REVIEW ARTICLE

Activation and assembly of the NADPH oxidase: a structural perspective

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The NADPH oxidase of professional phagocytes is a crucial component of the innate immune response due to its fundamental role in the production of reactive oxygen species that act as powerful microbicidal agents. The activity of this multi-protein enzyme is dependent on the regulated assembly of the six enzyme subunits at the membrane where oxygen is reduced to superoxide anions. In the resting state, four of the enzyme subunits are maintained in the cytosol, either through auto-inhibitory interactions or through complex formation with accessory proteins that are not part of the active enzyme complex. Multiple inputs are required to disrupt these inhibitory interactions and allow translocation to the membrane and association with the integral membrane components. Protein interaction modules are key regulators of NADPH oxidase assembly, and the protein–protein interactions mediated via these domains have been the target of numerous studies. Many models have been put forward to describe the intricate network of reversible protein interactions that regulate the activity of this enzyme, but an all-encompassing model has so far been elusive. An important step towards an understanding of the molecular basis of NADPH oxidase assembly and activity has been the recent solution of the three-dimensional structures of some of the oxidase components. We will discuss these structures in the present review and attempt to reconcile some of the conflicting models on the basis of the structural information available.

Key words: NADPH oxidase, oxidase assembly, phosphorylation, protein–protein interaction, reactive oxygen species.

INTRODUCTION

The production of superoxide anions (O_2^-) by neutrophils and other phagocytes is an important step in our body's innate immune response. O_2^- is the precursor of a range of chemicals generally referred to as ROS (reactive oxygen species). These act as microbicidal agents and kill invading micro-organisms either directly or through the activation of proteases [1–5]. O_2^- is produced by the NADPH oxidase, a multi-protein enzyme complex, which is inactive in resting phagocytes, but becomes activated after interaction of the phagocyte with pathogens and their subsequent engulfment in the phagosome [1,3,6]. Defects in the function of the NADPH oxidase result in a severe immunodeficiency, and individuals suffering from CGD (chronic granulomatous disease), a rare genetic disorder that is caused by mutations in NADPH oxidase genes, are highly susceptible to frequent and often life-threatening infections by bacteria and fungi [5,7–9]. The microbicidal activity of ROS has generally been seen as the only beneficial function of these chemicals, and uncontrolled production of ROS has been implicated in tissue destruction and a number of disease states [10,11]. However, over the last couple of years, it has become apparent that ROS produced by NADPH oxidase homologues in non-phagocytic cells also play an important role in the regulation of signal transduction, often via modulation of kinase and phosphatase activities or through gene transcription [12–14]. These NADPH oxidase homologues are referred to as Nox enzymes ($gp91^{phox}$ is specified as Nox2; where phox is phagocytic oxidase), and several members of this novel protein family have been identified so far (reviewed in [15–17]).

In the present review, we will focus on the phagocytic NADPH oxidase, with an emphasis on the molecular mechanisms that regulate the assembly process of this heterohexameric enzyme. In particular, we will describe the recent progress that has been made towards a structural description of NADPH oxidase function, and discuss the novel insights that have been gained through these structures.

THE PLAYERS: NADPH OXIDASE SUBUNITS

The NADPH oxidase consists of six hetero-subunits, which associate in a stimulus-dependent manner to form the active enzyme complex and produce O_2^-. This activity has to be spatially and temporally restricted to the closed phagosome in order to prevent destruction of host tissue in what has been previously described as 'collateral damage' [3]. Tight regulation of enzymatic activity is achieved by two mechanisms: separation of the oxidase subunits into different subcellular locations during the resting state (cytosolic and membrane-bound) and modulation of reversible protein–protein and protein–lipid interactions. These can either enforce the resting state or allow translocation to the membrane in response to appropriate stimuli. Two NADPH oxidase subunits, $gp91^{phox}$ and $p22^{phox}$, are integral membrane proteins. They form a heterodimeric flavocytochrome b_{558} ('cyt b_{558}') that constitutes the catalytic core of the enzyme, but exists in a dormant state in the absence of the other subunits. These play mostly regulatory roles, and are located in the cytosol during the resting state. They include the multidomain proteins $p67^{phox}$, $p47^{phox}$ and $p40^{phox}$, as well as the small GTPase Rac, which is a member of the Rho family of small GTPases.

Flavocytochrome b_{558}

The $gp91^{phox}$ subunit (also called the β-subunit of the cytochrome) consists of 570 amino acids and has a molecular mass of 65.3 kDa,

Abbreviations used: AC, acidic cluster; BC, basic cluster; CGD, chronic granulomatous disease; GAP, GTPase-activating protein; GDI, GDP-dissociation inhibitor; GEF, guanine-nucleotide-exchange factor; GST, glutathione S-transferase; ITC, isothermal titration calorimetry; MAPK, mitogen-activated protein kinase; PB1, Phox and Bem1; PC, phox and Cdc24; phox, phagocytic oxidase; PPII helix, polyproline type II helix; PX, Phox homology; PRR, proline-rich region; RMS, root mean square; ROS, reactive oxygen species; SH3, Src homology 3; SPR, surface plasmon resonance; TPR, tetratricopeptide repeat.

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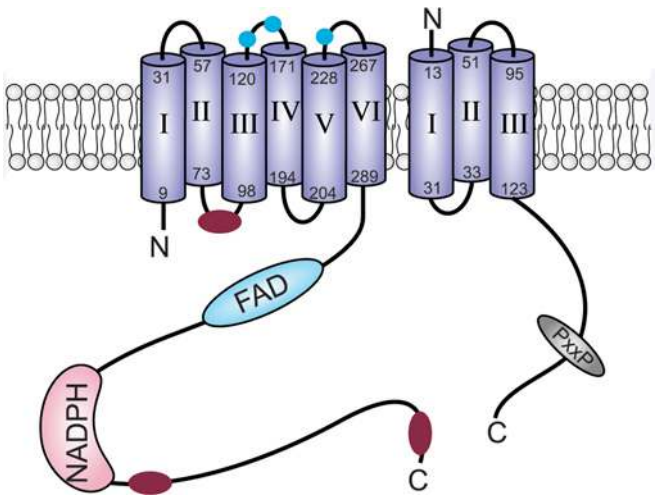

Figure 1 Model of the cytochrome b_{558}

The predicted transmembrane helices of gp91^{phox} and p22^{phox} are indicated. Glycosylation sites are indicated by cyan dots and regions that are believed to interact with p47^{phox} in the active state in red. The FAD- and NADPH-binding sites in gp91^{phox} are shown in cyan and pink respectively. The position of the consensus PxxP motif in the cytoplasmic region of p22^{phox} that interacts with p47^{phox} is indicated in grey.

but runs as a broad smear of approx. 91 kDa on SDS/polyacrylamide gels due to a heterogeneous glycosylation pattern of three asparagine residues (Asn^{132}, Asn^{149} and Asn^{240}) [18–20]. The N-terminal 300 amino acids are predicted to form six transmembrane α-helices, while the C-terminal cytoplasmic domain contains the binding sites for FAD and NADPH (Figure 1), shown experimentally through cross-linking studies and the observation that relipidated flavocytochrome alone can generate O_2^- [21–27]. In addition, gp91^{phox} is responsible for complexing the two non-identical haem groups of the NADPH oxidase via two histidine pairs [28–30]. Hence gp91^{phox} contains all co-factors required for the electron transfer reaction which occurs in two steps. First, electrons are transferred from NADPH on to FAD and then to the haem group in the second step to reduce O_2 to O_2^- in a one-electron-transfer reaction [31–34]. At present, no information is available on the three-dimensional structure of gp91^{phox} or fragments thereof, although a model for the structure of the cytoplasmic domain of gp91^{phox} has been suggested based on sequence homology with the FNR (ferredoxin–NADP reductase) family [35]. Significant insight into the topology of the cytochrome and the sites of interaction with other oxidase components has been gained through the use of a number of techniques, including epitope mapping or random sequence peptide phage analysis (Figure 1) [36–43]. Additionally, the study of cytochrome isolated from patients with X-linked CGD has contributed to our current understanding of its function [9,44–46].

p22^{phox} (also called the α-subunit) contains 195 amino acids and has a molecular mass of 21.0 kDa. It associates with gp91^{phox} in a 1:1 complex, and contributes to its maturation and stabilization [47–50]. Its N-terminal portion is predicted to contain three transmembrane α-helices, while the C-terminal cytoplasmic portion appears to be devoid of any secondary structure and its only recognizable motif is a PRR (proline-rich region) that contains a consensus PxxP (Pro-Xaa-Xaa-Pro) motif around Pro^{156} (Figure 1). This motif is known to be a target of the SH3 (Src homology 3) domains of p47^{phox} and Pro^{156} has been found mutated in a CGD patient [51–54]. Studies using a reconstituted cell-free system for NADPH oxidase activation show that p22^{phox} becomes phosphorylated in a phosphatidic-acid-dependent manner on a threonine residue (Thr^{132} or Thr^{147}) [55,56]. The physiological role of these events is not understood at present, but it is interesting to note that Thr^{147} is close to the region of p22^{phox} that is involved in the interaction with the cytosolic regulatory subunit p47^{phox}.

The GTPase Rac

It was first suspected in the late 1980s that a GTPase might play a role in NADPH oxidase activation when it was demonstrated that guanine nucleotides were able to stimulate oxidase activity [57–60]. The GTPase was subsequently identified as Rac1 or Rac2, and it is now clear that its presence is absolutely required for full oxidase function [61,62] (reviewed recently in [63,64]). Rac belongs to the Rho-family of small GTPases, which act as molecular switches and regulate a large variety of signalling pathways, including cytoskeletal remodelling and chemotaxis [65–67]. Their activity is determined by the type of guanine nucleotide to which they are bound: GDP maintains the protein in the inactive state, while GTP induces the active state, thereby allowing interaction with downstream effectors and propagation of a signalling response. The conversion between the active and inactive states is tightly regulated by GEFs (guanine-nucleotide-exchange factors), which promote the release of GDP and allow GTP to bind, and by GAPs (GTPase-activating proteins) that increase the rate of GTP hydrolysis by several orders of magnitude and hence down-regulate GTPase signalling [68,69].

Structural studies on many small GTPases over the last 15 years have shown that they all share a common fold consisting of a six-stranded β-sheet and five α-helices (reviewed in [70–72]). The conformational changes that occur during the interconversion between the active and the inactive states are by and large confined to two regions of the protein, which have been termed the switch I and switch II regions. These include amino acids 30–40 (Rac numbering scheme), also known as the effector loop, and amino acids 60–67 respectively. Not surprisingly, these regions are generally recognized by regulatory proteins [GDIs (GDP-dissociation inhibitors), GEFs and GAPs] or downstream effectors, and they can therefore be viewed as the docking stations for GTPase-binding proteins. The remainder of the GTPase, including the insertion helix (amino acids 123–135), which is found only in Rho-family GTPases, stays unaltered during GDP–GTP cycling. Rac, however, is an exception to this rule, because its switch II region does not change its conformation upon GTP binding, as shown by crystallographic studies on GTP- and GDP-bound forms of Rac [73–76]. Both Rac1 and Rac2 are geranylgeranylated at the C-terminus, which facilitates their association with membranes. Nevertheless, both isoforms are kept cytosolic in the resting state due to an association with the GDI protein RhoGDI [77], to which Rac binds mainly through its switch II region and a hydrophobic pocket of the GDI that accommodates the geranylgeranyl moiety of the GTPase [74,78]. Appropriate stimuli induce the dissociation from RhoGDI, allowing membrane translocation independent of the other oxidase subunits [79–81] and exchange of GDP against GTP catalysed by GEFs such as the phosphoinositide-activated exchange factor P-Rex1 and the haematopoietic cell-specific GEF Vav1 [82–84].

Two regions of Rac are of particular interest to NADPH oxidase regulation: the insertion helix and the hypervariable C-terminus, where most of the differences between Rac1 and Rac2 occur. Rac1 is expressed ubiquitously, while Rac2 expression is restricted to haematopoietic cells [85,86]. Both proteins consist of 192 amino acids and share 92 % sequence homology, and, importantly, no amino acid substitutions occur in the switch regions or the insertion helix. Their ability to support O_2^- production is similar in reconstituted cell-free systems using purified proteins. However,

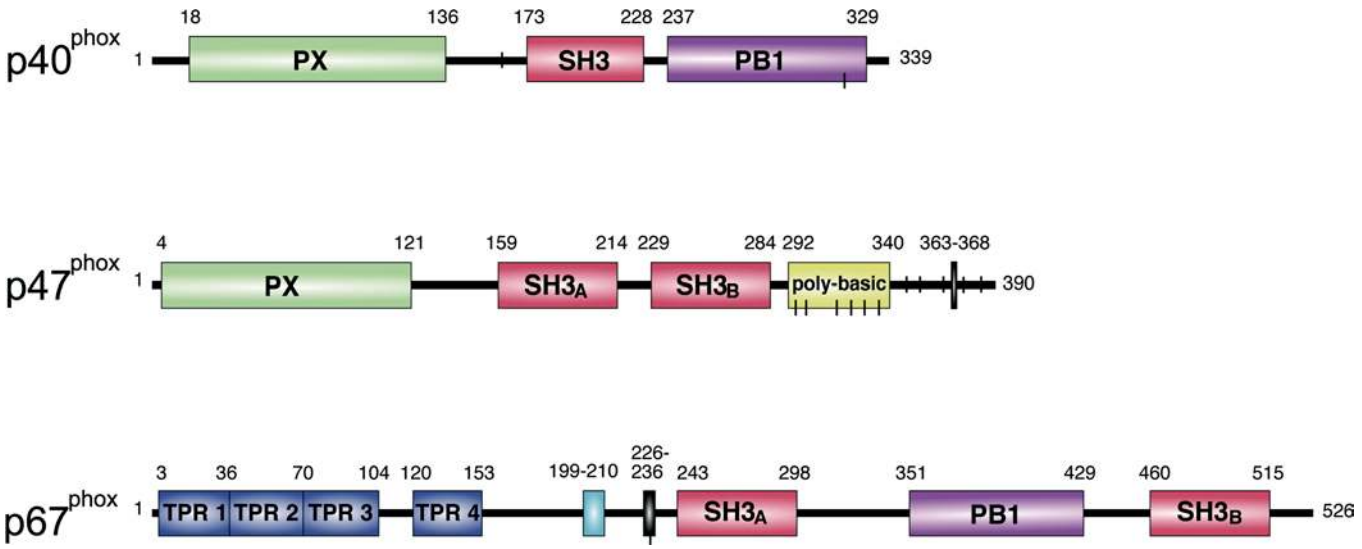

Figure 2 Domain structure of the cytosolic subunits p40^{phox}, p47^{phox} and p67^{phox}

The domain structure of the cytoplasmic components is shown as predicted by SMART (http://smart.embl-heidelberg.de/). The positions of consensus PxxP motifs in p47^{phox} (amino acids 363–368) and p67^{phox} (amino acids 226–236) are indicated by thick black bars. The locations of serine and threonine residues that become phosphorylated during activation are indicated by thin black bars.

functional differences between Rac1 and Rac2 have been found in assays using neutrophil cytosol, suggesting that regulatory, non-oxidase proteins have different effects on the two isoforms [61,83,87]. Interestingly, the two isoforms are found in different subcellular micro-environments in activated neutrophils. This distribution has been shown recently to be regulated by the hypervariable C-terminus and Asp^{150} of Rac2, and might explain apparent differences in oxidase regulation [88].

The cytosolic regulatory subunits

The activity of the phagocytic NADPH oxidase is tightly regulated by three cytosolic components p67^{phox}, p47^{phox} and p40^{phox}. These regulatory factors feature a number of protein–protein and protein–lipid interaction modules, sometimes in multiple copies, and undergo a variety of controlled protein–protein interactions at different stages during the activation process. Some of these interactions are modulated by reversible phosphorylation of serine or threonine residues, while others are targeted by phospholipids.

p47^{phox}

p47^{phox} is a 390-amino-acid protein with a molecular mass of 44.7 kDa that consists of a PX (Phox homology) domain, two adjacent SH3 domains, a region rich in arginine and lysine residues (the polybasic region) and a PRR (Figure 2). The PX domain was first identified in 1996 as a novel domain that is present in the NADPH oxidase subunits p40^{phox} and p47^{phox} [89], and has since been shown to specifically recognize phosphoinositides [90,91]. In the case of p47^{phox}, it recognizes preferentially PtdIns(3,4)P_2 and thereby contributes to membrane anchoring of p47^{phox} after activation-induced translocation [91]. The two SH3 domains of p47^{phox} have been shown to mediate a number of protein–protein interactions in both the resting and the active states, some of which are targeted by phosphorylation [1,6,92]. In fact, p47^{phox} is the most extensively phosphorylated subunit of the NADPH oxidase, and a total of 11 phosphorylation sites have been identified to date, all of which map to the region C-terminal of SH3 domain B [93–97]. Pure, recombinant p47^{phox} is a monomeric protein free in solution as shown by analytical ultracentrifugation and neutron scattering, and there is no indication that post-translational modifications might change this state [98,99]. A number of reports have suggested that the actin cytoskeleton might play a role in NADPH oxidase regulation, most likely through an interaction with p47^{phox} and possibly other oxidase components [100–104]. Specifically the PX domains of p47^{phox} and p40^{phox} have been shown to bind moesin, which belongs to the ERM (ezrin/radixin/moesin) family of actin-binding proteins [105]. The precise effect of association of phox components with the cytoskeleton remains unknown, but it has recently been suggested that the moesin–p47 PX domain interaction might be responsible for membrane translocation of p47^{phox} [106]. Such an interaction is difficult to reconcile with the phosphoinositide-binding function of PX domains, which is well documented by biochemical and structural evidence. Clearly, additional data are required to clarify the physiological role of the actin cytoskeleton in oxidase assembly.

p67^{phox}

p67^{phox} is a 526-amino-acid protein with a molecular mass of 59.8 kDa, which consists of a four TPR (tetratricopeptide repeat) motif-containing domain, a PRR and two SH3 domains that are separated by a PB1 (Phox and Bem1) domain (Figure 2). The N-terminal portion of p67^{phox} that encompasses the TPR domain is responsible for mediating the interaction with Rac in a GTP-dependent manner [107–109]. TPR domains are known to promote protein–protein interactions and are often found in proteins that are part of multi-protein assemblies [110–113]. In addition, recent reports have suggested that the TPR domain of p67^{phox} may also bind NADPH and exhibit weak dehydrogenase activity in spite of the absence of any homology with NADPH-binding sites in other proteins [114]. The significance of this observation is not understood at present, since it is generally accepted that the cytochrome contains the binding sites for all the co-factors that are necessary for efficient oxygen reduction. No binding partner has yet been identified for the PRR in p67^{phox} nor is it established if SH3 domain A participates in the regulation of NADPH oxidase activity. The PB1 domain is a novel protein–protein interaction module that interacts with other PB1 domains and

Table 1 PDB entries for structures of the NADPH oxidase subunits

This Table lists all the PDB entries of structures of NADPH oxidase components that are currently available. aa, amino acids.

Code	Protein	Binding partner	Reference	Method	Figure
1H6H	$p40^{phox}$ PX domain	di-C_4-PtdIns(3)*P*	[189]	X-ray	8
1GD5	$p47^{phox}$ PX domain		[194]	NMR	
1KQ6	$p47^{phox}$ PX domain		M. Wahl, H. Delbrueck, H. Oschkinat and U. Heinemann, published in PDB, 2003	X-ray	
1O7K	$p47^{phox}$ PX domain	Sulphate ions	[191]	X-ray	8
1K4U	$p47^{phox}$ aa 359–390	$p67^{phox}$ SH3B	[136]	NMR	4
1NG2	$p47^{phox}$ auto-inhibited (aa 156–340)		[144]	X-ray	5, 6
1UEC	$p47^{phox}$ auto-inhibited (aa 151–340)		[145]	X-ray	
1OV3	$p47^{phox}$ aa 156–285	$p22^{phox}$ peptide (aa 148–162)	[144]	X-ray	5
1OEY	$p67^{phox}$ PB1 domain	$p40^{phox}$ PB1 domain	[119]	X-ray	3
1HH8	$p67^{phox}$ TPR domain		[177]	X-ray	
1E96	$p67^{phox}$ TPR domain	Rac	[73]	X-ray	7

has been named after its occurrence in the phagocytic oxidase and Bem1 [115–117]. In the case of $p67^{phox}$, it forms a heterodimer with the PB1 domain of $p40^{phox}$ [118,119]. In addition to its protein interaction modules, $p67^{phox}$ contains an 'activation domain', which encompasses amino acids 199–210 and has been shown to be absolutely required for O_2^- production in a reconstituted cell-free system [120,121]. It is believed that this region might interact directly with the flavocytochrome and thereby participate in the regulation of electron transfer [122].

The shape and oligomerization state of $p67^{phox}$ in solution is controversial. Based on neutron scattering data in combination with analytical gel filtration, it was suggested that it exists as a dimer; however, the protein used in those studies had a propensity to aggregate even at low protein concentrations [99]. Analytical ultracentrifugation data instead indicated that it is an elongated monomer, which could explain its apparent high molecular mass on gel filtration [98]. On the other hand, phosphorylation studies of $p67^{phox}$ using different kinases including p38 MAPK (mitogen-activated protein kinase) and ERK1/2 (extracellular-signal-regulated kinase 1/2) suggest that it might exist in an auto-inhibited state. A new phosphorylation site in the C-terminal part (amino acids 244–526) of $p67^{phox}$ appears after removal of the N-terminal portion of the protein [123], suggesting a conformation in which the C-terminal phosphorylation site is masked by an N-terminal fragment containing the TPR domain. Inhibition of phosphorylation was also observed *in trans*, when N- and C-terminal fragments were mixed, indicating that the interaction between the two regions must be relatively tight. Taken together with the neutron scattering data, this may indicate that dimerization of $p67^{phox}$ occurs in an N- to C-terminal fashion. However, this model is in disagreement with studies showing that binding of Rac to the TPR domain of $p67^{phox}$ is not inhibited by the presence of the remainder of the protein [73,120]. The solution of the three-dimensional structure of full-length $p67^{phox}$ will be required to resolve this question.

$p40^{phox}$

$p40^{phox}$ is a 339-amino-acid protein with a molecular mass of 39.0 kDa and consists of a PX domain, an SH3 domain and a PB1 domain (in the context of $p40^{phox}$, previously described as a PC domain, where PC is phox and Cdc24) (Figure 2). $p40^{phox}$ was the last NADPH oxidase subunit to be identified by co-immunoprecipitation and co-purification with $p47^{phox}$ and $p67^{phox}$ [124–126]. It interacts with $p67^{phox}$ via its PB1 domain, while its SH3 domain has been suggested to interact with the PRR in $p47^{phox}$ [99,127]. However, this interaction is very weak in comparison with that between $p47^{phox}$ and $p67^{phox}$, and its physiological relevance is not clear at present. The PX domain of $p40^{phox}$ binds specifically to PtdIns(3)*P*, which accumulates in phagosomal membranes, and could thus facilitate oxidase assembly at this location [90,91]. The overall function of $p40^{phox}$ in oxidase regulation is still controversial, and it has been described as both activator and inhibitor [118,128–130].

PROTEIN–PROTEIN INTERACTIONS IN THE RESTING STATE

Reversible protein–protein interactions mediated by modular protein interaction domains are key to NADPH oxidase assembly, and much effort has been put into identifying the regions that are responsible for mediating complex formation during the different stages of the activation process. The interactions have been examined using a variety of techniques including phage display, yeast-two hybrid assays, oxidase reconstitution assays, GST (glutathione S-transferase) pull-down experiments, fluorescence spectroscopy and ITC (isothermal titration calorimetry). In addition, several crystal and NMR structures of NADPH oxidase fragments and complexes thereof have been solved recently. The PDB (Protein Data Bank) entries of these structures are listed in Table 1. We will first focus on the interactions of the cytosolic proteins in the resting state as these are the best characterized at present, and later give an overview of our current understanding of protein interactions occurring at the membrane in the fully assembled enzyme.

Early isolation of a complex of the regulatory oxidase subunits from the cytosol of resting neutrophils detected a molecular mass of 240–300 kDa by analytical gel filtration and showed that this complex contained $p47^{phox}$ and $p67^{phox}$ [131,132]. $p40^{phox}$ had not been identified at that time, and it was only appreciated later that it was part of the cytosolic complex. The large apparent molecular mass suggested that one or more oxidase subunits exist in multiple copies in this complex. However, recent biophysical studies employing ITC and analytical ultracentrifugation have shown that $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$ associate with a 1:1:1 stoichiometry (see also the model in Figure 6), and that the high molecular mass of the cytosolic complex is likely to be due to a non-globular shape [98]. This trimeric complex is generally believed to constitute the resting state of the cytosolic components and will be discussed as such in the present review. However, a recent report by Yaffe and co-workers suggested that $p47^{phox}$ may actually exist separately from the p40–$p67^{phox}$ complex in resting cells, and that formation

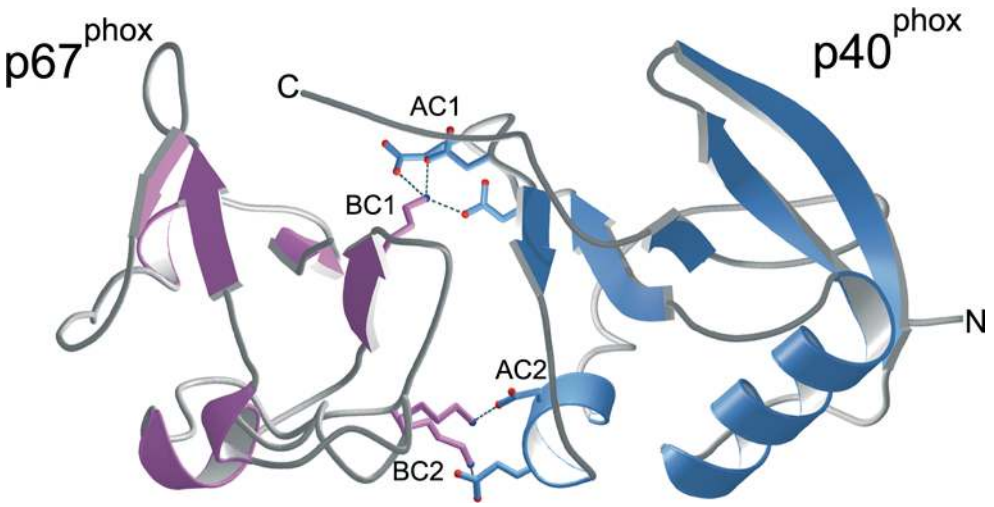

Figure 3 Complex between the PB1 domains of p40^{phox} and p67^{phox}

Ribbons representation of the complex between the PB1 domains of p40^{phox} in blue and p67^{phox} in magenta (PDB code 1OEY) [119]. Residues from the basic clusters (BC1 and BC2) and acidic clusters (AC1 and AC2), which mediate complex formation are indicated in a ball and stick representation.

of the trimeric complex requires stimulation [133]. Hence this would constitute the first step along the activation pathway. In the following sections, we will describe the architecture of the trimeric complex and will then discuss possible mechanisms which may prevent its formation.

The p40^{phox}–p67^{phox} interaction

The interaction between p40^{phox} and p67^{phox} is mediated by their respective PB1 domains [115,117]. Originally, it was believed that only p67^{phox} contained a PB1 domain, while its target region in p40^{phox} was designated the PC motif. However, recent sequence and structural analysis indicated that both domains are in fact members of the same family, and that both regions should be referred to as PB1 domains [116,119,134]. p40^{phox} and p67^{phox} form a very tight complex. Indeed, all of the binding affinity is contributed by the interaction between the two PB1 domains, as evidenced by the similar affinities for the isolated domains, $K_d = 4$ nM, and the full-length proteins, $K_d = 10$ nM [98,119]. This interaction is constitutive, and there is no evidence that it might be subject to regulation by post-translational modifications or competing proteins. In fact, it has been suggested that p67^{phox} might stabilize p40^{phox} and act as a kind of chaperone [127,135]. The latter was proposed due to the fact that p40^{phox} is not essential for oxidase function and that CGD patients who lack p67^{phox} showed reduced or no expression of p40^{phox}, implying that it is unstable in the absence of p67^{phox} [135].

The X-ray crystallographic structure of the complex between the PB1 domains of p40^{phox} (amino acids 237–339) and p67^{phox} (amino acids 352–429), shown in Figure 3, reveals how this domain can form heterodimeric complexes through a 'front-to-back' arrangement of the two domains [119]. Each PB1 domain has the same topology, consisting of a five-stranded β-sheet and two α-helices that superimpose with an RMS (root mean square) of 1.6 Å (1 Å = 0.1 nm). The complex is not symmetrical, and p67^{phox} uses its basic 'back' to bind the acidic 'front' of p40^{phox} (Figure 3). The basic surface on p67^{phox} consists of two clusters called BC (basic cluster) 1 and BC2, which contain Lys^{355} and Lys^{382}/Lys^{365} respectively. BC1 interacts with AC (acidic cluster) 1 of p40^{phox}, which is made up of Asp^{289}, Glu^{291} and Asp^{293}, while BC2 interacts with AC2, containing Glu^{301} and Asp^{302} (Figure 3). The importance of these acidic residues in p40^{phox} was suggested previously based on yeast two-hybrid data and *in vitro* pull-down assays [117]. These residues make up an acidic DX(D/E)GDX$_7$(D/E)D motif that is conserved in a large subset of PB1 domains and is called the OPCA [OPR (octicosapeptide repeat)/PC/AID (atypical protein kinase C interaction)] motif. A mutation within this motif, Asp^{289} → Ala, disrupts binding to p67^{phox} and abrogates enhancement of membrane translocation [118]. In addition, Lys^{355}, conserved among PB1 domains, constitutes an essential residue on the p67^{phox} side of the protein interaction interface. Alanine substitution of Lys^{355} eliminates heterodimer formation, observed in *in vitro* pull-down assays, and reduces NADPH oxidase activation *in vivo* [115,118]. The p40–p67^{phox} crystal structure rationalizes these observations as these two residues form an important salt bridge at the protein interface (Figure 3) [119]. In addition to the electrostatic interactions described above, a significant proportion of the protein–protein interface between p40^{phox} and p67^{phox} is contributed by a C-terminal extension of p40^{phox} that is not part of the conserved PB1 homology domain and whose deletion suppresses binding to p67^{phox} (Figure 3) [117]. These interactions are partly hydrophobic, partly hydrogen-bond-mediated and contribute to the specificity of this interaction [119].

The p47^{phox}–p67^{phox} interaction

p47^{phox} associates with the p40–p67^{phox} complex via its C-terminal consensus PxxP motif and the second SH3 domain of p67^{phox} (p67-SH3$_B$). The binding affinity of 20 nM for complex formation is atypically high for an SH3 domain/proline-rich target interaction, but can be explained by additional contacts made outside of the consensus PxxP motif [136]. The importance of these additional contacts is demonstrated by the low-affinity binding (K_d = 20 μM) of a peptide encompassing only the PxxP motif (amino acids 360–370) to p67-SH3$_B$. The remaining binding energy is contributed by the region C-terminal to this motif which, nevertheless, is not able to bind to p67^{phox} on its own. However, extension of this region to include Arg^{368} restores binding to p67-SH3$_B$ with an affinity of 10 μM. The NMR structure of a complex between p67-SH3$_B$ and p47^{phox} (amino acids 359–390), shown in Figure 4, rationalizes these observations and illustrates how amino

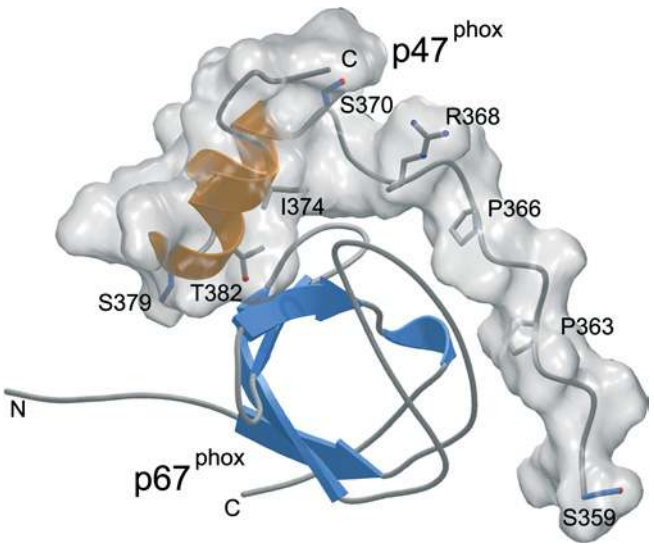

Figure 4 C-terminal SH3 domain of p67^{phox} in complex with the C-terminal region of p47^{phox}

p67-SH3B is shown in blue in a ribbon representation, while the C-terminal region of p47^{phox} encompassing amino acids 359–390 is shown in a surface representation with underlying ribbons (PDB code 1KU4) [136]. The side chains of Ser^{359}, Ser^{370} and Ser^{379}, which become phosphorylated during activation, and of Arg^{368}, Ile^{374} and Thr^{382}, which are important for complex formation, are shown in a ball and stick representation. Furthermore, the positions of Pro^{363} and Pro^{366}, which are part of the consensus PxxP motif are indicated.

acids 360–370 bind as a PPII (polyproline type II) helix in a typical class II orientation, while the region C-terminal to this motif forms two antiparallel α-helices that make extensive contacts with the SH3 domain [136]. Importantly, this region contacts a surface on p67-SH3$_B$ that has not been shown previously to mediate protein–protein interactions through SH3 domains. As a result of this additional binding site, the affinity is increased almost 1000-fold. Mutational analysis shows that Ile^{374} in helix α1 and Thr^{382} in helix α2 are very important for complex formation, and that mutation of either residue to alanine weakens the interactions to 3.0 μM and 1.1 μM respectively (Figure 4) [136].

The SH3 domain of p40^{phox} has also been suggested to interact with the PRR of p47^{phox} and thereby link p47^{phox} and p67^{phox} [99,137–139]. However, this interaction is relatively weak (K_d $\sim$5 μM), as estimated by analytical ultracentrifugation [98] and small-angle neutron scattering [99]. It would therefore not be expected to be able to compete with p67^{phox} that binds with a 250-fold higher affinity, even if complex formation can be observed using the isolated domains. Instead, p67^{phox} serves as a bridge between p47^{phox} and p40^{phox} by interacting with both co-regulators simultaneously as shown in the model in Figure 6 [98,118]. This model in which p67^{phox} is regarded as the central component of the trimeric complex is well supported by binding data and structural studies, yet still leaves a number of unanswered questions: what are the targets of the SH3 domain of p40^{phox}, the N-terminal SH3 domain of p67^{phox} and the PRR adjacent to this domain? Is it possible that these domains are 'orphans' in the resting state, but become involved in protein–protein interactions at later stages along the activation pathway? Or may they possibly interact with yet unidentified proteins and thereby modulate oxidase activity? Likewise, how can the recent suggestion that p47^{phox} is not associated with the p40–p67^{phox} complex in resting neutrophils [133] be reconciled with the fact that unmodified p47^{phox} and p67^{phox} interact with nanomolar affinity? Interestingly the PRR of p47^{phox} is surrounded by a number of serine residues (Ser^{359}, Ser^{370} and Ser^{379}), which are known to become phosphorylated during NADPH oxidase activation, as shown in Figure 4. Phosphorylation of these residues significantly weakens the interaction with p67^{phox} (F. Hussain and K. Rittinger, unpublished work), raising the possibility that there is a basal level of phosphorylation that could prevent the interaction between p47^{phox} and p67^{phox}. According to this model, NADPH oxidase assembly would then have to include activation of a phosphatase to allow formation of the trimeric complex. Alternatively p67-SH3$_B$ or the PRR of p47^{phox} may be associated with other molecules that prevent their association. More data are now required to decide if the p40–p67–p47^{phox} complex constitutes the true resting state or if this complex is only formed after initial activation.

The auto-inhibited conformation of p47^{phox} in the resting state

One of the main roles of p47^{phox} in NADPH oxidase function is to control and facilitate the translocation of the cytoplasmic p40–p67–p47^{phox} complex to the membrane and correctly position it with respect to the cytochrome. Translocation and anchoring to the membrane is achieved through an interaction between the SH3 domains of p47^{phox} and a conventional PxxP motif in the cytoplasmic portion of p22^{phox}. This interaction of the active state as well as its inhibition during the resting state has been the subject of many studies, and has led to a number of models for the different conformations of p47^{phox} during oxidase assembly. Initially, it was believed that the PRR in the C-terminus of p47^{phox} bound in an intramolecular fashion to its SH3 domains [53,140], thereby preventing them from interacting with p22^{phox}. However, more recent biochemical studies have suggested that the polybasic region, C-terminal to SH3$_B$, interacts with the SH3 domains instead [141–143]. Ago et al. [141] investigated which minimal fragment was responsible for masking the SH3 domains and found that while a core region (PPRR) is important, the highest affinity to the SH3 domains required the whole polybasic region (residues 296–340). Interestingly, both of the SH3 domains were required to achieve binding [141]. This observation has now been consolidated by the three-dimensional structure of the auto-inhibited core of p47^{phox} (comprising amino acids 156–340) that shows how the tandem SH3 domains of p47^{phox} interact with the polybasic region in a novel and unexpected fashion [144–146] (Figure 5).

Each SH3 domain adopts the conserved SH3 domain fold in the auto-inhibited structure; however, the two domains are arranged in such a fashion that their conserved ligand-binding surfaces are juxtaposed and contact one another across the interface, burying 579 Å^2 of solvent-accessible surface. This particular orientation of the two domains creates a novel ligand-binding surface that accommodates the sequence RGAPPRRSS (amino acids 296–304) in the N-terminal portion of the polybasic region in an arrangement that has been termed the 'SuperSH3 domain' (Figure 5) [144]. Residues GAPPR form a PPII helix, characteristic of SH3 domain ligands, in spite of the absence of a consensus PxxP motif. This structure represents a novel interaction between SH3 domains and binding partners as two SH3 domains bind a single target simultaneously. Apart from interactions made by the core peptide GAPPRR, an extensive network of interactions is generated by the rest of the polybasic region, with the linker connecting both SH3 domains and the back of SH3$_B$ (Figure 5). Binding studies using ITC revealed that these additional contacts contribute significantly to auto-inhibition, increasing the affinity of the polybasic region for the tandem SH3 domains by a factor of 20 compared with that of a peptide comprising only amino acids 296–304 [144]. This unexpected structure of the auto-inhibited fragment challenges some of the earlier suggestions about the

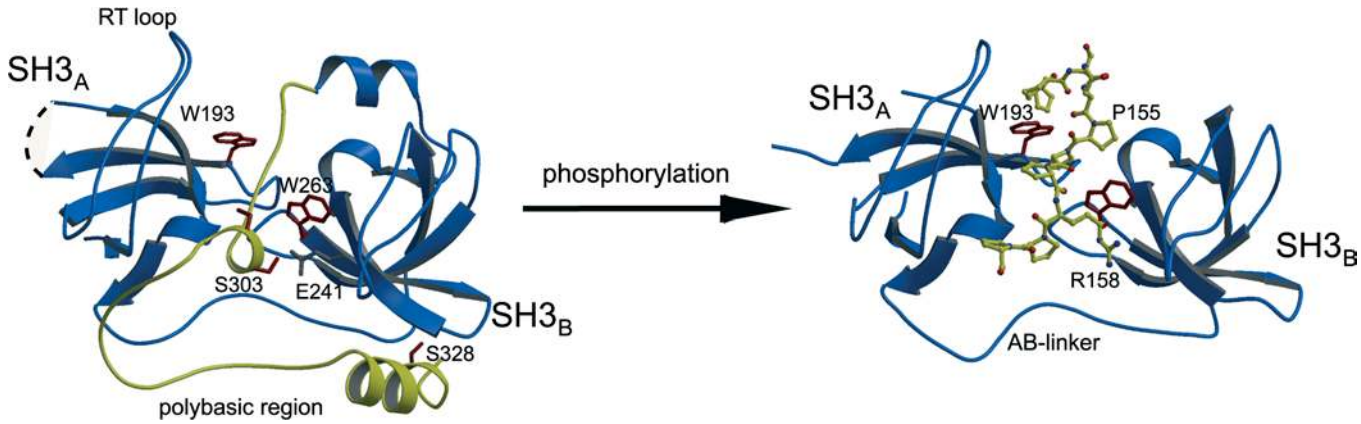

Figure 5 Regulation of p47^{phox} activity

Left-hand side: in the auto-inhibited conformation of p47^{phox}, the tandem SH3 domains (blue) are masked by an intramolecular interaction with the polybasic region (yellow) where a core region interacts with conserved Trp^{193} and Trp^{263} residues (red) (PDB code 1NG2) [144]. The positions of serine residues 303, 304 and 328, which are important for activation, are indicated in red. The auto-inhibited core of p47^{phox} crystallized as a domain-swapped dimer, but the monomer is shown in this Figure. Right-hand side: a proline-rich peptide from the cytoplasmic region of p22^{phox} encompassing residues 151–160 (yellow) binds to the same SuperSH3 arrangement as the core of the polybasic region (PDB code 1OV3) [144]. In addition to the consensus PxxP core that is involved in hydrophobic contacts with both conserved ligand-binding surfaces of the SH3 domains (blue), there are extensive additional contacts between $SH3_A$ and the p22 peptide.

interactions made by $SH3_B$, in particular with respect to the PX domain. This will be discussed in more detail in a later section.

PHOSPHORYLATION, OXIDASE ASSEMBLY AND PROTEIN–PROTEIN INTERACTIONS IN THE ACTIVE ENZYME COMPLEX

Phosphorylation

Phosphorylation has long been recognized as one of the key events in NADPH oxidase activation, and most oxidase components (apart from Rac and gp91^{phox}) have been shown to become phosphorylated to various degrees during the activation process. In the case of p47^{phox}, it is well established that multiple phosphorylation events are required to relieve auto-inhibition and allow translocation to the membrane. There association with the flavocytochrome occurs by virtue of an interaction between the tandem SH3 domains and a PRR in the C-terminal cytoplasmic tail of p22^{phox}. Neither p40^{phox} nor p67^{phox} is able to translocate in the absence of p47^{phox}, as evidenced by the cytoplasmic location of p40–p67^{phox} in stimulated cells from CGD patients that lack a functional p47^{phox} [135]. The phosphorylation of p47^{phox} is extensive, and 11 phosphorylation sites have been mapped including serine residues 303, 304, 310, 315, 320, 328, 345, 348, 359, 370 and 379 [93–97,147,148]. It is not clear if all serine residues become phosphorylated while the protein is still cytosolic, nor is the sequence of phosphorylation events known in detail. Several kinases have been shown to be involved in the phosphorylation of p47^{phox}. Protein kinase C plays a dominant role, mainly through the isoforms β, δ and ζ [96,149,150], but, additionally, p38-activated protein kinases [93–95,151], PAK (p21-activated kinase) [152], casein kinase 2 [153] and protein kinase B/Akt [154] were shown to contribute to phosphorylation of p47^{phox}. Serine residues 303, 304, 310, 315, 320 and 328 are located in the polybasic region, as indicated in Figure 2, and are hence part of the auto-inhibitory segment. Mutagenesis studies and cell-free NADPH oxidase activation assays have shown that phosphorylation of Ser^{303}, Ser^{304} and Ser^{328} are particularly important for activation [93–96,141,155], an observation that can easily be rationalized based on the structure of auto-inhibited p47^{phox} (Figure 5) [144]. Ser^{303} forms a hydrogen bond with the side chain of Glu^{241}, and phosphorylation of this serine residue will therefore lead to charge repulsion. Furthermore, its proximity to arginine residues in the polybasic region and the potential for electrostatic interactions may provide a means whereby the polybasic region could 'drag' the region around Ser^{303} and Ser^{304} away from the SuperSH3 domain, thereby vacating the binding site for p22^{phox}. Ser^{328} is involved in hydrogen bonding interactions with the side chain of Arg^{267} in $SH3_B$, and introduction of a bulky phosphate group might lead to a steric clash. Serine residues 345 and 348 are located within MAPK consensus sequences, and were shown to become phosphorylated during oxidase activation [93], yet the significance of phosphorylation at these positions is not well understood. In addition to phosphorylation of serine residues located within the polybasic region, three further phosphorylation sites were found within the binding site for p67-$SH3_B$, including serine residues 359, 370 and 379 (see Figures 2 and 4). Interestingly, mutation of Ser^{379} to alanine prevents membrane translocation and leads to a complete loss of NADPH oxidase activity, a behaviour that is similar to a mutant in which all serine residues that become phosphorylated have been replaced by alanine residues [156]. Similarly, substitution of alanine for Ser^{359} or Ser^{370} dramatically reduces phosphorylation of other residues, impairs protein translocation to the membrane and severely reduces O_2^- production [157]. Phosphorylation and translocation of these mutants is restored by the introduction of glutamate residues to mimic the effect of a phosphoserine, but oxidase activity was still severely impaired. This has led to a model in which phosphorylation of Ser^{359} or Ser^{370} initiates activation and precedes phosphorylation of other serine residues. It furthermore induces translocation to the membrane, an event that may happen before or after phosphorylation of the remaining serine residues [97,157]. Only the most extensively phosphorylated forms of p47^{phox} seem to associate with the membrane, and indeed membrane attachment might be required for some phosphorylation events [158,159]. On the other hand, given the large basis of biochemical and structural data supporting the notion that phosphorylation of serine residues 303, 304 and 328 in the polybasic region are required and sufficient for translocation to the membrane and for interaction with p22^{phox}, it is not obvious how phosphorylation of Ser^{359} and Ser^{370} might initiate activation. Furthermore, these residues are distant from the auto-inhibitory region that is responsible for

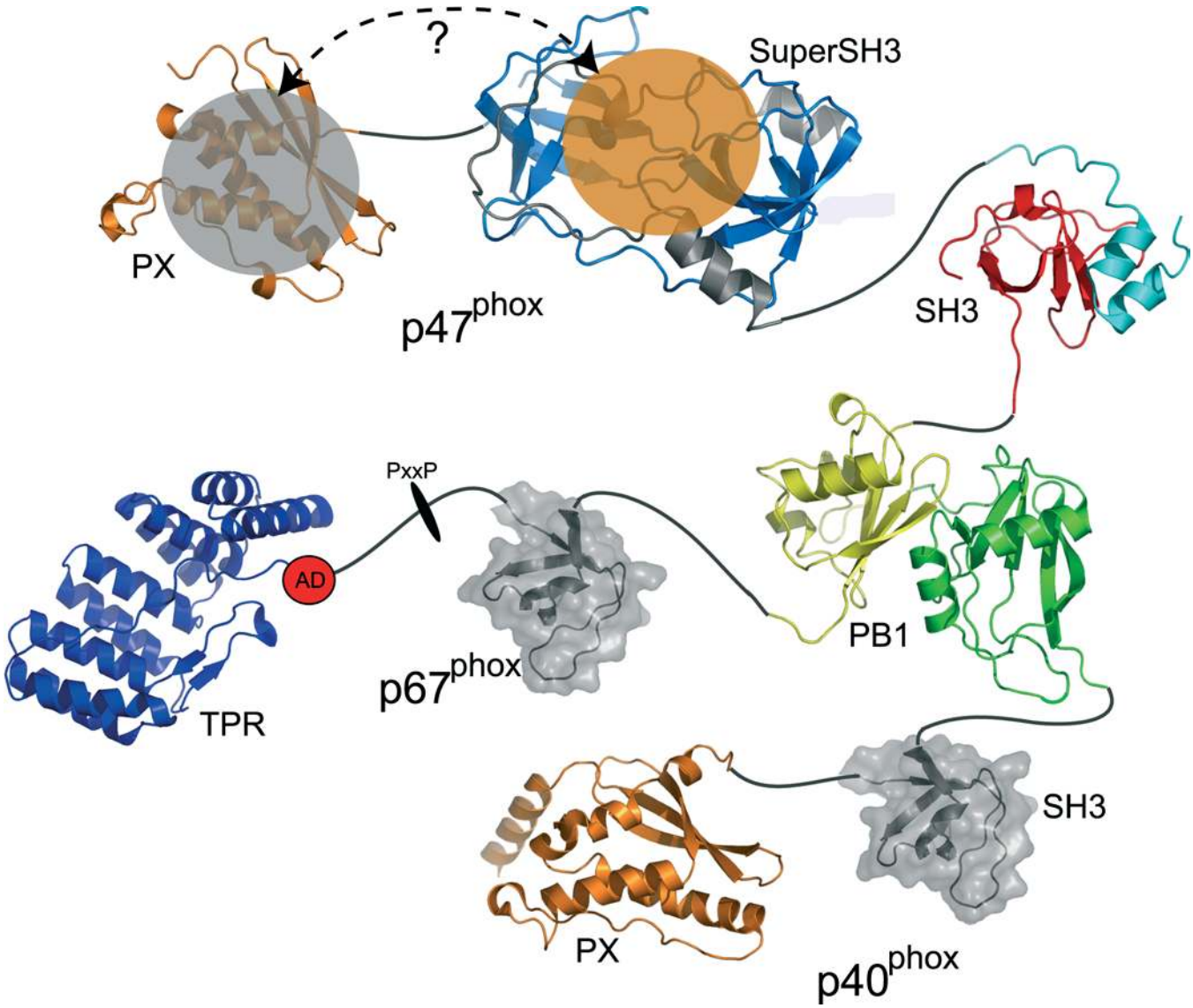

Figure 6 Model of the resting state of the NADPH oxidase

A model for the protein–protein interactions formed during the resting state of the NADPH oxidase, based on available X-ray and NMR structures (PDB codes 1O7K, 1NG2, 1KU4, 1OEY, 1E96 and 1H6H). The structures of the SH3 domains of $p67^{phox}$ (SH3A) and of $p40^{phox}$ have not yet been solved, nor have their binding partners been identified. In their place, a surface representation of the structure of an archetypal SH3 domain is shown in transparent grey (PDB code 2SEM). The positions of the activation domain (AD) and PxxP motif in $p67^{phox}$ are indicated in red and black respectively. At present, the molecular details of the cross-talk between the PX domain and tandem SH3 domains of $p47^{phox}$ are not understood (see text). This is represented by the grey and orange circles on top of the PX and SuperSH3 domains of $p47^{phox}$.

keeping $p47^{phox}$ in an inactive conformation (see Figure 2). Instead, they are part of the binding interface for $p67^{phox}$ and may regulate the interaction with this oxidase subunit.

The cytoplasmic regulatory proteins $p40^{phox}$ and $p67^{phox}$ are also known to become phosphorylated; however, the physiological role of these events is less clear [160–163]. The phosphorylation sites on $p40^{phox}$ have been mapped to Thr^{154}, which is situated close to its central SH3 domain, and Ser^{315} in the C-terminal portion of the PB1 domain (Figure 2) [164]. Based on cell-free oxidase assays with $p40^{phox}$ phosphorylated at Thr^{154}, it has been suggested that this phosphorylated form might act as an oxidase inhibitor, while the unphosphorylated form might act as an activator. The main difference between the two forms was speculated to be a conformational change that might possibly expose its SH3 domain. Further evidence is required to support such a model, in which phosphorylation induces conformational changes and thereby regulates the function of $p40^{phox}$. Nevertheless, this is an attractive hypothesis which might explain the controversial results obtained so far in search of the function of $p40^{phox}$. At present, only one phosphorylation site has been mapped on $p67^{phox}$, Thr^{233}, but the presence of further sites in the N- and C-terminal portions of $p67^{phox}$ has been suggested (Figure 2) [123,165].

Oxidase assembly

Activation of the NADPH oxidase requires conformational changes in the cytoplasmic complex to allow the assembly of the heterohexameric enzyme at the membrane. Docking of the p47–p67–$p40^{phox}$ complex to the membrane bound cytochrome b_{558} is supported by an interaction between the tandem SH3 domains of

p47^{phox} and the cytoplasmic tail of p22^{phox} [53,54,143,166,167]. The crystal structure of the tandem SH3 domains of p47^{phox} in complex with a peptide derived from the C-terminal tail of p22^{phox} (amino acids 149–166) revealed that both SH3 domains co-operate to mediate this interaction (Figure 5) [144]. As observed in the auto-inhibited structure of p47^{phox}, the tandem SH3 domains act in conjunction to form a SuperSH3 domain and bind the peptide simultaneously through conserved residues from both domains. SH3 domain A makes a larger contribution to complex formation, as demonstrated by its ability to interact with the peptide in the absence of $SH3_B$. However, the interaction between p47^{phox} and p22^{phox} is strengthened significantly through additional contacts made with $SH3_B$ [144].

Once at the membrane, additional contacts between p47^{phox} and the cytochrome take place, which are believed to either help position p67^{phox} correctly or possibly induce a conformational change within the cytochrome. These interactions have been mapped to the first cytoplasmic loop of gp91^{phox} [37,42], and to two regions in the cytoplasmic domain of gp91^{phox}: to amino acids 450–457 adjacent to the NADPH-binding site and to the extreme C-terminus of the molecule as highlighted in Figure 1 [37,44,168]. Studies using atomic force microscopy support the notion that oxidase assembly, and specifically association with p67^{phox}, induce a conformational change in the cytochrome [169]. In contrast, no evidence has yet been found for a direct interaction between p40^{phox} and the cytochrome.

The Rac–p67^{phox} complex

A crucial step in oxidase assembly is the interaction between p67^{phox} and active, GTP-bound Rac that occurs after both proteins have, independently from one another, translocated to the membrane. These two proteins, together with the cytochrome, are sufficient to induce electron transfer, although significantly higher concentrations of either are required in the absence of p47^{phox} [170,171]. The precise mechanism by which the Rac–p67^{phox} complex participates in oxygen reduction is controversial, and various models have been put forward that differ primarily in the role of Rac. It is either seen solely as a scaffold that ensures the correct positioning of p67^{phox} towards the cytochrome [81,172,173] or as a direct participant in the electron transfer reaction [174]. These different models have been discussed in detail in recent reviews [175,176]. In the present review, we will only comment on the models in light of the crystal structure of a complex between the four TPR motif-containing N-terminal domain of p67^{phox} and Rac.GTP, that is shown in Figure 7 [73]. The two proteins interact with an affinity of 2–3 μM as determined by ITC. The remainder of p67^{phox} neither positively nor negatively influences complex formation as indicated by the similar affinities of Rac1 for either the truncated or full-length protein, suggesting that the TPR domain is not involved in auto-inhibitory interactions with the C-terminal portion of the protein [73]. Furthermore, no conformational changes take place upon complex formation, as demonstrated by comparison of the complex with the apo structures of Rac and p67 TPR respectively [76,177]. The affinities of p67^{phox} for Rac1 and Rac2 are similar [73], in accordance with previous studies, which used a fluorescence-based assay to characterize the Rac–p67^{phox} interaction, although the dissociation constants determined in that study were approx. 20-fold lower overall [109]. The reason for these discrepancies is not clear at present.

The individual TPR motifs consist of two antiparallel α-helices that pack against one another in a regular fashion to create an extended structure with a right-handed superhelical twist. A 20-amino-acid insertion in p67^{phox} between TPR motifs three and four forms two antiparallel β-sheets, which have been called the β-hairpin insertion. This insertion, which is not present in other TPR proteins, together with the loops that connect TPR1 with TPR2 and TPR2 with TPR3, form the binding site for Rac and accounts for most of the protein–protein contacts (Figure 7) [73,108]. This binding mode is very different from other TPR-domain-mediated protein interactions, in which protein partners interact with the groove created by the superhelical twist of the domain [110]. Instead, this groove is occupied in an intramolecular interaction with amino acids 168–186 in p67^{phox}. The Rac binding site in p67^{phox} has previously been suggested to include amino acids 170–199 based on dot-blot assays [178]. However, the Rac–p67^{phox} complex structure clearly indicates that this is not the case and suggests that the results obtained in that study might be an artifact due to the exposure of a hydrophobic surface on p67^{phox} that interacts non-specifically with other proteins. The p67^{phox}-binding surface on Rac is formed by the highly conserved switch I region and a region in the C-terminal portion of the protein, including amino acids Ala^{159}, Leu^{160} and Gln^{162} (Figure 7). Rather surprisingly, the complex interface contains only two residues, Ala^{27} and Gly^{30}, that differ between Rac and its close homologue Cdc42, which is neither able to activate the oxidase nor able to interact with p67^{phox}. Mutation of both amino acids in Rac results in complete loss of binding [73] and oxidase activation [179]. In contrast, introduction of the corresponding residues from Rac into Cdc42 produces a protein which is able to interact with p67^{phox} and activate the NADPH oxidase [73,179], confirming that these two amino acids are sufficient to explain the biologically observed specificity.

Two regions in the Rac–p67^{phox} complex have particular significance for oxidase activity: the activation domain in p67^{phox} (amino acids 199–210) and the insertion helix in Rac (amino acids 123–135, highlighted in Figure 7). The activation domain of p67^{phox} has been shown by deletion and mutational analysis to be pivotal for regulating electron transfer in a cell-free system and has been suggested to interact directly with the cytochrome [120,122]. Such a direct interaction has since been shown by overlay techniques and GST pull-down assays. The activation domain is not absolutely required for complex formation [180,181], suggesting that other regions of p67^{phox} are responsible for mediating the interaction with the cytochrome. The fragment of p67^{phox} that was crystallized in complex with Rac.GTP contained only amino acids 1–204 and hence was missing part of the activation domain [73]. However, there was no electron density for amino acids 182–204, suggesting that this region might be flexible and disordered. The structure of the isolated TPR domain of p67^{phox} included the activation domain in the crystallized fragment, but, again, there was no electron density after amino acid 193 [177], suggesting that, even in the presence of the activation domain, it does not adopt a defined structure and might only do so upon interaction with the cytochrome.

The role of the insertion helix in Rac has been investigated by many groups, but its role in NADPH oxidase activity remains controversial. While some studies show a clear requirement of this region for oxidase activity, and in some cases even for complex formation with p67^{phox} [172,174], others do not see a decrease in O_2^- production upon deletion [182,183]. The Rac–p67^{phox} structure demonstrates that the insertion helix is not required for complex formation and is far away from the protein–protein interface, and hence fully accessible for a potential interaction with the cytochrome (Figure 7). An interesting study by Diebold and Bokoch [174] provides evidence that active GTP-bound Rac2 binds directly to the cytochrome in an insertion-helix-dependent fashion and thereby stimulates the first step of the electron transfer reaction. Only the second step of the reaction requires

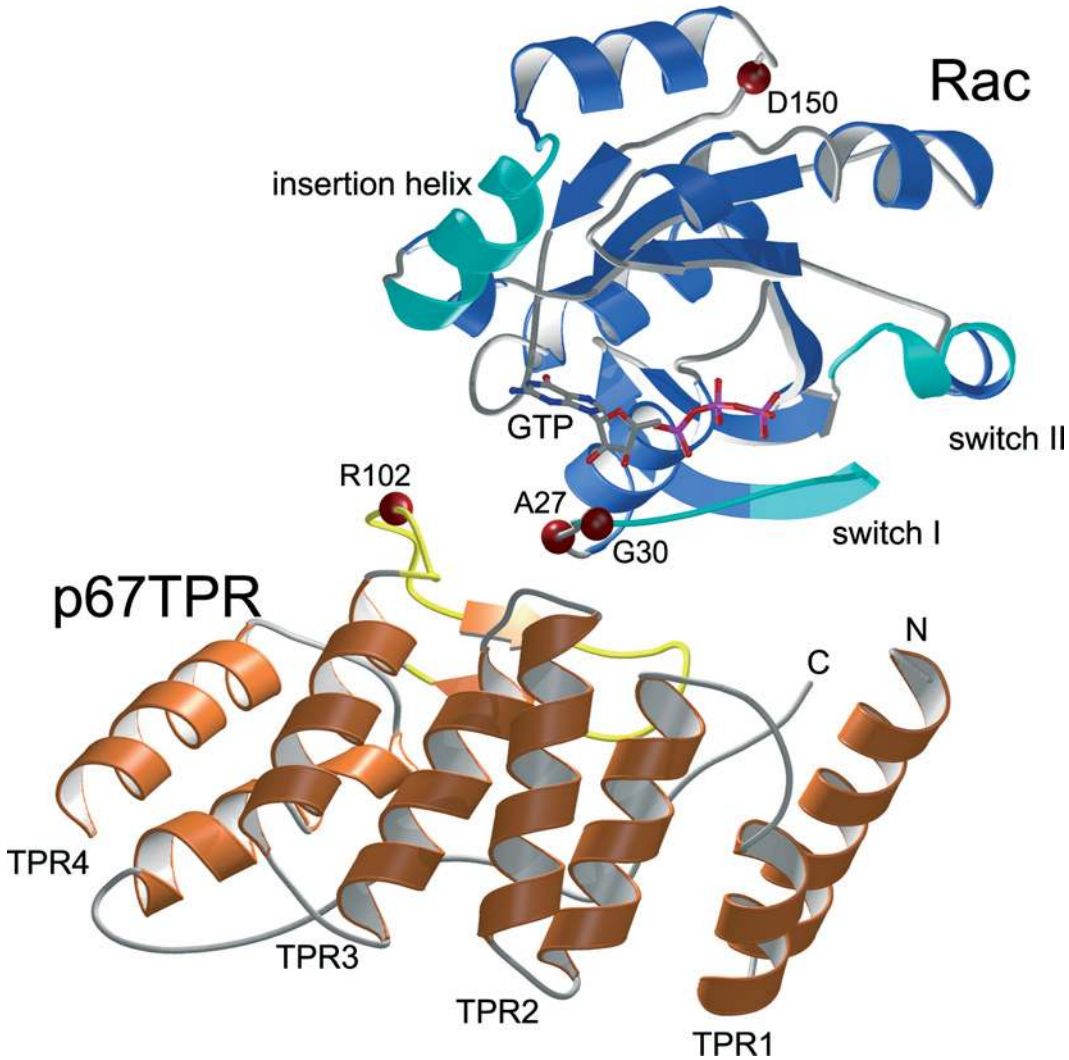

Figure 7 Complex formation between Rac and p67^{phox}

Complex formation between the active form of Rac in blue and the TPR domain of p67^{phox} in orange occurs at the membrane (PDB code 1E96) [73]. The β-hairpin insertion, which contains Arg^{102} that is crucial for complex formation, is highlighted in yellow. Switch I, switch II and the insertion helix of Rac are highlighted in light green. The positions of Ala^{27} and Gly^{30}, which determine specificity, and of Asp^{150}, which appears to be important for subcellular localization, are indicated by red balls.

complex formation between Rac and p67^{phox}. This model was originally based on experiments using a p67^{phox} deletion mutant (Δ178–184), which was believed not to interact with Rac. As discussed above, this region is not involved in complex formation, making the interpretation of these results difficult. However, these experiments have since been repeated with structure-based mutants in the Rac–p67^{phox} interface, and similar results have been obtained [175]. A recent study by Pick and colleagues using Rac–p67^{phox} chimaeras which are covalently linked, but contain mutations that prevent a functional Rac–p67^{phox} interaction, lends support to the idea that Rac is required to anchor p67^{phox} to the membrane on one hand, and also to promote an 'active form' of p67^{phox} [184]. Given the observation that complex formation alone does not appear to induce a conformational change in p67^{phox} (as judged by the similar affinities and energetics for the interaction between Rac and truncated or full-length p67^{phox}), this study strengthens further the idea that complex formation between membrane-anchored Rac and p67^{phox} is required to bring p67^{phox} into a correct position to interact productively with the cytochrome. Such a 'productive' interaction could then lead to changes in the structure of either p67^{phox} or the cytochrome.

LIPID BINDING AND OXIDASE ASSEMBLY

The interaction of the PX domains of p40^{phox} and p47^{phox} with phospholipids constitutes an additional mechanism to orchestrate the association of the cytoplasmic components with the membrane. PX domains are recently identified lipid-binding modules that are approx. 120 amino acids in length and recognize phosphoinositides with varying specificities (reviewed in [185–188]). The PX domain of p40^{phox} interacts selectively with PtdIns(3)*P* [91,189], while the PX domain of p47^{phox} preferentially recognizes PtdIns(3,4)P_2 [91,190–192]. Both recombinant full-length p40^{phox} and p47^{phox}, as well as the isolated PX domains, are monomers in solution and interact very tightly with their preferred lipids with affinities of 1.4 nM for the p40^{phox} PX domain and 38 nM for the p47^{phox} PX domain [189,191,193]. Interestingly the affinity of the p40^{phox} PX domain for the soluble phospholipid

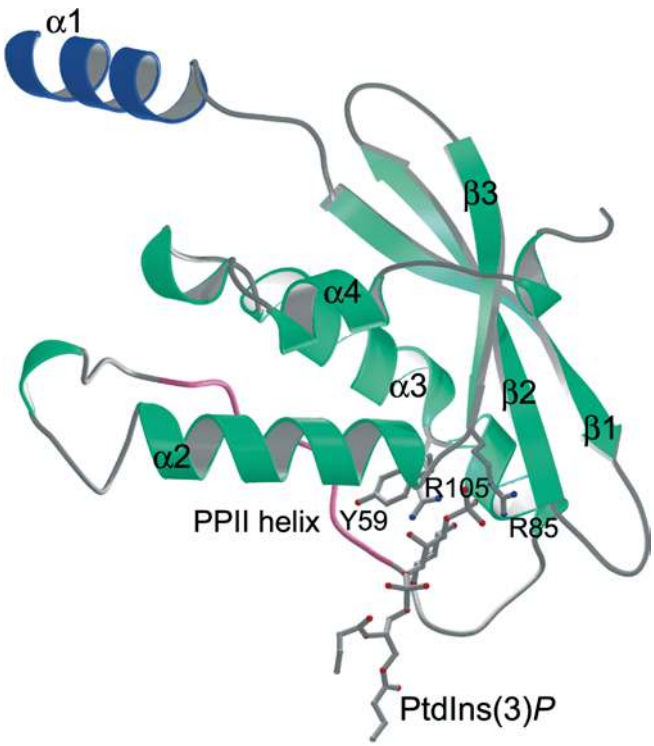

Figure 8 The PX domain as a phosphoinositide-binding module

The structure of the PX domain of p40^{phox} bound to di-C_4-PtdIns(3)*P* in a ribbon representation (PDB code 1H6H) [189]. Helix α1, which is important for solubility, but not part of the PX domain, is coloured blue. The phospholipid is shown in a ball-and-stick representation. The PPII helix is highlighted in pink. The positions of residues Tyr^{59}, Arg^{85} and Arg^{105}, which make important contacts with di-C_4-PtdIns(3)*P* are indicated.

di-C_4-PtdIns(3)*P* measured by ITC is significantly lower (5 μM), suggesting that other factors may contribute to tight interaction with the lipid bilayer [189]. The structures of a number of PX domains, including p47^{phox} and p40^{phox}, have been solved by NMR spectroscopy (p47PX) [194] and X-ray crystallography (p47PX and p40PX) [189,191]. These structures show that PX domains have a fairly flat shape and adopt a novel fold that consists of an N-terminal three-stranded antiparallel β-sheet (β1–3) that packs against a C-terminal α-helical domain, which consists of four α-helices (for which a varying nomenclature is used in the three structures of PX domains available). P40^{phox} contains an additional α-helix (called α1 in this structure) before β-sheet β1 that is not present in other PX domain structures solved so far, but whose presence is absolutely required for the solubility of the domain (Figure 8). The crystal structures of p40PX and p47PX are very similar and the Cα-positions superimpose with an RMS of 1.4 Å for 100 atoms. The major differences occur in the region following helix α2, including the PPII helix and the membrane interaction loop (see below). Interestingly, this is also the region where significant differences were found between the X-ray and NMR structures of p47PX. The structure of p40PX has been solved in complex with the ligand di-C_4-PtdIns(3)*P*, while the structure of p47PX is known in the apo form (NMR) and bound to two sulphate ions, which are believed to mimic the phosphate groups of phosphoinositides and phosphatidic acid respectively [191]. The two sulphate ions are found in basic pockets, and one of the sulphates binds in the position that is occupied by the phosphate in the phosphoinositide-bound p40PX structure, while the second sulphate is located in a previously unnoticed pocket. Biochemical studies show that both lipid-binding sites function independently, but synergistically, to increase the membrane affinity of p47^{phox} [191].

The crystal structure of the p40^{phox} PX domain in complex with di-C_4-PtdIns(3)*P* shows that the phosphoinositide-binding pocket is formed by residues from the N-terminal portions of strand β2 and helix α3, by the β3–α2 loop and by the loop connecting helices α2 and α3 that contains the PxxP motif (Figure 8) [189]. Two conserved arginine residues, Arg^{85} in the β3–α2 loop and Arg^{105} in helix α3, make extensive contacts with the phosphoinositide, and mutation of either abrogates complex formation. Additionally, Tyr^{59}, which is a tyrosine or phenylalanine residue in other PX domains, is involved in stacking interactions with the inositol ring and thereby protects one side of the carbohydrate from the solvent. Chemical shift changes have been detected in the loop connecting the proline-rich motif with helix α3 during micelle binding of the PX domain of VAM7p (vesicle-associated membrane protein 7). It was suggested that this loop may play the role of a 'membrane-interaction loop' and aid correct orientation of the PX domain with respect to the membrane [195]. Both p40PX and p47PX domains contain exposed hydrophobic residues in this loop, suggesting that they may also contain such a membrane-attachment loop.

Is the PX domain of p47^{phox} a dual protein interaction module?

Most PX domains contain a consensus PxxP motif between helices α2 and α3, and have therefore been predicted to be able to interact with SH3 domains. This motif is highlighted in Figure 8 in pink. Interestingly, some PX domains contain a basic residue in the P-3 position, suggesting that they would bind their target SH3 domain in a class I orientation, while many other PX domains contain a basic ligand C-terminal to the core PxxP motif, which would make them a class II ligand. A direct PX–SH3 domain interaction has been observed for the PX domain of p47^{phox} and its $SH3_B$ domain ($K_d = 50\ \mu$M, determined by NMR chemical shift perturbations) [194]. This observation led to a model in which an intramolecular PX–SH3 interaction regulates the lipid-binding ability of p47^{phox} and thereby helps to maintain p47^{phox} in the cytoplasm in the resting state. This model is supported by biochemical data showing that phospholipid binding to full-length p47^{phox} is 34-fold weaker than to the isolated PX domain (1.3 μM compared with 38 nM) [191]. Inhibition can be released by mutation of a conserved tryptophan residue in $SH3_B$, Trp^{263} → Arg, increasing the affinity to 2.4 nM, 15-fold higher than for the isolated PX domain. Importantly, phosphorylation of a number of serine residues in the polybasic region, which are known to induce oxidase activation, also restores phospholipid binding (serine to glutamate mutations to mimic phosphorylation increase the affinity to 13 nM). Similar data have been obtained from liposome-binding assays [155]. However, in spite of these convincing data indicating that the PX domain communicates with the remainder of the protein, the recently solved crystal structure of the auto-inhibited core of p47^{phox} clearly demonstrates that the model of a direct PX–$SH3_B$ interaction is too simple and that cross-talk between the different domains must occur in an unconventional manner [144]. As described above, both SH3 domains are occupied in an intramolecular interaction by the polybasic region of p47^{phox}, thereby preventing the conserved ligand-binding surface of $SH3_B$ from binding another ligand. Furthermore, differences in the affinities of the tandem SH3 domains for the PX domain and polybasic region clearly indicate that the polybasic region is the preferred target. It binds, even *in trans*, with an affinity of 1.5 μM to the tandem SH3 domains, which is not reduced by the presence of the PX domain (construct 1–295 binds with an affinity of 0.5 μM) [144]. In contrast, the PX–$SH3_B$

interaction is over 30-fold weaker, although it has been proposed that the presence of $SH3_A$ might increase this affinity. Taken together, these data suggest that the PX domain does indeed interact in an auto-inhibitory fashion with the remainder of $p47^{phox}$. However, the binding target of the PX domain seems to be the whole of the auto-inhibited core rather than the isolated $SH3_B$ domain, as shown schematically in Figure 6. This model is compatible with the results of the $Trp^{263} \rightarrow Arg$ mutation, as well as phosphorylation of serine residues 303, 304 and 328, as either will disrupt the tandem SH3–polybasic interaction and thereby the proposed PX-domain-binding surface. The three-dimensional structure of full-length $p47^{phox}$ is now required to fully resolve this issue.

Conclusions

The production of O_2^- anions through the multi-protein enzyme NADPH oxidase is crucial for our ability to fight invading micro-organisms, but can also induce tissue damage and promote inflammatory diseases. For this reason, an intricate system has evolved which ensures that oxidase subunits only assemble and form the active enzyme complex when appropriate signals have been received. Many of the interactions between oxidase components are mediated by modular protein interaction domains: relatively small, globular domains that are used extensively in signalling pathways to build multi-protein complexes and networks. Much has been learned about the interactions that connect NADPH oxidase components and the mechanisms that modulate these protein–protein interactions. In particular, phosphorylation has emerged as a major regulator of NADPH oxidase activation and assembly. Binding to phospholipids drives the activation process further and contributes to membrane association. In parallel, formation of active GTP-bound Rac is not only crucial for oxidase activity, but also plays key roles in the process of phagocytosis and contributes, directly and indirectly, to the activation of kinases that phosphorylate oxidase components. Structural studies of isolated domains or fragments of oxidase components and complexes (see Table 1) have helped immensely to extend our understanding of the molecular mechanisms that govern oxidase assembly. Yet many questions still remain, and the structures of full-length proteins and their complexes are now required to guide us along the activation pathway towards the cytochrome and the active membrane-associated enzyme.

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