New protein methods. The Humana Press, Clifton
- Hallett MB, Lloyds D (1995) Neutrophil priming: the cellular signals that say "amber" but not "green". *Immunology Today* 16(6):264-268
- Harbecke O, Lundqvist H, Dahlgren C (1996) Okadaic acid inhibits the signal responsible for activation of the NADPH-oxidase in neutrophils stimulated with serum-opsonised yeast. *J Leukoc Biol* 59:754-762
- Hardie DG (ed) (1993) *Protein phosphorylation: a practical approach*. Oxford University Press, New York
- Hardie DG, Haystead TAJ, Sim ATR (1991) Use of okadaic acid to inhibit protein phosphatases in intact cells. *Methods Enzymol* 201:469-476
- Harlow E, Lane D (eds) (1988) *Antibodies: a laboratory manual*. Cold Spring Harbor Laboratory Publications, New York
- Harper AM, Chaplin MF, Segal AW (1985) Cytochrome b₂₄₅ from human neutrophils is a glycoprotein. *Biochem J* 227:783-788
- Hata K, Takashige K, Sumimoto H (1997) Roles for proline-rich regions of p47^{phox} and p67^{phox} in the phagocyte NADPH oxidase activation *in vitro*. *Biochem Biophys Res Commun* 241:226-231
- Hayakawa T, Suzuki K, Suzuki S, Andrews PC, Babior BM (1986) A possible role for protein phosphorylation in the activation of the respiratory burst in human neutrophils: evidence from studies with cells from patients with chronic granulomatous disease. *J Biol Chem* 261:9109-9115
- Henderson JF, Paterson ARP (eds) (1973) *Nucleotide metabolism: an introduction*. Academic Press, New York

Heyworth PG, Badway JA (1990) Continuous phosphorylation of both the 47 and 49 kDa proteins occurs during superoxide production by neutrophils. *Biochim Biophys Acta* 1052:299-305

Heyworth PG, Segal AW (1986) Further evidence for the involvement of a phosphoprotein in the respiratory burst oxidase of human neutrophils. *Biochem J* 239:723-731

Heyworth PG, Karnovsky ML, Badway JA (1989) Protein phosphorylation associated with synergistic stimulation of neutrophils. *J Biol Chem* 264:14935-14939

Heyworth PG, Shrimpton CF, Segal AW (1989) Localisation of the 47 kDa phosphoprotein involved in the respiratory-burst NADPH oxidase of phagocytic cells. *Biochem J* 260:243-248

Heyworth PG, Curnutte JT, Nauseef WM, Volpp BD, Pearson DW, Rosen H, Clark RA (1991) Neutrophil nicotinamide adenine dinucleotide phosphate oxidase assembly: translocation of p47^{phox} and p67^{phox} requires interaction between p47^{phox} and cytochrome b₅₅₈. *J Clin Invest* 87:352-356

Heyworth PG, Knaus UG, Settleman J, Curnutte JT, Bokoch GM (1993a) Regulation of NADPH oxidase activity by Rac GTPase activity protein(s). *Mol Biol Cell* 4:1217-1223

Heyworth PG, Knaus UG, Xu X, Uhlinger DJ, Conroy L, Bokoch GM, Curnutte JT (1993b) Requirement for post-translational processing of Rac-GTP-binding proteins for activation of human neutrophil NADPH oxidase. *Mol Cell Biol* 4:261-269

Heyworth PG, Bohl BP, Bokoch GM, Curnutte JT (1994) Rac translocates independently of the neutrophil NADPH oxidase components p47^{phox} and p67^{phox}. *J Biol Chem* 269:30749-30752

Heyworth PG, Ding J, Erickson RW, Lu DJ, Curnutte JT, Badway JA (1996) Protein phosphorylation in neutrophils from patients with p67^{phox}-deficient Chronic Granulomatous Disease. *Blood* 87(10):4404-4410

Heyworth PG, Ellis BA, Curnutte JT (1997) Stoichiometry of the cytosolic NADPH oxidase components in their multiprotein complex in normal and chronic granulomatous disease (CGD) neutrophils. *Blood* 90(10):19a

Hirshberg M, Stockley RW, Dodson G, Webb MR (1997) The crystal structure of human Rac1, a member of the Rho-family complexed with a GTP analogue. *Nature Struct Biol* 4(2):147-152

Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* 77:51-59

Holmes B, Page AR, Good RA (1967) Studies of the metabolic activity of leukocyte from patients with a genetic abnormality of phagocyte function. *J Clin Invest* 46:1422-1432

Iacono VJ, MacKay B, DiRienzo S, Pollock JJ (1980) Selective antibacterial properties of lysozyme for oral microorganisms. *Infect Immun* 29:623-632

Ito T, Nakamura R, Sumimoto H, Takeshige K, Sakaki Y (1996) An SH3 domain-mediated interaction between the phagocyte NADPH oxidase factors p40^{phox} and p47^{phox}. FEBS Letters 385:229-232

Iyer GYN, Islam MF, Quastel JH (1961) Biochemical aspects of phagocytosis. Nature 192:535-541

Iyer SS, Pearson DW, Nauseef WM, Clark RA (1994) Evidence for a readily dissociable complex of p47^{phox} and p67^{phox} in cytosol of unstimulated human neutrophils. J Biol Chem 269:22405-22411

Jamuar MP, Cronkite EP (1980) The fate of granulocytes. Exp Hematol 8:884-893

Jancarik J, Kim S-H (1991) Sparse matrix sampling: a screening method for crystallisation of proteins. J Appl Cryst 24:409-411

Jones SA, Wood JD, Coffey MJ, Jones OTG (1994) The functional expression of p47^{phox} and p67^{phox} may contribute to the generation of superoxide by an NADPH oxidase-like system in human fibroblasts. FEBS Letters 355:178-182

Jones SA, Hancock JT, Jones OTG, Neubauer A (1995) The expression of NADPH oxidase components in human glomerular mesangial cells: detection of protein and mRNA for p47^{phox}, p67^{phox} and p22^{phox}. J Am Soc Nephrol 5:1483-1491

Jouan A, Dagher M-C, Fuchs A, Foucaud-Gamen J, Vignais PV (1993) Immunoaffinity purification of an oxidase-activating cytosolic complex from bovine neutrophils. Biochem Biophys Res Commun 197:1296-1302

Kalunta CI, Kaptein JS, Scott ST, Lin CK, Gu DG, Lad PM (1992) Induction of fMLP-mediated calcium mobilisation requires crosslinking of surface immunoglobulin in Daudi cells. Immunol Lett 32:167-174

Kamps MP (1991) Determination of phosphoamino acid composition by acid hydrolysis of protein blotted to immobilon. Methods Enzymol 201:21-27

Kansha M, Takeshige K, Minakami S (1993) Decrease in the phosphotyrosine phosphatase activity in the plasma membrane of human neutrophils on stimulated by phorbol 12-myristate 13-acetate. Biochim Biophys Acta 1179:189-196

Keep NH, Barnes M, Barsukov I, Badii R, Lian L-Y, Segal AW, Moody PCE, Roberts GCK (1997) A modulator of Rho family G proteins, RhoGDI, binds these G proteins via an immunoglobulin-like domain and a flexible N-terminal arm. Current Biology 5(15 May):623-633

Kenney RT, Malech HL, Epstein ND, Roberts RL, Leto TL (1993) Characterisation of the p67^{phox} gene: genomic organisation and restriction fragment length polymorphism analysis for prenatal diagnosis in chronic granulomatous disease. Blood 82(12):3739-3744

Kitchen E, Rossi AG, Condiff AM, Haslett C, Chilvers ER (1996) Demonstration of reversible priming of human neutrophils using platelet-activating factor. *Blood* 88(11):4330-4337

Kitsis E, Weissmann G (1991) The role of the neutrophil: rheumatoid arthritis. *Clinical Orthopaediatric Related Research*: 63-72

Klebanoff SJ (1975) Antimicrobial mechanisms in neutrophilic polymorphonuclear leukocytes. *Semin Hemat* 12:117-142

Kleinberg ME, Malech HL, Rotrosen D (1990) The phagocyte 47-kilodalton cytosolic oxidase protein is an early reactant in activation of the respiratory burst. *J Biol Chem* 265:15577-15583

Kleinberg ME, Mital D, Rotrosen D, Malech HL (1992) Characterisation of a phagocyte cytochrome b₅₅₈ 91-kilodalton subunit functional domain - identification of peptide sequence and amino acids essential for activity. *Biochemistry* 31:2686-2690

Knaus UG, Morris S, Dong H-J, Chernoff J, Bokock GM (1995) Regulation of human leukocyte p21-activated kinases through G protein-coupled receptors. *Science* 269:221-223

Koshkin V, Pick E (1994) Superoxide production by cytochrome b₅₅₉: mechanism of cytosol-independent activation. *FEBS Letters* 338:285-289

Koshkin V, Lotan O, Pick E (1996) The cytosolic component p47^{phox} is not a *sine qua non* participant in the activation of NADPH oxidase but is required for optimal superoxide production. *J Biol Chem* 271:30326-30329

Kozma LM, Rossomando AJ, Weber MJ (1991) Comparison of three methods for detecting tyrosine phosphorylated proteins. *Methods Enzymol* 201:28-43

Kramer IM, Verhoeven AJ, van der Bend RL, Weening RS, Roos D (1988) Purified protein kinase C phosphorylates ~47 kDa protein in control neutrophil cytoplasts but not in neutrophil cytoplasts from patients with the autosomal form of chronic granulomatous disease. *J Biol Chem* 263:2352-2357

Kreck ML, Uhlinger DJ, Tyagi SR, Inge KL, Lambeth JD (1994) Participation of the small molecular weight GTP-binding protein Rac1 in cell-free activation and assembly of the respiratory burst oxidase: inhibition by a carboxyl-terminal Rac peptide. *J Biol Chem* 269:4161-4168

Kreck ML, Freeman JL, Abo A, Lambeth JD (1996) Membrane association of Rac is required for high activity of the respiratory burst oxidase. *Biochemistry* 35:15683-15692

Kuo SC, Lampen JO (1972) Inhibition by 2-deoxy-D-glucose of synthesis of glycoprotein enzymes by protoplasts of *Saccharomyces*: relation to inhibition of sugar uptake and metabolism. *J Bacteriol* 111(2):419-429

Kuroki M, O'Flaherty JT (1997) Differential effects of a mitogen-activated protein kinase kinase inhibitor on human neutrophil responses to chemotactic factors. *Biochem Biophys Res Commun* 232:474-477

Kwong CH, Malech HL, Rotrosen D, Leto TL (1993) Regulation of the human neutrophil NADPH oxidase by Rho-related G-proteins. *Biochemistry* 32:5711-5717

Labadia ME, Zu Y-L, Huang C-K (1996) A synthetic peptide containing a predominant protein kinase C site within p47^{phox} inhibits the NADPH oxidase in intact neutrophils. *J Leukoc Biol* 59:16-124

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685

Lambeth JD (1988) Activation of the respiratory burst in neutrophils: on the role of membrane-derived second messengers, Ca²⁺ and protein kinase C. *J Bioenerg Biomembr* 20:709-733

Lehrer R, Ganz T, Selsted M (1991) Defensins: endogenous antibiotic peptides of animal cells. *Cell* 64:229-230

Leto TL, Lomax KJ, Volpp BD, Nunoi H, Sechler JMG, Nauseef WM, Clark RA, Gallin JJ, Malech HL (1990) Cloning of a 67-kD neutrophil oxidase factor with similarity to a noncatalytic region of p60c-src. *Science* 248:727-730

Leto TL, Garrett MC, Fujii H, Nunoi H (1991) Characterisation of neutrophil NADPH oxidase factors p47^{phox} and p67^{phox} from recombinant baculoviruses. *J Biol Chem* 266:19812-19818

Leto TL, Adams AG, de Mendez I (1994) Assembly of the phagocyte NADPH oxidase: Binding of Src homology 3 domains to proline-rich targets. *Proc Natl Acad Sci USA* 91:10650-10654

Leusen JHW, Bolscher BGJM, Hilarius PM, Weening RS, Kaulfersch W, Seger RA, Roos D, Verhoeven AJ (1994a) 156Pro-Gln substitution in the light chain of cytochrome b558 of the NADPH oxidase (p22^{phox}) leads to defective translocation of the cytosolic proteins p47^{phox} and p67^{phox}. *J Exp Med* 180:2329-2334

Leusen JHW, de Boer M, Bolscher BGJM, Hilarius PM, Weening RS, Ochs HD, Roos D, Verhoeven AJ (1994b) A point mutation in gp91^{phox} of cytochrome b₅₅₈ of the human NADPH oxidase leading to defective translocation of the cytosolic proteins p47^{phox} and p67^{phox}. *J Clin Invest* 94:2120-2126

Leusen JHW, Fluiter K, Hilarius PM, Roos D, Verhoeven A, Bolscher BGJM (1995) Interactions between the cytosolic components p47^{phox} and p67^{phox} of the human neutrophil NADPH oxidase that are not required for activation in the cell-free system. *J Biol Chem* 270:11216-11221

Leusen JHW, De Klein A, Hilarius PM, Ahlin A, Palmblad J, Smith CIE, Diekmann D, Hall A, Verhoeven AJ, Roos D (1996) Disturbed interaction of Rac with mutated p67^{phox} causes chronic granulomatous disease. *J Exp Med* 184:1243-1249

Levy R, Dana R, Leto TL, Malech HL (1994) The requirement of p47^{phox} phosphorylation for activation of NADPH oxidase by opsonised zymosan in human neutrophils. *Biochim Biophys Acta* 1220:253-260

Lew PD, Wollheim CB, Waldvogel FA, Pozzan T (1984) Modulation of cytosolic free calcium transients by changes in intracellular calcium-buffering capacity: correlation with exocytosis and O_2^- production in human neutrophils. *J Cell Biol* 99:1212-1220

Li F, Linton GF, Sekhasaia S, Whiting-Theobald N, Katlin JP, Gallin JI, Malech HL (1994) $CD34^+$ peripheral blood progenitors as a target for genetic correction of the two flavocytochrome b_{558} defective forms of chronic granulomatous disease. *Blood* 84(1):53-58

Ligeti E, Tardif M, Vignais PV (1989) Activation of O_2^- generating oxidase of bovine neutrophils in a cell-free system. Interaction of a cytosolic factor with the plasma membrane and control by G nucleotides. *Biochemistry* 28:7116-7123

Lomax KJ, Leto TL, Nunoi H, Gallin JI, Malech HL (1989) Recombinant 47-kilodalton cytosol factor restores NADPH oxidase in chronic granulomatous disease. *Science* 245:409-412

Lu DJ, Takai A, Leto TL, Grinstein S (1992) Modulation of neutrophil activation by okadaic acid, a protein phosphatase inhibitor. *Am J Physiol* 262:C39-49

Malech HL, Maples PB, Whiting-Theobald N, Linton GF, Sekhsaria S, Vowells SJ, Li F, Miller JA, DeCarlo E, Holland SM, Leitman SF, Carter CS, Butz RE, Read EJ, Fleisher TA, Schneiderman RD, Van Epps DE, Spratt SK, Maack CA, Rokovich JA, Cohen LK, Gallin JI (1997) Prolonged production of NADPH oxidase-corrected granulocytes after gene therapy of chronic granulomatous disease. *Proc Natl Acad Sci USA* 94:12133-12138

Manser E, Chong C, Zhao Z, Leung T, Michael G, Hall C, Lim L (1995) Molecular cloning of a new member of the p21-Cdc42/Rac-activated kinase family. *J Biol Chem* 270:25070-25078

Martin GA, Bollag G, McCormick F, Abo A (1995) A novel serine kinase activated by Rac1/Cdc42Hs-dependent autophosphorylation is related to PAK65 and STE20. *EMBO J* 14(9):1970-1978

Mayer BJ, Hamaguchi M, Hanafusa H (1988) A novel viral oncogene with structural similarity to phospholipase C. *Nature* 332:272-274

McPhail LC, Snyderman R (1983) Activation of the respiratory burst enzyme in human polymorphonuclear leukocytes by chemoattractants and other soluble stimuli. *J Clin Invest* 72:192-200

McPhail LC, Shirley PS, Clayton CC, Snyderman R (1985) Activation of the respiratory burst enzyme from human neutrophils in a cell-free system. *J Clin Invest* 75:1735-1739

McPhail LC, Harvath L (1993) Signal transduction in neutrophil oxidative metabolism and chemotaxis. In: Abramson JS, Wheeler JG (eds) *The neutrophil*. Oxford University Press, New York

McPhail LC, Qualliotine-Mann D, Waite KA (1995) Cell-free activation of neutrophil NADPH oxidase by a phosphatidic acid-regulated protein kinase. *Proc Natl Acad Sci USA* 92:7931-7935

McPhail LC, Clayton CC, Snyderman R (1984) The NADPH oxidase of human polymorphonuclear leukocytes: evidence for regulation by multiple signals. *J Biol Chem* 259:5768-5775

McPherson MJ, Hames BD, Taylor GR (eds) (1995) *PCR 2: a practical approach*. Oxford University Press, New York

Michl J, Ohlbaum DJ, Silverstein SC (1976) 2-deoxyglucose selectively inhibits Fc and complement receptor-mediated phagocytosis in mouse peritoneal macrophages: II Dissociation of the inhibitory effects of 2-deoxyglucose on phagocytosis and ATP generation. *J Exp Med* 144:1484-1493

Mizuki K, Kadomatsu K, Hata K, Ito T, Fan Q-W, Kage Y, Fukumaki Y, Sakaki Y, Takeshige K, Sumimoto H (1998) Functional modules and expression of mouse p40^{phox} and p67^{phox}, SH3-containing proteins involved in the phagocyte NADPH oxidase complex. *Eur J Biochem* 251:573-582

Morel F, Doussiere J, Vignais PV (1991) The superoxide-generating oxidase of phagocytic cells: physiological, molecular and pathological aspects. *Eur J Biochem* 201:523-546

Morel F, Cohner Tanugi Cholley L, Brandolin G, Dianoux AC, Martel C, Champelovier P, Seigneum JM, Francois P, Bost M, Vignais PV (1993) The O₂⁻ generating oxidase of B lymphocytes: Epstein-Barr virus-immortalised B lymphocytes as a tool for the identification of defective components of the oxidase in Chronic Granulomatous Disease. *Biochim Biophys Acta* 1182:101-109

Morimatsu T, Kawagoshi A, Yoshida K, Tamura M (1997) Actin enhances the activation of human neutrophil NADPH oxidase in a cell-free system. *Biochem Biophys Res Commun* 230:206-210

Murkerjee G, Quinn MT, Linner JG, Jesaitis AJ (1994) Remodelling of the plasma membrane after stimulation of neutrophils with f-Met-Leu-Phe and dihydrocytochalasin B: identification of membrane subdomains containing NADPH oxidase activity. *J Leukoc Biol* 55:685-694

Musacchio A, Noble M, Pauptit R, Wieranger R, Saraste M (1992) Crystal-structure of a Src-homology-3 (SH3) domain. *Nature* 359:851-855

Naccache PH, Gilbert C, Caon AC, Huang C-K, Bonak VA, Umezawa K, McColl SR (1990) Selective inhibition of human neutrophil functional responsiveness by erbstatin, an inhibitor of tyrosine protein kinase. *Blood* 76(10):2098-2104

Najmudin S, Bax B, Guillory RJ (1997) Structural analysis of the SH2 and SH3 domains: modules that regulate protein interactions. *J Biochem Mol Biol and Biophys* 1:73-88

Nakamura R, Sumimoto H, Mizuki K, Hata K, Ago T, Kitajima S, Takeshige K, Sakaki Y, Ito T (1998) The PC motif: a novel and evolutionarily conserved sequence involved in

interaction between p40^{phox} and p67^{phox}, SH3 domain-containing cytosolic factors of the phagocytic NADPH oxidase. *Eur J Biochem* 251:583-589

Nakanishi A, Imajoh-Ohmi S, Fijinawa T, Kikuchi H, Kanegasaki S (1992) Direct evidence for interaction between COOH-terminal regions of cytochrome b₅₅₈ subunits and cytosolic 47 kDa protein during activation of an O₂⁻ generating system in neutrophils. *J Biol Chem* 267:19072-19074

Nasmith PE, Mills GB, Grinstein S (1989) Guanine nucleotides induce tyrosine phosphorylation and activation of the respiratory burst in neutrophils. *Biochem J* 257:893-897

Nauseef WM, Volpp BD, Clark RA (1990) Immunochemical and electrophoretic analyses of phosphorylates native and recombinant neutrophil oxidase component p47^{phox}. *Blood* 76:2622-2629

Nauseef WM, Volpp BD, McCormick S, Leidal KG, Clark RA (1991) Assembly of the neutrophil respiratory burst oxidase: protein kinase C promotes cytoskeletal and membrane association of cytosolic oxidase components. *J Biol Chem* 266:5911-5917

Nauseef WM, McCormick S, Renee J, Leidal KG, Clark RA (1993) Functional domain in an arginine-rich carboxyl-terminal region of p47^{phox}. *J Biol Chem* 268:23646-23651

Newburger PE, Parmley RT (1995) Neutrophil structure and function. In: Hoffmann R, Benz EJ, Shattil SJ, Furie B, Cohen HJ, Silberstein LE (eds) *Haematology: basic principles and practice*, 2nd edn. Churchill Livingstone, New York

Nishizuka Y (1984) The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature* 308:693-698

Nisimoto Y, Freeman JLR, Motalebi SA, Hirshberg M, Lambeth JD (1997) Rac binding to p67^{phox}. *J Biol Chem* 272:18834-18841

Nobes CD, Hall A (1995) Rho, Rac and Cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibres, lamellipodia, and filopodia. *Cell* 81:53-62

Nunoi H, Malech HL (1988) Two forms of autosomal chronic granulomatous disease lack distinct neutrophil cytosol factors. *Science* 242:1298-1301

Nunoi H, Iwata M, Tatsuzawa S, Onoe Y, Shimizu S, Kanegasaki S, Matsuda I (1995) AG dinucleotide insertion in a patient with chronic granulomatous disease lacking cytosolic 67-kDa protein. *Blood* 86(1):329-333

Odeberg H, Olsson I (1975) Antibacterial activity of cationic proteins from human granulocytes. *J Clin Invest* 56:1118-1124

Odell EW, Segal AW (1991) Killing of pathogens associated with chronic granulomatous disease by the non-oxidative microbial mechanisms of human neutrophils. *J Med Microbiol* 43:129-135

Okamura N, Curnutte JT, Roberts RL, Babior BM (1988) Relationship of protein phosphorylation to the activation of the respiratory burst in human neutrophils: defects in the phosphorylation of a group of closely related 48-kDa proteins in two forms of chronic granulomatous disease. *J Biol Chem* 263:6777-6782

Okamura N, Babior BM, Mayo LA, Peveri P, Smith RM, Curnutte JT (1990) The p67^{phox} cytosolic peptide of the respiratory burst oxidase from human neutrophils: functional aspects. *J Clin Invest* 85:1583-1587

Okuyama M, Sakon M, Kambayashi J, Kawasaki T, Monden M (1996) Involvement of protein phosphatase 2A in PKC-independent pathway of neutrophil superoxide generation by fMLP. *J Cell Biochem* 60:279-288

O'Reilly DR, Miller LK, Luckow VA (eds) (1994) *Baculovirus expression vectors: a laboratory manual*. Oxford University Press, New York

Pang L, Sawada T, Decker SJ, Saltiel AR (1995) Inhibition of MAP kinase kinase blocks the differentiation of PC-12 cells induced by nerve growth factor. *J Biol Chem* 270:13585-13588

Park J-W, Ahn SM (1995) Translocation of recombinant p47^{phox} cytosolic component of the phagocyte oxidase by *in vitro* phosphorylation. *Biochem Biophys Res Commun* 211:410-416

Park J-W, Ma M, Reudi JM, Smith RM, Babior BM (1992) The cytosolic components of the respiratory burst oxidase exist as a Mr ~240,000 complex that acquires a membrane-binding site during activation of the oxidase in a cell-free system. *J Biol Chem* 267:17327-17332

Park J-W, El Benna J, Scott KE, Christensen BL, Chanock SJ, Babior BM (1994) Isolation of a complex of respiratory burst oxidase components from resting neutrophil cytosol. *Biochemistry* 33:2907-2911

Park J-W, Babior BM (1997) Activation of the leukocyte NADPH oxidase subunit p47^{phox} by protein kinase C: a phosphorylation-dependent change in the conformation of the C-terminal end of p47^{phox}. *Biochemistry* 36:7474-7480

Park J-W, Hoyal CR, El Benna J, Babior BM (1997) Kinase-dependent activation of the leukocyte NADPH oxidase in a cell-free system. *J Biol Chem* 272:11035-11043

Park M-Y, Imajoh-Ohmi S, Nunoi H, Kanegasaki S (1997) Synthetic peptides corresponding to various hydrophilic regions of the large subunit of cytochrome b₅₅₈ inhibit superoxide generation in a cell-free system from neutrophils. *Biochem Biophys Res Commun* 234:531-536

Parkos CA, Allen RA, Cochrane CG, Jesaitis AJ (1987) Purified cytochrome b from human granulocyte plasma membrane is comprised of two polypeptides with relative molecular weights of 91,000 and 22,000. *J Clin Invest* 80:732-742

Parkos CA, Dinauer MC, Walker LE, Allen RA, Jesaitis AJ, Orkin SH (1988) Primary structure and unique expression of the 22 kilodalton light chain of human neutrophil cytochrome b. *Proc Natl Acad Sci USA* 85:3319-3323

- Pawson T, Gish G (1992) SH2 and SH3 domains: from structure to function. *Cell* 71:359-362
- Pinna LA (1990) Casein kinase 2: an "eminence grise" in cellular regulation? *Biochim Biophys Acta* 1054:267-284
- Ponting CP (1996) Novel domains in NADPH oxidase subunits, sorting nexins, and PtdIns 3-kinases: binding partners of SH3 domains? *Protein Science* 5:2353-2357
- Porter CD, Parkar MH, Levinsky RL, Collins MKL, Kinnon C (1993) X-linked chronic granulomatous disease: correction of NADPH oxidase defect by retrovirus-mediated expression of gp91^{phox}. *Blood* 82(7):2196-2202
- Prigmore E, Ahmed S, Best A, Kozma R, Manser E, Segal AW, Lim L (1995) A 68 kD kinase and NADPH oxidase component p67^{phox} are targets for Cdc42Hs and Rac1 in neutrophils. *J Biol Chem* 270:10717-10722
- Pryzwansky KB, Wyatt TA, Nichols H, Lincoln TM (1990) Compartmentalisation of cyclic GMP-dependent protein kinase in formyl-peptide stimulated neutrophils. *Blood* 76(3):612-618
- Quie PG, White JG, Holmes B, Good RA (1967) *In vitro* bacterial capacity of human polymorphonuclear leukocytes: diminished activity in chronic granulomatous disease in childhood. *J Clin Invest* 46:668-679
- Quilliam LA, Mueller H, Bohl BP, Prossnitz V, Sklar LA, Der CJ, Bokoch GM (1991) Rap 1A is a substrate for cyclic AMP-dependent protein kinase in human neutrophils. *J Immunol* 147:1628-1635
- Quinn MT, Bokoch GM (1995) Measurement of Rac translocation from cytosol to membranes in activated neutrophils. *Methods Enzymol* 256:256-267
- Quinn MT, Parkos CA, Walker L, Orkin SH, Dinanier MC, Jesaitis AJ (1989) Association of a Ras-related protein with cytochrome b of human neutrophils. *Nature* 342:198-200
- Quinn MT, Evans T, Loetterle LR, Jesaitis AJ, Bokoch GM (1993) Translocation of Rac correlates with NADPH oxidase activation: evidence for equimolar translocation of oxidase components. *J Biol Chem* 268:20983-20987
- Ravel P, Lederer F (1993) Affinity-labeling of an NADPH-binding site on the heavy subunit of flavocytochrome b₅₅₈ in particulate NADPH oxidase from activated neutrophils. *Biochem Biophys Res Commun* 196:543-552
- Ren R, Mayer BJ, Cicchetti P, Baltimore D (1993) Identification of a ten-amino acid proline-rich SH3 binding site. *Science* 259:1157
- Roberts PJ, Cross AR, Jones OT, Segal AW (1982) Development of cytochrome b and an active oxidase system in association with maturation of a human promyelocytic (HL-60) cell line. *J Cell Biol* 95:720-726

Robinson JM, Heyworth PG, Badway JA (1990) Utility of staurosporine in uncovering differences in the signal transduction pathways for superoxide production in neutrophils. *Biochim Biophys Acta* 1055:55-62

Rodaway AF, Teahan CG, Casimir CM, Segal AW, Bentley DL (1990) Characterisation of the 47 kilodalton autosomal chronic granulomatous disease protein: tissue-specific expression and transcriptional control by retinoic acid. *Mol Cell Biol* 10:5388-5396

Rollet E, Caon AC, Roberge CJ, Liao NW, Malawista SE, McCall SR, Naccache PH (1994) Tyrosine phosphorylation in activated human neutrophils: comparison of the effects of different classes of agonists and identification of the signalling pathways involved. *J Immunol* 153:353-363

Roos D, de Boer M, Kuribayashi F, Meischl C, Weening RS, Segal AW, Ahlin A, Nemet K, Hossle JP, Bernatowska-Matuskiewicz E, Middleton-Price H (1996) Mutations in the X-linked and autosomal recessive forms of chronic granulomatous disease. *Blood* 87(5):1663-1681

Rossi F (1986) The O_2^- forming NADPH oxidase of the phagocytes: nature, mechanisms of activation and function. *Biochim Biophys Acta* 853:65-89

Rossi F, Zatti M (1964) Changes in the metabolism pattern of polymorphonuclear leukocytes during phagocytosis. *Br J Exp Pathol* 45:548-559

Rossi F, De Togni P, Bellavite P, Della Bianca V, Grzeskowiak M (1983) Relationship between the binding of N-formylmethionylleucylphenylalanine and the respiratory response of human neutrophils. *Biochim Biophys Acta* 758:168-175

Rotrosen D, Leto TL (1990) Phosphorylation of neutrophil 47-kDa cytosolic oxidase factor: translocation to membrane is associated with distinct phosphorylation events. *J Biol Chem* 265:19910-19915

Rotrosen D, Kleinberg ME, Nunoi H, Leto T, Gallin JJ, Malech HL (1990) Evidence for a functional cytoplasmic domain of phagocyte oxidase cytochrome b_{558} . *J Biol Chem* 265:8745-8750

Rotrosen D, Yeung CL, Leto TL, Malech HL, Kwong CH (1992) Cytochrome b_{558} : the flavin-binding component of the phagocyte NADPH oxidase. *Science* 256:1459-1462

Rotrosen D, Yeung CL, Katkin JP (1993) Production of recombinant cytochrome b_{558} allows reconstitution of the phagocyte NADPH oxidase solely from recombinant proteins. *J Biol Chem* 268:14256-14260

Royer-Pokora B, Kunkel LM, Monaco AP, Goff SC, Newburger PE, Baehner RL, Cole FS, Curnutte JT, Orkin SH (1986) Cloning the gene for an inherited human disorder-chronic granulomatous disease-on the basis of its chromosomal location. *Nature* 322:32-38

Sambrook J, Fritsch EF, Maniatis T (eds) (1989) *Molecular cloning: a laboratory manual*, 2nd edn. Cold Spring Harbor Laboratory Press, New York

Sathyamoorthy M, De Mendez I, Adams AG, Leto TL (1997) p40^{phox} down-regulates NADPH oxidase activity through interactions with its SH3 domain. *J Biol Chem* 272:9141-9146

Sbarra AJ, Karnovsky ML (1957) The biochemical basis of phagocytosis: 1. Metabolic changes during the investigation of particles by polymorphonuclear leukocytes. *J Biol Chem* 234:1355-1362

Schagger H, Von Jagow G (1987) Tricine-sodium dodecyl sulphate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem* 166:368-379

Schneider C, Zanetti M, Romeo D (1981) Surface-reactive stimuli selectively increase protein phosphorylation in human neutrophils. *FEBS Letters* 127:4-8

Sergeant S, McPhail LC (1997) Opsonised zymosan stimulates the redistribution of protein kinase C isoforms in human neutrophils. *J Immunol* 159:2877-2885

Segal AW, Coade SB (1978) Kinetics of oxygen consumption by phagocytosing human neutrophils. *Biochem Biophys Res Commun* 84:611-617

Segal AW (1987) Absence of both cytochrome b₂₄₅ subunits from neutrophils in X-linked chronic granulomatous disease. *Nature* 326:88-91

Segal AW, Jones OTG (1978) Novel cytochrome b system in phagocytic vacuoles of human granulocytes. *Nature* 276:515-517

Segal AW, Jones OTG (1979a) Reduction and subsequent oxidation of a cytochrome b of human neutrophils after stimulation with phorbol myristate acetate. *Biochem Biophys Res Commun* 88:130-134

Segal AW, Jones OTG (1979b) The subcellular distribution and some properties of the cytochrome b component of the microbicidal oxidase system of human neutrophils. *Biochem J* 182:181-188

Segal AW, Jones OTG (1980a) Absence of cytochrome b reduction in stimulated neutrophils from both female and male patients with chronic granulomatous disease. *FEBS Letters* 110:111-114

Segal AW, Jones OTG (1980b) Rapid incorporation of the human neutrophil plasma membrane cytochrome b into phagocytic vacuoles. *Biochem Biophys Res Commun* 92:710-715

Segal AW, Dorling J, Coade S (1980c) Kinetics of fusion of the cytoplasmic granules with phagocytic vacuoles in human polymorphonuclear leukocytes: biochemical and morphological studies. *J Cell Biol* 85:42-59

Segal AW, Garcia R, Goldstone H, Cross AR, Jones OT (1981a) Cytochrome b₂₄₅ of neutrophils is also present in human monocytes, macrophages and eosinophils. *Biochem J* 196:363-367

Segal AW, Geisow M, Garcia R, Harper A, Miller R (1981b) The respiratory burst of phagocytic cells is associated with a rise in vacuolar pH. *Nature* 290:406-409

Segal AW, Cross AR, Garcia RC, Borregaard N, Valerius NH, Soothill JF, Jones OT (1983) Absence of cytochrome b₂₄₅ in chronic granulomatous disease: a multicentre European evaluation of its incidence and relevance. *New Engl J Med* 308:245-251

Segal AW, Heyworth PG, Cockcroft S, Barrowman MM (1985) Stimulated neutrophils from patients with autosomal recessive chronic granulomatous disease fail to phosphorylate a Mr-44,000 protein. *Nature* 316:547-549

Segal AW, West I, Wientjes F, Nugent JHA, Chavan AJ, Haley B, Garcia RC, Rosen H, Scrase G (1992) Cytochrome b₂₄₅ is a flavocytochrome containing FAD and the NADPH-binding site of the microbicidal oxidase of phagocytes. *Biochem J* 284:781-788

Sekhsaria S, Gallin JI, Linton GF, Mallory RM, Mulligan RC, Malech HL (1993) Peripheral blood progenitors as a target for genetic correction of p47^{phox}-deficient chronic granulomatous disease. *Proc Natl Acad Sci USA* 90:7446-7450

Selvaraj RJ, Sbarra AJ (1966) Relationship of glycolytic and oxidative metabolism to particle entry and destruction in phagocytosing cells. *Nature* 211:1272-1276

Sikorski RS, Bogurski MS, Goebel M, Hieter P (1990) A repeating amino acid motif in CDC23 defines a family of proteins and a new relationship among genes required for mitosis and RNA synthesis. *Cell* 60:307-317

Skalnik DG, Strauss EC, Orkin SH (1991) CCAAT displacement protein as a repressor of the myelomonocytic-specific gp91^{phox} gene promoter. *J Biol Chem* 266:16736-16744

Smith DB, Johnson KS (1988) Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene* 67:31-40

Smith RM, Connor JA, Chen M, Babior BM (1996) The cytosolic subunit p67^{phox} contains an NADPH-binding site that participates in catalysis by the leukocyte NADPH oxidase. *J Clin Invest* 98:977-983

Snyderman R, Pike MC (1984) Chemoattractant receptors on phagocytic cells. *Ann Rev Immunol* 2:257-281

Someya A, Nagaoka I, Yamashita T (1993) Purification of the 260 kDa cytosolic complex involved in the superoxide production of guinea pig neutrophils. *FEBS Letters* 330:215-218

Someya A, Nagaoka I, Nunoi H, Yamashita T (1996) Translocation of guinea pig p40^{phox} during activation of NADPH oxidase. *Biochim Biophys Acta* 1277:217-225

Staehelin H, Karnovsky ML, Farnham AE, Suter E (1957) Studies on the interaction between phagocytes and *tubercle bacilli*. *J Exp Med* 105:265-277

Sumimoto H, Sakamoto N, Nozaki M, Sakai Y, Takeshige K, Minkami S (1992) Cytochrome b₅₅₈, a component of the phagocyte NADPH oxidase, is a flavoprotein. *Biochem Biophys Res Commun* 186:1868-1875

Sumimoto H, Kage Y, Nunoi H, Sasaki H, Nose T, Fukumaki Y, Ohno M, Minakami S, Takeshige K (1994) Role of Src homology 3 domains in assembly and activation of the phagocyte NADPH oxidase. *Proc Natl Acad Sci USA* 91:5345-5349

Sumimoto H, Hata K, Mizuki K, Ito T, Kage Y, Sakaki Y, Fukumaki Y, Nakamura M, Takeshige K (1996) Assembly and activation of the phagocyte NADPH oxidase: specific interaction of the N-terminal Src homology 3 domain of p47^{phox} with p22^{phox} is required for activation of the NADPH oxidase. *J Biol Chem* 271:22152-22158

Suzuki K, Yamaguchi T, Oshizawa T, Yamamoto Y, Nishimaki-Mogami T, Hayakawa T, Takahashi A (1995) Okadaic acid induces both augmentation and inhibition of opsonised zymosan-stimulated superoxide production by differentiated HL-60 cells. Possible involvement of dephosphorylation of a cytosolic 21 K protein in respiratory burst. *Biochim Biophys Acta* 1266:261-267

Swain SD, Helgersen SM, Davis AR, Nelson LK, Quinn MT (1997) Analysis of activation-induced conformational changes in p47^{phox} using tryptophan fluorescence spectroscopy. *J Biol Chem* 272:29502-29510

Takahashi K, Sasaki T, Mammoto A, Takaishi K, Kameyama T, Tsukita S, Tsukita S, Takai Y (1997) Direct interaction of the Rho GDP dissociation inhibitor with ezrin/radixin/moesin initiates the activation of the Rho small G protein. *J Biol Chem* 272:23371-23375

Tamaoki T (1991) Use and specificity of staurosporine, UCN-01 and calphostin C as protein kinase inhibitors. *Methods Enzymol* 201:340-347

Tanugi-Cholley LC, Issartel JP, Lunardi J, Freycon F, Morel F, Vignais PV (1995) A mutation located at the 5' splice junction sequence of intron 3 in the p67^{phox} gene causes the lack of p67^{phox} mRNA in a patient with chronic granulomatous disease. *Blood* 85(1):242-249

Taylor WR, Jones DT, Segal AW (1993) A structural model for the nucleotide binding domain of the flavocytochrome b₂₄₅ β -chain. *Protein Science* 2:1675-1685

Tenenhouse HS, Scriver CR (1975) Orthophosphate transport in the erythrocyte of normal subjects and of patients with X-linked hypophosphataemia. *J Clin Invest* 55:644-654

Test ST, Lampert MB, Ossanna PJ, Thoene JG, Weiss SJ (1984) Generation of nitrogen-chlorine oxidants by human phagocytes. *J Clin Invest* 74:1341-1349

Thrasher A, Chetty M, Casimir C, Segal AW (1992) Restoration of superoxide generation to a chronic granulomatous disease-derived B-cell line by retrovirus mediated gene transfer. *Blood* 80(5):1125-1129

Thrasher AJ, Keep NH, Wientjes F, Segal AW (1994) Chronic granulomatous disease: a review. *Biochim Biophys Acta* 1227:1-24

Thrasher AJ, Casimir CM, Kinnon C, Morgan G, Segal AW, Levinsky RJ (1995a) Gene transfer to primary chronic granulomatous disease monocytes. *Lancet* 346:92-93

Thrasher AJ, de Alwis M, Casimir CM, Kinnon C, Page K, Lebhowski J, Segal AW, Levinsky RJ (1995b) Functional reconstitution of the NADPH-oxidase by adeno-associated virus gene transfer. *Blood* 86(2):761-765

Thrasher AJ, de Alwis M, Casimir CM, Kinnon C, Page K, Lebhowski J, Segal AW, Levinsky RJ (1995c) Generation of recombinant adeno-associated virus (rAAV) from an adenoviral vector and functional reconstitution of the NADPH oxidase. *Gene Therapy* 2:481-485

Torres M, Hall FL, O'Neill K (1993) Stimulation of human neutrophils with formyl-methionyl-leucyl-phenylalanine induces tyrosine phosphorylation and activation of two distinct mitogen-activated protein kinases. *J Immunol* 150:1563-1578

Tsunawaki S, Mizunari H, Nagata M, Tatsuzawa O, Kuratsuji T (1994) A novel cytosolic component, p40^{phox}, of respiratory burst oxidase associates with p67^{phox} and is absent in patients with chronic granulomatous disease who lack p67^{phox}. *Biochem Biophys Res Commun* 199:1378-1387

Tsunawaki S, Kagara S, Yoshikawa K, Yoshida LS, Kuratsuji T, Namiki H (1996) Involvement of p40^{phox} in activation of phagocyte NADPH oxidase through association of its carboxyl-terminal, but not its amino-terminal, with p67^{phox}. *J Exp Med* 184:893-902

Uhlinger DJ, Perry DK (1992) A carboxy-terminal peptide from p47^{phox} is a substrate for phosphorylation by protein kinase C and by a neutrophil protein kinase. *Biochem Biophys Res Commun* 187:940-948

Uhlinger DJ, Inge KL, Krech ML, Tyagi SR, Neckelmann N, Lambeth JD (1992) Reconstitution and characterisation of the human neutrophil respiratory burst oxidase using recombinant p47^{phox}, p67^{phox} and plasma membrane. *Biochem Biophys Res Commun* 186:509-516

Uhlinger DJ, Tyagi SR, Inge KL, Lambeth JD (1993) The respiratory burst oxidase of human neutrophils: guanine nucleotides and arachidonate regulate the assembly of a multicomponent complex in a semirecombinant cell-free system. *J Biol Chem* 268:8624-8631

Uhlinger DJ, Taylor KT, Lambeth JD (1994) p67^{phox} enhances the binding of p47^{phox} to the human neutrophil respiratory burst oxidase complex. *J Biol Chem* 269:22095-22098

Umei T, Takeshige K, Minakami S (1986) NADPH binding component of neutrophil superoxide-generating oxidase. *J Biol Chem* 261:5229-5232

Valerius NH, Stendahl O, Hartwig JH, Stossel TP (1981) Distribution of actin-binding protein myosin in polymorphonuclear leukocyte during locomotion and phagocytosis. *Cell* 24:195-202

Van Gelder BF, Slater EC (1962) The extinction coefficient of cytochrome c. *Biochim Biophys Acta* 58:593-595

Verhoef K, Koken SEC, Berhout B (1993) Electroporation of the HIV Tat trans-activator protein into cells. *Anal Biochem* 210:210-214

Vestergaard-Bogind B (1963) The transport of phosphate ions across the human red cell membrane: II The influence of the concentration of inorganic phosphate on the kinetics of the uptake of (³²P) phosphate ions. *Biochim Biophys Acta* 66:93-109

Vlahos CJ, Matter WF, Brown RF, Traynor-Kaplan AE, Heyworth PG, Prossnitz ER, Ye RD, Marder P, Schlem JA, Rothfuss KJ, Serlin BS, Simpson PJ (1995) Investigation of neutrophil signal transduction using a specific inhibitor of phosphatidylinositol 3-kinase. *J Immunol* 154:2413-2422

Volkman DJ, Buescher ES, Gallin JI, Fauci AS (1984) B cells lines as models for inherited phagocytic diseases: abnormal superoxide generation in chronic granulomatous disease and giant granules in Chediak-Higashi syndrome. *J Immunol* 133:3006-3009

Volpp BD, Nauseef WM, Clark RA (1988) Two cytosolic neutrophil oxidase components absent in autosomal Chronic Granulomatous Disease. *Science* 242:1295-1297

Volpp BD, Nauseef WM, Donelson JE, Moser DR, Clark RA (1989) Cloning of the cDNA and functional expression of the 47-kilodalton cytosolic component of human neutrophil respiratory burst oxidase. *Proc Natl Acad Sci USA* 86:7195-7199 (with corrections in *Proc Natl Acad Sci USA* 86:9563)

Volpp BD, Lin Y (1993) *In vitro* molecular reconstitution of the respiratory burst in B lymphoblasts from p47^{phox}-deficient chronic granulomatous disease. *J Clin Invest* 91:201-207

Waite KA, Wallin R, Qualliotine-Mann D, McPhail LC (1997) Phosphatidic acid-mediated phosphorylation of the NADPH oxidase component p47^{phox}. *J Biol Chem* 272:15569-15578

Walker JE, Saraste M, Runswick MJ, Gay NJ (1982) Distantly related sequences in the α - and β - subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J* 1:945-951

Wallach TM, Segal AW (1996) Stoichiometry of the subunits of flavocytochrome b₅₅₈ of the NADPH oxidase of phagocytes. *Biochem J* 320:33-38

Wallach TM, Segal AW (1997) Analysis of glycosylation sites on gp91^{phox}, the flavocytochrome of the NADPH oxidase, by site-directed mutagenesis and translation *in vitro*. *Biochem J* 321:583-585

Watson F, Robinson J, Edwards SW (1991) Protein kinase C-dependent and -independent activation of the NADPH oxidase of human neutrophils. *J Biol Chem* 266:7432-7439

Weil WM, Linton GF, Whiting-Theobald N, Vowells SJ, Rafferty SP, Li F, Malech HL (1997) Genetic correction of p67^{phox} deficient chronic granulomatous disease using peripheral blood progenitor cells as a target for retrovirus mediated gene transfer. *Blood* 89(5):1754-1761

- Weiss SJ (1989) Tissue destruction by neutrophils. *New Engl J Med* 320:365-376
- Wick AN, Drury DR, Nakada HI, Wolfe JB (1957) Localisation of the primary metabolic block produced by 2-deoxyglucose. *J Biol Chem* 224:963-969
- Wientjes FB, Hsuan JJ, Totty NF, Segal AW (1993) p40^{phox}, a third cytosolic component of the activation complex of the NADPH oxidase to contain Src homology 3 domains. *Biochem J* 296:557-561
- Wientjes FB, Panayotou G, Reeves E, Segal AW (1996) Interactions between cytosolic components of the NADPH oxidase: p40^{phox} interacts with both p67^{phox} and p47^{phox}. *Biochem J* 317:919-924
- Wientjes FB, Segal AW, Hartwig JH (1997) Immunoelectron microscopy shows a clustered distribution of NADPH oxidase components in the human neutrophil plasma membrane. *J Leukoc Biol* 61:303-312
- Woodgett JR, Gould KL, Hunter T (1986) Substrate specificity of protein kinase C: use of synthetic peptides corresponding to physiological sites as probes for substrate recognition requirements. *Eur J Biochem* 161:177-184
- Woodgett JR, Hunter A (1987) Isolation and characterisation of two distinct forms of protein kinase C. *J Biol Chem* 262:4836-4843
- Woodman RC, Ruedi JM, Jesaitis AJ, Okamura N, Quinn MT, Smith RM, Curnette JT, Babior BM (1991) Respiratory burst oxidase and three of four oxidase-related polypeptides are associated with the cytoskeleton of human neutrophils. *J Clin Invest* 87:1345-1351
- Worthen GS, Avdi N, Buhl AM, Suzuki N, Johnson GL (1994) fMLP activates Ras and Raf in human neutrophils: potential role in activation of MAP kinase. *J Clin Invest* 94:815-823
- Wymann MP, Kernen P, Deranleau DA, Baggiolini M (1989) Respiratory burst oscillations in human neutrophils and their correlation with fluctuations in apparent cell shape. *J Biol Chem* 264:15827-15834
- Yamaguchi M, Sasaki J-I, Kuwana M, Sakai M, Okamura N (1993) Cytosolic protein phosphatase may turn off activated NADPH oxidase in guinea pig neutrophils. *Arch Biochem Biophys* 306(1):209-214
- Yamaguchi M, Oishi H, Araki S, Saeki S, Yamane H, Okamura N, Ishibashi S (1995) Respiratory burst and tyrosine phosphorylation by vanadate. *Arch Biochem Biophys* 323(2):382-386
- Yamaguchi M, Saeki S, Yamane H, Okamura N, Ishibashi S (1996) Involvement of several protein kinases in the phosphorylation of p47^{phox}. *Biochem Biophys Res Commun* 220:891-895

Yu H, Suchard SJ, Nairn R, Jove R (1995) Dissociation of mitogen-activated protein kinase activation from the oxidative burst in differentiated HL-60 cells and human neutrophils. *J Biol Chem* 270:15719-15724

Yu HT, Rosen MK, Shin TB, Seideldugan C, Brugge JS, Schreiber SL (1992) Solution structure of the SH3 domain of Src and identification of its ligand-binding site. *Science* 258:1665-1668

Zhan S, Vazquez N, Zhan S, Wientjes FB, Budarf ML, Schrock E, Ried T, Green ED, Chanock SJ (1996) Genomic structure, chromosomal localisation, start of transcription, and tissue expression of the human p40^{phox}, a new component of the nicotinamide adenine dinucleotide phosphate-oxidase complex. *Blood* 88(7):2714-2721

Zicha D, Dunn GA, Segal AW (1997) Deficiency of p67^{phox}, p47^{phox} or gp91^{phox} in chronic granulomatous disease does not impair leukocyte chemotaxis or motility. *Brit J Haem* 96:543-550

Zimmerman BJ, Granger DN (1994) Mechanism of reperfusion injury. *Am J Med Sci* 307:284-292