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Review

The superoxide-generating NADPH oxidase: structural aspects and activation mechanism

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Abstract. Flavocytochrome b_{558} is the catalytic core of the respiratory-burst oxidase, an enzyme complex that catalyzes the NADPH-dependent reduction of O_2 into the superoxide anion O_2^- in phagocytic cells. Flavocytochrome b_{558} is anchored in the plasma membrane. It is a heterodimer that consists of a large glycoprotein gp91phox (phox for *ph*agocyte *ox*idase) (β subunit) and a small protein p22phox (α subunit). The other components of the respiratory-burst oxidase are water-soluble proteins of cytosolic origin, namely p67phox, p47phox, p40phox and Rac. Upon cell stimulation, they assemble with the membrane-bound flavocytochrome b_{558} which becomes activated and generates O_2^-. A defect in any of the genes encoding gp91phox, p22phox, p67phox or p47phox results in chronic granulomatous disease, a genetic disorder characterized by severe and recurrent infections, illustrating the role of O_2^- and the derived metabolites H_2O_2 and HOCl in host defense against invading microorganisms. The electron carriers, FAD and hemes *b*, and the binding site for NADPH are confined to the gp91phox subunit of flavocytochrome b_{558}. The p22phox subunit serves as a docking site for the cytosolic phox proteins. This review provides an overview of current knowledge on the structural organization of the O_2^--generating flavocytochrome b_{558}, its kinetics, its mechanism of activation and the regulation of its biosynthesis. Homologues of gp91phox, called Nox and Duox, are present in a large variety of non-phagocytic cells. They exhibit modest O_2^--generating oxidase activity, and some act as proton channels. Their role in various aspects of signal transduction is currently under investigation and is briefly discussed.

Key words. Neutrophil flavocytochrome *b*; phagocyte oxidase; respiratory burst; superoxide anion; NOX protein.

Historical background

The NADPH oxidase of professional phagocytic cells (neutrophils, macrophages and eosinophils) is a multicomponent complex, dormant in non-activated cells, that catalyzes during phagocytosis the production of superoxide anion O_2^-, according to the reaction:

$$NADPH + 2\ O_2 \rightarrow 2\ O_2^- + NADP^+ + H^+$$

The term respiratory burst is often used to describe the abrupt and marked increase in O_2 consumption, which characterizes the activation of NADPH oxidase and is accompanied by the release of O_2^- to the extracellular medium or into the phagocytic vacuole. In the resting cell, the components of the NADPH oxidase are present in two different compartments, the plasma membrane and the cytosol. The membrane-bound component, a flavocytochrome *b*, is the catalytic core of the NADPH oxidase. The cytosolic components, termed cytosolic phox (*ph*agocytic *ox*idase) proteins or cytosolic factors of oxidase activation are translocated to the membrane-bound flavocytochrome *b* in stimulated phagocytes. The flavocytochrome *b* of the oxidase complex is referred to either as flavocytochrome b_{558}, because of the heme absorbance peak at 558 nm in the reduced state, or as flavocytochrome b_{-245} because of the midpoint potential of

–245 mV at pH 7.0 of the heme component, close to that of the O_2/O_2^- couple (–160 mV) [1, 2]. The midpoint potential of the flavin component, FAD, was averaged to –280 mV, a value intermediate between that of the NADP/NADPH couple, –320 mV, and that of heme *b* [3]. Flavocytochrome b_{558} is a heterodimer consisting of a large glycoprotein of 90–100 kDa (gp91phox or β subunit) and a small protein of 22 kDa (p22phox or α subunit). Associated proteins of cytosolic origin promote the transition of flavocytochrome b_{558} from a resting to an active state. Three of them, p47phox, p67phox and a small GTP protein, either Rac1 or Rac2, are necessary and sufficient, when used in a cell-free assay, to activate the membrane-bound flavocytochrome *b* [4]. Another cytosolic phox protein, p40phox, coimmunoprecipitates with p47phox and p67phox [5] and may play a role in the regulation of the NADPH oxidase activity [6, 7]. The superoxide anion O_2^- generated through the NADPH oxidase reaction is rapidly converted inside the cell to hydrogen peroxide (H_2O_2) by dismutation. In the presence of traces of iron salt, O_2^- and H_2O_2 interact to form the hydroxyl radical $OH^\bullet$ via the Haber-Weiss reaction. H_2O_2 reacts with chloride anions to generate hypochlorous acid. Because of their potent antibacterial properties, these oxygen derivatives, called reactive oxygen species (ROS), participate in the killing of microorganisms during phagocytosis. In routine assays, O_2^- production is monitored by the superoxide dismutase-inhibitable reduction of cytochrome *c*, following oxidase activation upon addition of phorbol myristate acetate, an activator of protein kinase C (PKC). Other methods are sometimes used to determine the NADPH oxidase activity, including the polarographic measurement of oxygen consumption by the Clark electrode, or the detection of ROS by lucigenin/luminol-enhanced chemiluminescence [8].

The central importance of the phagocyte NADPH oxidase to innate host defense is illustrated in chronic granulomatous disease (CGD), a rare genetic disorder characterized by severe and recurrent infections (skin and liver abcesses, mouth ulcers, lymphadenitis) due to the inability of neutrophils and macrophages to mount a respiratory burst and kill invading bacteria, and also by granulomas that obstruct vital structures such as the gastrointestinal and genitourinary tracts [for a review see ref. 9]. A number of defects resulting in CGD have been identified in the genes encoding gp91phox, p22phox, p67phox and p47phox (http://www.ncbi.nlm.nih.gov). The incidence of CGD is approximately 1 in 250,000 persons. The genetic heterogeneity in CGD was discovered in the late 1980s, the mutation being transmitted in an X-linked fashion in some relations and in an autosomal form in others. This genetic heterogeneity reflected the fact that CGD is the result of defects in different components of the NADPH oxidase complex. Defects in the X-linked gene encoding the gp91 subunit of flavocytochrome b_{558} are the most prevalent, accounting for about two-thirds of the cases of CGD. Next in importance are the mutations in p47phox, responsible for about one-fourth of CGD cases. Mutations in p67phox and p22phox leading to CGD are rare (5–6%). No case has so far been associated with a lack of p40phox, and only in one case has a mutation in Rac2 been found to be associated with a neutrophil immunodeficiency syndrome [10, 11].

The X-linked defective gene was cloned by reverse genetics from a cDNA library of differentiated human promyelocytic HL60 cells [12]. In the meantime, a molecular species was purified and characterized as a heterodimer consisting of a large subunit, now called gp91phox, [13] and a small subunit, p22phox [14]. The heterodimer was identical to a molecular entity identified several years before as a novel cytochrome *b* in phagocytic vacuoles of human neutrophils [15]. The low midpoint potential of this cytochrome, –245 mV at pH 7.0, was a peculiarity that allowed discrimination from otherwise similar redox molecules [16]. A large variety of mutations responsible for X-CGD have been characterized. They consist of missense, nonsense, and splice site mutations, deletions, and insertions, and affect to different degrees the structure of the gp91phox subunit of flavocytochrome b_{558} [17, 18]. The standard notations for differentiating the various phenotypes of X-linked CGD are X91°, $X91^-$ and $X91^+$, where the superscript denotes whether the level of gp91phox is undetectable (°) (the most frequent case), diminished (–), or normal (+), as determined by immunological or spectral analysis. In contrast to the large spectrum of mutations distributed over the gp91phox gene, the lack of functional p47phox in the secondmost common form of CGD (47° CGD) is caused by an unusual uniform mutation pattern, namely a GT deletion, which results in a premature stop codon at amino acid 51 (more than 90% of the cases). This defect is probably due to recombination events between the p47phox gene and highly homologous pseudogenes [19]. B lymphocytes harbor the same oxidase complex as that found in neutrophils. Although their oxidase activity is modest compared to neutrophils, they can be propagated indefinitely following transformation by the Epstein-Barr virus (EBV). This peculiarity makes the EBV-B lymphocytes a convenient system for exploring the molecular defects responsible for CGD [20, 21].

Due to advances in recombinant retrovirus technology, gene replacement therapy for CGD was attempted in the early 1990s [9] and now appears to be feasible. Retroviral vectors have been recently used for transfer of the gp91phox cDNA into murine X-CGD bone marrow cells which were later transplanted into lethally irradiated X-CGD mice. Despite a low level of expression of recombinant gp91phox (5–10% of wild type), the oxidase func-

tion of the neutrophils was substantially improved and, most importantly, lasted for several months [22, 23]. Similar assays were carried out in p47phox knockout CGD mice [24]. Clinical trials in patients with CGD are currently underway in Europe and the United States [for a review, see ref. 25].

The study of mutations in human CGD has provided valuable information about the components of the NADPH oxidase complex and their mode of interaction. In parallel, and sometimes complementary to mutation analysis, the development of a cell-free system of oxidase activation, initially discovered by Bromberg and Pick [26], was another breakthrough that helped to decipher the basic features of activation of NADPH oxidase. This technique consists of incubating membranes from resting neutrophils or macrophages (or purified flavocytochrome b_{558}) and cytosol from the same cells (or purified cytosolic phox proteins) in the presence of an amphiphile (one of the most effective being arachidonic acid) for a few minutes before assaying the oxidase activity. In contemporary experiments, neutrophils are commonly used because they are easy to isolate in large amounts.

Activation of NADPH oxidase in phagocytic cells depends on the binding of specific ligands to receptors expressed in the plasma membrane. In some cases, during exposure of neutrophils to agonists, such as granulocyte-macrophage-colony-stimulating factor (GM-CSF), granulocyte-colony-stimulating factor (G-CSF) or tumor necrosis factor α (TNF-α), present at substimulatory concentrations unable to elicit the respiratory burst, neutrophils are primed, i.e., they are brought to a state of enhanced responsiveness that is reflected by a marked increase in the respiratory burst upon further addition of soluble or particulate stimuli. Priming probably involves the rearrangement of surface receptors and assembly of active signal transduction pathways [27, 28].

Serpentine receptors, the prototype of which is the formyl-methionyl-leucyl-phenylalanine (fMLP) receptor [29, 30], are involved in the chemotaxis of neutrophils, i.e., their migration, following extravasation, toward the site of infection and inflammation [31]. Chemotaxis occurs when neutrophils are challenged by agonists such as formyl peptides at nanomolar concentrations. When the agonist concentration reaches the micromolar range, at the site where microorganisms proliferate, phagocytosis of microorganisms is engaged. At the onset of phagocytosis, a number of enzymes in the neutrophil cell are activated, including tyrosine protein kinases and serine threonine protein kinases [for reviews, see refs 32–35], phosphatidylinositol-specific phospholipase C (PLC), phospholipase D (PLD) [36] activated by a small G protein of the ARF or Rho family, possibly also by PKCα and phosphatidyl inositol 4,5-bisphosphate [PtdIns(4,5)P2] [37], and a set of phosphoinositide kinases, among them the well-studied phosphoinositide 3-kinase (Pi3K) and phosphoinositide phosphatases [for a review, see ref. 38]. The product of Pi3K, phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3), accumulates in the plasma membrane at the level of the developing cup during internalization of opsonized microorganisms. Following sealing of the phagosomal vacuole, the concentration of phosphatidylinositol 3-phosphate (PtdIns(3)P) increases rapidly [39–41]. At the same time, the NADPH oxidase undergoes a transition from the resting to the active state, and generates O_2^-. Accumulation of endogenous ceramide through the sphingomyelinase pathway may contribute to the termination of the respiratory burst by inhibition of PLD activity [42].

Until recently, the prevailing idea about phagocytosis was that the ROS derived from O_2^- generated by the activated NADPH oxidase were essentially responsible for the destruction of internalized microorganisms. Apparently, in addition to ROS, granule proteases present in the phagocytic vacuole, such as elastase and cathepsin G, are required [43]. At the onset of phagocytosis, granules fuse with the phagocytic vacuole. Cathepsin G and elastase, cationic proteins firmly attached to a strongly anionic sulfated proteoglycan matrix within the granules, are released into the phagocytic vacuole, concomitant with an influx of H^+ and K^+. The rise in K^+ concentration in the vacuole generates hypertonicity that solubilizes and activates the proteases. In this process, O_2^- serves as a signal that triggers the vectorial transport of H^+ and K^+, resulting in protease activation. This elaborate process of protease activation combined with the highly regulated oxidase activation mirrors the complexity of the innate immune system.

Generation of O_2^- by NADPH oxidase is not an exclusive attribute of phagocytic cells from animal tissues. It has been demonstrated in B lymphocytes [20, 44, 45], fibroblasts [46], vascular smooth muscles [47], endothelial cells [48, 49], the carotid body [50], lung [51, 52], kidney [53, 54], as well as plant tissues [55–58]. In non-phagocytic cells, NADPH oxidase activity may be used for cell signaling, O_2 sensing or cross-linking reactions. In parallel to the demonstration of an O_2^--generating oxidase activity in non-phagocytic cells, a number of cDNAs encoding homologues of human gp91phox have been cloned. They are named Nox or Duox [for reviews, see refs 59, 60].

Interest of researchers in the field of the O_2^--generating NADPH oxidase is reflected by the growing number of reviews published in the last 10 years [9, 61–70]. The present article centers on the structural characteristics, activation, kinetics and regulation of biosynthesis of the flavocytochrome *b* component of the phagocyte NADPH oxidase. Recent findings on homologues of human flavocytochrome b_{558} are also discussed.

General features of flavocytochrome b_{558}: localization, amino acid sequence, subunit stoichiometry and redox carrier composition

In resting neutrophils, flavocytochrome b_{558} is mostly localized in the membrane of specific granules, a small fraction (10–20%) being present in the plasma membrane. Upon activation, some of the specific granules move to the cell membrane and fuse with it, so that this membrane becomes enriched in flavocytochrome b_{558} [71, 72]. Following internalization of microorganisms, flavocytochrome b_{558} appears in the membrane of the phagocytic vacuoles. A refined cytochemical technique applied to phorbol ester-stimulated neutrophils has recently revealed production of O_2^- in small vesicles, close to, but not at the plasma membrane, suggesting that O_2^- might be released to the outside of the cell by exocytosis [73]. Further investigations are needed to clarify this point. In contrast to neutrophils, the O_2^--generating oxidase of macrophages is located essentially in the plasma membrane, due to the absence of a granule-localized pool of flavocytochrome *b* [74]. The promyelocytic cell line HL60 acquires an oxidase activity after differentiation induced by addition of dimethyl sulfoxide or cyclic AMP. This oxidase activity, dependent on activated flavocytochrome b_{558}, is confined to the plasma membrane, since HL60 cells lack specific granules, and thereby granule-bound flavocytochrome b_{558} [75]. The B lymphocyte flavocytochrome b_{558} is also localized in the plasma membrane. Due to its low levels compared to the cytosolic phox proteins, flavocytochrome b_{558} is a limiting factor in the oxidase activity of B cells [76, 77].

The predicted amino acid contents of gp91phox and p22phox (including the encoded N-terminal methionine for the numbering of amino acids) are 570 and 195 amino acids, respectively (figs 1, 2). The gp91phox subunit is highly glycosylated, the mass of the bound polysaccharide amounting to one-third of the total mass of the subunit. The broad smear of gp91phox seen on gels following sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) reflects the heterogeneity of glycosylation [78]. p22phox contains near the C terminus a proline-rich sequence (residues 155–160) representing a putative binding site for SH3 domain(s) (see section on Interactions of flavocytochrome b_{558} with the cytosolic phox proteins).

The gp91phox subunit is encoded by a gene (CYBB) located on the short arm of the X chromosome at locus Xp21.1. The gene encoding p22phox (CYBA) is localized on chromosome 16 at 16q24. The CYBB gene com-

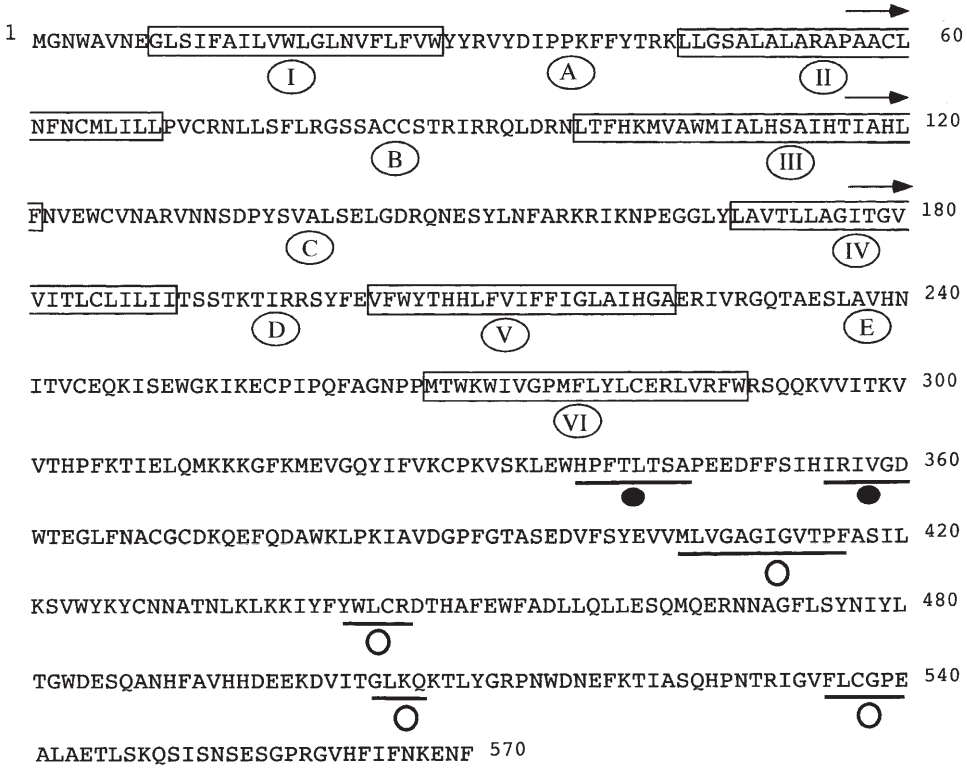

Figure 1. Sequence of the gp91phox subunit of human flavocytochrome b_{558}. The hydrophobic sequences that may be transmembrane α helices are boxed and numbered I–VI. The sites of interactions with NADPH (open circles) and with FAD (black ovals) are indicated. Letters A–E designate the loops corresponding to the extramembrane portions of the hydrophobic sequence of the gp91phox polypeptide chain (see fig. 3).

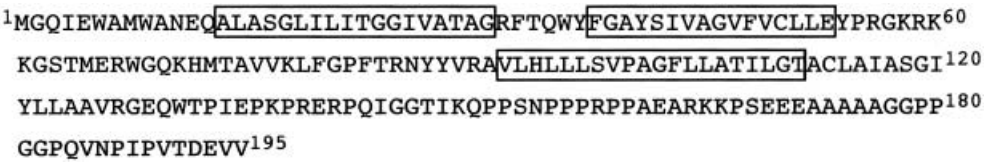

Figure 2. Sequence of the p22phox subunit of human flavocytochrome b_{558}. The hydrophobic sequences that may be transmembrane α helices are boxed.

prises 13 exons spread out over a total of 30 kb. The CYBA gene is divided in 6 exons spanning 8.5 kb [for reviews, see refs 79–81]. The hydropathy profiles deduced from the gene sequences are compatible with six transmembrane α helices in gp91phox and at least two (possibly three) transmembrane helices in p22phox (figs 1, 2) [for a review, see ref. 82].

The two subunits of flavocytochrome b_{558}, gp91phox and p22phox, are in a molar ratio of 1 [83]. The contention that flavocytochrome b_{558} is a multiheme protein was based first on spectrophotometric measurements [84], and later on redox potential measurements [85]. The heme potential that had been previously averaged to –245 mV [16] was recently reassessed [85]; two distinct mid-potentials of –225 mV and –265 mV at pH 7.0 were determined, in agreement with the presence of two hemes in flavocytochrome b_{558}. Definite evidence for the localization of the two hemes in the gp91phox subunit of flavocytochrome b_{558} stems from spectral and potential measurements made on membranes of monkey kidney cells (COS 7 cells) transfected with cDNA encoding gp91phox [86]. The redox spectrum was identical to that of neutrophil membranes, and the two measured redox potentials at –233 and –264 mV were close to values reported for neutrophil flavocytochrome b_{558} [85].

The idea that the phagocyte NADPH oxidase contains a functional FAD prosthetic group originated from the early report that the oxidase activity of a Triton extract of neutrophil membranes is markedly increased by added FAD [87]. Soon afterwards, 5-deaza FAD, which carries electrons by pairs, was found to inhibit the NADPH oxidase activity [88]; this was consistent with the interference of 5-deaza FAD with FAD, since electrons are transferred one by one from $FADH_2$ to the heme component of flavocytochrome b_{558}, as demonstrated in an EPR study by the presence of a semi-quinone form of FAD in membranes of activated neutrophils [3]. Other lines of evidence for the presence of FAD in the phagocyte NADPH oxidase are as follows. (i) Purified flavocytochrome b_{558} has virtually no NADPH oxidase activity, because most of its bound FAD has been lost. Oxidase activity is recovered upon addition of FAD to the purified flavocytochrome b_{558} activated either by crude neutrophil cytosol [89] or by purified recombinant cytosolic factors p47phox, p67phox and Rac [90, 91]. (ii) FAD is also needed for elicitation of oxidase activity in purified flavocytochrome b_{558} relipidated with phosphatidylcholine or phosphatidic acid [92, 93]. (iii) Further evidence for the flavoprotein nature of the membrane component of the NADPH oxidase comes from reflavination with a flavin analogue, 8-mercapto-FAD [94]. The binding of 8-mercapto-FAD to FAD-depleted flavocytochrome b_{558} was accompanied by a shift in the mercapto-FAD optical absorbance from 520 to 580 nm. (iv) Photolabeling with a radiolabeled azido derivative of FAD concomitant with photoinactivation of the oxidase provided definite evidence for the presence of a specific and functional FAD-binding site in flavocytochrome b_{558} [95].

Different approaches have been used to establish the FAD and heme *b* stoichiometry in neutrophil flavocytochrome b_{558}. In an early study, flavocytochrome-bound FAD was assessed as the difference between the amount of FAD present in neutrophil membranes of healthy subjects and that present in neutrophil membranes of X-CGD patients [96]. The difference was compared to the heme concentration in normal flavocytochrome b_{558}. In another approach, the production of O_2^- by purified and then relipidated flavocytochrome b_{558} was titrated with increasing amounts of FAD, until saturation was attained [92, 97]. In a third approach, neutrophil plasma membranes were photolabeled with a radiolabeled azido derivative of FAD, and the extent of covalently bound azido-FAD was determined as a function of the percentage of inactivation of the oxidase activity [95]. Concordant results obtained by these different approaches indicate that FAD and the heme *b* are present in functional flavocytochrome b_{558} in a molar ratio of 1 : 2.

Arrangement of the polypeptide chains of the two subunits of flavocytochrome b_{558}, gp91phox and p22phox, in the plasma membrane of neutrophils

In early studies, specific surface features of the membrane-bound flavocytochrome b_{558} were mapped with sequence-specific antibodies raised in rabbits against synthetic peptides corresponding to various regions of the two subunits of the flavocytochrome (figs 1, 2). In addition, synthetic peptides mimicking short amino acid se-

quences in flavocytochrome b_{558} were assayed as inhibitors of NADPH oxidase activation. Rotrosen et al. [98] found (i) that antibodies directed against the carboxyl-terminal 13 amino acid residues of the gp91phox subunit blocked the production of O_2^- in a cell-free system and (ii) that synthetic peptides mimicking the same region of gp91phox inhibited the respiratory burst of neutrophils electrically permeabilized to provide access of the peptides to the cytoplasm of the cells. These results defined a functional cytoplasmic domain of gp91phox that was postulated to mediate interactions with the cytosolic phox proteins. Nakanishi et al. [99] reported that synthetic peptides of 20 amino acid residues corresponding to the C-terminal region of the two subunits of flavocytochrome b_{558} inhibited NADPH oxidase activation in a cell-free system. The same peptides could be easily cross-linked with p47phox, leading to the conclusion that the C-terminal region of the two subunits of flavocytochrome b_{558} contained binding sites for p47phox and that binding occurred on the cytosolic face of the membrane during oxidase activation. Using antibodies raised against residues 151–173 of gp91phox, Imajoh-Ohmi et al. [100] demonstrated that these antibodies bound to intact neutrophils. This showed that the sequence 151–173 of gp91phox is exposed to the outside of the cell, close to the potential glycosylation sites corresponding to Asn132 and Asn149. On the other hand, antibodies directed against the carboxyl-terminal region of gp91phox prevented oxidase activation, only after the cells were permeabilized by freezing and thawing, indicating that this region is exposed to the cytosolic side of the plasma membrane. By the same approach, the conclusion was drawn that the N and C termini of the p22phox subunit are also exposed to the cytosol [100]. Consonant with these results were mutagenesis experiments showing that interactions between cytosolic phox proteins and the C-terminal regions of gp91phox and p22phox are essential for NADPH oxidase assembly (see section on Interaction of flavocytochrome b_{558} and cytosolic phox proteins).

In the mid 1990s, a novel mapping technique, random-sequence peptide phage analysis, was used to screen cytoplasmic regions of flavocytochrome b_{558} interacting with specific sequences of p47phox. Using random-sequence peptide phage libraries of hexa- and nonapeptides, De Leo et al. [101] were able to identify three sites of interaction of gp91phox with p47phox, encompassing residues ^{87}STRVRRQL, ^{451}FEWFADLL and ^{555}ESPRGVHFIF. The predicted arrangement of the gp91phox peptide chain (fig. 3) indicated that the first sequence ^{87}STRVRRQL is situated in a cytoplasmic loop of gp91phox and the other two in the cytoplasmic tail of the protein. The same mapping technique allowed the identification in p22phox of two sites of interaction with p47phox, corresponding to residues ^{156}PPRPP and ^{177}GGPPGGP, located in the proline-rich region of p22phox, near the C terminus [101]. Burritt et al. [102] combined the use of monoclonal antibodies (mAbs) with that of random peptide phage display libraries to identify antibody epitope. Bacteriophage epitope mapping exploits the specificity of mAbs and the unique sequences expressed on selected phages. Phages selected by mAb 44.1, specific for p22phox, expressed a peptide corresponding to the C-terminal sequence ^{181}GGPQVNPI of p22phox. Flow cytometric analysis of saponin-permeabilized neutrophils and intact neutrophils indicated that the ^{181}GGPQVNPI epitope of p22phox was accessible on the cytosolic, but not the external side of the plasma membrane, in agreement with previous studies [100, 101]. On the other hand, phages selected by mAb 54.1 expressed the peptidic fragment ^{382}PKIAVDGP of gp91phox. Since this epitope was not accessible to its specific antibody in intact neutrophils, it could not be located on the membrane surface.

In a recent mapping analysis, immunological detection was combined with modern techniques of nuclear magnetic resonance (NMR) spectroscopy, based on the transferred nuclear Overhauser effect and the transferred rotating frame Overhauser effect. The mAb utilized, mAb 44.1, recognized two distinct sequences ^{181}GGPQVNPI and ^{29}TAGRF in p22phox [103]. These sequences were inferred to combine to form a complex epitope on the cytosolic surface of the flavocytochrome. The NMR data supported the phage display analysis and provided a low-resolution view of the tertiary structure of the p22phox protein. The phage display technique was also used to map the epitope recognized by another mAb, 7D5, known to bind to an extracellular epitope of flavocytochrome b_{558} in neutrophils. Two extracytoplasmic regions of gp91phox, corresponding to residues ^{160}IKNP and ^{226}RIVRG, emerging from two transmembrane helices, were found to react with mAb 7D5 [104]. These data provided direct evidence for the identity of two juxtaposed transmembrane helices in gp91phox (helices IV and V; figs 1, 3).

In summary, in the absence of crystallographic data, a variety of experimental approaches have been utilized to explore the arrangement of the polypeptide chains of the two subunits of flavocytochrome b_{558}. They include the access to target sites of antibodies directed against specific sequences and synthetic peptides mimicking some regions of gp91phox and p22phox, the use of random peptide phage display libraries and, in a more sophisticated fashion, NMR techniques. Recently, atomic-force microscopy was used to assess conformational changes in flavocytochrome b_{558} occurring during the activation process [105]. Despite some conflicting results, these different approaches have led to a tentative model of the topology of flavocytochrome b_{558} that can be used to plan future investigations (fig. 3).

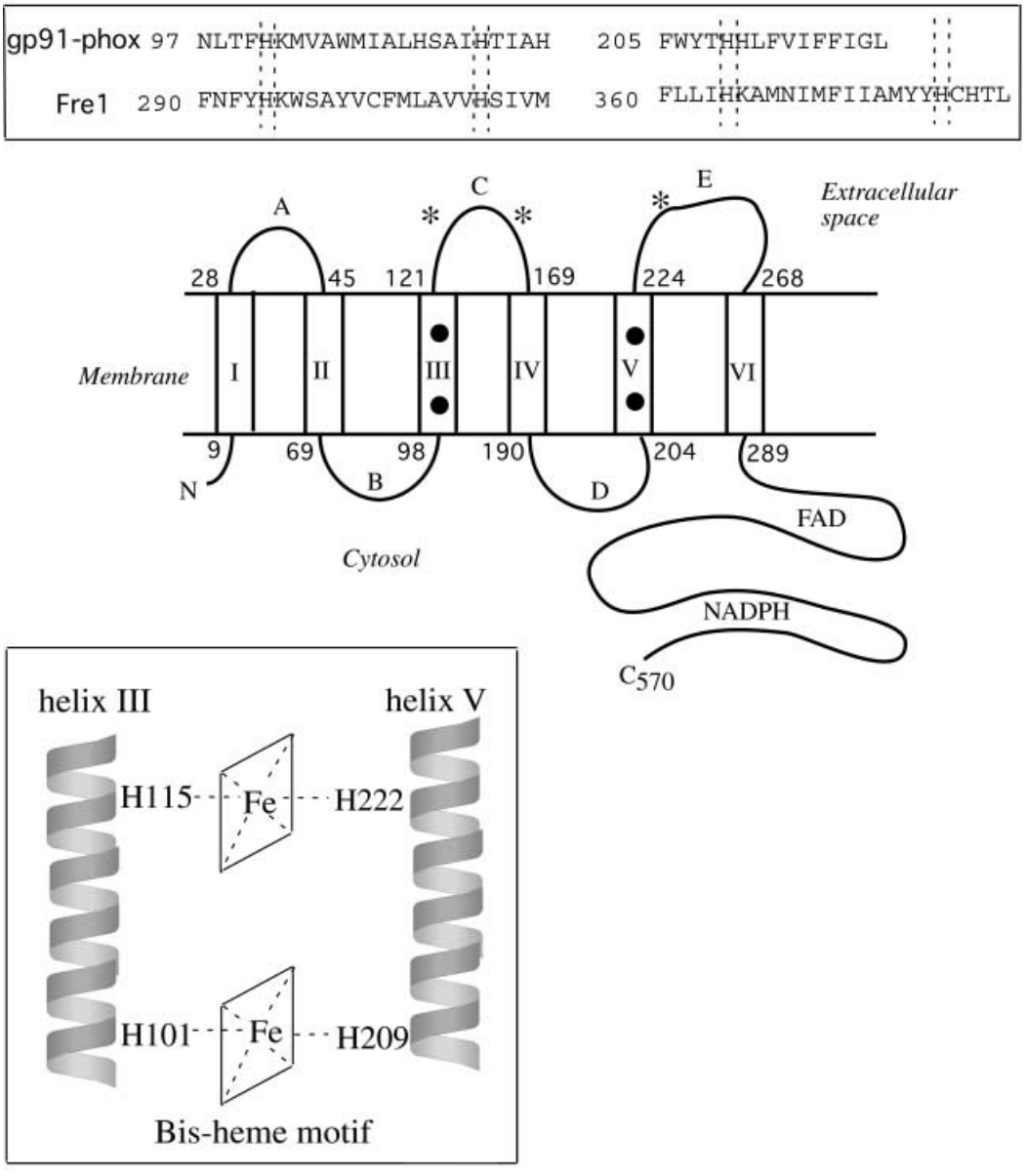

Figure 3. The heme-binding domain of human gp91phox. Localization of histidine residues that serve as ligands for the two hemes of human gp91phox was deduced from comparison of the sequences of gp91phox and yeast Fre1 (upper panel). The figure shows the putative organization of the polypeptide chain of gp91phox with a pair of heme irons liganded by histidine residues, His101 and His209, His115 and His222, in transmembrane α helices III and V. The positions of Asn132, Asn149 and Asn240 on loops C and E are indicated by asterisks.

Mapping of NADPH, FAD and heme-binding sites in the gp91phox subunit of flavocytochrome b_{558}

The NADPH- and FAD-binding sites of flavocytochrome b_{558} are localized in the gp91phox subunit, as demonstrated by photolabeling with photoactivatable azido derivatives of NADPH [96, 106] and FAD [95] and also by affinity labeling, using pyridoxal-5′-diphospho-5-adenosine [107, 108]. The crystal structure of several NADPH-dependent flavoproteins is known, and the regions of their polypeptide chains that participate in binding NADPH and FAD have been characterized. The prototype is the ferredoxin NADPH reductase (FNR) [109]. gp91phox contains NADPH- and FAD-binding sites that show similarities to those of the FNR family [89, 90, 96]. Earlier comparison of sequences led to the conclusion that the sequence ^{218}GLAIHG of gp91phox (fig. 1) was similar to the canonical ADP-binding fingerprint GXGXXG of flavoenzymes. This sequence is localized in a predicted $\beta\alpha\beta$ fold of the peptide chain of gp91phox which spans residues 214–246. Recently, these conclusions were questioned, based on comparison of the

aligned sequences of human, bovine, murine and porcine gp91phox proteins [82, 110]. In fact, bovine gp91phox shows significant divergence from the human protein in the second β sheet of the $\beta\alpha\beta$ fold (residues 239–246). In addition, Glu245 in human gp91phox, which is suggested to form a hydrogen bond with the 2′-hydroxyl group of the ADP-ribose moiety of FAD, is absent in bovine, murine and porcine gp91phox. Another convincing argument against the participation of residues 214–246 in the FAD-binding site of gp91phox relies on the putative organization of the polypeptide chain of gp91phox. As shown in figure 3, residues 214–246 are located on the E loop on the outer face of the plasma membrane, whereas the FAD-binding site is situated in a region of gp91phox exposed to the inside of the cell. In contrast, other sequences of gp91phox, ^{337}HPFTLSA and ^{355}IRIVGD, that are similar to sequences involved in the binding of the isoalloxazin ring and the ribityl chain of FAD in FNR and glutathione reductase [89], are virtually identical in the four gp91phox species. In agreement with their participation in the binding of FAD is the report of a rare X-linked CGD case characterized by complete depletion of gp91phox in FAD. This was due to a mutation replacing His338 by Tyr in gp91phox [111]. The deficient neutrophils were unable to generate O_2^- and the defect could not be corrected by addition of FAD, indicating that His338 is a critical residue for incorporation of FAD in gp91phox. Four sequences of gp91phox, namely ^{405}MLVGAGIGVTPF, ^{442}YWLCR, ^{504}GLKQ and ^{535}FLCGPE are considered to be binding sites for the pyrophosphate, ribose, adenine and nicotinamide units of NADPH, respectively. These sequences are identical in human, bovine, murine and porcine gp91phox proteins. In addition, comparison with FNR [109] makes it likely that the cysteine residue juxtaposed to glycine 538 in all four gp91phox proteins is close to the C4 atom of nicotinamide. A model of the C-terminal region of gp91phox, with specific emphasis on the FAD- and NADPH-binding sites, has been constructed, based on the crystal structure of FNR [112]. In this model, a loop of 20 amino acids (Asp 484–Thr 503) covers the cleft in which NADPH binds. The loop is thought to move aside when the oxidase is activated, allowing NADPH to reach its binding site and deliver electrons to FAD.

The hemes of flavocytochrome b_{558} are situated in the N-terminal half of the molecule, as shown in a recent study in which flavocytochrome b_{558} was partially digested by a bacterial proteinase [113]. The undigested product retained the heme absorbance spectrum of flavocytochrome b_{558}. It contained, in particular, the N-terminal 336 or 363 residues of gp91phox that correspond to a hydrophobic region in which the transmembrane helices are located. The sequence of the amino-terminal region of gp91phox has been compared to that of Fre1, an iron reductase that, in the yeast cell, is required for iron acquisition. By analogy, a model of coordination of the two hemes of gp91phox has been proposed (fig. 3), in which the two hemes are liganded by two pairs of histidine residues separated by 13–14 amino acids and located in the transmembrane helices III and V of gp91phox [114, 115]. In this bis-heme model (fig. 3), one of the two hemes is positioned close to the cytosolic space, in the neighborhood of the FAD-binding site. The second heme is on the opposite side of the membrane, close to the extracellular space. Similar heme arrangements have been described for the mitochondrial cytochrome *b* and the chloroplastic cytochrome b_6. The bis-heme model was recently validated by experiments carried out with COS cells transfected with a mutant of gp91phox in which the four critical histidine residues 101, 115, 209 and 222 had been replaced by leucine or arginine residues [116]. Mutations of His101 or His115 resulted in complete loss of the heme spectrum, suggesting that these residues are necessary for acquisition and stabilization of both hemes. Conversely, mutations of either His209 or 222 decreased the heme content only partially, which may reflect the ability of one of these two histidine residues to bind heme weakly. The functional implication of His101, His115, His209 and His222 in heme binding is illustrated by a number of cases of X-CGD caused by missense mutations at these residues [116]. p22phox possesses a histidine residue (His94) that is conserved among a number of species. The absence in p22phox from rabbit and other species of a second histidine residue (His72), which is polymorphic in human p22phox [117], supports the idea that p22phox alone is not capable of coordinating heme.

Post-translational modifications of flavocytochrome b_{558}

Since the molecular mass of gp91phox is averaged to 91 kDa and cleavage of the bound carbohydrate with N glycosidase results in a product that migrates in SDS-PAGE with an apparent mass of 50–55 kDa [118], carbohydrates must represent at least one-third of the mass of gp91phox. Carbohydrates are bound to asparagine residues of the protein core by N linkages. The majority are N-acetyl glucosamine and galactose, while a minority are sugars like fucose, mannose and glucose. There are five consensus sites for N-linked glycosylation (N-X-S/T) at asparagine positions 97, 132, 149, 240 and 430 in human gp91phox. The mutagenesis approach based on the replacement of Asn residues by Thr residues demonstrated that glycosylation of human gp91phox is restricted to Asn132, Asn149 and Asn240 [119]. Loops C and E of the polypeptide chain of gp91phox, that contain the glycosylated sites, are exposed to the extracellular space (fig. 3). Comparison of sequences of gp91phox from different species [110, 120] showed that Asn132 and

Asn149 are missing in gp91phox from mouse and dolphin, respectively. The putative external glycosylation site Asn240 in human gp91phox is at Asn247 in bovine, dolphin and pig gp91phox. It is absent in rabbit gp91phox. Differences in the apparent molecular mass of gp91phox in neutrophils from different species are explained by differences in the amount of bound polysaccharide exposed to the outside at the level of glycosylation sites. For example, the murine homologue of gp91phox migrates with an apparent molecular mass of 58 kDa, compared with 91 kDa for the human protein [110]. Examination of the sequences of the two proteins shows that three glycosylation sites located in loops C and E of the human gp91phox (fig. 3) are missing in the murine protein. The size modification brought about by the heavy glycosylation of the human gp91phox was recently investigated by atomic-force microscopy, using purified flavocytochrome b_{558} incorporated in liposomes [121]. Analysis of images of flavocytochrome b_{558} isolated from B lymphocytes and incorporated in liposomes revealed a mass of protruding carbohydrate significantly more important than in the neutrophil counterpart.

p22phox was found to be phosphorylated in vitro by conventional PKC isoforms and a novel phosphatidic acid-activated protein kinase [122]. Activation of phospholipase D (PLD) which converts phosphatidyl choline to phosphatidic acid (PA) is mediated through the activation of the fMLP receptor at the onset of the respiratory burst. The data suggested that NADPH oxidase activity and p22phox phosphorylation are correlated [122].

Rap1A, a flavocytochrome *b*-associated small G protein in search of a function

Ten years ago, a series of papers appeared concerning Rap1A, a small G protein closely related to Ras, abundant in neutrophils and localized in the plasma membrane and the specific granules [123, 124]. Conclusions drawn about this protein were as follows. (i) Rap1A copurifies with flavocytochrome b_{558} from a detergent extract of human neutrophils [125]. (ii) Rap1A is colocalized and translocated with flavocytochrome *b* in resting and activated neutrophils [123 , 124]. (iii) Rap1A serves as a substrate for cyclic AMP-dependent protein kinase (PKA) both in vitro, in electroporated neutrophils, and in differentiated HL60 cells [126]. (iv) Association of Rap1A with flavocytochrome b_{558} is stoichiometric and inhibited by phosphorylation of Rap1A [127]. (v) Immunodepletion of Rap1A in a cell-free system of oxidase activation results in loss of oxidase activity; moreover, antibodies raised against Rap1A inhibit cell-free NADPH oxidase activation [128]. These reports concurred to support a role for Rap1A, through its association with flavocytochrome b_{558}, in the regulation of the NADPH oxidase activation or the elicited oxidase activity [reviewed in ref. 129]. Doubt was cast on this view with the demonstration that functional NADPH oxidase can be reconstructed in vitro from recombinant proteins in the absence of Rap1A [4]. Nevertheless, the intriguing presence of large amounts of Rap1A in neutrophils continued to raise questions. The finding that, in the presence of Rap1A, the phosphorylation of PKC substrates is significantly enhanced suggested that Rap1A might be involved in PKC-dependent signal transduction [130]. A possible implication of Rap1A in translocation of flavocytochrome b_{558} from specific granules to the plasma membrane was also envisaged on the basis that both activated and inhibitory mutants of Rap1A decreased the respiratory burst of stimulated B lymphocytes [131] and differentiated HL60 [132], a behavior similar to that of the Rab proteins in membrane trafficking [133].

Recent development in understanding the functioning of Rap1A in neutrophils was made possible through the use of a novel assay to measure activation of Rap1A based on the high affinity of the Rap1-binding domain of Rapl-GDS for Rap1-GTP, but not for Rap1-GDP [134]. A variety of stimuli involved in neutrophil activation, including fMLP, platelet-activating factor, GM-CSF and IgG-coated particles, induced a rapid and transient Rap1A activation. Rap1A was also found to be normally activated, in a GTP-bound form, in X-CGD neutrophils which lack flavocytochrome b_{558} and have defective oxidase activity. Thus, in neutrophils, Rap1A is apparently activated by multiple signaling pathways, independently of both a functional NADPH oxidase complex and the presence of flavocytochrome b_{558}.

A survey of the cytosolic phox proteins: p47phox, p67phox and p40phox

Only three cytosolic proteins, p47phox, p67phox and Rac1/2, are needed in the cell-free system to activate flavocytochrome b_{558} [4]. However, a distinct protein, p40phox, copurifies and coimmunoprecipitates with p67phox and p47phox [5], suggesting that p40phox may be a regulatory protein. The three cytosolic phox proteins, p47phox, p67phox and p40phox harbor domains and motifs homologous to the homology domain 3 (SH3 domain) of p60-Src as well as proline-rich sequences. Since SH3 domains have a propensity to interact with polyproline motifs [135], SH3-polyproline interactions between the proteins of the cytosolic phox triad were the first to be studied [for a review, see ref. 68]. Later, other domains and motifs were recognized as potential sites for transient associations (Pfam; http://www.sanger.ac.uk), namely PX (*P*ho*X* domain, Pfam 787) in p47phox and p40phox, TPR (*T*etratrico *P*eptide *R*epeat, Pfam 515) in p67phox and OPR (*O*cticosa *P*eptide *R*epeat, Pfam 564) in p40phox (fig. 4).

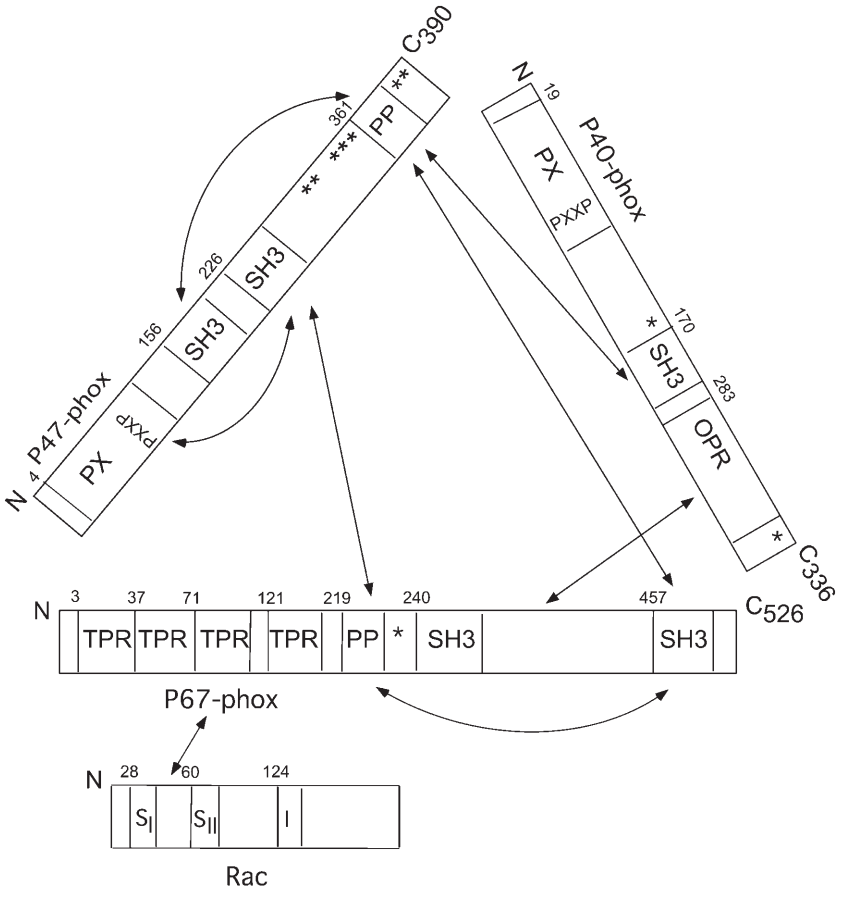

Figure 4. Rac and the triad of cytosolic phox proteins, p47phox, p67phox and p40phox, in the human NADPH oxidase. Potential interactions between specific sites of the three cytosolic phox proteins are represented by arrows. PP, polyproline motif; SH3, src homology domain 3; TPR, tetratrico peptide repeat; PX, phox homology domain; OPR, octicosa peptide repeat. Phosphorylation sites are represented by an asterisk. S_I and S_{II} in Rac denote the 'switch' regions I and II, and I, the insert region. Multiple hydrogen bonds occur between Rac and p67phox [162] (see text).

Human p47phox is encoded by a gene located on chromosome 7 (locus 7q11.23), whose mutations account for nearly 30% of CGD cases [for reviews, see refs 9, 68]. p47phox comprises 390 amino acids, with a large excess of positively charged residues concentrated in the C-terminal region, which explains the basic pI 9.1 of the protein. Two SH3 domains are situated in the middle of the molecule (residues 156–213 and 226–283), and there is a polyproline motif at the C terminus (residues 361–371) (fig. 4). The N-terminal region of p47phox contains a PX domain (residues 4–125) [136]. Interaction between phosphoinositides and the PX domain in a wide variety of proteins is well documented [137]. In the case of p47phox, there are divergent opinions as to the propensity of the PX domain to interact with phosphoinositides. One report [138] concluded that it binds selectively to phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P2); another [139] argued that the binding was weak and not selective. Based on the finding that the Arg42 → Gln mutation in p47phox abolishes phosphoinositide binding [138], a hypothesis was proposed [140] that the inhibition of O_2^- production in cases of CGD due to this mutation [141] was the result of a structural modification in the PX domain of p47phox. However, neutrophils from these CGD patients have no detectable p47phox, rendering the above hypothesis no longer tenable [142]. Another property of the PX domain, shared by many proteins, is to harbor a PxxP motif capable of interacting with an SH3 domain. In the case of p47phox, the PxxP motif corresponds to residues 73–76. Through the use of the isolated PX domain of p47phox and SH3 domains of p47phox produced separately as glutathione S-transferase fusion proteins, the p47phox PX domain was shown to bind to the C-terminal SH3 domain of p47phox [136].

Upon stimulation of neutrophils, p47phox undergoes extensive phosphorylation [143, 144] and moves to the membrane-bound flavocytochrome *b* [145–147] (see section on Interaction of flavocytochrome b_{558} with the cytosolic phox proteins). In vitro, p47phox can be phosphorylated by a number of protein kinases, including conventional, novel and atypical PKCs as well as mitogen-activated kinases and p21Rac-activated kinases [for

a review, see ref. 148]. Affinity chromatography of the β and δ isoforms of PKC from activated neutrophil cytosol, using a p47phox matrix, provided evidence for the propensity of the β isoform to interact with, and to phosphorylate p47phox [149]. Consistent with this finding was the report that PKCβ antisense oligonucleotides inhibit the NADPH oxidase activity and the phosphorylation of p47phox in differentiated HL60 cells [150]. The serine residues Ser345 and Ser348 of human p47phox, which are located in consensus sites recognized by MAP kinase, are not conserved in murine, bovine and rabbit p47phox [120]. A strategic function for these serine residues in oxidase activation is therefore unlikely. Phosphorylation of p47phox is concentrated in a cluster of serine residues from Ser303 through Ser379 at the C terminus of the molecule [151, 152]. Phosphorylation occurs in a sequential manner, Ser359 and Ser370 being phosphorylated prior to Ser303 and Ser304 [153, 154]. Whereas the replacement of Ser303 and Ser304 by alanine destroyed the activity of p47phox, their replacement by glutamate did not change the efficiency of p47phox. A plausible explanation is that oxidase activation requires a negative electrostatic potential in the vicinity of Ser303 and Ser304 [154]. Ser379 is of special interest because the mutated p47phox (Ser379 $\rightarrow$ Ala) is functionless [151]. Conformational changes in p47phox undergoing phosphorylation have been demonstrated by monitoring the intrinsic fluorescence of the molecule [155] or the extrinsic fluorescence of covalently attached probe [156]. Based on mutagenesis experiments, models were proposed to explain the transition of p47phox from a resting to an activated state. In the resting state, the N-terminal SH3 domain of p47phox is masked, due to its interaction with one of two well-defined regions in the same molecule, namely the polyproline motif at the C terminus [157] (fig. 4) or the arginine + lysine-rich region corresponding to residues 301–305 [158]. The p47phox molecule thus adopts a folded shape. Serine phosphorylation, essentially on residues 303, 304 and 328, disrupts the SH3-mediated interaction, and the unmasked SH3 domain becomes capable of interacting with the polyproline motif of p22phox [157, 158]. The phosphorylation-dependent unmasking of the SH3 domains of p47phox is mimicked by arachidonic acid. The transition of p47phox from a resting to an active conformation has therefore been proposed to be governed synergistically by serine phosphorylation and binding of arachidonic acid released from phospholipids by activated cytosolic PLA2 [159].

Human p67phox contains 526 amino acids and is encoded by a gene located on chromosome 1 (locus 1q25) [for reviews, see 9, 68]. The dimeric form of isolated p67phox previously observed by gel filtration [160] was recently confirmed by small-angle neutron scattering analysis [161]. p67phox contains two SH3 domains (residues 240–297 and 457–514) and a polyproline motif (residues 219–231) (fig. 4). As in p47phox, an intramolecular interaction might exist between the polyproline motif and the C-terminal SH3 domain of p67phox (fig. 4). TPR motifs (34 amino acid residues each, organized in two antiparallel α helices) are located in the N-terminal region [162, 163]. The first three repeats (residues 3–36, 37–70 and 71–104) are tandemly arranged. Sixteen extra residues are located between the third and the fourth repeat (residues 121–154). The early report that binding of Rac to p67phox is essential to oxidase activation [164] was recently refined with the demonstration that the TPR motif of p67phox functions as a Rac target [165]. Crystallographic data indicated that Arg102 in p67phox plays a key role in complex formation with Rac through hydrogen binding with several residues of Rac (see section on Rac, a small G protein with a prominent function in NADPH oxidase activation) [162]. The N-terminal region of p67phox also contains an activation domain localized to amino acid residues 199–210 and essential for NADPH oxidase activation [166]. Curiously, rabbit p67phox differs from other species by an extension of 17 additional amino acids at the C terminus [120]. This additional sequence displays homology to proteins capable of interacting with calcineurin, a Ca^{2+}-binding protein, suggesting in vivo association of rabbit p67phox with calcineurin or a closely related protein in an integrated complex. The central function of the N-terminal region of p67phox in oxidase activation is illustrated by the fact that most of the p67phox mutations responsible for CGD are localized in this region. During oxidase activation, p67phox is phosphorylated [167]. The phosphorylation is on Thr233 [168].

Through the study of the binding of [^{32}P]NADPH dialdehyde to p67phox, p67phox has been concluded to contain an NADPH-binding site [169] located in the TPR domains at the C terminus of the molecule [170]. The redox function of the NADPH-binding site in p67phox remains, however, to be elucidated, since purified and reflavined flavocytochrome b_{558} is able to catalyze the transfer of electrons from NADPH to O_2, by itself, in the absence of the cytosolic phox proteins [92]. Not excluded is that the p67phox-bound NADPH might serve as an allosteric regulatory ligand for flavocytochrome b_{558}.

The two cytosolic activating factors p47phox and p67phox have been immunodetected in soybean, cowpea, tobacco and cotton extracts with human-protein-directed antibodies [56], and a homologue of gp91phox is present in plant cells (see section on Homologues of the gp91phox subunit of flavocytochrome b_{558}). This suggests that plant cells harbor an NADPH oxidase complex closely related to that of mammalian cells.

p40phox is a protein of 336 amino acids. The human p40phox gene spans approximately 18 kb and has ten exons. It is located on chromosome 22 (locus 22q13.1) [171]. Like p47phox, p40phox possesses an N-terminal

PX domain (residues 24–143) that shows a high propensity to bind PtdIns(3)P [139, 172]. The crystal structure of the PX domain of p40phox bound to a soluble derivative of PtdIns(3)P revealed key interactions between Lys92 and Arg58 of p40phox and the 1-phosphate and the 3-phosphate of the phosphoinositide, respectively [173]. p40phox also contains an SH3 domain (residues 170–227) and in the C-terminal region, a sequence of 28 amino acids (residues 283–310), the OPR [174] or PC (Phox Cdc 24p) [175] (cdc 24p is an exchange factor for the small G protein cdc42 in yeast). In the course of oxidase activation, Thr154 and Ser315 of p40phox are phosphorylated by PKC [176, 177]. Upon stimulation of differentiated HL60 cells, the onset of O_2^- production coincides with p40phox phosphorylation, and at the end of the respiratory burst, p40phox is dephosphorylated [176], indicating a correlation between the kinetics of O_2^- production and p40phox phosphorylation. An increased level of phosphorylation of p40phox at serine and threonine residues was demonstrated in stimulated B lymphocytes subjected to an oxidative stress, induced by addition of H_2O_2 [178]. The overphosphorylation of p40phox is catalyzed by an endogenous PKCδ activated through the H_2O_2-dependent phosphorylation of tyrosine residue(s). Worth noting is that both p40phox and p47phox contain consensus motifs for casein kinase II (CK II). Phosphorylation of p40phox [176] and p47phox [179] by purified CK II in vitro have been demonstrated. Yet, in the case of p40phox, in vivo phosphorylation, using stimulated neutrophils, was exclusively confined to sites specifically recognized by PKC [176, 177]. This suggests that in vivo phosphorylation of p40phox by endogenous PKC is prevented by some unknown mechanism.

The triad p47phox, p67phox and p40phox forms a complex that participates in oxidase activation by translocation to the membrane-bound flavocytochrome b_{558}. In the mid-1990s, through different approaches, namely the two-hybrid system [180, 181] and the use of truncated and mutated proteins in binding assays [145, 146, 182–186], a number of potential interactions between the components of the phox triad were delineated, among which the interactions of polyproline motifs with the SH3 domains were preeminent (fig. 4). Thus, the C-terminal polyproline region of p47phox (residues 361–371) interacts with the C-terminal SH3 domain of p67 phox (residues 457–514), and also with the SH3 domain of p40phox (residues 170–227). It may even bind to the N-terminal SH3 domain in its own polypeptide chain (residues 156–213), conferring on it a bent shape [157]. The interaction of the SH3 region of p40phox with p47phox has been suggested to indirectly increase the affinity of p47phox for flavocytochrome b_{558} [7]. Through the phage display technique, another region of p47phox, encompassing residues 323–332, was found to interact with p67phox [187]. On the other hand, the proline-rich motif of p67phox (residues 219–231) has been proposed to mediate binding to the second SH3 domain of p47phox (residues 226–283) during NADPH oxidase assembly [145]. Data on interactions between the three cytosolic phox proteins, p47phox, p67phox and p40phox, accumulated between 1994 and 1996, leading to the formulation of models. One of these [180, 181] suggested that, in the resting state, p40phox acts as a bridging molecule between p47phox and p67phox, and that, upon activation, p40phox is dislodged, allowing direct interaction between p47phox and p67phox. Through the use of isothermal titration calorimetry that allows direct measurement of equilibrium binding constants, a novel model has been proposed, with p67phox being the primary binding partner even in the resting state [188].

The peptide-walking technique [189] was recently applied to inventorize more precisely functional domains in p47phox that participate in oxidase assembly [190]. This technique is based on testing a large set of overlapping synthetic peptides, spanning the entire sequence of the phox protein to be investigated (here p47phox) for an inhibitory effect on oxidase activation in a cell-free system. In the present case, assuming that p47phox interacts with other phox proteins through short sequences of amino acids to form a complex essential for oxidase activation, then the peptides that best mimic these sequences will be the most effective inhibitors of oxidase activation. By this approach, eight potential domains of interaction in p47phox were delineated. Four corresponded to sequences already shown to participate in NADPH oxidase assembly [145, 182–185]. The other four sites (residues 21–35, 105–119, 305–319 and 373–387) represented newly identified entities.

The cell-free system approach and the two-hybrid technique essentially provide information on potential sites of interaction. The physiologically relevant binding sites need to be confirmed by an in vivo approach. In this respect, some aspects of the reported interactions between the phox proteins have been critically reviewed [68]. Attention was drawn to the potential non-specific binding of SH3 domains to a number of proline-rich targets, especially in truncated proteins. As an example, in stimulated intact B lymphocytes, the two SH3 domains of p67phox are required for O_2^- production whereas in the cell-free system, the truncation of the SH_3 domains does not interfere with oxidase activation [183].

Rac, a small G protein with a prominent function in NADPH oxidase activation

In addition to the triad p47phox, p67phox and p40phox, a small G protein, Rac, is involved in the activation of NADPH oxidase. The implication of a G protein in neutrophil oxidase activation stems from observations show-

ing that, in the cell-free sytem, oxidase activity is increased in the presence of GTPγS and decreased upon addition of GDPγS [191–193]. This G protein was identified as Rac through the use of purified recombinant proteins in cell-free assays from macrophages [194] and neutrophils [195]. Rac participation in oxidase activation was supported by the finding that transfection of B lymphocytes with Rac antisense oligonucleotides [196] and expression of a dominant negative form of Rac [132] inhibit the production of O_2^- in stimulated cells. Two Rac isoforms, Rac1 and Rac2, activate NADPH oxidase. Rac1 and Rac2, which are 92% identical, differ in only 14 of 192 residues [197]. Rac2 was reported to be a better activator than Rac1 [198], despite the fact that all residues on Rac1 and Rac2 found to be involved in bonds with p67phox are identical [162]. The N-terminal half of Rac contains amino acid residues to which GTP or GDP is attached, and, in addition, two short sequences termed 'switch' regions I (residues 28–40) and II (residues 60–75), because they adopt different conformations depending on the nature of the bound nucleotide (GDP or GTP). Rac proteins have a basic C-terminal region (more basic in Rac1 than in Rac2) with a cysteine residue to which a geranylgeranyl group can be attached through a thioether linkage. Like other G proteins, Rac cycles between an inactive GDP-bound form and an active GTP-bound form. This cycle is regulated by GDP/GTP exchange factors (GEF proteins) that catalyze the replacement of bound GDP by GTP, and by GTPase-activating proteins (GAPs) that enhance the endogenous GTPase activity of the G protein and may participate in termination of the respiratory burst [199]. GAP activities have been localized in the plasma membrane and in the cytosol of neutrophils [200].

In resting cells, Rac is essentially located in the cytosol, associated with a protein, the GDP dissociation inhibitor of Rho family proteins (RhoGDI), that prevents the exchange of GDP for GTP. Structural analysis of Rac2-RhoGDI [201] and Rac1-RhoGDI [202] has provided a certain amount of information on the sites of interaction of the partners in these heterodimers. The geranylgeranyl tail of Rac plunges into a pocket of Rho GDI, which consists of conserved hydrophobic residues. In addition to this lipid-protein interaction, protein-protein interactions between Rac1 and RhoGDI have been identified and deciphered. The implicated Rac1 residues are Tyr64, Arg66, His 103and His104 [202]. Through the use of Rac1 mutants, prenylated or not, the predominant role of protein-lipid over protein-protein interaction in the Rac1-RhoGDI complex was confirmed, as was the involvement of Arg66-Leu67, His103 and the C-terminal polybasic region of Rac in complex formation with RhoGDI [203].

The central control played by Rac in NADPH oxidase activation is achieved through interaction with p67phox, more particularly with its N-terminal half (residues 1–199) [164]. Another line of evidence is that prenylated Rac recruits p67phox to the membrane and elicits O_2^- production in the absence of p47phox [204]. Binding of Rac to p67phox might be facilitated by the release of the intramolecular interaction between the polyproline motif and the C-terminal SH3 domain of p67phox [205]. In fact, deletion of either of these two regions leads to a marked increase in Rac binding. In early studies, site-directed mutagenesis was applied to locate in Rac amino acid residues playing a role in oxidase activation. In a cell-free semi-recombinant system, consisting of neutrophil membranes and recombinant cytosolic phox proteins including Rac1, the following Rac mutations Asn26 → His, Ile33 → Asn, Asp38 → Asn and Met45 → Thr led to a strongly reduced activity in O_2^- generation [206]. Through the same approach, modified forms of Rac with the following substitutions Thr35 → Ala, Asp38 → Ala and Tyr40 → Lys were found unable to sustain O_2^- production [164]. Interestingly, the above mutations are confined to a short sequence of Rac that was first considered as the main effector site of Rac and is now termed the 'switch I' region (see above). Another effector site in Rac (residues 143–175), important for interaction with p67phox, was subsequently reported [207]. Rac is also endowed with a specific sequence (residues 124–135), called the 'insert' region [208], which is absent in Ras, and is implicated in oxidase activation, not through binding to p67phox, but possibly through binding to flavocytochrome b_{558} [209, 210]. In addition to the mutagenesis approach, the peptide-walking technique applied to Rac allowed the detection in this molecule of four distinct sites needed for oxidase activation (residues 73–81, 103–107, 123–133 and 163–169) [189]. The third and fourth sites confirmed previous identifications by mutagenesis. Through the use of Rac1/HRas chimeras, domain deletions and point mutations, the physiological relevance of the second and fourth sites detected by peptide walking in Rac was ascertained, and His103 as well as Lys166 were pinpointed as critical residues in these two strategic sites [211]. Most of the above-mentioned studies have been rationalized through the examination of crystallographic data, and the specification of contact regions between Rac and p67phox [162]. Thus, hydrogen bonds occur between Arg102 in p67phox and at least four residues in Rac (Ser22, Asn26, Ala159 and Leu160). Gln162 and Asn26 in Rac form hydrogen bonds with the side chain of Asn104 in p67phox. There is also a contact between Asp108 in p67phox and Thr25 in Rac. In addition, Gly30 and Glu31 located in the switch I region of Rac interact with Asp67 and Ser37 in p67phox, respectively. Finally, the functional role of the carboxyl-terminal region of Rac in oxidase activation was examined in mutation and truncation experiments [212]. The results supported an essential requirement for the polybasic C terminus of Rac in binding to the membrane associated

with the insertion of the geranylgeranyl tail into the phospholipid bilayer.

The binding parameters of Rac to p67phox have been quantified through the use of a fluorescent analogue of GTP attached to Rac as a reporter group [209]. The stoichiometry was 1:1, and the K_d values were 120 and 60 nM for Rac1 and Rac2, respectively. Site-directed mutations in the 'switch I' region, namely Asn26 → His, Ile33 → Asn and Asp38 → Asn, decreased the affinity of Rac for p67phox [209]. Another phox partner, p40phox, might play a role in controling the Rac/p67phox interaction. In fact, p40phox binds rather firmly to p67phox [213], and the Rac/p67phox association is disrupted by Rac-GTP, not by Rac-GDP [214].

Interaction of flavocytochrome b_{558} with the cytosolic phox proteins

During the course of oxidase activation in phagocytic cells, cytosolic phox proteins are translocated to the membrane-bound flavocytochrome *b*. An early report concluded that participation of p47phox precedes that of p67phox in the formation of the active NADPH oxidase complex by association with flavocytochrome b_{558} [215]. Consistent with this report was the finding that p67phox translocation to the membrane-bound flavocytochrome b_{558} does not occur in p47phox-deficient CGD, whereas, in the genetic absence of p67phox, translocation of p47phox is not impaired [216]. These peculiarities could be explained by a readily dissociable complex of p47phox and p67phox found in the cytosol of unstimulated neutrophils [217]. While p47phox appears to be essential for the respiratory burst of intact cells, its requirement in in vitro oxidase activation can be overcome under specific conditions. Thus, in a reconstituted sytem, oxidase activation is achieved in the absence of p47phox provided that Rac and excess p67phox are present [218, 219]. However, the addition of p47phox dramatically lowers EC_{50} values for Rac and p67phox [219]. A fusion construction between the N-terminal regions of p47phox (residues 1–286) and p67phox (residues 1–210) proved to be much more efficient than the individual proteins in oxidase activation [220], a result consistent with the idea that, in vivo, p47phox is an efficient adjuvant in oxidase activation. Direct interaction between p67phox and flavocytochrome b_{558} was demonstrated through overlay, dot blot and affinity precipiation studies [221]. The interaction was increased in the presence of phosphorylated p47phox or phosphorylated p40phox, leading to the suggestion that phosphorylated p47phox serves as an adapter protein, bringing p67phox into proximity with flavocytochrome b_{558} [222].

Despite the fact that isolated Rac and p67phox are prone to interact [164, 198, 209, 223], during oxidase activation, Rac translocates to the membrane-bound flavocytochrome *b* as a separate unit from p47phox and p67phox [224, 225]. Rac translocation occurs even in the absence of p47phox and p67phox as shown with CGD neutrophils deficient in p47phox and p67phox [226]. Conformational changes of flavoprotein b_{558} essentially dependent on p67phox and Rac have been detected, using the technique of atomic-force microscopy [105]. Complementation experiments carried out with CGD p67phox-deficient cytosol confirmed that p67phox is a limiting factor in reconstitution of the oxidase activity [227]. Recombinant chimeric proteins joining various domains of truncated p67phox and non-prenylated Rac1 have been produced and assayed in a cell-free system supplemented with p47phox and amphiphile [228, 229]. The data confirmed that the N-terminal region of p67phox (residues 1–212) is essential for oxidase activation, in agreement with the presence of an activation domain in residues 199–210 of p67phox [166]. A subsequent study [230] revealed that a chimeric protein consisting of prenylated Rac1 joined to truncated p67phox (residues 1–212) was not only more effective than the non-prenylated Rac1-p67phox chimera, but also quite effective in the absence of p47phox and amphiphile. On this basis, the in vitro basic requirements for oxidase activation were postulated to be (i) a motif in Rac comprising the C-terminal basic sequence with the attached prenyl group and (ii) some protein interaction between Rac and p67phox causing a conformational change in the activation domain of p67phox. In summary, p67phox in association with Rac appears to be the essential factor that activates electron transfer within flavocytochrome b_{558}.

Of note is that Rac2, specific to the myeloid lineage, exerts its effect not only on the oxidase complex, but also in the preceding steps leading to the assembly of the oxidase components. In fact, experiments performed with Rac2 $^{-/-}$ neutrophils prepared from mice with a targeted disruption of Rac2 have shown that Rac2 is a critical regulator of signaling pathways that activate NADPH oxidase dowstream of chemoattractant receptors and Fcγ receptors [231]. Along this line, a GEF protein named P-Rex1 (for *P*tdIns(3,4,5)P3-dependent *R*ac *ex*changer), relatively abundant in neutrophils, was reported to be involved in receptor-stimulated activation of Rac [232]. P-Rex1 was activated synergistically by PtdIns(3,4,5)P3 and the $\beta\gamma$ subunits of trimeric G protein bound to membrane receptors, suggesting that G$\beta\gamma$ and PtdIns(3,4,5)P3 can bind simultaneously to independent sites on P-Rex1 and that P-Rex1 is directly under the control of G$\beta\gamma$ subunits in neutrophils.

Each of the two subunits of flavocytochrome b_{558} is endowed with specialized functions. The view that the p22phox subunit of flavocytochrome b_{558} serves as a docking site for p47phox, and accessorily for p67phox, is based on a series of convergent observations. (i) A syn-

thetic peptide corresponding to residues 175–194 of p22phox prevents activation of NADPH oxidase and exhibits a propensity to bind to p47phox [99]. (ii) Another synthetic peptide corresponding to residues 149–162 of p22phox interferes with the binding of the p47phox SH3 domain to p22phox [233]. (iii) At the onset of the respiratory burst in intact neutrophils, phosphorylation of p47phox on serine residues in the C-terminal region is accompanied by translocation of the phosphorylated p47phox to the cytoplasmic region of p22phox [145, 146, 157, 158]. (iv) A glutathione S transferase (GTS) fusion protein containing the two SH3 domains of p47phox (residues 154–285) binds to the proline-rich motif of p22phox (residues 151–160) [182]. (v) The random-sequence peptide display technique indicated the presence of two potential sites of interaction with p47phox in the C-terminal region of p22phox, corresponding to the sequences ^{156}PPRPP and ^{177}GGPPGP [101]. (vi) The interaction between p47phox and the polyproline motif of p22phox was corroborated by mutagenesis experiments [182, 185, 233], and the N-terminal SH3 domain of p47phox was found to be essentially responsible for the p47phox-p22phox interaction [146]. (vii) The peptide-walking approach revealed that p47phox binds not only to the proline-rich region at the C terminus of p22phox, but also to a region located in the N-terminal half of p22phox encompassing residues 51–63, and that p67phox shares with p47phox the ability to interact with p22phox [234].
In keeping with the inventory of strategic sequences in p22phox, rabbit p22phox has been reported to exhibit a high degree of variability in two areas located at residues 58–90 and 168–198, suggesting that these areas do not play a significant role in the structure and function of p22phox [120].
Like p22phox, gp91phox contains binding sites for p47phox. An early report concluded that the synthetic peptide RGVHFIF, corresponding to residues 559–565 of gp91phox, inhibits oxidase activation in a cell-free system by preventing the access of p47phox to the C-terminal region of gp91phox [99]. The phage display technique confirmed the interactions of p47phox with residues 559–565 of gp91phox [101]. These conclusions were, however, questioned following the finding that mutations in residues 559–565 of gp91phox had no deleterious effect on the elicited oxidase activity of mutated granulocytes [235]. In contrast, mutation of gp91phox in X-CGD neutrophils due to the substitution Asp500 → Gly resulted in defective translocation of cytosolic phox proteins to the membrane in stimulated neutrophils, and the synthetic peptide corresponding to residues 491–504 of gp91phox that contains Asp500 blocked oxidase activation in a cell-free system [236]. Two other sites of interaction of gp91phox with p47phox have been identified by the phage display technique. They encompass residues ^{87}STRVRRQL and ^{451}FEWFADLL of gp91phox [101]. The positively charged doublet Arg91/Arg92 in the ^{87}STRVRRQL sequence is likely to have a strategic function in the respiratory burst. In fact, PLB985 cells (from a human myeloid leukemia cell line), in which Arg91/Arg92 in gp91phox had been replaced by two glutamic acids, were unable to mount a respiratory burst upon stimulation [237].
In an attempt to locate more precisely the sites of interaction of the cytosolic phox proteins in each of the two subunits of flavocytochrome b_{558}, B lymphocytes obtained from CGD patients were used for translocation studies [238]. In stimulated B lymphocytes from X-CGD patients expressing p22phox in the absence of gp91phox, p47phox, p67phox and p40phox did not translocate to the membrane, indicating that gp91phox is required for the binding of the cytosolic phox proteins to flavocytochrome b_{558}. With B lymphocytes from an X91$^+$ CGD patient in whom p22phox was normally expressed and gp91phox was present but lacked five amino acids, translocation of p47phox was not affected, but p40phox and p67phox were poorly translocated, indicating that p47phox associates with flavocytochrome b_{558} (at the level of p22phox), independently of p67phox and p40phox, and that gp91phox is involved in the binding of p67phox and p40phox. Another report [166] provided evidence that the activation domain of p67phox controls the translocation of this protein.
A minimal conclusion emerging from all these data is that oxidase activation in vivo proceeds through a series of transitory interactions between the cytosolic phox proteins and the membrane-bound flavocytochrome *b*. The precise sequence of these interactions has not yet been fully elucidated. In this respect, it should be noted that a number of conclusions bearing on kinase-dependent phosphorylations and on the effect of arachidonic acid come from in vitro assays, using, in particular, the cell-free system of oxidase activation. The physiological relevance of these conclusions should be carefully assessed.

Electron transfer across the redox carriers of flavocytochrome b_{558}

The redox components present in the gp91phox subunit of flavocytochrome b_{558} are characterized by unusually low redox potentials (fig. 5). In stimulated neutrophils, FAD reacts readily with NADPH, and a relatively high amount of a neutral semi-quinonic form of FAD is detected under steady-state conditions [3]. The presence of a topologically distinct hydrophilic domain in gp91phox, harboring NADPH- and FAD-binding sites (fig. 3) led to the hypothesis that this domain could be endowed with an intrinsic diaphorase activity. Electron transfer from NADPH via FAD to appropriate electron acceptors like p-iodonitrotetrazolium violet (INT) [239] or dichlorophe-

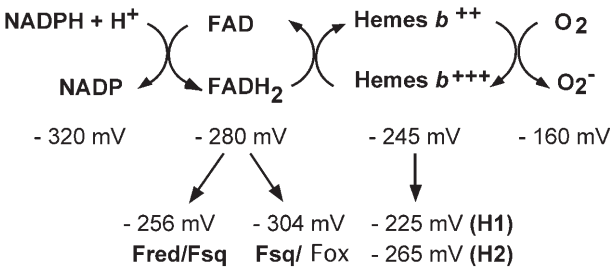

Figure 5. The redox carriers of the NADPH oxidase. The midpoint potentials of the redox carriers at pH 7 are indicated (for details see text). H1 and H2 designate the two hemes *b* that transport electrons from $FADH_2$ to O_2.

nol indophenol (DCPIP) in the presence of detergent [240] was in fact reported. Not only the O_2^--generating oxidase activity, but also the apparent diaphorase activity required activation to be fully elicitated [239, 240]. Supporting the idea of regulation at the FAD level was the report that stimulated neutrophils from a CGD ($X91^+$) patient with a point mutation (Arg54 → Ser) in gp91phox, although unable to reduce O_2 into O_2^- despite the presence of heme *b*, retained the capacity to reduce INT [241]. Another line of evidence was that the electron transfer from NADPH to FAD is dependent on p67phox, but not on p47phox, and that, in contrast, the electron transfer from reduced FAD to O_2 via the hemes is dependent on p47phox [239, 242]. These conclusions should be reassessed in the light of recent experiments showing that INT reacts preferentially with the heme *b* component of flavocytochrome *b*, and is not, as currently believed, a specific probe of diaphorase [243].

Early investigations on electron transfer in the O_2^--generating NADPH oxidase revealed some unexpected peculiarities of this enzyme. Under anaerobic conditions, the NADPH-dependent reduction of heme *b* in stimulated neutrophils was slow and only 50% complete after several minutes [244, 245]. Under aerobic conditions, although the extent of heme *b* reduction in an extract of stimulated neutrophils was less than 10%, the rate of heme reduction was fast and virtually equal to the rate of O_2^- production [246]. The conclusion was drawn that the heme component of the oxidase complex is competent as an electron carrier and that O_2 is required for rapid electron flow. More recent kinetic data confirmed that O_2 facilitates electron transfer between NADPH and FAD [247]. In another experiment, the large degree of reduction of FAD compared to that of heme *b* (38 vs 8%) in an extract of neutrophils supplemented with NADPH was explained by a kinetic barrier between FAD and heme *b* [248]. However, in a kinetically competent cell-free system consisting of purified and relipidated flavocytochrome b_{558}, supplemented with p47phox, p67phox and Rac1, the levels of reduction of FAD and heme *b* were virtually equal (about 10%) [97]. The kinetic barrier at the FAD level therefore appears to be overcome under appropriate conditions, and some of the unusual kinetic properties of flavocytochrome b_{558} are apparently linked to topological constraints.

A constraint of a different nature exists at the level of interaction between O_2 and heme *b*. The paradox that developed around the electron transfer from heme *b* to O_2 stems from the observation that the reduced heme *b* is rapidly reoxidized by O_2 [249], but is virtually unreactive to CO [245], in contrast to classical hemoproteins such as cytochrome oxidase and cytochrome P450 which combine with CO as well as with O_2. On the other hand, optical spectroscopic studies demonstrate that heme ligands such as butyl isocyanide, imidazole and pyridine [250] bind easily to flavocytochrome b_{558} solubilized in detergent. Both cytochrome oxidase and cytochrome P450 have five of the heme iron coordination sites occupied by intrinsic ligands while the sixth coordination site is opened for the binding of O_2 or other extrinsic ligands. In contrast, neutrophil flavocytochrome *b* apparently behaves in EPR as a low-spin hexacoordinated hemoprotein [251–253], and in flavocytochrome b_{558} electrons were hypothesized to be transferred from the heme to O_2 at, or near, the heme edge without formation of an heme iron-O_2 complex [254]. The elusive reactivity of the heme component of the neutrophil flavocytochrome *b* was finally explained when arachidonic acid, an activator of NADPH oxidase, was found to promote the transition of the heme iron from a hexacoordinated form to a pentacoordinated form capable of reacting with O_2 or another extrinsic ligand like butyl isocyanide [255]. Upon addition of arachidonic acid, a blue shift of the Soret peak of 2.5 nm was observed in low-temperature spectra, consistent with the emergence of a high-spin pentacoordinated configuration of the heme iron. The subsequent addition of butyl isocyanide resulted in a red shift of the Soret peak from 425 to 434 nm, indicating the binding of this ligand to the pentacoordinated heme iron. A similar red shift was observed when butyl isocyanide was added to stimulated neutrophils. Thus, the reversible transition of the coordination state of the heme iron of flavocytochrome b_{558} demonstrated in vitro also occurs in vivo in stimulated neutrophils [255]. Arachidonic acid induces typical concentration-dependent modifications of the kinetics of the elicited NADPH oxidase activity in cell-free system [256]. At concentrations lower than 0.6 µmol/mg protein, arachidonic acid increases the affinity of flavocytochrome b_{558} for O_2; at higher concentrations, it promotes the interaction between the cytosolic phox proteins and the membrane-bound flavocytochrome *b*, and the result is an increase in the catalytic efficiency of flavocytochrome b_{558} in the production of O_2^-.

Since non-biological amphiphiles, like sodium dodecyl sulfate, mimic the effect of arachidonic acid, albeit with a lower efficiency, the physiological relevance of arachi-

donic acid as an activator of the respiratory burst has occasionally been questioned. The following data favor the view that arachidonic acid is a physiological activator of the respiratory burst, which acts primarily at the level of the membrane-bound flavocytochrome *b*. (i) In stimulated neutrophils, arachidonic acid is released from phospholipids by activated cPLA2 [257]. (ii) Human myeloid neutrophils deficient in cPLA2, which are unable to generate arachidonic acid, fail to activate the NADPH oxidase in response to soluble and particulate stimuli; addition of arachidonic acid to cPLA2-deficient neutrophils restores the production of O_2^- [258–260]. (iii) The S100A8/A9 protein (MRP8/MRP14), present in large amounts in neutrophils [261], has the potential to bind arachidonic acid selectively, in large amounts and in a saturable and reversible manner [262–264]. It is also a positive regulator of NADPH oxidase activation in stimulated neutrophils [265].

Whereas NADPH oxidase activation is promoted by arachidonic acid, it is inhibited by phenylarsine oxide (PAO), a trivalent arsenic derivative that forms ring complexes with vicinal or neighboring thiols [266, 267]. Interestingly, PAO exerts opposite effects to those of arachidonic acid [268]. Through the use of a photolabeled derivative, the PAO target was shown to be located within gp91phox [268]. Two binding sites for PAO have been postulated. One corresponds to the vicinal Cys85 and Cys86, close to the sequence ^{87}STRVRRQL known to interact with p47phox [101]; the other is localized at the level of the proximal Cys369 and 371, close to the FAD- and NADPH-binding sites of gp91phox. These cysteine residues are conserved in gp91phox from different species [82]. Whether or not they participate in oxidase activation by reacting with O_2 and by cycling between reduced (thiols) and oxidized (disulfide) forms remains to be determined.

Two oxidase inhibitors, diphenylene iodonium (DPI) and iodonium biphenyl (IBP) [240, 269–273] are currently used in the study of electron transfer in flavocytochrome b_{558}. These inhibitors were first characterized as arylating agents reacting with nucleophiles to form covalent adducts. From the demonstration of an adduct of DPI with reduced FAD, inhibition of the neutrophil NADPH oxidase by DPI was inferred to result from the binding of DPI to the FAD prosthetic group of gp91phox [271]. However, monitoring the IBP- and DPI-induced modifications of the optical and EPR spectra of neutrophil membranes revealed that the IBP- and DPI-dependent inhibition of oxidase activity was directly correlated with the spectral modifications of heme *b* [273]. Clearly, the binding of arylazonium derivatives to both the FAD and heme *b* redox centers of gp91phox contributes to inhibition of the NADPH oxidase.

Based on the established molar ratio of FAD to heme *b* of 1:2 in flavocytochrome b_{558} and on the observation that

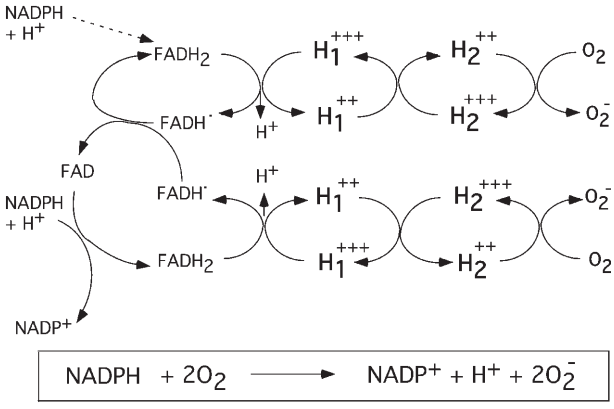

Figure 6. A possible mechanism for electron transfer in flavocytochrome b_{558}. Under steady-state conditions, only one molecule of NADPH is required to deliver electrons to two molecules of O_2 through the redox centers of gp91phox organized as a dimer. For details, see text.

low-potential flavoproteins cycle as one-electron carriers between the semi-quinone and hydroquinone forms [for reviews, see refs 274, 275], a tentative model for electron transfer in flavocytochrome b_{558} is illustrated in figure 6. The theoretical premises are as follows: (i) Flavocytochrome b_{558} is supposed to be organized as a dimer. (ii) Two pairs of hemes *b* (H_1 and H_2) per dimer are reduced by one pair of FAD cycling between the semiquinone $FADH^\bullet$ and the fully reduced form $FADH_2$. (iii) Two semi-quinones $FADH^\bullet$ generate by disproportionation one FAD and one $FADH_2$. The newly generated FAD is, in turn, fully reduced to $FADH_2$ by one NADPH + H^+. (iv) The two generated $FADH_2$ are reintroduced in a new cycle. (v) To initiate the electron cycling, two NADPH are needed. However, under steady state conditions, only one NADPH is necessary to supply the oxidase with electrons, in agreement with the stoichiometry of the oxidase reaction: NADPH + 2 O_2 → $NADP^+$ + H^+ + 2 O_2^-. The model in figure 6 implies that, under steady-state conditions, the NADPH-binding site is transitorily masked and non-functional in one of the two gp91phox chains. Masking and unmasking of the NADPH-binding site in each of the two gp91phox chains might alternate, possibly under the control of the cytosolic phox proteins.

Electrogenicity of the O_2^--generating NADPH oxidase and associated proton conductance

Generation of O_2^- by activated NADPH oxidase as a result of the transmembrane electron transfer of cytosolic NADPH either to external O_2 or to O_2 present in the medium of the phagosomal vacuole is accompanied by rapid depolarization of the membrane [276; for a review, see ref. 67]. Through the use of a non-invasive and accurate measurement procedure, a depolarization as high as

100 mV has been determined [277]. This large variation in membrane potential at the onset of the respiratory burst is likely to influence the redox potential of the two hemes of flavocytochrome b_{558}, and consequently the kinetics of electron transfer within the flavocytochrome. Definite evidence for the generation of electron currents across the plasma membrane of phagocytic cells, specific to the NADPH oxidase activity, was recently obtained by applying the patch clamp technique to eosinophil cells [278]. All these data support the view that the O_2^--generating NADPH oxidase is electrogenic.

The electrogenic action of NADPH oxidase in phagocytic cells is rapidly compensated by a proton efflux inhibitable by Cd^{2+} and Zn^{2+} [276] and by diethylpyrocarbonate (DEPC), a histidine-modifying agent [279]. It is activated by arachidonate [280, 281]. The tight coupling of the oxidase activity and the proton conductance is artificially overcome by the provision of an alternative charge compensation, brought about, for example, by addition of K^+ and valinomycin, an ionophorous antibiotic that specifically carries K^+ across phospholipid bilayers [for a review, see ref. 67].

In the mid-1990s, while the existence of a proton movement associated with the respiratory burst was well recognized, controversy started to develop about the identity of the protein component acting as a proton channel. The belief that gp91phox is a proton channel was supported by a number of experiments. (i) The expression of H^+ channel activity increases in parallel with that of the stimulatable generation of O_2^- during differentiation of HL60 cells. Moreover, undifferentiated HL60 cells transfected with gp91phox cDNA express an H^+ channel activity correlating with the expression of gp91phox [282]. (ii) Transfection of Chinese hamster ovary (CHO) cells with a 230-amino-acid N-terminal fragment of gp91phox containing the sites of ligation of hemes *b* rendered the cells competent to exhibit an arachidonate-activable proton flux in response to an imposed proton motive force [283]. (iii) Proton conductance was no longer observed in CHO cells transfected with gp91phox bearing point mutations on the histidine residues 111, 115 and 119, thought to be heme iron ligands [284, 285]. In the past, some doubts about gp91phox being a necessary component of the H^+ conductive pathway associated with the respiratory burst have been expressed, based on measurement of H^+ conductance in normal and CGD phagocytes [286]. These doubts were recently reiterated and supported by experimental data obtained with the human myelocytic cell line, PLB-985, in which gp91phox was knocked out (KO) by gene targeting [287]. In unstimulated PLB-KO cells, H^+ currents had amplitude and gating kinetics similar to those of wild-type cells. Cell stimulation increased H^+ currents to a similar extent in X-CGD, PLB-KO and wild-type PLB cells. Thus, whereas gp91phox appears to function as an H^+ channel when expressed in non-phagocytic cells, it may not contribute significantly to the total H^+ conductance in phagocytic cells.

Regulation of flavocytochrome b_{558} synthesis

The following components of the O_2^--generating NADPH oxidase, gp91phox, p47phox, p67phox and p40phox, are synthesized in the lymphoid and myeloid lineages, including neutrophils, eosinophils, monocytes, macrophages and B lymphocytes. Rac and p22phox are more broadly distributed. In contrast to gp91phox, p67phox and p47phox are not expressed in myeloid precursor cells, but are induced during differentiation and maturation processes [288]. p40phox is present in myeloid precursor cells, but its expression is increased during granulocyte differentiation [171]. Taken together, these facts suggest coordinated regulation of the expression of the genes encoding the NADPH oxidase components. Unexpectedly, hybridization analysis of mice tissues has revealed that p40phox mRNA is expressed in brain neurons located in the hippocampus and the cerebellum [289]. Thus, in addition to its participation in the NADPH oxidase complex, p40phox is likely to have other functions.

Hematopoietic development is controlled by specific transcription factors. A few years ago, an unusual form of X-linked CGD was identified in two kindreds, with 5–10% of neutrophils expressing normal levels of flavocytochrome b_{558} and normal oxidase activity [290]. A genetic defect in gp91phox gene regulation was suspected as the cause of this form of CGD. Genomic sequencing demonstrated two neighboring mutations in the upstream promoter region of the gp91phox gene in these kindreds. The consequence of these mutations was impaired transcription of the gp91phox gene. Among the transcription factors that regulate the expression of the phox genes, PU.1, which is synthesized exclusively in hematopoietic cells, has been particularly well studied. PU.1 was initially characterized as a regulatory factor allowing the growth of an SV4 variant in lymphoid cells, and binds to a purine-rich sequence (5′GAGGAA3′) [291, 292]. PU.1 binds to a sequence located in the proximal region (bp –57 to –50) of the gp91 phox promoter. Its implication in the expression of gp91phox [293] is illustrated by the finding that, in a patient with X-linked CGD, a single base mutation at position –53 of the promoter of the CYBB gene inhibited the binding of PU.1 [294]. The PU.1-dependent positive regulation of gp91phox expression appears to implicate an array of interactions of PU.1 with a number of other factors, including the interferon-regulating factor (IRF), the interferon-consensus-sequence-binding protein (ICSBP), the CREB-binding protein (CBP) and hematopoietic factor 1 (HAF-I) [293, 295]. Another positive regulator of gp91phox expression

is the transcription factor CP1, which binds to the CCAAT box motif in the promoter region. This interaction is prevented by the CCAAT displacement protein (CDP) [296–298]. The DNA-binding activity of CDP is down-regulated during phagocyte differentiation, coincident with induction of gp91phox transcription. PU.1 also controls the expression of the cytosolic phox proteins. However, whereas the transcription of p47phox and p40phox requires essentially PU.1 [299], that of p67phox is under the control of additional factors, including AP-1, Sp1 and HAF1 [300].

Maturation of the protein core of gp91phox (58 kDa) to form the highly glycosylated gp91phox protein (90–100 kDa) proceeds first in the endoplasmic reticulum and then in the Golgi apparatus. A putative, mannosylated precursor of 65 kDa (p65) was identified some years ago in B lymphocytes from a CGD patient deficient in p22phox by immunodetection with anti-gp91phox antibodies [301, 302]. Introduction of a p22phox transgene into the p22phox-deficient cells resulted in generation of a fully processed flavocytochrome *b* containing both gp91phox and p22phox [301]. Incorporation of heme *b* is a decisive step in the assembly of the subunits of flavocytochrome b_{558}, as illustrated with PLB-985 myeloid cells in which heme synthesis was prevented by addition of succinyl acetone to the cell culture [303, 304]. Succinyl acetone decreased the expression of both p22phox and the mature 91-kDa form of gp91phox, but not that of the putative precursor of 65 kDa (p65). Mutagenesis experiments [116] further highlight the role of heme ligation in the processing of pg91phox. When the His residues 101, 115, 209 and 222, that serve as ligands for the two hemes in gp91phox, are replaced by Leu or Arg, p65 is no longer processed, the heterodimer p65phox/p22phox is not formed and the cells lack flavocytochrome b_{558}.

Homologues of the gp91phox subunit of flavocytochrome b_{558}

In 1999, a human expressed sequence tag (EST) that showed homology to human gp91phox was identified, and complete sequencing revealed a predicted amino acid sequence homologous to the carboxyl-terminal half of gp91phox [305]. Sequencing was completed by rapid amplification of complementary DNA ends, using human colon cDNA. The predicted protein was 564 amino acids long, and its calculated mass was about 65 kDa. It showed 56% identity to human gp91phox, and, like gp91phox, it contained binding sites for FAD and NADPH. The hydropathy profile resembled that of gp91phox, and five or six hydrophobic stretches could be identified in the N-terminal half of the molecule. The conserved histidine residues in this region were considered candidates for heme ligation. The protein lacked the asparagine-linked glycosylation sites typical of the highly glycosylated human gp91phox. The coding gene was located at Xq22.2, not far from the locus of the CYBB gene encoding gp91phox located at Xp21.1. Tissue screening showed high expression of the protein in human colon epithelial cells, in human colon carcinoma cells Caco2, as well as in cultures of rat aortic vascular smooth-muscle cells proliferating in the presence of platelet-derived growth factor (PGDF). Because of its suspected relationship with cell division, this p91phox homologue was first termed Mox-1 (for mitogenic oxidase) [305]. NIH 3T3 cells transfected with Mox-1 DNA were able to generate O_2^- at a rate of 0.3–0.6 nmol/min per 10^6 cells, i.e. about ten times less than the rate of O_2^- production in activated neutrophils. Mox-1-dependent production of O_2^- was not enhanced by addition of p47phox, p67phox or Rac in a cell-free system. When overexpressed in fibroblasts, Mox-1 induced malignant transformations, rendering these cells highly tumorigenic following injection to athymic mice [306]. The induced tumors were highly vascularized, indicating that Mox-1 triggers the angiogenic switch typical of tumorigenesis [307]. Coexpression of catalase with Mox-1 reversed the phenotype. Thus H_2O_2, generated by dismutation of O_2^-, is a critical product of the activity of Mox-1. Interestingly, the aconitase activity of 3T3 cells transfected with Mox-1 was significantly altered. Since aconitase contains an iron-sulfur cluster highly sensitive to O_2^- and O_2^- derivatives, similarly sensitive proteins were inferred to be affected by the Mox-1 activity [306].

The same gp91phox homologue was identified at about the same time by another group and named gp91-2 [308]. The term NOH-1 was also used for the same molecule [309]. With the discovery of other homologues of gp91phox, unified terminology had to be adopted. The proposal was made that the 65-kDa homologues of gp91phox should be designated by the common name Nox (for *N*ADPH *ox*idase), and that Mox-1, gp91-2 and NOH-1 should be called Nox-1 [59, 60, 310].

The Nox proteins all show five or six conserved predicted transmembrane α helices with putative heme-binding His residues and FAD and NADPH-binding sites. Apart from their potential ability to transport electrons, the Nox proteins function as H^+ channels. Proton conduction might even be the only function of a short form of Nox1 (Nox1S) derived from the long form Nox1L by alternative splicing [309]. Nox1S contains the membrane domain of Nox1, but is devoid of binding sites for NADPH and FAD. The Nox proteins have a relatively specific tissue distribution. Nox1 is predominantly expressed in colon [305, 308]. Nox2 which designates the classical gp91phox is present in phagocytic cells. Nox3 was cloned from fetal kidney [310], Nox4 also called Renox was initially found in kidney cortex [311, 312], and later in osteoclasts [313]. In HeLa cells transfected with Nox4 cDNA, immunodetection revealed in the membrane frac-

tion a protein band of 75 kDa in addition to the main protein band of 66 kDa [312]. The 75-kDa protein could be a glycosylated form of Nox4. Relatively distinct from the other Nox proteins, Nox5 is expressed in a variety of fetal tissues and in adult spleen and uterus. As a peculiarity, Nox5 possesses in its N-terminal region three EF-hand motifs and a proline-arginine-rich domain [314]. Despite its evolutionary distance, functionally Nox5 resembles the neutrophil gp91phox more closely than the other Nox proteins. In fact, Nox5 is a second-messenger-activated enzyme, while Nox1 and Nox3 are constitutively active. Furthermore, heterogeneously expressed Nox5 generates larger amounts of O_2^- than Nox1 and Nox4. However, whereas the elicited oxidase activity of neutrophil gp91phox needs the assembly of several other subunits, in Nox5, the catalytic and regulatory modules are combined within one molecule.

The Duox proteins are a well-individualized group of gp91phox homologues, whose molecular mass (175–180 kDa) is three times as high as that of the Nox proteins. They possess an N-terminal domain homologous to peroxidases with several Ca^{2+}-binding sites, hence their name of dual oxidases or Duox. They comprise Duox1 (also termed Thox1) present in the thyroid gland [315, 316] and Duox2 (also termed Thox2), present not only in the thyroid, but also in the small intestine and colon [316, 317]. The corresponding genes of Thox1 and Thox2 are closely linked on chromosome 15q15.3 [316]. Thox1 and Thox2 display 83% sequence similarity and are clearly related to gp91phox (53 and 47% similarity, respectively). A protein referred as $p138^{Tox}$ [315] appears to correspond to the carboxyl fragment of Thox2 [316]. The Thox proteins are mostly present in the cytoplasm, while a fraction are found at the cell surface in thyrocytes [318]. As shown by a survey of genome data, the Nox and Duox proteins are widely distributed in nature. Nox orthologues have been identified in *Drosophila* and in the simple eukaryote *Dictyostelium discoideum* [60]. A protein related to Duox1 has been found in *Caenorhabditis elegans* [319]. A schematic representation of topology models for the Nox and Duox proteins is illustrated in figure 7. Except for the truncated form of Nox1 (Nox1-S), these homologues share a C-terminal domain that contains NADPH- and FAD-binding sites and a transmembrane domain comprised of six hydrophobic segments that are predicted to be α helices. Their differences resides in the N-terminal region.

Although the primary structures of the Nox and Duox proteins are well delineated, their functions remain speculative. Different scenarios have, however, been proposed [60]. Nox1 and Nox4, present in colon and kidney tubular cells, respectively, might serve in host defense against microbial infections, although their O_2^--generating oxidase activity is relatively modest. The Nox proteins might also participate in signal transduction, related to mitogenic regulation. H_2O_2 generated by dismutation of O_2^- could serve as a control signal for redox-regulated enzyme activities. Because both Nox4 and erythropoietin are expressed in kidney tubules, Nox4 has been proposed to regulate the synthesis of erythropoietin indirectly by control of oxygen sensing at the level of the heterodimeric transcription factor HIF1 [311]. Under hypoxic conditions, an elevated concentration of HIF1 favors the synthesis of erythropoietin. On the other hand, under normoxic conditions, the concentration of HIF1 decreases due to oxidation of one of its subunits which becomes sensitive to proteolysis. A metabolic role for the Nox proteins has also been envisaged. This possibility was based on the role of ROS in bone remodeling, a mechanism controlled by Nox4 [313] and also on the observation that the suppression of Duox in *C. elegans* leads to defects in cuticle proteins that form the outer covering of the nematode, possibly by lack of Duox-generated tyrosine cross-links [319].

An evolutionary tree, taking into account the amino acid sequences of Nox and Duox proteins, has been constructed [60]. It shows that Nox5 is highly divergent from other members of the family, and probably the closest to the primordial Nox. Apart from Nox5, two subgroupings have been postulated in relation to the sequence data [60]. One of them assembles Nox1, Nox2 (gp91phox), Nox3 and Nox4, all these proteins being characterized by similar molecular masses (~65 kDa). The other subgrouping assembles the Duox proteins (~180 kDa) that are characterized by an extended N-terminal sequence harboring a peroxidase domain and an EF-hand Ca^{2+}-binding domain. Precise information on the functions of the Nox proteins clearly needs the development of cell lines or animals with targeted deletions or disruption of the corresponding genes.

As mentioned in this review, plants possess an NADPH oxidase system similar to that of neutrophils, with cytosolic proteins immunologically related to p47phox and p67phox [56] and homologues of the membrane-bound gp91phox. Specifically, in the case of *Arabidopsis thaliana*, a gene called RBOHA (for *R*espiratory *B*urst *O*xidase *H*omology A) encodes a protein of 108 kDa [57]. The C-terminal region of this protein is highly similar to the human neutrophil gp91phox. The RbohA protein has a large hydrophilic N-terminal domain, not present in human gp91phox. This domain contains two Ca^{2+}-binding EF-hand motifs, and has an extended similarity to the human Ran-GTPase activating protein (fig. 7). The rice gp91phox homologue is shorter than that of *Arabidopsis* (~69 kDa) and it lacks the Ran-GTPase-activating domain [57]. A subsequent study revealed the presence in *A. thaliana* of five other genes encoding homologues of the human gp91phox protein [320]. These homologues are of approximately the same size (between 897 and 948 amino acids), whereas gp91phox is 570 amino acids in length.

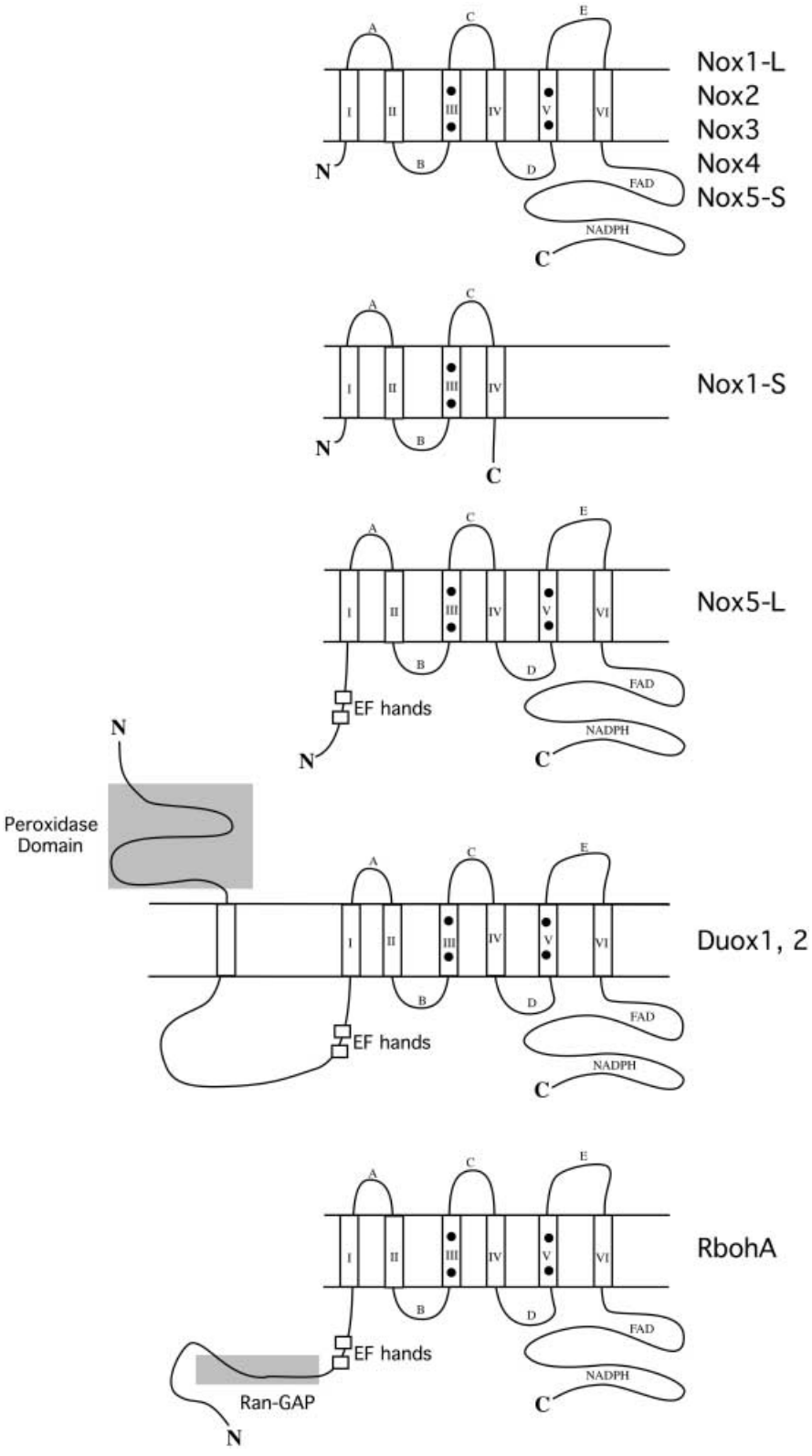

Figure 7. Models of transmembrane topology of gp91phox homologues. The numbers I–VI denote putative membrane-spanning α helices. Closed circles indicate histidine residues in helices III and V to which two pairs of heme *b* are attached (except for Nox1-S). In all the gp91phox homologues shown in the figure (except Nox1-S), the C-terminal region contains the NADPH- and FAD-binding sites. An additional transmembrane α helix is present in the Duox proteins followed by an extended N-terminal domain homologous to peroxidase. EF motifs in Nox5-L, Duox and RbohA are indicated, as well as a Ran-GTPase-activating domain (GAP) in RbohA.

Apart from their N-terminal extension, they are 60% similar to gp91phox. They contain six potential transmembrane hydrophobic α helices with four conserved histidine residues postulated to bind two hemes. They all carry a large N-terminal extension with Ca^{2+}-binding motifs. The apparent conservation of the membrane topology between the Rboh proteins in plant cells and gp91phox suggests that the products of the NADPH oxidase reaction accumulate on the external face of the plasma membrane [321], in keeping with the function of ROS in wall cross-linking and cellular signaling [for a review, see ref. 58].

Concluding remarks

In phagocytic cells, the O_2^--generating NADPH oxidase is now currently generally acknowledged to be an enzyme complex comprising a membrane-bound redox protein, flavocytochrome b_{558}, which is the catalytic core of the oxidase, and associated proteins of cytosolic origin, namely the small G protein Rac, p47phox and p67phox. Upon binding to flavocytochrome b_{558}, these cytosolic proteins activate the production of O_2^-. For this reason, they are called cytosolic factors of oxidase activation. An additional cytosolic protein, p40phox, which copurifies and coimmunoprecipitates with p47phox and p67phox, is not essential to activate the membrane-bound flavocytochrome b_{558} in a cell-free system, but it might, under appropriate conditions in a cell-free system, modulate the activation of the NADPH oxidase.

During the last decade, research on the phagocyte oxidase complex has developed along three main limes, namely structural characterization of the components of the oxidase complex, the mechanism of oxidase activation, and, more recently, identification and characterization of a number of homologues of flavocytochrome b_{558} in non-phagocytic cells. Flavocytochrome b_{558} is now definitely established to be a heterodimer, consisting of a large glycoprotein gp91phox and a small protein p22phox associated at a molar ratio of 1:1. The large subunit gp91phox is believed to contain the redox carriers, one FAD and two hemes *b*, needed for the transfer of electrons from NADPH to O_2. The small subunit p22phox is considered as a docking protein for the cytosolic phox proteins, more particularly for p47phox. Despite the absence of crystallographic data, a low-resolution view of the tertiary structure of flavocytochrome b_{558} is now emerging, resulting from different approaches, including hydropathy analysis of the amino acid sequence, immunochemical reactivity of accessible regions, eventually combined with the phage display approach and search for structural homology to related proteins in combination with computer modeling. Furthermore, valuable insights into the organization of the cytosolic phox protein complex have been obtained using the small-angle neutron-scattering technique, and crystal structures of Rac and p67phox have been reported. A number of potential protein-protein interactions between the components of the NADPH oxidase complex have been identified, using binding assays, the double-hybrid technique and the mutagenesis approach. NADPH oxidase activation appears to be triggered by two regulatory pathways: (i) the phosphorylation of the cytosolic phox proteins and their translocation with Rac to the membrane-bound flavocytochrome b_{558} and (ii) the arachidonate-dependent transition of the hexacoordinated form of the heme component in gp91phox to a pentacoordinated form capable of reacting with O_2. The binding of Rac and the cytosolic phox proteins to flavocytochrome b_{558} presumably results in a modification of the conformation of the gp91phox subunit of the flavocytochrome, favoring the efficiency of electron transfer from NADPH to heme *b*. In this process, p67phox and Rac have a pre-eminent function, the role of p47phox being to facilitate the binding of p67phox and Rac to flavocytochrome *b*. The hierarchical mode of phosphorylation of serine residues clustered in the C-terminal region of p47phox during the course of NADPH oxidase activation provides evidence for the multistep mode of regulation of the NADPH oxidase. Whereas the main events of NADPH oxidase activation are partially unveiled, the fine regulation including the priming and termination of the respiratory burst remains virtually unknown. The electrogenic nature of the NADPH oxidase has been ascertained; however, the location and the nature of the H^+ channel associated with the oxidase activity remain unsolved. In particular, the functioning of gp91phox as a proton channel remains a matter of debate.

Cell-free systems of oxidase activation have been intensively used to study oxidase activation. They have been particularly useful for identifying the essential components of the oxidase complex and characterizing their interacting domains. However, oxidase activation in a cell-free system clearly takes place in a non-physiological environment and extrapolation of the conclusions to whole phagocytic cells may be misleading. The present trend is to develop whole-cell systems consisting of non-hematopoietic cells that can be transfected with cDNAs encoding the components of the NADPH oxidase. Along this line, a recent report describes the successful transfection of COS-7 cells with all the components of the oxidase complex [322]. Upon stimulation with phorbol ester or arachidonic acid, the transfected COS cells generated O_2^- at a rate similar to that measured in stimulated neutrophils. Pursuing this investigation, the authors showed that the transient expression of constitutively active Rac1 derivatives (dominant positive) was sufficient to induce O_2^--generating NADPH oxidase activity in Cos7

cells in which human cDNAs for essential components, gp91phox, p22phox, p47phox and p67phox, were expressed as stable transgenes [323].

Evidence is increasing for the existence of a complex network of interactions in intact cells between the cytosolic phox proteins and a few molecular entities related to the cytoskeleton including coronin [324], actin [325], moesin [326], and the Ca^{2+}-binding protein S100A8/A9 [327]. Coronin binds p40phox and p67phox [324], actin binds p47phox [325], moesin binds the Px domain of p47phox and p40phox [326], and the S100A8/A protein binds Rac and p67phox [327]. The cytoskeleton proteins are not required for elicitation of the production of O_2^- by the NADPH oxidase in a cell-free system; however, in vivo these proteins may exert some control of the kinetics of the respiratory burst, concomitant with particle internalization. Unexpectedly, in B lymphocytes, the cofactor of the DNA protein kinase Ku70 was found to interact with p40phox [328]. Ku70 and the triad p40phox, p47phox and p67phox were also found to be present in both the cytoplasm and the nucleus of B cells; moreover, p47phox and p67phox are phosphorylated in vitro by the DNA protein kinase [328]. The recently discovered overphosphorylation of p40phox that occurs under conditions of oxidative stress [178] might influence the affinity of p40phox for Ku70 and modulate the propensity of the cytosolic phox protein triad to migrate to the nucleus of B cells. Taken together, these data suggest novel functions of p40phox and the associated cytosolic phox proteins in the immune system response.

In the late 1990s, a number of homologues of gp91phox were discovered in non-phagocytic cells and cloned. These homologues generate low amounts of ROS, insufficient to produce extensive oxidative damage to cells, but sufficient to trigger the activity of key redox-sensitive proteins. They are referred to as Nox and Duox proteins. They probably play crucial functions in the cell economy, although their precise involvement is still speculative. Much remains to be learned about these enigmatic proteins.

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