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INTERACTIONS BETWEEN THE COMPONENTS OF THE HUMAN NADPH OXIDASE: A REVIEW ABOUT THE INTRIGUES IN THE *PHOX* FAMILY

Jeanette H. W. Leusen[1,2], Arthur J. Verhoeven[1] and Dirk Roos[3]

[1]Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and Laboratory for Experimental and Clinical Immunology, University of Amsterdam, Plesmanlaan 125, 1066 CX Amsterdam, The Netherlands. [2]Department of Pediatrics, Emma's Children Hospital, Academic Medical Center, Amsterdam, The Netherlands.

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1. ABSTRACT

When microorganisms invade the body, they encounter a large asssortment of defense mechanisms. Among these, phagocytes play an important role in the process of killing pathogens. This event is mediated by two important processes, *viz*. activation of the NADPH oxidase enzyme, which leads to the production of toxic oxygen metabolites, and fusion of intracellular granules with the phagosome (the vesicle that contains the ingested micro-organisms), which causes release of the toxic granule contents into this vesicle. The human NADPH oxidase is a very complex enzyme, in two ways: 1. it exists of at least 6 components: cytochrome b_{558} (a heterodimer comprised of gp91-*phox* and p22-*phox*), p47-*phox*, p67-*phox*, p40-*phox*, *rac* and Rap1A, and 2. there are multiple signal transduction pathways leading to activation of the NADPH oxidase.

The most likely reason for this complexity is the toxicity of the oxygen radicals produced by the active NADPH oxidase; these compounds are not only harmful to the invading pathogens, but also to the surrounding tissues. This latter effect is enforced by the activation of metalloproteases released by neutrophils and by oxidation of protease inhibitors by oxygen metabolites (1). Therefore, an improper activation of the NADPH oxidase must be prevented at all costs and, when the infection has been cleared, a rapid deactivation mechanism is imperative.

In this review, the interaction between the different components of the NADPH oxidase and the activation of these proteins will be discussed.

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[3] To whom correspondence should be addressed at, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and Laboratory for Experimental and Clinical Immunology, University of Amsterdam, Plesmanlaan 125, 1066 CX Amsterdam, The Netherlands. Tel #: 31-20-5123110. Fax #: 31-20-5123310 E-mail: clbkvi@xs4all.nl

2. NADPH oxidase

The NADPH oxidase is a multi-component enzyme, localized in the plasma membrane of phagocytic leukocytes (2). It accepts electrons from NADPH at the cytosolic side of the membrane and donates these to molecular oxygen at the other side of the membrane,

either on the outside of the cells or in the phagosome that contains ingested micro-organisms (3). In this way, a one-electron reduction of oxygen to superoxide anion is catalyzed, at the expense of NADPH.

$$2\ O_2 + NADPH \rightarrow 2\ O_2^{\bullet -} + NADP^+ + H^+$$

The superoxide produced is subsequently converted to hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and other microbicidal products.

In resting, non-phagocytosing leukocytes, the NADPH oxidase is inactive. In this form, the enzyme components are localized in different parts of the cell. Phagocyte activation, *e.g.* by binding of opsonized micro-organisms to cell surface receptors, leads to assembly of the active enzyme complex.

2.1. Mechanisms of activation of the oxidase

The oxidase can be activated by receptor-mediated and by receptor-independent mechanisms. Typical receptor-dependent stimuli are the complement fragment C5a, the chemotactic tripeptide *N*-formyl-Met-Leu-Phe (fMLP)(for reviews see (4, 5)), and immune complexes (for reviews, see (6-9)). Receptor-independent stimuli include long-chain unsaturated fatty acids and phorbol 12-myristate 13-acetate (PMA). Oxidase activation by receptor-mediated stimuli usually lasts less than 5 min, while receptor-independent stimuli activate the enzyme for a much longer period, but only when the stimulus remains present. It appears that in intact cells the activated oxidase is undergoing a continuous process of activation and deactivation.

3. Chronic Granulomatous Disease (CGD)

The importance of the NADPH oxidase in human host defense is exemplified by patients suffering from chronic granulomatous disease (CGD). CGD is an immunodeficiency syndrome characterized clinically by severe recurrent bacterial and fungal infections. These infections typically consist of pneumonia, lymphadenitis and abcesses that involve the liver, the subcutaneous tissues and the bones. Control of the infections can usually be achieved with appropriate antibiotic therapy, but eradication of the infecting organisms is often slow and attained with great difficulty. The persistence of micro-organisms, often within the phagosomal vacuoles of neutrophils or macrophages, is the stimulus to a chronic inflammatory state with granuloma formation.

Biochemically, CGD is characterized by the inability of phagocytic leukocytes (neutrophils, eosinophils, monocytes and macrophages) to activate the NADPH oxidase and to generate the reactive oxygen compounds needed for the killing of phagocytosed micro-organisms (10, 11). Therefore, the most common pathogens encountered in CGD patients are catalase-positive organisms, because catalase prevents the CGD phagocytes from using microbial hydrogen peroxide for killing these pathogens. Predominant are *Staphylococcus aureus*, *Aspergillus* species and a variety of gram-negative enteric bacilli.

CGD is a rare disease, with an estimated incidence of 1 in about 250,000 individuals, without any ethnic preference. It usually manifests itself in early childhood and is predominantly found in boys (12). CGD is a very heterogeneous disorder; clinically because of many antimicrobial systems that can partially compensate for the defect in oxygen-dependent killing systems, and biochemically because of the complicated genetic origin of CGD (13). Characterizing the mutations that lead to CGD is important for improved diagnosis and treatment of CGD patients, but also provides a better understanding of the functional domains within the oxidase components.

4. The components of the NADPH oxidase.

Four oxidase components have been identified through studies with CGD cells (14-20): two membrane-bound components, gp91-*phox* and p22-*phox*, which together comprise cytochrome b_{558}, and two cytosolic components called p47-*phox* and p67-*phox*. Upon cell activation, the latter two proteins, together with a third cytosolic p40-*phox* component, translocate to cytochrome b_{558} in the plasma membrane to form the active enzyme (21, 22). Also, two low-molecular-weight GTP-binding proteins are involved in the activation of the NADPH oxidase, *viz*. Rap 1A and *rac*. The components of the NADPH oxidase and the assembly of this enzyme in the plasma membrane of a phagocyte are depicted in Fig. 1.

4.1. Cytochrome b_{558}

The membrane-bound gp91-*phox*/p22-*phox* heterodimer is designated cytochrome b_{558} because of its optical spectrum with an a absorbance peak at 558 nm (23-25). This protein is also known as cytochrome b_{-245} for its unusual low midpoint potential at -245 mV (26). Both a flavin (27-29) and two heme redox centers (30) are contained within the cytochrome heterodimer. Electrons supplied by NADPH are transferred to oxygen as follows (25,31)

$$\begin{array}{lllllll} & & & & e^- & & \\ & & & & \text{Heme} & \rightarrow & O_2 \rightarrow O_2^{\bullet -} \\ & 2e^- & & 2e^- & & & \\ NADPH & \rightarrow & \text{Flavin (FAD)} & \rightarrow & \text{Heme} & \rightarrow & O_2 \rightarrow O_2^{\bullet -} \\ & & & & e^- & & \\ & & & & \overline{\qquad\qquad} & & \\ & & & & \text{cytochrome } b_{558} & & \end{array}$$

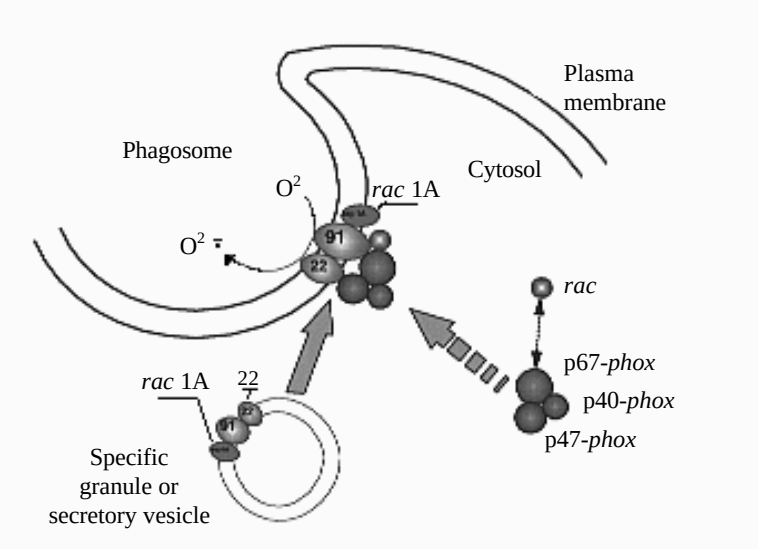

Figure 1. Assembly of the NADPH oxidase in phagocytic cells. In resting phagocytes, the cytochrome b_{558} subunits p22-*phox* and gp91-*phox*, in association with Rap1A, are located in the membranes of specific granules and of secretory vesicles. Upon cell activation, these organelles fuse with the plasma membrane, which results in expression of cytochrome b_{558} in this membrane. At the same time, a complex of p47-*phox*, p67-*phox* and p40-*phox* translocates from the cytosol to the plasma membrane and forms a complex with cytochrome b_{558}. This translocation is facilitated by simultaneous redistribution of *rac* to the plasma membrane.

In resting neutrophils, this flavocytochrome is localized in the membranes of specific granules and of secretory vesicles (26, 32-34). During cell activation, fusion of these organelles with the plasma membrane leads to re-allocation of the oxidase (34, 35).

The cDNA's of both subunits of cytochrome b_{558} have been cloned and sequenced, and the genes encoding these proteins have been localized and characterized (15, 36-38). The alpha subunit p22-*phox* contains 195 amino acids, with hydrophobic helices in the N-terminal half of the protein that could serve as membrane-spanning domains (38). The CYBA gene encoding p22-*phox* is located on chromosome 16 (15).

The beta subunit gp91-*phox* of cytochrome b_{558} contains 570 amino acids, with four or five transmembrane helices and five potential N-linked glycosylation sites in the amino-terminal region (37, 39, 40). The topology of gp91-*phox* has been determined with anti-peptide antibodies and partial proteolysis (40). This study showed that residues 150-172 are exposed on the outside, and an 18-kD C-terminal fragment is cytosolic. Furthermore, there is evidence that residue 240 is glycosylated, thus extracellular (A.W. Segal, unpublished results). The CYBB gene for gp91-*phox* is located on the X chromosome (37, 39). Mutations in this gene account for all cases of X-linked CGD (X91 CGD), which is the most common form of the disease (about 70% of all patients). In most cases of X-linked CGD, gp91-*phox* cannot be detected on immunoblot; this is referred to as $X91^0$ CGD. In some cases, residual gp91-*phox* protein and oxidase activity are present ($X91^-$ CGD) (41), and in a few exceptional cases the (non-functional) protein is present in normal amounts ($X91^+$ CGD) (42-44). In p22-*phox*-affected CGD patients, the protein is typically absent (15, 38, 45), except in one patient with $A22^+$ CGD (46). The mutation in the CYBA gene of this last patient is predicted to lead to a proline-to-glutamine substitution at residue 156 in p22-*phox*. This residue resides in a proline-rich region that could be an important counter structure for *src* homology region 3 (SH3) domains (47-49) (see Fig. 2). The functional defect in this patient has been studied in ref. (50). Both subunits of cytochrome b_{558} are usually missing in $A22^0$ CGD as well as in $X91^0$ CGD (24, 38, 51, 52). This indicates that these subunits stabilize each other (53). It is not known, however, which regions in p22-*phox* and gp91-*phox* are involved in this mutual stabilization.

4.2. Rap1A

A 22-kD low-molecular-weight GTP-binding protein co-purifies with cytochrome b_{558} and remains associated with the cytochrome even after immunoaffinity purification on matrices composed of antibodies to cytochrome b_{558} (54, 55). This protein was identified as Rap1A by immunostaining. Rap1A has been shown to bind specifically to cytochrome b_{558}, with a one-to-one stoichiometry. In neutrophils, Rap1A becomes phosphorylated by the cAMP-dependent kinase PKA (56). A serine residue (^{180}Ser) at the

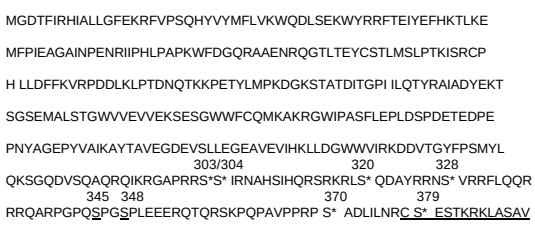

Figure 2. Putative phosphorylation sites in p47-phox. The 390 amino-acid sequence of p47-*phox* is given. In the last quarter of the protein, six putative PKC phosphorylation sites are localised (marked with asterisk *) and two putative MAP kinase sites (underlined). The consensus sequence for a PKC site is R-X-S-X-(R-R), with R for arginine or another positively charged amino acid (*i.e*. lysine) and X for a non-charged amino acid. The last two positively charged amino acids are not essential for a PKC site. The consensus sequence for a MAP kinase site is P-X-S/T-P or even X-P, with P for proline, S/T for serine or threonine and X for any amino acid. Whether these sites actually become phosphorylated is discussed in the text. The importance of the C-terminal sequence (also underlined) is to be discussed in the text.

COOH-terminal region of Rap1A has been identified as a site of phosphorylation by PKA (56), and phosphorylation of Rap1A abrogates the interaction with cytochrome b_{558} (57). It is possible that (phosphorylation of) Rap1A regulates the deactivation of the NADPH oxidase.

4.3 p47-phox

The majority of patients with CGD due to a deficiency of cytosolic NADPH oxidase components lacks detectable levels of p47-phox by immunoblotting; this defectaccounts for about 30% of all patients with CGD (58). Segel *et al.* have shown that a 47-kD phosphoprotein is absent in the neutrophils from certain patients with autosomal recessive inheritance of CGD who have normal levels of cytochrome b_{558} (59). Subcellular fractionation techniques have shown that this 47-kD phosphoprotein (now recognized as p47-*phox*) is present in the cytosol and in the plasma membrane, but not in granule fractions of PMA-stimulated neutrophils (60, 61). An explanation for this dual location was provided by Heyworth *et al.*, who showed that phosphorylation of p47-*phox* initially occurs in the cytosol, before transloction of p47-*phox* to the membrane, and continues after membrane association (62). Two-dimensional electrophoresis revealed that the two most acidic isoforms are not found in stimulated neutrophils from CGD patients lacking cytochrome b_{558} (63, 64). Normal cells, as well as cells that contain normal levels of dysfunctional cytochrome b_{558} (bearing a ^{415}Pro⌧His substitution in gp91-*phox*) contain all phosphorylated isoforms (64). Thus, the final phosphorylations of p47-*phox* occur only when an intact cytochrome b_{558} is present and are likely to take place after translocation of p47-*phox* to the membrane.

The cDNA for p47-*phox* has been cloned, and the gene has been localized and characterized (18, 19). The NCF1 gene encoding p47-*phox* is located on chromosome 7 (65); mutations in this gene cause the A47 type of CGD. Recombinant p47-*phox* as well as purified cytosol fractions containing p47-*phox* are able to restore oxidase-supporting activity in cytosol from A47 CGD neutrophils (17-19, 66-68). The deduced amino-acid sequence (390 residues) of p47-*phox* contains at least six potential serine phosphorylation sites for protein kinase C (PKC), in good agreement with the apparent number of phosphorylated isoforms seen on two-dimensional electrophoresis. However, the actual kinase responsible for p47-*phox* phosphorylation in intact cells is not yet known. A schematic representation of the phosphorylation sites in p47-*phox* is shown in Fig. 2.

Furthermore, the amino-acid sequence of p47-*phox* contains two SH3 motifs and at least one proline-rich region (Fig. 3). These domains probably play a role in the interaction between the cytosolic components and in the assembly of the NADPH oxidase (see below).

4.4. p67-phox

A minority of patients with cytosol-deficient CGD are lacking p67-*phox*, as determined by immunoblotting; this defect accounts for less than 5% of all cases of CGD (58). A full-length cDNA has been obtained and sequenced (20); p67-*phox* is encoded by the NCF2 gene that is located on chromosome 1 (65). The predicted 526-amino-acid sequence of p67-*phox* also contains two SH3 domains an at least one proline-rich region, as depicted in Fig. 3. Translocation experiments have revealed that p67-*phox* is unable to translocate to the plasma membrane in cells of p47-*phox*-deficient patients (69). In contrast, p47-*phox* appeared to translocate normally in cells of a p67-*phox*-deficient donor (69-71). However, we observed otherwise (almost no translocation of p47-*phox* in p67-*phox* deficient patients, J. Leusen *et al*., manuscript submitted).

4.5. p40-phox

A third cytolic *phox* protein was shown to reside in a complex with p67-*phox* in the cytosol of resting neutrophils (72, 73). The cDNA of this 40-kD protein has also been cloned, and the predicted protein of 339 amino acids contains one SH3 domain (73). Although p40-*phox* is not required for activity in the cell-free assay (see Section 5.1 'Cell-free system'), it is thought to play a role in stabilizing p67-*phox* in intact cells.

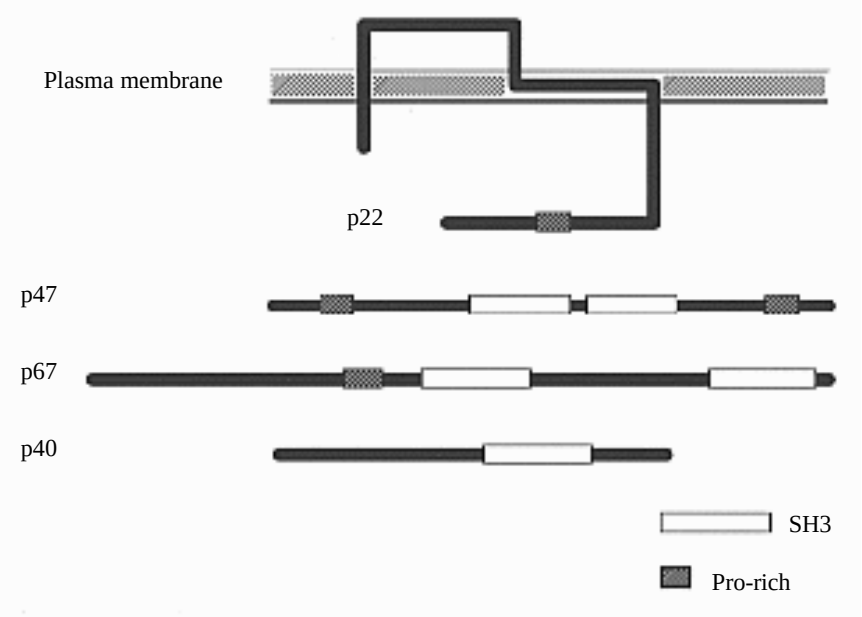

Figure 3. SH3 domains and proline-rich regions in the components of the NADPH oxidase. The membrane-bound p22-*phox*, the light subunit of cytochrome b_{558}, contains one proline-rich region, a putative counter structure for one of the SH3 domains in the cytosolic proteins. P47-*phox* and p67-*phox* each contain two SH3 domains and one proline-rich region, p40-*phox* contains only one SH3 domain.

4.6. p21-rac

The last cytosolic protein required for the activity of the NADPH oxidase is a *ras*-related protein called *rac* (74-78). This protein family has a wide tissue distribution; in neutrophils p21-*rac*2 is the most abundant *rac* protein, but p21-*rac*1 is also present (79). Probably, *rac* functions by changing from an inactive, GDP-bound state to an active, GTP-bound state in which it can mediate the activation of the NADPH oxidase. Fine-tuning of this process may be mediated by the regulation of GTP/GDP exchange of *rac* by GDP-dissociation inhibitor (GDI) protein and GDP-dissociation stimulator (GDS) protein (75-78, 80).

CGD patients with decreased NADPH oxidase activity due to mutations in *rac* or GTP/GDP exchange-regulating proteins are not known, probably due to the fact that these proteins are involved in several other (essential) cellular functions, such as vesicular transport and cytoskeleton dynamics. Mutations in such proteins may be incompatible with life.

5. Activation of the NADPH oxidase

5.1. Cell-free system

Many of the recent advances in our understanding of the components of the NADPH oxidase have been the result of studies employing a cell-freeactivation system, first developed by Heyneman and Vercauteren (81) and Bromberg and Pick (82), and later extended by other investigators (83-88). The cell-free NADPH system is comprised of neutrophil membranes (containing cytochrome b_{558}), and three neutrophil cytosolic fractions containing p47-*phox*, p67-*phox* and *rac* (89). The system is activated by GTP or its non-hydrolysable derivative GTPgS and an amphiphilic agent (SDS or arachidonic acid). NADPH is needed as a substrate for the NADPH oxidase. Unlike in intact cells, phosphorylation of p47-*phox* is not required in this system: inhibitors of PKC have no inhibitory effect in the cell-free system.

5.2. Important domains within cytochrome b_{558}

CGD patients with a single amino-acid substitution in one of the subunits of cytochrome b_{558} have provided useful information about the structure and function of the cytochrome. For the $A22^{+}$ patient described in ref. (50) the functional defect was obvious: the ^{156}Pro⌫Gln substitution in the proline-rich region of p22-*phox* disrupts a putative counter structure for SH3 domains in p47-*phox*. Indeed, we found no translocation of p47-*phox* and p67-*phox* to the plasma membrane in either intact neutrophils or in the cell-free system of this patient (50). We also showed a normal electron flow within the cytochrome b_{558} of this patient when neutrophil membranes were tested in a cell-free system without p47-*phox* and p67-*phox*, in which negatively charged phospholipids were added as

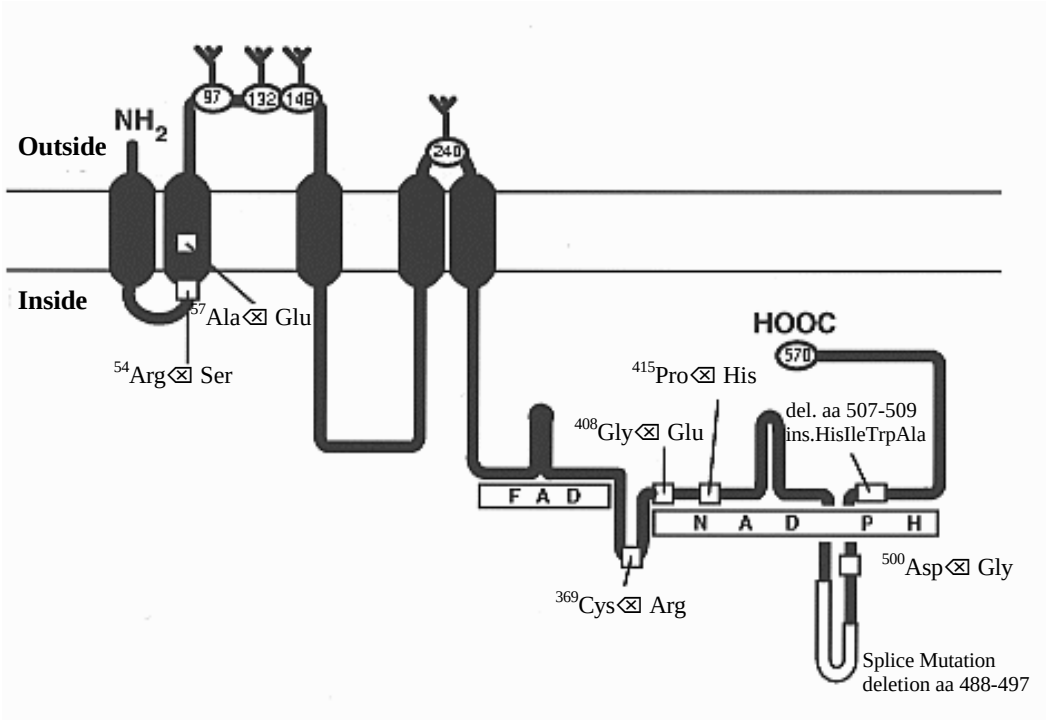

Figure 4. Localization of X91$^+$ CGD mutations in gp91-phox. Schematic representation of the gp91-*phox protein*. Indicated are the possible oritentation of the peptide in the membrane, the N and C termini, the possible glycosylation sites (Y), the putative binding regions for FAD and NADPH, and the positions of the mutations that induce the X91$^+$ CGD phenotype (normal expression of gp91-*phox* protein, no oxidase activity).

activators for cytochrome b_{558} (90, 91). This proves that 1) the mutation in p22-*phox* in the neutrophil membranes does not affect the transfer of electrons from NADPH via FAD and heme to oxygen, and 2) the cytosolic proteins act only as activators of the NADPH oxidase, as was already stated by Koshkin and Pick (90, 91). This means that the superoxide-generating capacity is fully contained within the cytochrome b_{558}. Most likely, the cytosolic proteins p47-*phox* and p67-*phox* induce by their interaction with cytochrome b_{558} a conformational change in the cytochrome that permits binding of NADPH and start of the electron flow in this flavocytochrome. The neutrophil membranes from all four X91$^+$ patients tested by us in this system (with negatively charged phospholipids but without p47-*phox* and p67-*phox* as activators of the oxidase) were incapable of generating any superoxide, regardless of the ability of the cytosolic proteins to bind to cytochrome b_{558} in the usual cell-free system. This may implicate that any mutation in gp91-*phox* destroys the catalytic activity of the flavocytochrome under all conditions tested, suggesting that gp91-*phox* alone is the catalytic unit, and that p22-*phox* is only required for stabilization of gp91-*phox*. Indeed, sequence-homology studies between the C-terminal half of gp91-*phox* and the ferredoxin-NADP$^+$ reductase flavoenzyme family imply that both FAD and NADPH can be bound by this part of gp91-*phox*, and the location of these putative FAD- and NADPH-binding domains within gp91-*phox* have been deduced (27-29) (see Fig. 4).

The model described above does not predict the position of the heme groups in cytochrome b_{558}, because ferridoxin reductases do not contain heme moieties. Also, the model only describes the C-terminal part of gp91-*phox*, and the hemes are most probably located in the N-terminal half of gp91-*phox*. In fact, thereare indications for two hemes for each cytochrome b_{558} unit; one liganded by two histidines of gp91-*phox* (92) and another shared between the two cytochrome b_{558} subunits (30), possibly involving histidine-239 of gp91-*phox* and histidine-94 of p22-*phox* (C.D. Porter *et al*., in press). A recent report on an X91$^+$ CGD patient with an ^{54}Arg⌫Ser substitution has substantiated the two-heme hypothesis (93, 94). This mutation abolishes the electron attraction of the positively charged arginine and perhaps disrupts a hydrogen bond between the arginine and a heme propionate side chain. As a result, the mutant form of the cytochrome contains two non-identical hemes with midpoint potentials of E_{m7} = -220 and E_{m7} = -300 mV. In the light of this information, the investigators reanalyzed the wild-type cytochrome b_{558} and concluded that it also contains two separate heme centers, with midpoint potentials of E_{m7} = -225 mV and E_{m7} = -265 mV (94).

It is generally believed that the cytosolic components p47-*phox* and p67-*phox* have to interact with the membane-bound p22-*phox* and gp91-*phox* to induce NADPH oxidase activity. In the neutrophils of the patient with the ^{54}Arg⌧Ser substitution in the N-terminal part of gp91-*phox* the cytosolic proteins translocated normally (93). Two other X91^{+} patients are known thus far with normal translocation of p47-*phox* and p67-*phox*: patient R.C. (42, 44, 64, 69) and a patient described in ref. (95). Patient R.C. carries a ^{415}Pro⌧His substitution in a putative NADPH-binding region in gp91-*phox* (Fig. 4), which leads to decreased binding of NADPH to the oxidase (27). The second patient has a deletion of five nucleotides and an insertion of eight nucleotides in exon 12 of the CYBB gene, resulting in a replacement of ^{507}Gln-^{508}Lys-^{509}Thr by His-Ile-Trp-Ala in gp91-*phox*. This mutation also resides in a putative NADPH-binding region (Fig. 4).

We have characterized three X91^{+} patients with a defective translocation of p47-*phox* and p67-*phox*: patient D.S., described in ref. (44), patient C.E. and patient N.K. (J. Leusen *et al.*, manuscript in preparation). All three patients had point mutations leading to an amino-acid substitution in the cytosolic, C-terminal part of gp91-*phox*. In two of the patients, the substitutions are situated in protein loops exposed to the cytosol, as predicted by a computer model of the C-terminal half of gp91-*phox* (96), viz. ^{500}Asp⌧Gly in patient D.S. and ^{369}Cys⌧Arg in patient C.E. (Figs. 4 and 5). We have established the domain around residue 500 as a binding region for either p47-*phox* or p67-*phox* by blocking the translocation of both cytosolic proteins in a cell-free assay with a synthetic peptide with the sequence of this domain (44).

Previously, we have postulated (44) that p47-*phox* is the most likely candidate for interaction with the domain surrounding ^{500}Asp of gp91-*phox*, because this protein has been described to translocate without p67-*phox* (69, 71), while the opposite (translocation of p47-*phox* independently of p67-*phox*) did not seem to occur (69). Because we found no translocation of *either* cytosolic protein to the neutrophil plasma membranes of patient D.S., it seemed logical to suppose a disturbed interaction of this gp91-*phox* domain with p47-*phox*. Furthermore, p47-*phox* contains several basic regions that might act as counter structures for the negatively charged ^{500}Asp; it was observed that positively charged peptides also block NADPH oxidase assembly (97). However, upon neutrophil activation, p47-*phox* becomes phosphorylated at 6-8 serine residues, and only the two most acidic isoforms are associated with the membrane. Also, we found recently that p47-*phox* did *not* translocate in an A67^{0} patient (J. Leusen *et al.*, submitted). Furthermore, there is good evidence that p47-*phox* interacts with p22-*phox* after activation of the oxidase, mediated via the SH3 domains of p47-*phox* (see next section) and the proline-rich region in p22-*phox* mutated in the A22^{+} patient (50). These observations render p67-*phox* a better candidate for binding to the domain in gp91-*phox* surrounding residue 500 (see also next paragraph). The loop containing residue 369 is involved in this interaction, because the two domains are quite close in the 3D model of gp91-*phox* (96) (Fig. 5).

Recently, it has been shown that p47-*phox* and p67-*phox* have distinct roles in the regulation of electron flow in cytochrome b_{558} (98, 99). In the absence of p47-*phox*, p67-*phox* alone can facilitate electron flow from NADPH to the flavin center, resulting in the reduction of FAD, whereas the presence of p47-*phox* is required for electron transfer to proceed beyond the flavin center to the hemes in cytochrome b_{558} and then to oxygen. This fits with the idea suggested above that p67-*phox* is the cytosolic component that binds to the protein loops mutated in patients D.S. and C.E., because p67-*phox* would then be in close proximity to the NADPH-binding regions and the flavin center of gp91-*phox*, enabling it to perform a regulatory function in this part of the protein.

The ^{408}Gly, substituted for Glu in patient N.K., is localized between the two protein loops containing residue 500 and 369, and predicted to be buried in the protein (Fig. 5). Therefore, it is not a likely binding site for one of the cytosolic proteins. However, it is possible that the mutation changes the folding of the protein, perhaps leading to another orientation of the two loops with respect to each other. If this is true and one protein (p67-*phox*) binds to both loops, this interaction might be disturbed by the ^{408}Gly⌧Glu substitution.

A number of additional regions within gp91-*phox* have been identified as binding regions of p47-*phox* by inhibition studies with synthetic peptides in the cell-free system (oxidase activity or translocation) (70, 100) or by phage display and panning system with purified or recombinant proteins (101, 102). To substantiate the importance of these domains, synthetic peptides were used to assess inhibition of oxidase activation. However, the IC_{50} of some of these peptides was sometimes relatively high (>10 μM), and scrambled peptides were not always used as a control. It is known that positively charged peptides are inhibitory under cell-free activation conditions with IC_{50} values <2 μM (97, 103). Possibly, the effect of positively charged peptides is specific in the sense that they block the binding of p47-*phox* to domain ^{86}TRVRRQL^{93} of gp91-*phox* (101). This domain, however, has been postulated by others to reside in an extracellular loop of the protein (see Fig. 4). Therefore, the specificity of these interactions remains to be proven, and genetic studies still provide the most convincing evidence for physiologically relevant interactions within the NADPH oxidase system.

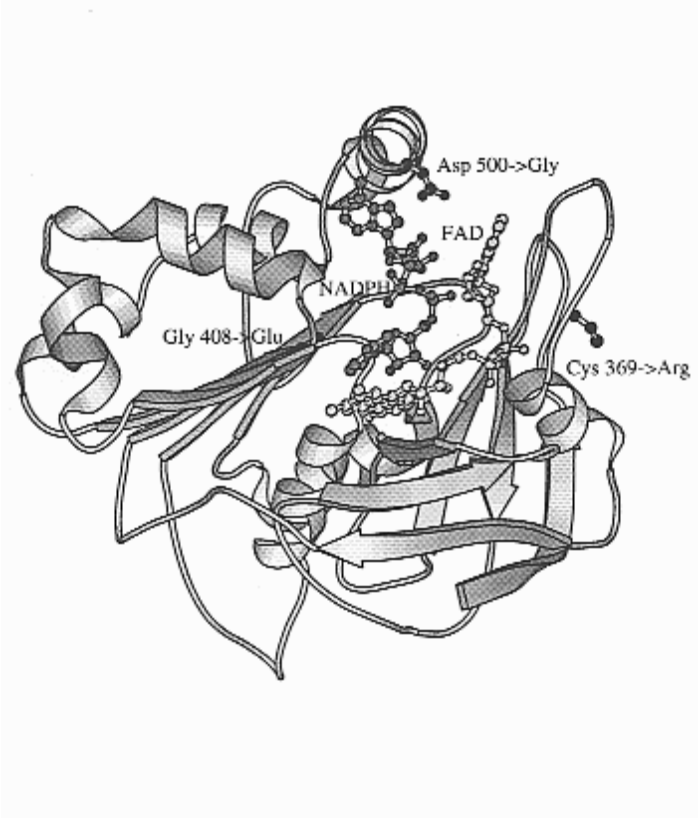

Figure 5. Model of the cytosolic part of gp91-phox in the activated, NADPH-bound state. The protein is modelled on Spinach ferrodoxin (157) as described by Taylor et al. (96). The figure was drawn with MOLSCRIPT (158), and kindly provided by dr Nicholas Keep of prof. Segal's group (University College, London, G.B.). The position of the substitutions in patients C.E. (^{369}Cys⌧Arg), patient N.K. (^{408}Gly⌧Glu) and patient D.S. (^{500}Asp⌧Gly) are shown. Residues 369 and 500 are situated in protein loops exposed to the cytosol, but residue 408 is buried in the protein between these loops.

5.3. Interactions between the cytosolic components

The cytosolic components of the NADPH oxidase appear to reside in a complex of 240-300 kD in the cytosol of resting neutrophils (104-106). Both p47-*phox* and p67-*phox* contain two SH3 domains, known to be involved in protein-protein interactions (47, 48, 107). P40-*phox* contains one SH3 domain (38). SH3 domains bind to proline-rich regions (49). Both p47-*phox* and p67-*phox* contain at least one such putative SH3-binding domain. As described in (108), p47-*phox* binds to p67-*phox* blotted onto nitrocellulose, and p67-*phox* binds to blotted p47-*phox* and p40-*phox*. We found that the proline-rich region in p67-*phox* is not required for interaction with either p47-*phox* or p40-*phox*. In contrast, the proline-rich region of p47-*phox* is essential for the binding to p67-*phox*, also when both proteins are in solution. In addition, the last 13 amino acids of p47-*phox* seem to be involved in exposing this proline-rich region, because an antibody directed against the C-terminal 13 amino acids of p47-*phox* blocks the interaction between p47-*phox* and p67-*phox*, but only when p47-*phox* is in solution. A truncated p47-*phox* confirmed these findings. In Fig. 6, a hypothetical model is given of the interactions between the oxidase components, based on the data described in this section.

It is of interest to note that a synthetic peptide mimicking the last 13 amino acids of p47-*phox* becomes phosphorylated *in vitro* by PKC and other serine kinases (109). In combination with our findings, this indicates that the phosphorylation of the C-terminus could be an important switch for repositioning the cytosolic *phox* proteins, leading to their interaction with the membrane-bound oxidase components and thus to an active NADPH oxidase. Other investigators have shown that the C-terminal SH3 domain of p67-*phox* is the counter structure for the proline-rich region in p47-*phox* (110, 111) and that the SH3 domains of p47-*phox* can bind to proline-rich sequences in p47-*phox* itself and to the proline-rich sequence in p22-*phox* (111, 112). Addition of SDS or arachidonic acid, activators of the cell-free system, leads to exposure of the SH3 domains in p47-*phox* (112). In the cell-free system, the p47-*phox* without its proline-rich region (108) and p67-*phox*

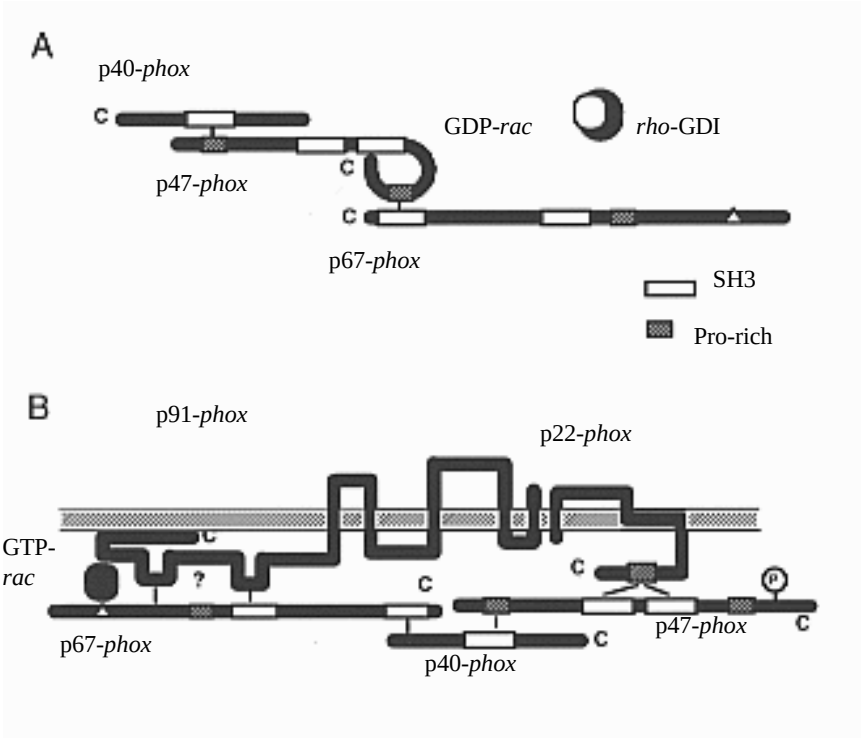

Figure 6. Hypothetical model of the interactions between the NADPH oxidase components. The interactions between the cytosolic components are represented for the resting situation in panel A. For the activated state, the interactions between the cytosolic components and cytochrome b_{558} are depicted in panel B. It is postulated that in the resting state, the C-terminus of p47-*phox* is folded backwards, binding or covering its SH3 domains and exposing the C-terminal proline-rich region, allowing it to bind to the C-terminal SH3 domain of p67-*phox*. The other two binding domains (one proline-rich region and one SH3 domain) of p67-*phox* are not involved in binding to one of the cytosolic proteins, but possibly interact with the cytoskeleton. It is known that the SH3 domain of p40-*phox* interacts with p47-*phox* (114), perhaps to its N-terminal proline-rich region, as is shown here. Upon cell activation, p47-*phox* becomes phosphorylated, and the phosphorylation of ^{379}Ser has been shown to be crucial for activity and translocation (133). Therefore, we suggest here that phosphorylation of this site leads to uncovering of the SH3 domains of p47-*phox*, making them available for interaction with the proline-rich region of p22-*phox* (the idea that phosphorylation of p47-*phox* leads to exposure of SH3 domains was already suggested by Leto *et al.* (111)). This also disrupts the interaction with p67-*phox*. However, via binding to p40-*phox*, p67-*phox* is still retained in the complex. We also hypothesize that p67-*phox* binds to two protein loops containing residues 369 and 500 of gp91-*phox*, although the binding sites on p67-*phox* are still unknown. *Rac* can only bind after dissociation from *rho*-GDI and loading with GTP. To which domain of cytochrome b_{558} *rac* binds, is unknown.

without its SH3 domains (113) can still induce superoxide production. Hence, the interaction between p47-*phox* and p67-*phox* is *not* required for cell-free oxidase activity (108,113)). However, in intact cells (EBV transformed B-cell lines of CGD patients transfected with cDNA encoding the missing cytosolic component), the second SH3 domain of p67-*phox* that interacts with p47-*phox* is indispensable for oxidase activity (113), suggesting that a complex between p47-*phox* and p67-*phox* is required in intact cells.

With the use of the two-hybrid system the binding domains of p40-*phox* have also been mapped: the SH3 domain of p40-*phox* interacts with p47-*phox*, and its last 36 amino acids interact with p67-*phox* (114). The same method revealed that the C-terminal SH3 domain of p67-*phox* is crucial for the interaction with p40-*phox* and p47-*phox* (A. Fuchs, unpublished results). Thus far, there are no domains of p67-*phox* known to interact with cytochrome b_{558} after activation.

5.4. Role of phosphorylations in activation of the oxidase

Neutrophils stimulated with PMA or fMLP exhibit rapid phosphorylation of p47-*phox* on multiple serine residues (115), which accompanies translocation of this protein to the submembraneous cytoskeleton. Protein kinase antagonists that inhibit phosphorylation of p47-*phox* (*e.g.* H-7, staurosporine or 1-*O*-hexadecyl-2-*O*-methylglycerol (AMG)) block translocation of this protein to the detergent-insoluble fraction (116, 117), and, concomitantly, block superoxide anion release (62,

118-120). Six to eight isoforms of p47-*phox* can be observed in stimulated cells that result from different numbers of phosphorylated serine residues (64, 121). Only the most heavily phosphorylated species are observed in the cytoskeletal fraction, which suggests that extensive phosphorylation of this protein is required for superoxide anion production when PMA is the stimulus (122). Interestingly, p47-*phox* undergoes continuous cycling between phosphorylation and dephosphorylation in PMA-stimulated cells, with the phosphorylation reaction predominating. Interruption of the phosphorylation of p47-*phox* results in rapid dephosphorylation of p47-*phox* and in dissociation of this protein from the cytoskeletal fraction (117, 123, 124). Thus, the activity of p47-*phox* can be regulated by a phosphorylation-dephosphorylation cycle that governs association-dissociation of this protein with cytochrome b_{558} (125). Protein kinase C may form a stable complex with p47-*phox* when PMA, but not when fMLP is the stimulus (117). Dephosphorylation of p47-*phox* is catalyzed by a phosphatase type 1 and/or 2A (124, 126).

All of the phosphorylations of p47-*phox* in neutrophils stimulated with PMA or fMLP occur in the C-terminal region between ^{303}Ser and ^{379}Ser (122). Edman degradation of ^{32}P-labeled p47-*phox* revealed that ^{303}Ser, ^{304}Ser, ^{320}Ser, ^{328}Ser, ^{345}Ser, ^{348}Ser and at least one of the three ^{359}Ser, ^{370}Ser, and ^{379}Ser were phosphorylated. The first four serine residues listed are flanked by basic groups and exhibit consensus sequences for phosphorylation by PKC (123) or one or more novel protein kinases described in neutrophils (127, 128). These novel kinases appear to function in a stimulatory pathway downstream of the phosphatidylinositol (PI) 3-kinase (129). The sequence around ^{345}Ser and ^{348}Ser (PGPQSPGSP) constitutes a consensus sequence for proline-directed protein kinases (*e.g.* MAP kinases) (130, 131). However, we tested whether recombinant p47-*phox* was a substrate for the ERK2 MAP kinase, immunoprecipitated from 3T3 fibroblasts transfected with the insulin receptor and stimulated with insulin, and observed no difference in labeling between wild-type p47-*phox* and a deletion mutant of p47-*phox* lacking the putative MAP kinase phosphorylation site. Moreover, a classical substrate for MAP kinase, myelin basic protein (MBP), was phosphorylated about 100 times better (J. Leusen *et al*., unpublished results). Besides, the putative MAP kinase phosphorylation site is not conserved in the mouse sequence of p47-*phox* (132).

Only recently, Faust *et al.* (133) found an answer to the question which residue(s) in p47-*phox* need to become phosphorylated for NADPH oxidase activation. This was performed by mutating all the putative serine phosphorylation sites of p47-*phox* (between ^{303}Ser and ^{379}Ser) and monitoring these mutants in intact, transfected cells (EBV-transformed B lymphocytes of a CGD patient deficient for p47-*phox*) for superoxide-inducing capacity and for translocation to the plasma membrane upon cell activation. In this study it was demonstrated that only the phosphorylation of ^{379}Ser is essential for oxidase activity and membrane association (133). This is a very intriguing result, because apparently all phosphorylations of p47-*phox* can be reduced to one essential phosphorylation, and this phosphorylation happens to be in the C-terminal domain that we postulate to be an important switch for NADPH oxidase activation.

5.6. Role of rac and Rap1A in activating the oxidase

The absolute requirement for *rac* in the NADPH oxidase was shown by cell-free studies in which cytosol could be replaced with recombinant p47-*phox*, p67-*phox*, and *rac*1 or *rac*2 (134-136); similar studies in which the plasma membrane was replaced by purified cytochrome b_{558} demonstrated that these four components were the minimum proteins required to obtain a fully active NADPH oxidase (28, 89). The cytosolic p40-*phox* and the cytochrome-associated Rap1A protein, absent from these purified preparations, appeared not to be necessary for oxidase function under the cell-free conditions utilized. Also, recombinant Rap1A was unable to support NADPH oxidase activity in the cell-free system (135). Transfection studies of dominant-negative (GDP-bound) and dominant-positive (GTP-bound) Rap1A in human B lymphocytes (137) or in differentiated HL60 cells (138) revealed that overexpression of either of these mutant forms of Rap1A suppressed oxidase activity. GTP-bound Rap1A is associated with purified cytochrome b_{558} and GDP-bound Rap1A is not (56). Perhaps GTP-rap1A activates cytochrome b_{558}, by inducing a phosphorylation or a conformational change. As mentioned earlier, also phosphorylation of Rap1A disrupts the association with cytochrome b_{558}. Taken together, it is likely that Rap1A in its GTP-bound state activates and in its GDP-bound state deactivates the NADPH oxidase in a dynamic cycle in intact cells.

Rac is normally found in the cytosolic fraction of phagocytes, in a complex with the GDP dissociation inhibitor *Rho*-GDI (75-77, 139). Rac is maintained in its inactive form in this complex, but upon cell activation, biologically active lipids, such as arachidonic acid, phosphatidic acid and phosphatidylinositols, can disrupt the binding of *rac* to *Rho*-GDI (139, 140). In fibroblasts, it has been shown that PI 3-kinase acts upstream of *rac* to mediate PDGF-induced lamellipodia formation (141). In neutrophils, PI 3-kinase and the NADPH oxidase are simultaneously activated by several stimuli; furthermore, wortmannin, an inhibitor of PI 3-kinase, can prevent NADPH oxidase activation (142, 143). PI 3-kinase may therefore act upstream of *rac* in this system as well.

Two potential targets for *rac*1 have been discovered: p67-*phox* and the serine kinase p68-*pak*, related to the brain p65-*pak* (144,145). The GTP-bound *rac* activates p68-*pak*, which in turn phosphorylates p47-*phox* on ^{328}Ser (146). However, according to the findings

of Faust et al. (133), this phosphorylation is not essential for oxidase activation. In contrast to p47-*phox*, p67-*phox* is recovered predominantly in the Triton X-100 insoluble fraction of both stimulated and unstimulated neutrophils (116, 117). This property is consistent with p67-*phox* being bound to the cytoskeleton (147). Activated *rac* is known to be involved in cytoskeletal reorganization and could thus play a role in the function of p67-*phox* or mediate the interaction between p67-*phox* and other components of the NADPH oxidase. Some investigators have shown that *rac* associates with the plasma membrane upon cell activation (148, 149). However, we and others (150) could not reproduce these results. *Rac* translocation has also been reported to occur independently of p47-*phox* and p67-*phox* (151, 152). However, we have demonstrated with neutrophils from a CGD patient bearing a deletion of a lysine at position 58 in p67-*phox* that p47-*phox* and p67-*phox* failed to translocate in intact cells, whereas they did interact with cytochrome b_{558} in the cell-free system, but still without inducing superoxide production. The disease in this patient was caused by a disrupted interaction of p67-*phox*-Δ^{58}Lys with *rac* (J. Leusen *et al*, submitted). This indicates that the binding of *rac* to p67-*phox* is essential for the activity of the NADPH oxidase in both intact cells and the cell-free system; it is possible that *rac* induces a conformational change or phosphorylation of p67-*phox*. Furthermore, a *rac*/p67-*phox* interaction is needed for the translocation of p47-*phox* and p67-*phox* in intact cells. Most likely, *rac* functions as a 'shuttle protein', carrying p67-*phox* to the membrane in its GTP-bound state, and dissociating in its GDP-bound state and returning to the cytosol. Conversely, *rac* might induce a change in the submembraneous cytoskeleton (like membrane ruffling (153)) and create a docking site for p67-*phox*. Thus, *rac* probably mediates translocation of p67-*phox* and, as a consequence, also the translocation of p47-*phox*.

5.6. NADPH oxidase: cell-free system vs intact cells

It is evident from several observations discussed above that there are some important differences between the cell-free system and intact cells concerning the activation of the NADPH oxidase. In intact cells, the NADPH oxidase is a dynamic enzyme that needs incessant stimulation to remain active (continuous phosphorylation of p47-*phox*, GTP loading of *rac* and cycling of Rap1A). In contrast, once the NADPH oxidase is assembled under cell-free conditions with SDS or arachidonic acid and with GTPgS, it remains active. For instance, the sulfhydryl reagent N-ethylmaleimide (NEM) inhibits ongoing oxidase activity in activated neutrophils, whereas in the cell-free system it has no effect once the oxidase has been assembled (154). Also synthetic peptides that interfere with the assembly of the NADPH oxidase are only effective in the cell-free system when added prior to activation (44, 70, 97, 101, 102, 155, 156).

Another difference between the cell-free system and intact neutrophils is that in the cell-free system the oxidase components p40-*phox* and Rap1A are not required, but Rap1A is responsible for at least a four-fold increase in superoxide production when overexpressed in differentiated HL-60 cells (138). Also, a complex between p47-*phox* and p67-*phox* is not mandatory for cell-free oxidase activity, as shown with mutated p47-*phox* proteins in (108). In addition, interaction of *rac* with p67-*phox* is not necessary for translocation of the cytosolic components in the cell-free system (but it is for oxidase *activity* in this system) (J.Leusen *et al.*,submitted). We can conclude from these data that although the cell-free system is very useful for a number of applications, results obtained in this system cannot be directly extrapolated to intact cells.

To recapitulate: the human NADPH oxidase is a very intriguing enzyme; although its catalytic unit is retained within cytochrome b_{558}, various additional proteins are required for activity of the NADPH oxidase. In the past few years, substantial progress has been made to elucidate the protein-protein interactions and the activation events involved. It has become evident that 1) activation of *rac* and subsequent interaction with p67-*phox* is crucial for the interaction of p67-*phox* with cytochrome b_{558}, probably with gp91-*phox*, 2) p47-*phox* interacts with p22-*phox*, and phosphorylation of ^{379}Ser of p47-*phox* is obligatory for this event, and 3) p47-*phox* and p67-*phox* regulate each others translocation in a positive sense (see also ref. (71)). To put it differently: it is vital to gain insight in the intrigues within the *phox* family and associated characters to fully understand NADPH oxidase activation.

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