Journal of Biochemistry and Molecular Biology, Vol. 33, No. 6, November 2000, pp. 427-439

JOURNAL OF
Biochemistry
and
Molecular Biology

Review

Regulation of the Phagocyte Respiratory Burst Oxidase by Protein Interactions

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Received 5 September 2000

The activity of the phagocyte respiratory burst oxidase is regulated by complex and dynamic alterations in protein-protein interactions that result in the rapid assembly of an active multicomponent NADPH oxidase enzyme on the plasma membrane. While the enzymatic activity has been studied for the past 20 years, the past decade has seen remarkable progress in our understanding of the enzyme and its activation at the molecular level. This article describes the current state of knowledge, and proposes a model for the mechanism by which protein-protein interactions regulate enzyme activity in this system.

Keywords: NADPH oxidase, respiratory burst, superoxide, Rac, protein interaction

Introduction

The evolution of antibiotic resistance and the dissemination of resistant organisms into the human population has led to a resurgence of bacterial infection as an important health problem for the new millennium. Neutrophils provide the predominant host defense against bacterial and other infections. One of the major mechanisms by which these cells kill bacteria is the "respiratory burst" in which marked consumption of O_2 is seen. While this reaction was initially thought to be due to mitochondrial respiration, it was later found to be insensitive to the mitochondrial respiration inhibitor cyanide. It was subsequently discovered that the initial product formed from molecular oxygen is superoxide ($O_2^{\cdot-}$). In secondary reactions, superoxide then reacts to generate hydrogen peroxide (H_2O_2), hydroxyl radical, and HOCl. Together these oxygen-derived species participate in bacterial killing. The biological importance of the respiratory burst is illustrated by the rare inherited disorder Chronic Granulomatous Disease (CGD) (Smith and Curnutte, 1991; Roos *et al.*, 1996), a condition in which a failure of the respiratory burst is associated with an inability to fight infections. At the other extreme are inflammatory diseases (e.g., shock lung, rheumatoid arthritis, Crohn's disease) and ischemia/reperfusion injury (e.g., after a myocardial infarction) in which pathological activation of the respiratory burst damages host tissues. These examples illustrate the importance of understanding and eventually manipulating the oxidative defense mechanisms of the host.

Superoxide generation is activated by exposure of neutrophils to bacteria or by chemical stimuli such as phorbol esters and chemoattractants. These stimuli activate a complex cascade of signalling events whose direct connection to the respiratory burst enzymatic machinery remains incompletely understood, but includes G protein coupled chemotactic receptors, phospholipase C, phospholipase D, protein kinase C and other signalling enzymes (McPhail and Snyderman, 1984; Tauber, 1987; Lambeth, 1988a; Lambeth, 1988b). Following activation of neutrophils with the protein kinase C activator PMA (phorbol 12-myristate, 13-acetate), superoxide-generating activity can be recovered in an isolated plasma membrane fraction (Babior *et al.*, 1976; Dewald *et al.*, 1979). Studies in the 1980's focused on such preparations to evaluate the catalytic properties of the superoxide generating enzyme. NADPH was established as the preferred pyridine nucleotide electron donor, with a Km around 50 μm compared with nearly 1 mM for NADPH (Takanaka and O'Brien, 1975; Tauber and Goetzl, 1979; Cross *et al.*, 1984). The enzyme catalyzes the following reaction (Green and Shanguan, 1993) and has therefore been referred to as the "phagocyte NADPH-oxidase" or "respiratory burst oxidase":

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

Despite their early utility, such preparations did not prove to be useful for detailed characterization of the enzyme. The enzymatic activity was highly labile to attempts to solubilize it from its membrane environment using detergents (Tauber and Goetzl, 1979; Bellavite *et al.*, 1983; Cross *et al.*, 1984). Tamura *et al.* (1989) found that the activity could be stabilized

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using protein crosslinkers, but these preparations were difficult to characterize, because they formed large molecular weight complexes in which the cross-linking could not be reversed. It is now clear that the lability was due to dissociation of activating proteins from the multicomponent oxidase, which is prevented by cross-linkers. Recent studies have shown that these large molecular weight complexes contain components of the cytoskeleton, and that these components can influence the activity of the enzyme (Morimatsu *et al.*, 1997; Tamura *et al.*, 2000).

Identification of Cytochrome b_{558} as a Component of the Respiratory Burst Oxidase Segal and coworkers (Segal *et al.*, 1978) noted that a heme absorption spectrum in membrane preparations from human neutrophils was absent in a patient with Chronic Granulomatous Disease (CGD). When the membrane preparation was chemically reduced, it showed a sharp alpha absorbance band at 558 nm (Segal and Jones, 1979) and has therefore been referred to as cytochrome b_{558}. The oxidation-reduction potential of the cytochrome was around -245 mV (Cross *et al.*, 1981), and the cytochrome is sometimes referred to by this unusually low redox potential, cytochrome b_{-245}. The relationship between cytochrome b_{558} and the respiratory burst oxidase was firmly established based on the finding (Segal *et al.*, 1983) that the characteristic spectrum was absent in membrane preparations from a majority of patients with an X-linked form of CGD. Hence, the cytochrome was proposed to be a component of the respiratory burst oxidase. However, it was subsequently shown that the cytochrome spectrum was present in autosomal recessive forms of chronic granulomatous disease, implying that there are one or more additional protein components of the respiratory burst oxidase.

Cell-Free Activation of the Respiratory Burst A major technical advance that has permitted a more complete understanding of the proteins that comprise the respiratory burst oxidase came with the establishment in the 1980's by several laboratories of cell-free activation systems. These systems originally used cytosol plus plasma membrane together with an activating anionic amphiphile such as arachidonate or SDS (Bromberg and Pick, 1984; Curnutte, 1985; Agwu *et al.*, 1991) or, in some later studies, phosphatidic acid (Qualliotine-Mann *et al.*, 1993). GTP analogs (Seifert and Schultz, 1987; Ishida *et al.*, 1989; Uhlinger *et al.*, 1991; Uhlinger *et al.*, 1993) and diacylglycerol (Uhlinger *et al.*, 1991) synergize with the anionic amphiphile to greatly augment $O_2^{\cdot-}$ generation. Recently, cell-free methods have been described to activate the burst in response to phosphorylation by protein kinases (El Benna *et al.*, 1995; Park *et al.*, 1997; Lopes *et al.*, 1999), confirming an earlier pioneering study by Tauber's group (Cox *et al.*, 1985). Cell-free studies established that in addition to the membrane-associated cytochrome b_{558}, one or more proteins present in the cytosol are essential for observing optimal activity. Complementation studies (Nunoi *et al.*, 1988) using cytosol from CGD patients in a cell-free activity system established that a minimum of two additional cytosolic proteins were needed for NADPH-oxidase activity. Fractionation of the cytosol then provided the essential methodology which subsequently led to the identification of cytosolic regulatory proteins for the oxidase.

Cytochrome b_{558}, the Catalytic Moiety of the Respiratory Burst Oxidase Based on its absorption properties, cytochrome b_{558} was purified and characterized by several groups (Pember *et al.*, 1984; Harper *et al.*, 1984; Parkos *et al.*, 1987; Knoller *et al.*, 1991). The hemoprotein consists of two subunits, gp91*phox*, a glycoprotein that migrates as a diffuse band centering around 91 kDa, and p22*phox*, a 22 kDa protein. In addition, early preparations also contained the small GTPase Rap1a (Quinn *et al.*, 1989). Full-length Rap1a does not affect activity in cell-free systems. Nevertheless, using a cell transfection approach, a dominant negative forms of Rap1a inhibited (Maly *et al.*, 1994) and wild type Rap1a (Gabig *et al.*, 1995) stimulated reactive oxygen production. Thus, the involvement of Rap1a as a component of cytochrome b_{558} is incompletely understood, and additional studies are needed to resolve this question.

The gene that is absent or mutated in the X-linked form of the phagocytic disorder chronic granulomatous disease was cloned based on its chromosomal map position by Orkin and colleagues in 1986 (Royer-Pokora *et al.*, 1986). Subsequent studies (Dinauer *et al.*, 1987; Seedorf *et al.*, 1994) established that the protein encoded by this gene is a component of cytochrome b_{558}, and that the gene encodes a protein of ~65 kDa which runs anomalously on SDS gels due to glycosylation. The second component of cytochrome b_{558}, p22*phox*, was subsequently cloned (Parkos *et al.*, 1988). The two subunits of cytochrome b_{558} were shown to stabilize one another (Segal, 1996; Yu *et al.*, 1997), so that in the absence of either subunit, there is decreased expression of its partner protein. Analysis of preparations of cytochrome b_{558} revealed 2-3 hemes (Quinn *et al.*, 1992), identified as protoporphyrin IX (Pember *et al.*, 1984). Subsequent studies demonstrated a stoichiometry of 2 non-identical hemes per cytochrome (Cross *et al.*, 1995; Nisimoto *et al.*, 1995). While early studies suggested that one of the hemes was shared between the two subunits (Quinn *et al.*, 1992), it was later shown that gp91*phox* expressed in the absence of p22*phox* retained the heme groups (Yu *et al.*, 1998), indicating a localization in gp91*phox*.

Based on biochemical precedent and effects of flavin or flavin analogs on activity or stability (Babior and Kipnes, 1977; Light *et al.*, 1987), the phagocyte NADPH-oxidase was predicted to include a flavoprotein component which accepts electrons from NADPH and transfers them to the hemes in cytochrome b_{558}. Because the isolated cytochrome preparations were inactive either alone or in combination with cytosol and because purified preparations of cytochrome b_{558}

did not contain any FAD or FMN, it was assumed that a flavoprotein component of the oxidase was missing. Therefore, a great deal of effort was expended attempting to identify a distinct membrane-associated NADPH-specific flavoprotein that could transfer electrons to cytochrome b_{558}. However, studies by Pick and coworkers (Knoller *et al.*, 1991) established definitively by purification that the membrane associated component of the NADPH oxidase is cytochrome b_{588} and that there are no additional membrane components. It is now clear that cytochrome b_{558} itself is the long-sought flavoprotein (Rotrosen *et al.*, 1992; Segal *et al.*, 1992; Takeshige and Sumimoto, 1994) and that the absence of activity in purified preparations resulted from loss of FAD during the isolation procedures. A careful examination of the sequence of cytochrome b_{558} revealed a weak homology with a number of known flavoproteins including cytochrome P450 reductase, ferredoxin-$NADP^+$ reductase, and others, and active preparations of cytochrome b_{558} could be obtained by incubating purified cytochrome with a combination of FAD and phospholipid. Following this procedure, retention of FAD with the cytochrome was seen upon gel filtration chromatography. Under optimized reconstitution conditions, using both native FAD and FAD analog, a stoichiometry of approximately 1 FAD per 2 hemes was obtained (Doussiere *et al.*, 1995; Nisimoto *et al.*, 1995). In addition, the gp91*phox* contains regions that are homologous to pyridine nucleotide binding sites in known proteins. Thus, gp91*phox* is a flavocytochrome, and contains the binding sites and prosthetic groups that are needed for transfer of electrons from NADPH to oxygen.

A hydropathy model of flavocytochrome b_{558} is shown in Fig. 1. The N-terminal half of flavocytochrome b_{558} is extremely hydrophobic, and contains 5-6 predicted transmembrane alpha helices. This region participates in anchoring the enzyme to the plasma membrane as indicated in Fig. 1. The C-terminal half of the molecule is homologous to known flavoproteins, as described above, and contains the predicted FAD and NADPH binding regions. The location of the heme groups is less well established. The iron of the heme groups was shown based on physical studies to be low spin, hexacoordinate, and ligated by histidyl nitrogens (Hurst *et al.*, 1991; Miki *et al.*, 1992; Isogai *et al.*, 1993). Thus, the location of the hemes can be predicted based on the presence of conserved histidines. While gp91*phox* in several species shows numerous conserved histidines, in a recent development, multiple homologies of gp91*phox* have been identified (Suh *et al.*, 1999; Lambeth *et al.*, 2000). Alignment of these sequences reveals the presence of 6 absolutely conserved histidine residues, one of which is present in the flavoprotein domain and can be eliminated from consideration since it constitutes part of the predicted FAD binding site. The remaining 5 conserved histidines (Histidines 101, 115, 119, 209, and 222 are present in the N-terminal half of the molecule, and 4 of these must ligand to the two heme irons. This interpretation is consistent with a study (Cross *et al.*, 1995) showing that an Arg54→ Ser substitution in gp91*phox* from a patient with CGD caused a perturbation of the oxidation-reduction potential of one of the heme groups. A topology model of flavocytochrome b_{558} is shown in Fig. 1, and includes an N-terminal transmembrane region that contains the two hemes (which are predicted to reside approximately within in the two leaflets of the plasma membrane) and a C-terminal flavoprotein domain which is located on the cytosolic side of the membrane. Such a model is consistent with recent studies from our laboratory (Han, C. H. and Lambeth, J. D., unpublished) that show that the flavoprotein domain expressed in *E. coli* shows NADPH-dependent diaphorase activity. This topology accounts for the transmembrane generation of superoxide using reducing equivalents (NADPH) from the cytosol, and is likely to be important for delivering reactive oxygen species to the phagosome in which captured microbes reside following phagocytosis.

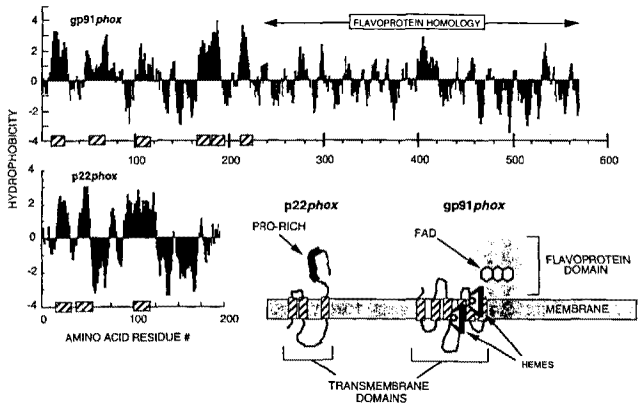


Fig. 1. Hydropathy plots and transmembrane models for gp91*phox* and p22*phox*. Hydropathy plots are shown for gp91*phox* and p22*phox*. Hydrophobic regions that are predicted to be transmembrane regions are shown as hashed boxes. A region that is homologous to known flavoprotein dehydrogenases including P-450 reductase and ferredoxin-$NADP^+$ oxidoreductase is indicated. Inset: Models for gp91*phox* indicate predicted transmembrane region including two bound heme groups and the flavoprotein domain containing bound FAD and the NADPH binding site. A transmembrane model for p22*phox* is also shown, and includes a proline-rich region near the C-terminus that serves as a binding site for p47*phox*.

Also shown in Fig. 1 is the proposed topology of p22*phox*, the second membrane-associated component of flavocytochrome b_{558}. This small protein consists of three N-terminal predicted transmembrane alpha helices, and a C-terminal region that contains two proline-rich sequences. As discussed below, these proline-rich sequences in p22*phox* provide a docking site for cytosolic regulatory proteins.

Cytosolic Regulatory Proteins p47*phox* and p67*phox*, and p40*phox* Several groups identified a 47 kDa phosphoprotein

which was absent in some forms of chronic granulomatous disease (Roos *et al.*, 1996; Segal, 1996). An activating fraction from human neutrophil cytosol obtained by chromatography on GTP sepharose revealed two major proteins of apparent molecular sizes 47 kDa and 67 kDa (Volpp *et al.*, 1988). An antibody made against this fraction reacted with both proteins, and this antibody later proved to be the key reagent to screen an expression library from a human phagocyte cell line, resulting in the molecular cloning of cDNAs for p47*phox* (Lomax *et al.*, 1989; Volpp *et al.*, 1989) and p67*phox* (Leto *et al.*, 1990). These two proteins migrate on gel filtration columns as part of a large molecular weight (240-260 kDa) cytosolic complex (Park *et al.*, 1992). The complex also contains a 40 kDa protein (Someya *et al.*, 1993) which was cloned and is referred to as p40*phox* (Wientjes *et al.*, 1993; Sathyamoorthy *et al.*, 1997). Although a large complex is seen by gel filtration, the complex is readily dissociated by increased temperature, high osmolarity or prolonged incubation (Iyer *et al.*, 1994), and it is not clear whether the complex remains associated during cell activation. Both p47*phox* and p67*phox* have been expressed in insect cells using baculovirus technology, and the expressed proteins can partially substitute in activity assays for the cytosolic fraction in cell-free assays (Leto *et al.*, 1991; Abo *et al.*, 1992; Uhlinger *et al.*, 1992). Recombinant p40*phox* inhibits NADPH oxidase activity (Sathyamoorthy *et al.*, 1997), suggesting a negative regulatory role, but other reports also indicate a modest stimulatory function (Cross, 2000).

Role for the Small GTPase Rac in Activating the Respiratory Burst Oxidase p47*phox* plus p67*phox* alone are insufficient to fully reconstitute the NADPH-oxidase activity of plasma membrane or purified flavocytochrome b_{558}. Before p47*phox* and p67*phox* were identified, several groups (Seifert *et al.*, 1987; Ishida *et al.*, 1989; Bolscher *et al.*, 1990) observed high activity of crude preparations (lysate or plasma membrane/cytosol) from phagocytes in the presence of the hydrolysis-resistant guanine nucleotides GTPγS and GppNHp, and the activity was suppressed by GDP or GDPβS. ATPγS also stimulated activity, but the effects were traced to a nucleoside diphosphate kinase activity that allowed formation of the true activating factor, GTPγS from endogenous GDP (Uhlinger *et al.*, 1991; Peveri *et al.*, 1992). These data implied that an unknown guanine nucleotide regulatory protein regulates the NADPH-oxidase activity.

Fractionation of cytosol from guinea pig macrophages (Abo and Pick, 1991a) or from human neutrophils was used to isolate and identify the factor that was responsible for conferring stimulation by guanine nucleotides. The activating factor was identified as Rac1 in macrophages (Abo *et al.*, 1991b) and Rac2 in human neutrophils (Knaus *et al.*, 1991; Knaus *et al.*, 1992; Mizuno *et al.*, 1992). Rac2 expression is limited to phagocytic cells, whereas Rac1 is widely expressed in a variety of tissues (Didsbury *et al.*, 1989). Rac1 and Rac2 are members of the Rho family of small GTPases, which are around 21 kDa in sizes and are posttranslationally modified by a geranyl-geranyl lipid group at their C-termini (Didsbury *et al.*, 1990). This moiety participates in membrane localization. Rac1 and Rac2 are approximately 90% identical at the amino acid level, and both are capable of restoring activity in cell-free systems (Abo *et al.*, 1991b; Ando *et al.*, 1992), suggesting some redundancy in their function in regulating NADPH-oxidase activity. However, a mutation in Rac2 [Rac2(D57N)] has been described in a patient with an immune disorder characterized by multiple defects in phagocyte function including defective superoxide generation (Ambruso *et al.*, 2000). A Rac2 knockout mouse (Roberts *et al.*, 1999) shows diminished NADPH-oxidase activity in bone marrow neutrophils, but normal activity in neutrophils from peritoneal exudates. These data imply that Rac2 can be the predominant isoform that regulates NADPH-oxidase activity, but that under some conditions Rac1 or another factor can compensate for an absence or mutation in Rac2.

Assembly of Cytosolic Proteins with Flavocytochrome b_{558} Regulates the Activity of the Respiratory Burst Oxidase Both p47*phox* and p67*phox* translocate from the cytosol to the plasma membrane upon cell activation (Clark *et al.*, 1990; Doussiere *et al.*, 1990; Ohtsuka *et al.*, 1990), and translocation fails to occur when flavocytochrome b_{558} is absent (Uhlinger *et al.*, 1993). In intact cells, both proteins move to the plasma membrane with similar kinetics (Quinn *et al.*, 1993), and it has been suggested that p47*phox* and p67*phox* translocate to the membrane without dissociation from their pre-existing complex (Park and Babior, 1992). p40*phox* also translocates to the membrane (Dusi *et al.*, 1996), but whether it is bound at the plasma membrane within the same activated complex as the other components is not certain. Using neutrophils from patients with variants of CGD in which either p67*phox* or p47*phox* is absent, translocation of p47*phox* occurred whether or not p67*phox* was present, but translocation of p67*phox* did not occur when p47*phox* was absent (Kleinberg *et al.*, 1990). Translocation of recombinant p47*phox* and p67*phox* to the membrane has also been demonstrated in a cell-free system activated by anionic amphiphile (Strum *et al.*, 1990; Tyagi *et al.*, 1992; Uhlinger *et al.*, 1993). A stoichiometry of 1 : 1 : 1 was observed for binding of p47*phox* : p67*phox* : flavocytochrome b_{558} indicating a ternary complex (Uhlinger *et al.*, 1993). p47*phox* was shown to be an early reactant, and was needed for 67*phox* binding (Kleinberg *et al.*, 1990; Uhlinger *et al.*, 1993). However, subsequent kinetic studies (Uhlinger *et al.*, 1994) revealed that the binding of p67*phox* and p47*phox* to flavocytochrome b_{558} is synergistic, suggesting that a sequential binding model may be an oversimplification. In addition, GTPγS enhances and GDPβS inhibits the binding of $p67_{phox}$ (Uhlinger *et al.*, 1993). These data pointed to a role for Rac in the binding of cytosolic proteins, and are consistent with a model in which multivalent cooperative interactions participate in the assembly of NADPH-oxidase components.

Rac1/2 in resting phagocytes is found largely in a complex with an inhibitory protein, RhoGDI (GDP Dissociation Inhibitor). The geranyl-geranyl group of Rho type proteins participates in binding to RhoGDI, and this binding masks this hydrophobic group from interactions with solvent and membrane (Hoffman *et al.*, 2000). The RhoGDI binds preferentially to Rho proteins in their inactive GDP-associated form (Sasaki *et al.*, 1993). Activation of small GTPases involves the catalytic activity of exchange factors, which permit dissociation of GDP and binding of GTP, resulting in an active conformation of the small GTPase, its dissociation from RhoGDI, and its interaction with the membrane (Abo *et al.*, 1994; Bokoch *et al.*, 1994). Such a mechanism regulates the activation of Rac1/2 in phagocytes and its translocation from cytosol to the membrane. Under some conditions, the GDP-associated form of Rac can also activate the guinea pig macrophage NADPH-oxidase activity (Bromberg *et al.*, 1994). However, the human enzyme is markedly and preferentially stimulated by the GTP-associated form (Uhlinger *et al.*, 1991; Ando *et al.*, 1992).

Although Rac is not detected in the same cytosolic complex along with p47*phox* and p67*phox*, translocation of Rac in intact cells occurs at a similar rate and with similar stoichiometry as p47*phox* and p67*phox* (Quinn *et al.*, 1993). In experiments using neutrophils obtained from flavocytochrome b_{558}-deficient CGD patients, translocation of Rac2 to the plasma membrane was reduced to 25% of the control value (Heyworth *et al.*, 1994). When either p47*phox* or p67*phox* was absent, Rac2 translocation occurred normally. Thus, Rac translocates independently of both p67*phox* and p47*phox*. While translocation is partially independent of flavocytochromes b_{558}, interaction with this component stabilizes the membrane interaction. We suggest that Rac1/2 anchored to the plasma membrane via its geranyl-geranyl group interacts with the NADPH-oxidase complex within the plane of the membrane. For some small GTPases, some protein-protein interactions are mediated by the attached farnesyl or geranyl-geranyl group. However, studies in a cell-free system revealed that while membrane association is essential, the geranylgeranyl group itself is not needed, and high activity was also achieved through ionic interactions with the membrane mediated by the a non-isoprenylated polybasic C-terminal region in Rac1 (Kreck *et al.*, 1996). Thus, in a cell-free system, membrane association of Rac1 is critical for activity, but the specific mechanism for membrane attachment is not critical, and the geranyl-geranyl group is therefore unlikely to mediate specific protein-protein interactions.

Phosphorylation of Components of the Respiratory Burst Oxidase Several proteins of the phagocyte NADPH-oxidase become phosphorylated upon cell activation, and phosphorylation of at least two of these is involved in enzyme activation. p47*phox* becomes phosphorylated on multiple sites following treatment of neutrophils with phorbol esters or chemotactic peptide (El Benna *et al.*, 1994). Phosphorylation sites include sites for protein kinase C, protein kinase A and MAP Kinase (Beena *et al.*, 1996), and all are located in the C-terminal 1/3 of the molecule, which includes an autoinhibitory domain (see below). Serine residues 303/304, 328, 359 and 370 have been shown through mutagenesis to be important for enzyme activation (Inanami *et al.*, 1998; Johnson *et al.*, 1998; Ago *et al.*, 1999). Kinases in both the cytosol and the plasma membrane carry out kinetically distinguishable phosphorylation of p47*phox* (Rotrosen and Leto, 1990; Johnson *et al.*, 1998). Functionally, phosphorylation induces a conformational change in p47*phox*, which unmasks a binding site for p22*phox* (Ago *et al.*, 1999; Huang and Kleinberg, 1999; Park *et al.*, 1999).

In addition to p47*phox*, phosphorylation of p67*phox*, p40*phox*, and p22*phox* occurs upon cell activation. p67*phox* becomes phosphorylated following treatment of cells with phorbol myristate acetate or a chemotactic peptide (Dusi and Rossi, 1993), and phosphorylated p67*phox* forms a complex with phosphorylated p47*phox* (El Benna *et al.*, 1997). The phosphorylation occurs in the cytosol (Forbes *et al.*, 1999a) on residue 233, which is within a proline-rich sequence on p67*phox* (Forbes *et al.*, 1999b). p22*phox* becomes stoichiometrically phosphorylated in a cell-free system by a phosphatidic acid-activated kinase (Regier *et al.*, 1999), and phosphorylation of p22*phox* also occurs in intact cells in response to various agonists (Regier *et al.*, 2000). As described above, NADPH-oxidase activity can be reconstituted in the absence of anionic amphiphiles in a cell-free system by phosphorylation with protein kinase C (El Benna *et al.*, 1995; Park *et al.*, 1997). Activation requires phosphorylated p47*phox* and a phosphorylated membrane component, currently not identified, along with cytosol. P40*phox* also becomes phosphorylated *in vivo* in response to cell activation (Bouin *et al.*, 1998). Thus, multiple components of the respiratory burst oxidase are subject to regulation by phosphorylation.

Functional Domains in p47*phox*, p40*phox*, and p67*phox* As discussed above, assembly of cytosolic components with flavocytochrome b_{558} correlates with activation. The assembly can be partially understood based on specific protein-protein interactions that occur within the activated NADPH-oxidase complex (de Mendez *et al.*, 1994; Sumimoto *et al.*, 1994; Leusen *et al.*, 1995; DeLeo and Quinn, 1996; Leo *et al.*, 1996; Sumimoto *et al.*, 1996; de Mendez *et al.*, 1997). Src-homology 3 (SH3) domains have been shown in a number of proteins to participate in either intramolecular or intermolecular protein-protein interactions by binding to proline-rich target sequences (Gale *et al.*, 1993). p47*phox* contains tandem SH3 domains located near the center of the molecule, diagrammed in Fig. 2. The p47*phox* sequence also contains a proline-rich domain (PRD) near the C-terminus (Fig. 2). The tandem SH3 region ($SH3_{AB}$) of p47*phox* can interact intramolecularly with an arginine/lysine rich region in the C-terminus of p47*phox* (see Fig. 1), and phosphorylation

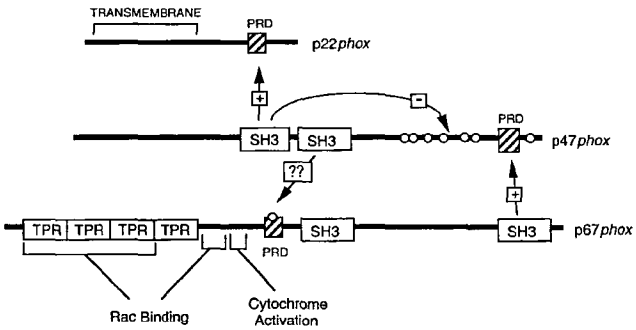


Fig. 2. Domain structure and protein-protein interactions among cytosolic factors and p22*phox*. Src-homology 3 domains (SH3) are indicated, and arrows point to documented interactions with proline-rich domains (PRD, hashed boxes) or other regions. Interactions that are thought to participate in the activated complex are indicated by "+", while an internal inhibitory interaction in p47*phox* is indicated by "-". Open circles mark sites that can become phosphorylated upon cell activation. TPR refers to tetratricodecapeptide repeat. The flavocytochrome activation domain (residues 199-210) in p67*phox* is also indicated.

of residues in this C-terminal region disrupts this interaction, leading to exposure of the $SH3_A$ domain (Huang and Kleinberg, 1999). p22*phox* contains a PRD near its C-terminus, and this binds to the exposed SH3 domain A ($SH3_A$) of p47*phox* (Sumimoto *et al.*, 1994; Sumimoto *et al.*, 1996; de Mendez *et al.*, 1997). These interactions provide a plausible mechanism by which phosphorylation of p47*phox* disrupts autoinhibitory intramolecular interactions, triggering assembly with flavocytochrome b_{558}. The model has been tested by replacing Ser-303, Ser-304 and Ser-328 on $p47_{phox}$ with aspartate residues to mimic phosphorylation of these residues (Ago *et al.*, 1999). As predicted by this model, the triply mutated protein binds to p22*phox* and activates cell-free superoxide generation in the absence of *in vitro* activators.

p67*phox*, like p47*phox*, also contains two SH3 domains. $SH3_A$ is located near the center of the molecule, while $SH3_B$ is located near the C-terminus. $SH3_B$ binds tightly to the C-terminal PRD of p47*phox* (Fig. 2). The SH3 domains are not essential for *in vitro* activity (Leusen *et al.*, 1995; Han *et al.*, 1998; Hata *et al.*, 1998), but are needed to observe activity *in vivo* (de Mendez *et al.*, 1994). The role of a proline-rich domain near the center of p67*phox* is unknown, but as described above, this region contains a site that becomes phosphorylated upon cell activation.

Another striking feature of the p67*phox* structure is the presence of four TPR (tetratricodecapeptide repeat) motifs comprising the N-terminal 1/3 of the molecule (Ponting, 1996). These motifs consist of a degenerate 34-amino acid repeat and are seen in a number of proteins of diverse biological function (Lamb *et al.*, 1995). In some cases, the motif may be involved in protein-protein interactions (Ponting, 1996). In protein phosphatase 5, the TPR motif binds arachidonate, inducing an activating conformational change (Das *et al.*, 1998). This may be significant, given the activating effect of arachidonate on the NADPH-oxidase. The TPR region is indispensable for *in vitro* activation of the NADPH-oxidase, and disruption of any of the first three TPR repeats disrupts the binding to Rac (Koga *et al.*, 1999). Immediately C-terminal to the TPR repeats is a region (residues 170-199) that is also implicated in binding to the small GTPase Rac (see Fig. 2) (Ahmed *et al.*, 1998). Some evidence suggests that p67*phox* contains the NADPH binding site of the oxidase (Smith *et al.*, 1996), based upon derivatization of p67*phox* by a chemically reactive NADPH analog. However, NADPH binding consensus sequences within p67*phox* are not convincing, and the presence of a more convincing pyridine nucleotide binding consensus sequence within gp91*phox* would seem to account fully for an NADPH binding site on the respiratory burst oxidase.

Molecular Interactions of the small GTPase Rac with NADPH oxidase components Data support a model in which activated Rac utilizes multiple binding surfaces for simultaneous interactions with two or more protein partners within the NADPH-oxidase complex and with the membrane (Nisimoto *et al.*, 1997; Toporik *et al.*, 1998). This represents a departure from the conventional view of small GTPases in which the small GTPase is thought to bind to a single effector target (e.g., Ras interactions with Raf-1), e.g. to localize it to a membrane and/or to activate the target by allosteric mechanisms. One of these surfaces is the "effector region" (amino acid residues 26-45) which is homologous to a corresponding region in Ras that is involved in GTP-dependent binding to its downstream effector Raf-1, a kinase that becomes activated following this interactions. Studies from several laboratories point to the importance of this region in NADPH-oxidase activity (Diekmann, *et al.*, 1994; Freeman *et al.*, 1994; Kwong *et al.*, 1995). Kinetic studies in a cell free system (Freeman *et al.*, 1994) showed that point mutations in this region resulted in up to 100-fold decreased binding of Rac to the oxidase complex.

The effector region binds to p67*phox* (Diekmann, *et al.*, 1994; Nisimoto *et al.*, 1997). To study this interaction quantitatively, a fluorescent analog of GTP was used as a reporter group (Nisimoto *et al.*, 1997). This analog binds tightly to Rac1, and undergoes a change in its fluorescence upon binding to p67*phox*. This interaction was moderately tight, and showed a 1 : 1 stoichiometry. Point mutations in the effector region diminished the binding affinity by up to 50-fold.

Rho family proteins including Rac also contain a region (the "insert region", amino acid residues 124-135) that has no counterpart in Ras. Point mutations as well as a deletion mutation demonstrated (Freeman *et al.*, 1996) that this region is important for the interaction of Rac with the NADPH-oxidase complex. Mutations in this region decreased the apparent affinity for the complex, based on EC_{50} values. The insert region in Rho family proteins is also important for

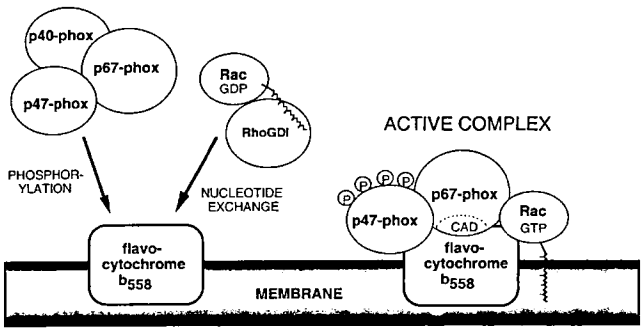


Fig. 3. Model for activation of flavocytochrome b_{558} by assembly with cytosolic regulatory proteins. According to the model, assembly is triggered by: a) phosphorylation of p47*phox* (and perhaps p22*phox* and p67*phox*) leading to translocation of p47*phox*, and b) exchange of GDP for GTP on Rac, leading to dissociation of Rac from RhoGDI and exposure of the lipophilic geranyl-geranyl group. Both p47*phox* and Rac provide binding sites for p67*phox*, and bring its cytochrome activation domain (CAD) into contact with flavocytochrome b_{558}. CAD then regulates the reduction of FAD by NADPH.

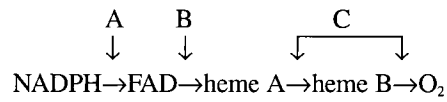
binding to RhoGDI (Nomanbhoy and Cerione, 1996). The target for binding to the insert region has not been identified directly, but both p47*phox* and p67*phox* can be eliminated based on kinetics and binding data (Freeman *et al.*, 1996). Thus, flavocytochrome b_{588} is the most likely candidate for interaction with the insert region.

An Activation Domain in p67*phox* An activation domain on p67*phox* (amino acid residues 199-210) was recently identified using truncation and point mutations (Han *et al.*, 1998). Mutations in this region reduce or eliminate the ability of p67*phox* to support NADPH-oxidase activity in a cell-free system. In particular, a point mutation (V204A) completely eliminated the activating function of p67*phox*. The interaction of p67*phox* with p47*phox* or Rac1 was not affected, and the mutant protein bound normally as part of the activated NADPH-oxidase complex. We interpret these data to indicate that the activation domain in p67*phox* regulates the activity of the gp91*phox* moiety. It has not been possible to demonstrate direct binding of this region in p67*phox* to other components of the NADPH-oxidase complex. However, we propose that the cooperative binding of the other oxidase proteins contributes the majority the binding energy towards juxtaposing the activation domain with its target. Thus, the affinity of the activation domain for gp91*phox* need not be high; indeed this region may contribute very little binding energy to the assembly of the active NADPHoxidase.

A model for assembly and activation of the NADPH-oxidase complex Based upon the evidence described above, we propose the following model for assembly and activation of the phagocyte respiratory burst oxidase (Fig. 3). Phosphorylation of p47*phox* by upstream kinase triggers translocation by eliminating internal interactions with $SH3_A$, allowing this domain to bind to p22*phox* in the plasma membrane (this interaction may also require phosphorylation of p22*phox*). This tight interaction is supplemented by other interactions with both the large and small subunit (DeLeo *et al.*, 1995; DeLeo and Quinn, 1996; Biberstine-Kinkade *et al.*, 1999). p47*phox* provides a binding site for p67*phox*, which translocates from cytosol to the flavocytochrome either independently, or as part of the same complex. Guanine nucleotide exchange triggers dissociation from RhoGDI and permits the translocation of Rac-GTP to the membrane where it is tethered by its geranyl-geranyl group to the membrane and can associate with the oxidase complex within the plane of the membrane. The guanine nucleotide exchange factor for Rac in phagocytes is currently unknown, but is probably regulated directly or indirectly by phosphatidylinositol 3-kinase (Okada *et al.*, 1994; Parker, 1995). Rac interacts, probably through its insert region, with flavocytochrome b_{558}. Such an interaction within the plane of the membrane is expected to be energetically favorable, requiring only a few kcal of binding energy. Like p47*phox*, Rac also provides a binding site for p67*phox*. Thus, we propose that these two interactions bind and properly orient p67*phox* in such a way that its activation domain is in contact with gp91*phox*, thus regulating NADPH-oxidase activity.

Mechanism of Regulation of NADPH-oxidase activity by p67*phox* According to the model above, p67*phox* is the key regulator of electron flow from NADPH to molecular oxygen, and both p47*phox* and Rac play supporting roles in the proper binding and orientation of p67*phox*. Consistent with this interpretation, full NADPH-oxidase activity can be reconstituted *in vitro* in the absence of p47*phox* when high concentrations of p67*phox* and Rac are used (Freeman and Lambeth, 1996; Koshkin *et al.*, 1996). The sole effect of p47*phox* was to greatly increase the affinity of the oxidase for p67*phox* and Rac (Freeman and Lambeth, 1996). Also consistent with this interpretation, mutations in Rac binding regions had only minor effects on the V_{max} when other components were present in excess (Freeman *et al.*, 1994; Freeman *et al.*, 1996); rather, the major effect was on binding to the NADPH-oxidase complex. Thus, only mutations in the activation domain of p67*phox* affected the V_{max}, reflecting regulation of electron flux from NADPH to molecular oxygen.

The kinetic mechanism by which the activation domain on p67*phox* regulates electron flow was recently described (Nisimoto *et al.*, 1999). The pathway for electron flow within flavocytochrome b_{558} is as follows:



The reoxidation of both hemes by molecular oxygen (steps C) is extremely rapid (Cross *et al.*, 1985; Isogai *et al.*, 1993),

making this an unlikely point for regulation. Using an analog of FAD that is fluorescent in its oxidized state and non-fluorescent in its reduced state, the steady state reduction level of FAD was monitored in the presence of native p67*phox* and p67*phox* that had been mutated in its activation domain so as to give partial activation. The steady state % reduction of FAD was affected by p67*phox*, but not by p47*phox*. Forms of p67*phox* that produced higher rates of superoxide generation also produced a greater % reduction of FAD. These data prove that reduction of FAD by NADPH (step A above), rather than its reoxidation (step B) is regulated by the activation domain of p67*phox*.

Reduction of FAD by NADPH consists of two steps: binding of NADPH and hydride transfer from NADPH to FAD. Kinetic deuterium isotope effects were used, using R- and S- NADPD as substrates, to explore which of these steps is regulated, utilizing the kinetic treatment of Klinman and Matthews, 1985. This approach permits calculation of the actual K_d for substrate binding (rather than a kinetic constant) from kinetic isotope data. Using this approach, there was very little effect of p67*phox* on the K_d for NADPH (step A). Thus, the regulated step is not binding (step A), but is the hydride transfer, (Step B) shown below:

$$\begin{array}{ccc} & \text{A} & \text{B} \\ & \downarrow & \downarrow \\ \text{NADPH+H}^+\text{+E-FAD} & \rightarrow & \text{NADPH-E-FAD} \rightarrow \text{NADP}^+\text{-E-FADH}_2 \end{array}$$

The above studies imply that the physical target for p67*phox* is the flavoprotein domain of gp91*phox*. We have expressed this domain in *E. coli* and investigated its catalytic properties and regulation (Han, C.-H. and Lambeth, J. D., unpublished). The domain is inactive in reducing molecular oxygen. However, when reconstituted with FAD, the flavoprotein domain shows a very low NADPH-dependent diaphorase activity towards nitroblue tetrazolium and other electron acceptors. The diaphorase activity is stimulated by p67*phox* when Rac is present, but is not affected by p47*phox*. These data indicate that the physical target of p67*phox* is the flavoprotein domain (Fig. 1).

Concluding Remarks

A great deal has been learned over the past decade about the mechanism by which protein-protein interactions regulate the activity of the phagocyte respiratory burst oxidase. According to the data and models presented here, both phosphorylation and guanine nucleotide exchange trigger the assembly of cytosolic factors with the catalytic moiety of the phagocyte oxidase, flavocytochrome b_{558}. The rationale for the assembly is to bring the activation domain of p67*phox* into proper contact and orientation with the flavoprotein domain of gp91*phox* in such a way as to promote hydride transfer from NADPH to FAD, which occurs slowly if at all in the absence of p67*phox*. While a great deal has been learned, there remain significant gaps in our knowledge. The following areas stand out as topics for future investigation.

1. *Structure*. Except for Rac, there is no available structural information for any of the other components. Ideally, such structural information should be available not only for the individual components, but also for the protein complexes, in order to provide a more complete picture of regulation.
2. *Dynamics of protein-protein interactions*. Little is currently known regarding the dynamics of protein-protein interactions during the activation process. How do protein-protein interactions change in the transition from soluble cytosolic complexes to the membrane-associated, active complex?
3. *Role of p40phox*. Data concerning the role of p40*phox* are contradictory, and its role needs to be resolved.
4. *Roles of phosphorylation*. While the phosphorylation of p47*phox* has been extensively studied, the role for phosphorylation of other oxidase component is not yet clear.
5. *Upstream signal transduction*. The upstream signalling systems that ultimately result in activation of the oxidase are incompletely understood. Which kinases function in individual phosphorylation reactions, and what is the pathway from receptor to guanine nucleotide exchange factors? What is the role of phospholipase D, diacylglycerol and phosphatidic acid in regulation?

Thus, the field of NADPH oxidase biology remains a fruitful area for future investigation.

Acknowledgment Thanks to Yukio Nisimoto for reading and commenting on this manuscript.

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