Full-length p40^{phox} structure suggests a basis for regulation mechanism of its membrane binding

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The superoxide-producing phagocyte NADPH oxidase is activated during phagocytosis to destroy ingested microbes. The adaptor protein p40^{phox} associates via the PB1 domain with the essential oxidase activator p67^{phox}, and is considered to function by recruiting p67^{phox} to phagosomes; in this process, the PX domain of p40^{phox} binds to phosphatidylinositol 3-phosphate [PtdIns(3)P], a lipid abundant in the phagosomal membrane. Here we show that the PtdIns(3)P-binding activity of p40^{phox} is normally inhibited by the PB1 domain both *in vivo* and *in vitro*. The crystal structure of the full-length p40^{phox} reveals that the inhibition is mediated via intramolecular interaction between the PB1 and PX domains. The interface of the p40^{phox} PB1 domain for the PX domain localizes on the opposite side of that for the p67^{phox} PB1 domain, and thus the PB1-mediated PX regulation occurs without preventing the PB1–PB1 association with p67^{phox}.

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Introduction

Upon cell stimulation, a variety of proteins are recruited to subcellular compartments, where they play their specific roles. Increasing attention has been focused on the mechanisms for stimulus-induced membrane targeting of proteins, a process that involves interactions of protein modules with membrane phospholipids (Cullen *et al*, 2001; Hurley and Meyer, 2001; Itoh and Takenawa, 2002; Lemmon, 2003). Among them, pleckstrin homology (PH) and phagocyte

oxidase (phox) homology (PX) domains recognize specific phosphoinositides to recruit proteins to appropriate cell membranes. In general, interactions between phosphoinositides and proteins are regulated by kinases or phosphatases for inositol-containing lipids via producing specific phosphoinositides (Cullen *et al*, 2001; Hurley and Meyer, 2001; Itoh and Takenawa, 2002; Lemmon, 2003). For example, in response to cell stimulants, type I phosphatidylinositol 3-kinase (PI3K) is activated, leading to the synthesis of phosphatidylinositol-3,4,5-trisphosphate [PtdIns(3,4,5)P₃], and thus proteins containing a PH domain that specifically binds to PtdIns(3,4,5)P₃, such as the protein kinase Akt, are recruited to the membrane (Cullen *et al*, 2001; Hurley and Meyer, 2001; Itoh and Takenawa, 2002; Lemmon, 2003). On the other hand, there exist few examples in which changes in the protein rather than the lipids trigger the protein–lipid interaction (Karathanassis *et al*, 2002; Ago *et al*, 2003).

The phagocyte NADPH oxidase, dormant in resting cells, becomes activated during phagocytosis to produce superoxide, a precursor of microbicidal oxidants (Cross and Segal, 2004; Lambeth, 2004; Nauseef, 2004; Quinn and Gauss, 2004; Sumimoto *et al*, 2005). This enzyme plays a crucial role in host defense, as is exemplified by recurrent and life-threatening infections that occur in patients with chronic granulomatous disease because of the lack of the superoxide-producing system in phagocytes. The catalytic center of the phagocyte oxidase is the membrane-integrated protein gp91^{phox} (also known as Nox2), which contains a complete electron-transporting apparatus from NADPH to molecular oxygen. Activation of gp91^{phox}, tightly complexed with the membrane-bound protein p22^{phox}, requires proteins present in the cytoplasm of resting cells: the small GTPase Rac and the SH3 domain-containing proteins p47^{phox}, p67^{phox}, and p40^{phox} (Cross and Segal, 2004; Lambeth, 2004; Nauseef, 2004; Quinn and Gauss, 2004; Sumimoto *et al*, 2005). These proteins translocate upon cell stimulation to the membrane and interact with the gp91^{phox}–p22^{phox} complex, leading to superoxide production. In this process, p47^{phox} localizes to the membrane via an SH3-mediated interaction with p22^{phox} (Leto *et al*, 1994; Sumimoto *et al*, 1994; Shiose and Sumimoto, 2000) and a PX-mediated association with phosphoinositides (Ago *et al*, 2003), whereas the membrane recruitment of p67^{phox} is mediated via its binding to p47^{phox} (Heyworth *et al*, 1991; Kami *et al*, 2002). In contrast, Rac is independently targeted to the membrane and thus participates in the oxidase assembly (Heyworth *et al*, 1994).

The adaptor protein p40^{phox}, starting from the N-terminus, possesses a PX domain, an SH3 domain, and a PB1 (phox and Bem 1) domain (Figure 1A): the PB1 domain mediates constitutive association with p67^{phox} via a PB1–PB1 interaction (Ito *et al*, 2001; Noda *et al*, 2003). Although p40^{phox} is dispensable to phagocyte oxidase activation *in vitro*, p40^{phox} enhances the activation by facilitating membrane localization of the essential protein p67^{phox} (and its associated protein p47^{phox}) (Kuribayashi *et al*, 2002). The PX domain of p40^{phox}

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Figure 1 Role of the PB1 domain in p40^{phox} binding to PtdIns(3)P. (A) A schematic diagram of p40^{phox}. The PX, SH3, and PB1 domain are colored in yellow, green, and magenta. (B) Subcellular distribution of p40^{phox} and its truncated proteins in transiently transfected HeLa cells. Left panel, distribution of GFP-tagged p40^{phox} in fixed HeLa cells (green); right panel, distribution of endogenous EEA-1 (red) and Hoechst staining of the nucleus (blue) in the same field of fixed HeLa cells. The insets show the magnified views. Scale bar, 5 μ m. (C) Localization of ectopically expressed p40^{phox} and its truncated proteins in RAW264.7 cells ingesting IgG-coated beads. Scale bar, 5 μ m. (D) SDS-PAGE analysis of GST-tagged p40^{phox} proteins used in the following lipid-binding assays (E, F). Proteins were stained with Coomassie brilliant blue. (E) ELISA-format lipid-binding assay. Each well of a titer plate coated with phospholipids containing 10% of PI or PtdIns(3)P was incubated with the indicated p40^{phox} proteins. The bound proteins were stained as described under Materials and methods. Each value represents the mean of data with bars representing s.d. in more than three independent experiments. (F) Co-sedimentation assay with liposomes. Liposomes containing 5% of PI or PtdIns(3)P were incubated with the indicated p40^{phox} proteins. After centrifugation, proteins in the supernatant (S) and precipitate (P) fractions were analyzed by SDS-PAGE. The experiments have been repeated more than three times with similar results.

specifically and strongly binds to phosphatidylinositol-3-phosphate [PtdIns(3)P], whereas the p47^{phox} PX domain prefers other phosphoinositides such as phosphatidylinositol-3,4-bisphosphate [PtdIns(3,4)P₂] but with a much lower affinity (Ago *et al*, 2001; Ellson *et al*, 2001a; Kanai *et al*, 2001). As PtdIns(3)P is enriched in the membranes of not only early endosomes but also phagosomes (Gillooly *et al*, 2000; Vieira *et al*, 2001; Ellson *et al*, 2001b), it is likely that p40^{phox} participates in activation of the NADPH oxidase at the phagosomal membrane. Consistent with this, recent studies have shown that p40^{phox} is crucial for phagocytosis-mediated activation of the oxidase (Ellson *et al*, 2006; Suh *et al*, 2006).

The phosphoinositide-binding activity of the p47^{phox} PX domain is known to be regulated by protein modification although the PX domain is normally inaccessible to phosphoinositides because of its possible intramolecular interaction with the SH3 domains (Karathanassis *et al*, 2002; Ago *et al*, 2003). On the other hand, it has remained to be elucidated whether the p40^{phox} PX domain binds to PtdIns(3)P in a regulated manner. Here we show that the PB1 domain of p40^{phox} prevents the PX domain from interacting with PtdIns(3)P, both *in vivo* and *in vitro*: the full-length wild-type p40^{phox}, but not the PB1-truncated one, is incapable of localizing to early endosomes or phagosomes. We have also determined the crystal structure of the full-length p40^{phox}, which reveals that the inhibition of the PtdIns(3)P-binding activity is mediated via an intramolecular interaction between the PB1 and PX domains.

Results

Role for the PB1 domain in the recruitment of p40^{phox} to early endosomes and phagosomes

As shown in Figure 1A, p40^{phox} contains PX, SH3, and PB1 domains from the N-terminus. The isolated PX domain of p40^{phox} is known to be targeted to early endosomes, abundant in PtdIns(3)P, in a manner dependent on its PtdIns(3)P-binding activity (Ago *et al*, 2001; Ellson *et al*, 2001a; Kanai *et al*, 2001). However, it has remained unknown whether the full-length p40^{phox} is also recruited to early endosomes. To address this question, we expressed the full-length wild-type p40^{phox} as a fusion-protein containing green fluorescence protein (GFP) at the C-terminus (p40-F-GFP) as well as the PX domain alone (p40-PX-GFP) in HeLa cells. As shown in Figure 1B and in agreement with previous observations (Ago *et al*, 2001), p40-PX-GFP co-localized with EEA1 (early endosome autoantigen 1), a marker protein of early endosomes. In contrast, p40-F-GFP was not targeted to early endosomes, suggesting that the PX-mediated PtdIns(3)P-binding activity of p40^{phox} is suppressed by other regions of this protein. To test this possibility, we expressed a mutant protein lacking the SH3 or PB1 domain and analyzed their localization in HeLa cells. Although deletion of the SH3 domain did not lead to recruitment of the protein to early endosomes, p40-ΔPB1-GFP was targeted to early endosomes (Figure 1B). The endosomal localization is likely driven by the PtdIns(3)P-binding activity of the PX domain, as p40-ΔPB1(R105K)-GFP carrying the R105K substitution, a mutation resulting in a loss of the PtdIns(3)P-binding activity (Ago *et al*, 2001), failed to localize to early endosomes. The same results were obtained when N-terminally GFP-fused proteins were used instead of C-terminally GFP-fused ones (data not shown).

These findings indicate that the PB1 domain, but not the SH3 domain, appears to block the endosomal localization of p40^{phox} by preventing the PX domain from interacting with PtdIns(3)P.

It is established that PtdIns(3)P also accumulates in the phagosomal membrane in mouse macrophage-like RAW264.7 cells (Ellson *et al*, 2001b; Vieira *et al*, 2001). To investigate the role of the p40^{phox} PB1 domain on phagosomal localization, we transfected RAW264.7 cells with cDNAs encoding the wild-type or mutant p40^{phox}. When the transfected cells were ingesting IgG-coated Latex beads, p40-PX-GFP, but not p40-F-GFP, translocated to the phagosomal membrane (Figure 1C), suggesting that the ability to bind to PtdIns(3)P is attenuated in the full-length p40^{phox} also in phagocytes. In addition, p40-ΔPB1-GFP was recruited to the phagosomal membrane in a manner that requires the PX-mediated binding to PtdIns(3)P, whereas p40-ΔSH3-GFP failed to localize to phagosomes (Figure 1C). Thus, localization of p40^{phox} to phagosomes as well as to early endosomes seems to be negatively regulated by the PB1 domain. Taken together, the present findings indicate that, in cells, the PX-mediated PtdIns(3)P-binding activity of p40^{phox} is inhibited by the PB1 domain but not by the SH3 domain.

Role for the PB1 domain in the PtdIns(3)P-binding activity of p40^{phox} *in vitro*

To determine whether the p40^{phox} PB1 domain directly inhibits the PX-mediated PtdIns(3)P-binding activity, we prepared recombinant proteins and tested their activities by two different *in vitro* lipid-binding assays (Figure 1D–F). First, we performed an enzyme-linked immunosorbent assay (ELISA)-format lipid-binding assay (Ghosh *et al*, 1996; Oikawa *et al*, 2004). As expected from the findings using intact cells, the full-length p40^{phox} was incapable of binding to PtdIns(3)P coated on wells under the conditions where the isolated PX domain fully bound (Figure 1E). Although a mutant p40^{phox} lacking the SH3 domain failed to interact with PtdIns(3)P, deletion of the PB1 domain allowed effective binding to PtdIns(3)P. This binding seems to be specific, because the PB1-truncated mutant protein did not bind to wells coated with phosphatidylinositol (PI) instead of PtdIns(3)P. Essentially the same results were obtained by a liposome co-sedimentation: the isolated PX domain and PB1-truncated p40^{phox} co-precipitated with liposomes containing PtdIns(3)P but not with those containing PI, whereas the full-length or SH3-truncated proteins were recovered exclusively in the supernatant, indicating the lack of the PtdIns(3)P-binding activity (Figure 1F). Thus it is likely that the PB1 domain but not the SH3 domain directly interferes with the PX-mediated PtdIns(3)P-binding activity of p40^{phox}.

Overall structure of the full-length p40^{phox}

The present functional analyses indicate that the PX domain in intact p40^{phox} is blocked by the PB1 domain but not by the SH3 domain both *in vivo* and *in vitro*. To directly examine this possibility, we crystallized the full-length p40^{phox} for structure determination by X-ray diffraction studies (Honbou *et al*, 2006). The crystal of intact p40^{phox} revealed that there are two p40^{phox} molecules in the asymmetric unit (Figure 2A). One molecule has a relatively clear electron density (hereafter referred to this molecule as molecule A), but the electron

Figure 2 The overall structure of p40^{phox}. (A) Structure of the two molecules of p40^{phox} in the asymmetric unit. Molecule A, which has a clear electron density, is colored blue, and molecule B, which has a poor electron density, is colored red. (B) A stereo pair of a ribbon diagram of p40^{phox}. Molecule B is superimposed onto Molecule A by the PX domain. Each molecule is color coded as in (A). (C) Experimental and calculated small-angle X-ray scattering curves for p40^{phox}. The smooth curve in red corresponds to the scattering curve calculated from the dummy atom model derived using the program DAMMIN, which is superposed on the experimental curve (dots with error bars). (D) The crystal structure of p40^{phox} (Molecule A, ribbon diagram) is superimposed onto the low-resolution model restored by the DAMMIN, shown as green surface representation. The right model is rotated by 90° around the y-axis and the upper model is rotated by 90° around the x-axis according to the arrows.

density of the other molecule is sparse especially for the SH3 and PB1 domains (hereafter referred to this molecule as molecule B). For molecule A, the N-terminus 8 residues (1–8), the C-terminus 9 residues (331–339), the linker region between the SH3 and PB1 domains (154–168), and some residues in the loop regions were not observed because of the structural disorder, and thus could not be modeled in the electron density map (see Structural statistics in Table I). To investigate the relative domain orientation between molecules A and B, we superimposed the PX domain of molecule B onto that of molecule A (red and blue, respectively, in Figure 2B). The overlay of the two molecules shows that the relative position of the PX domain to the PB1 domain is fixed in p40^{phox}, although that to the SH3 domain is not restricted. The finding suggests that the interaction between the PX and PB1 domains is not due to crystallization artifact. To our knowledge, this is the first full-length structure that has been determined among the NADPH oxidase proteins.

Comparison of the molecular shapes of p40^{phox} in solution and in crystal

To determine the global structure of p40^{phox} in solution, we performed small-angle X-ray scattering (SAXS) experiments. A low-resolution model of p40^{phox} was determined by *ab initio* molecular shape analysis using the simulated annealing program DAMMIN (Svergun, 1999). Ten independent simulated annealing calculations were carried out for small angle X-ray scattering data, and the models were superimposed to construct an averaged model. Although all the calculated models yielded nearly identical scattering curves, the best

Table I Data collection and refinement statistics

p40 ^{phox}	
<i>Data collection</i>	
Space group	C222 ₁
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	146.27, 189.81, 79.88
Resolution (Å)	50.0–3.00 (3.11–3.00)
<i>R</i> _{merge}	7.1 (47.9)
<i>I</i> / <i>σI</i>	20.3 (3.7)
Completeness (%)	99.5 (98.5)
Redundancy	7.1
<i>Refinement</i>	
Resolution (Å)	40.8–3.15
No. of reflections (total)	19197
No. of reflections (test set)	1912
<i>R</i> _{work} / <i>R</i> _{free}	26.3/30.2
No. of atoms	
Protein	4252
Ligand/ion	—
Water	—
<i>B</i> -factors	
Protein	82.3
R.m.s deviations	
Bond lengths (Å)	0.010
Bond angles (deg)	1.6

Highest-resolution shell is shown in parentheses.

model provided a fit to the experimental data with $\chi^2 = 0.41$, as shown in Figure 2C. The averaged low-resolution model roughly fits the molecular shape and dimension of molecule A in the crystal structure, with respect to the molecular shape

and dimension (Figure 2D). There were some slight differences between the crystal structure and the low-resolution model, probably due to the mobile nature of the SH3 domain. Thus the SAXS data support the idea that the PX–PB1 interaction is not due to the artifact of crystal packing, but rather represents an intrinsic property of intact p40^{phox}.

Interface between the PX and PB1 domains of p40^{phox}

The overall structure of p40^{phox} in molecule A is shown in Figure 3A, where the PX domain is colored yellow, the PB1 domain pink, and the SH3 domain green, respectively. A close-up view of the interface between the PX and PB1 domains, which is enclosed by a rectangle in the overall structure, is displayed as a stereo in Figure 3B. The total buried surface area in the interface of about 1300 Å² is slightly smaller than that of the average value observed for various protein–protein complexes (1600 ± 400 Å²) (Lo Conte *et al*, 1999). In Figure 3B, we showed the side chains of

amino-acid residues involved in the interface between the PX and PB1 domains in a stick model: F35, located on the loop between β1 and β2 of the PX domain, protrudes into the hydrophobic pocket lined by V257, P265, L273, F320, and W322 of the PB1 domain, which forms a critical interaction between the two domains. Moreover, there are ionic interactions between the basic cluster of H38, R58, and R60 in the PX domain and the acidic cluster of E259 and D269 in the PB1 domain. Intriguingly, these amino acids are evolutionarily conserved from fish to mammals (Inoue *et al*, 2004).

To elucidate how the PB1 domain affects the binding of the PX domain to PtdIns(3)P, we superimposed the structure of the p40^{phox} PX domain (blue in Figure 3C) complexed with a soluble PtdIns(3)P (Bravo *et al*, 2001) onto that of the full-length p40^{phox} (yellow, molecule A in Figure 3C). We observed a significant structural difference between the two structures in the loop region around 31–38 of the PX domain. This structural difference enables the aromatic ring of F35 in

Figure 3 The interface between the PX domain and the PB1 domain. (A) A ribbon diagram of Molecule A. Each domain of p40^{phox} is colored coded as in Figure 1A. (B) Stereo diagram of a close-up view (the region corresponds to the rectangle in (A)) of the interface between the PX domain and the PB1 domain. Amino-acid residues involved in the interaction are shown in a stick representation. (C) Superposition of the p40^{phox} PX domain complexed with PtdIns(3)P (blue; PDB code 1H6H) onto that of crystal structure of p40^{phox} (yellow). The SH3 domain is removed for clarity. PtdIns(3)P, R58, R60, and R105 of the each PX domain, and E259 and D269 of the PB1 domain, which interact with R58 and R60 respectively, are shown in a stick representation. (D) The model of the interaction between the full-length p40^{phox} and membrane-bound PtdIns(3)P. SH3 domain is connected by dashed line, since relative position of the SH3 domain does not seem to be restricted.

intact p40^{phox} to protrude into the hydrophobic pocket in the PB1 domain as indicated in Figure 3B. Although the PtdIns(3)P-binding site on the PX domain is close to the interface with the PB1 domain, the PB1 domain does not seem to restrict the access of PtdIns(3)P in intact p40^{phox} (Figure 3C). R58 and R105 in the PX domain are reported to be specificity determinants for the recognition of PtdIns(3)P. The side chains of R58 and R105 do not change their position in either structure and thus can recognize the 3-phosphate group and the 4- and 5-OH groups of PtdIns(3)P, respectively. On the other hand, the side-chain orientation of R60 is appreciably changed to interact with the 1-phosphate group of PtdIns(3)P in the complex; however, considering that R60 plays a minor role in the binding to PtdIns(3)P (Bravo *et al*, 2001), the salt bridge between R60 and D269 may be maintained in the p40^{phox}-PtdIns(3)P interaction. Thus, as shown in the model proposed here (Figure 3C), the PB1 domain does

not directly interfere with binding of the polar head of PtdIns(3)P to the PX domain. Instead the PB1 domain in p40^{phox} causes a steric clash with the membrane when the PX domain interacts with membrane-embedded PtdIns(3)P (Figure 3D). As a result, the PX domain in p40^{phox} cannot be tethered to the membrane.

To verify the significance of specific amino-acid residues on the PX-PB1 interface, we substituted alanine for E259, D269, and F320 in the PB1 domain. As shown in Figure 4A, the three mutant proteins p40-F(E259A)-GFP, p40-F(D269A)-GFP, and p40-F(F320A)-GFP were all targeted to early endosomes. The effects of the mutations were abrogated by further introduction of the R105K substitution, indicating the requirement for the PX-mediated PtdIns(3)P-binding activity. Similarly, when expressed in RAW264.7 cells, the three mutant proteins translocated to phagosomes in a manner dependent on the binding of PtdIns(3)P (Figure 4B). In

Figure 4 Role of E259, D269, and F320, residues in the PB1 interface for the PX domain, in the PtdIns(3)P-binding activity. (A) Subcellular distribution of p40^{phox} and its mutant proteins carrying the indicated amino-acid substitution in transiently transfected HeLa cells. Left panel, distribution of GFP-tagged p40^{phox} in fixed HeLa cells (green); right panel, distribution of endogenous EEA-1 (red) and Hoechst staining of the nucleus (blue) in the same field of fixed HeLa cells. The insets show the magnified views. Scale bar, 5 μ m. (B) Localization of ectopically expressed p40^{phox} and its mutant proteins in RAW264.7 cells ingesting IgG-coated beads. Scale bar, 5 μ m. (C) SDS-PAGE analysis of GST-tagged p40^{phox} and its mutant proteins carrying the indicated amino-acid substitution, which were used in the following lipid-binding assays (D, E). (D) The PtdIns(3)P-binding activity estimated by the ELISA-format phosphoinositide-binding assay as in Figure 1E. (E) The PtdIns(3)P-binding activity estimated by the liposome co-sedimentation assay as in Figure 1F.

addition, mutant proteins carrying the E259A, D269A, or F320A substitution in the PB1 domain were capable of binding to PtdIns(3)P *in vitro* via the PX domain, as estimated by the two different lipid-binding assays: ELISA-format one (Figure 4D) and liposome co-sedimentation one (Figure 4E). Thus, E259, D269, and F320 directly interact with the PX domain, whose interaction prevents this domain from binding to PtdIns(3)P both *in vivo* and *in vitro*.

Influence of the p40^{phox} PX domain on the PB1–PB1 interaction between p40^{phox} and p67^{phox}

The PB1 domains of p40^{phox} and p67^{phox} are considered to constitutively form a heterodimer. The complex formation involves direct interaction of the OPCA motif presented on the p40^{phox} PB1 domain with the conserved lysine residue on the p67^{phox} PB1 domain (Ito *et al*, 2001; Noda *et al*, 2003). To clarify the influence of the PX domain on the heterodimerization of the PB1 domains, we superimposed the structure of the p40^{phox} PB1 domain in the p40^{phox}–p67^{phox} PB1 complex onto that of intact p40^{phox}. As shown in Figure 5A, the OPCA motif of p40^{phox} is located on the opposite surface of the PX-binding region and therefore there is no steric clash between the p40^{phox} PX domain and the p67^{phox} PB1 domain. There is

little structural difference except for structural disorders at the C-terminus (encircled by dotted lines in Figure 5A) and at several loop regions. Because the C-terminal region of p40^{phox} is not included in the canonical PB1 domain but participates in the heterodimeric interaction, the structural disorder observed at the C-terminus is probably due to the absence of the p67^{phox} PB1 domain.

To test whether the interaction of the p40^{phox} PB1 domain with the p67^{phox} PB1 domain occurs independently of that with the p40^{phox} PX domain *in vivo*, we expressed both GFP-fused p40^{phox} and HA-tagged p67^{phox} proteins in HeLa cells. When the full-length p40^{phox} was co-expressed, both p67^{phox} and p40^{phox} were found throughout the cytoplasm (Figure 5B). In cells co-expressing p40-ΔPB1-GFP, a mutant defective in p67^{phox} binding, p67^{phox} retained in the cytoplasm despite the endosomal localization of p40-ΔPB1-GFP (Figure 5B). On the other hand, p67^{phox} co-localizes at early endosomes with mutant p40^{phox} proteins carrying a substitution that disrupts the intramolecular PX–PB1 interaction (E259A, D269A, and F320A). Thus these mutations do not seem to affect the PB1-mediated association with p67^{phox}, which is confirmed by the following observations: the mutant p40^{phox} proteins (p40-F(E259A)-GFP, p40-F(D269A)-GFP, and

Figure 5 Dual roles for the PB1 domain of p40^{phox}. (A) Ribbon diagram of the superposition of p40^{phox}/p67^{phox} PB1 heterodimer (blue is p67^{phox} PB1 and orange is p40^{phox} PB1; PDB code 1OEY) onto that of the full-length p40^{phox} (color coded as in Figure 1A). A C-terminal extension region is shown in dashed circle. (B) Subcellular distribution of GFP-tagged p40^{phox} and HA-tagged p67^{phox} in transiently transfected HeLa cells. Left panel, distribution of GFP-tagged p40^{phox} in fixed HeLa cells (green); right panel, distribution of endogenous HA-tagged p67^{phox} (red) and Hoechst staining of the nucleus (blue) in the same field of fixed HeLa cells. The insets show the magnified views. Scale bar, 5 μm. (C) Proteins of lysates prepared from HeLa cells expressing both p40^{phox} and p67^{phox} were immunoprecipitated (IP) with the anti-GFP or control IgG, and then analyzed by immunoblot (Blot) with the anti-HA or anti-GFP antibodies. (D) *In vitro* interaction between purified GST-p40^{phox} and His-tagged p67^{phox} in the presence or absence of PtdIns(3)P.

p40-F(F320A)-GFP), but not p40-ΔPB1-GFP, were co-immunoprecipitated with HA-tagged p67^{phox} to an extent similar to the wild-type p40^{phox} (Figure 5C). These findings indicate that the integrity of the mutant proteins carrying an amino-acid substitution in the PB1 domain is maintained, and that the p40^{phox} PB1 domain constitutively associates with the p67^{phox} PB1 domain in a manner independent of the p40^{phox} PX domain. Consistent with this, direct interaction between the full-length p40^{phox} and p67^{phox} was not affected by PtdIns(3)P as indicated by an *in vitro* pull-down assay using purified proteins (Figure 5D). In addition, the presence of the full-length p67^{phox} did not induce the binding of the full-length p40^{phox} to PtdIns(3)P in the liposome co-sedimentation assay (data not shown), supporting the idea that the heterodimerization of the PB1 domains does not affect the intramolecular interaction between the PB1 and PX domains in p40^{phox}.

Discussion

The present structural and functional analyses reveal that binding of the p40^{phox} PX domain to PtdIns(3)P is negatively regulated by its intramolecular interaction with the PB1 domain both *in vivo* and *in vitro*. It is generally considered that interactions between phosphoinositides and proteins are regulated by kinases or phosphatases for inositol-containing lipids via producing specific phosphoinositides, rather than by protein modification (Cullen *et al*, 2001; Hurley and Meyer, 2001; Itoh and Takenawa, 2002; Lemmon, 2003). Indeed, several proteins containing a PX domain constitutively associate with early endosomes (Xu *et al*, 2001a,b). In contrast, the PX domain of p47^{phox} is likely regulated by stimulus-induced phosphorylation of this protein (Karathanassis *et al*, 2002; Ago *et al*, 2003). In the resting state, the p47^{phox} PX domain seems to be inhibited by its interaction with the SH3 domains; a phosphorylation-driven conformational change of p47^{phox} renders the PX domain in a state accessible to phosphoinositides. This induced-binding plays a crucial role in both membrane translocation of p47^{phox} and activation of the phagocyte NADPH oxidase (Ago *et al*, 2003). On the other hand, we showed here that, although p40^{phox} also harbors an SH3 domain, the p40^{phox} PX domain is regulated by directly interacting with the PB1 domain but not with the SH3 domain, as shown in the present structure-based analyses. It is known that two types of PI3K are activated during phagosome formation: type I PI3K is initially activated at the phagocytic cup to produce PtdIns(3,4,5)P₃ and its metabolite PtdIns(3,4)P₂; and after phagosome closure, when the activity of the type I enzyme is attenuated, type III PI3K becomes active to synthesize PtdIns(3)P (Ellson *et al*, 2001b; Vieira *et al*, 2001). Interestingly, the p47^{phox} PX domain has a preference for PtdIns(3,4)P₂ as its target (Ago *et al*, 2001, 2003; Kanai *et al*, 2001; Karathanassis *et al*, 2002), whereas the p40^{phox} PX domain specifically binds to PtdIns(3)P. Thus the contents of these phosphoinositides and the binding activities of the PX domains are both regulated during phagocytosis. Activation of the NADPH oxidase at the phagosome may require coordinated functions of the PX domains of p47^{phox} and p40^{phox}: the initial oxidase complex assembles at the phagocytic cup in a manner dependent on the p47^{phox} PX domain and PtdIns(3,4)P₂, whereas the activated oxidase localizes to the closed phagosome via interaction of the

p40^{phox} PX domain with PtdIns(3)P. The strict control of the protein–lipid interaction by both phosphoinositide synthesis and protein conformational change appears to be particularly important for the phagocyte oxidase, as superoxide produced by the oxidase and its derivatives are toxic not only to invading microbes but also to host cells.

Based on the crystal structure of the full-length p40^{phox}, we propose a model where the PX domain is inaccessible to the membrane-embedded PtdIns(3)P. The inaccessibility is due to the fact that the PB1 domain sterically hinders the access of the PtdIns(3)P-binding site towards the surface of the membrane and thus towards the membrane-embedded PtdIns(3)P, rather than due to the fact that the PB1 domain directly masks the binding site or stabilizes a conformation in which the polar head of PtdIns(3)P is unable to bind (Figure 3C and D). This model explains well the previous observation that the full-length p40^{phox} is capable of binding to a soluble PtdIns(3)P, harboring C4 fatty acids, to the same extent as the isolated PX domain of p40^{phox} (Bravo *et al*, 2001). On the other hand, the SH3 domain is mobile in p40^{phox}, which is consistent with the present observations that this domain is not involved in inhibition of the PX domain binding to PtdIns(3)P (Figure 1). It has been reported that the full-length p40^{phox} binds to PtdIns(3)P as well as the isolated PX domain, using surface plasmon resonance/lipid monolayer assays (Stahelin *et al*, 2003). Although the reason for the discrepancy is presently unknown, it might be possible that the position of PtdIns(3)P is relatively flexible in the lipid monolayer, and thus the PX domain of the full-length p40^{phox} is accessible to the polar head of PtdIns(3)P.

The PB1 domain is a protein module that mediates a protein–protein interaction by directly binding to a PB1 domain on another protein (Ito *et al*, 2001; Terasawa *et al*, 2001; Kuribayashi *et al*, 2002; Noda *et al*, 2003; Wilson *et al*, 2003; Yoshinaga *et al*, 2003). It is well established that, in the resting state, p40^{phox} tightly associates with p67^{phox} via the PB1–PB1 interaction (Ito *et al*, 2001; Wilson *et al*, 2003). This implies, together with the present findings, that the *intermolecular* PB1–PB1 and *intramolecular* PX–PB1 interactions are not mutually exclusive but occur simultaneously. This is well explicable by the present crystal structure, in which the interface between the p40^{phox} PB1 and PX domains is on the side opposite to its interface with the p67^{phox} PB1 domain (Figure 5A). In addition, binding of p40^{phox} to PtdIns(3)P, which is induced by disruption of the PX–PB1 interaction, does not result in an impaired association with p67^{phox} (Figure 5B–D). Thus the PB1-mediated interaction of p40^{phox} with p67^{phox} is likely maintained independently of the PX-mediated binding to PtdIns(3)P. This allows p40^{phox} to recruit the essential oxidase activator p67^{phox} to PtdIns(3)P-rich phagosomal membranes, so that the components of the phagocyte NADPH oxidase can be assembled, producing a large amount of superoxide for microbial killing (Cross and Segal, 2004; Lambeth, 2004; Nauseef, 2004; Quinn and Gauss, 2004; Sumimoto *et al*, 2005). This scenario is supported by the following previous observations. Using K562 cells ectopically expressing oxidase proteins, we have previously shown that p40^{phox}, albeit not essential for the oxidase activity, enhances oxidase activation by facilitating the membrane translocation of the indispensable proteins p67^{phox} and p47^{phox}, and that this enhancement requires the PB1-mediated interaction between p40^{phox} and p67^{phox}.

(Kuribayashi *et al*, 2002). The positive role of p40^{phox} has recently been confirmed by experiments using neutrophils from p40^{phox}-deficient mice (Ellson *et al*, 2006). Intriguingly, phagocytosis-induced oxidase activation and bacterial killing are incompletely but severely impaired in p40^{phox}-deficient neutrophils (Ellson *et al*, 2006). Another recent study has shown that p40^{phox} plays an essential role in oxidase activation during phagocytosis of IgG-coated particles in the cells ectopically expressing other oxidase proteins and the FcγIIA receptor; the effect of p40^{phox} is totally dependent on the PX-mediated PtdIns(3)P-binding activity (Suh *et al*, 2006). Taken together, our and others' findings suggest that p40^{phox} participates in oxidase activation at phagosomes by tethering p67^{phox} (and its associated protein p47^{phox}) to PtdIns(3)P-containing phagosomes, a process that requires the simultaneous functions of the PB1 and PX domains.

It has been shown that, during ingestion of IgG-coated particles, the full-length p40^{phox} fused to yellow fluorescent protein, as well as to the isolated p40^{phox} PX domain, is recruited to phagosomes of human neutrophil-like PLB-985 cells or those of the cells with exogenous gene expression for other oxidase proteins and the FcγIIA receptor (Suh *et al*, 2006). In contrast, in the present study, the GFP-fused full-length p40^{phox} expressed in RAW264.7 macrophages did not translocate to phagosomes. This may suggest that a limiting quantity of an interacting partner is required to activate the 'PtdIns(3)P-binding switch'. However, co-expression of p47^{phox} and p67^{phox} in RAW264.7 macrophages does not force p40^{phox}-GFP to recognize PtdIns(3)P-rich phagosomes (R Minakami and H Sumimoto, unpublished observation). It seems thus possible that a heretofore unidentified partner of p40^{phox}, which assists a conformational change to allow the PX domain to become accessible to PtdIns(3)P, is abundant in neutrophils but not in macrophages.

In addition, disruption of the PX–PB1 interaction in p40^{phox}, leading to binding of the PX domain to PtdIns(3)P, might involve phosphorylation of p40^{phox}. It has been shown that p40^{phox} becomes phosphorylated at T154 and S315 by protein kinase C (PKC) (Bouin *et al*, 1998), and that PKC is activated at the phagosomal membrane (Ueyama *et al*, 2004); however, since T154 and S315 are not located near the PX–PB1 interface, it seems unlikely that the phosphorylation of these residues participates in the disruption of the PX–PB1 interaction. Consistent with this, the addition of phorbol myristate acetate, a potent activator of PKC, to RAW264.7 cells ingesting IgG-coated beads does not induce recruitment of the full-length p40^{phox} to phagosomes (R Minakami and H Sumimoto, unpublished observation). Further studies are needed to determine the mechanism underlying the disruption of the PX–PB1 interaction in p40^{phox}, which is considered to play a crucial role in assembly of the NADPH oxidase at phagosomes.

Materials and methods

Plasmid construction

The DNA fragments encoding the full-length of p40^{phox} (p40-F; amino-acid residues 1–339), p40-PX (1–167), p40-ΔSH3 (the full-length lacking amino acids 167–233), and p40-ΔPB1 (1–233) were amplified from the human p40^{phox} cDNA by polymerase chain reaction (PCR) with specific primers. Mutations leading to the indicated amino-acid substitutions were introduced by PCR-mediated site-directed mutagenesis. The PCR product was ligated

to pGEX-6P (GE Healthcare Bio-Sciences) for bacterial expression as a protein fused to glutathione S-transferase (GST), to pEGFP-C1 (Clontech) for expression as an N-terminally GFP-tagged protein in mammalian cells, or to a modified pEGFP vector for expression as a C-terminally GFP-tagged protein in mammalian cells. For expression of HA-tagged p67^{phox}, the cDNA was ligated to pEF-BOS as previously described (Miyano *et al*, 2006). All the constructs were sequenced for confirmation of their identities.

Cell culture, transfection, and confocal imaging

HeLa and mouse macrophage-like RAW264.7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. HeLa cells were transfected using Lipofect-AMINE and Plus reagent (Invitrogen) and cultured for 18–24 h. Cells were fixed in 1.2% formaldehyde, permeabilized in 0.1% Triton X-100, blocked with 3% bovine serum albumin (BSA), and stained with anti-early endosome antigen 1 (EEA1) monoclonal antibody (Transduction Laboratories) or anti-HA monoclonal antibody (HA.11; Covance). Alexa Fluor 594-labeled goat anti-mouse IgG antibody (Invitrogen) was used as a secondary antibody. Cell nuclei were stained with Hoechst 33342.

Transfection of RAW264.7 cells with cDNAs for the wild-type and mutant p40^{phox} was performed with Cell Line Nucleofector™ Kit V (amaxa) using the Nucleofector™ apparatus (amaxa), according to the manufacturer's instruction. The transfected RAW264.7 cells were plated for 3 h on 35-mm glass-bottom dishes (MatTek). For phagocytosis assay, latex beads (Polybead Carboxylate Microspheres, 4.5 μm; Polysciences) were opsonized by sequential incubation with BSA and mouse-derived anti-BSA IgG (Sigma). After the culture medium was replaced with HEPES-buffered saline (120 mM NaCl, 5 mM KCl, 5 mM glucose, 1 mM MgCl₂, 1 mM CaCl₂ and 17 mM HEPES, pH 7.4), cells were incubated with IgG-opsonized beads for 10 min at 37°C, and fixed in 1.2% formaldehyde. Samples were analyzed with a laser-scanning confocal microscope LSM510 (Carl Zeiss). For live phagocytosis assays, cells were maintained at 37°C on a microscope stage incubator. Phagocytosis was initiated by adding of IgG-opsonized beads. Confocal fluorescence images of live cells were captured every 20 s with the LSM510 microscope.

In vitro lipid-binding assays

The wild-type and mutant p40^{phox} were expressed as proteins fused to GST in the *Escherichia coli* strain BL21, and purified using glutathione-Sepharose-4B (GE Healthcare Bio-Sciences), as previously described (Ito *et al*, 2001; Ago *et al*, 2003). Synthetic PI or PtdIns(3)P with C16 fatty acids were purchased from Echelon Biosciences Inc.; phosphatidylethanolamine (PE) and phosphatidylcholine (PC) prepared from bovine brain were obtained from Sigma.

The ELISA-format lipid-binding assay for lipid binding was performed by the method of Ghosh *et al* (1996) with minor modifications (Oikawa *et al*, 2004). Briefly, phospholipid solutions containing PE (72%) and PC (18%) with 10% of PI, or PtdIns(3)P in ethanol, were applied to the wells of a 96-well titer plate (0.1 μg lipid for each well), and air dried for 12 h at room temperature. The remaining binding sites on the wells were blocked for 1 h with 5% BSA in PBS (137 mM NaCl, 2.68 mM KCl, 8.1 mM Na₂HPO₄, and 1.47 mM KH₂PO₄, pH 7.4). GST-fusion proteins (1.0 μg ml⁻¹) in PBS containing 5% BSA were incubated for 45 min in the wells coated with phospholipids. The wells were washed three times with PBS containing 0.05% Tween-20, and overlaid with glutathione-conjugated peroxidase (Sigma). The bound GST-fusion proteins were measured by reaction with *o*-phenylenediamine dihydrochloride: after the termination of the reaction with HCl, the absorbance at 490 nm was determined with a Wallac 1420 ARVosx ELISA plate reader (Wallac).

The co-sedimentation assay with liposomes was performed as previously described (Ago *et al*, 2001, 2003) with minor modifications. Briefly, liposomes were prepared by mixing PE (85%) and PC (10%) with 5% PI or PtdIns(3)P, drying the mixture under a stream of nitrogen, and resuspending it in a sample buffer (150 mM NaCl, 20 mM HEPES, pH 7.2). Liposomes (50 μM) were incubated for 10 min on ice with the indicated GST-fusion proteins (5 μg) in 50 μl of the sample buffer. The liposomes were precipitated for 20 min at 15 000g, and bound proteins were subjected to 12% SDS-PAGE followed by staining with Coomassie brilliant blue. For estimation

of the amount of proteins on the gel, densitometric analysis was performed using a LAS-1000plus image analyzer (Fuji photo film).

Structure determination of p40^{phox}

The construct encoding the full-length wild-type p40^{phox} (amino acids 1–339) was used for structure determination. Protein expression, purification, crystallization, data collection, and processing were performed as previously described (Honbou *et al*, 2006). Briefly, the full-length p40^{phox} was expressed as a TEV-cleavable 6 × His-tagged protein in *E. coli* strain BL21. The protein was purified by affinity chromatography, and 6 × His tag was cleaved by digestion with TEV protease. After digestion, further purification was performed using SourceQ column (GE Healthcare) followed by Superdex 75 gel filtration column (GE Healthcare). Purified protein solution was concentrated to 10 mg ml⁻¹ and crystallized using sitting-drop vapor diffusion against 5% PEG 20 000, 200 mM bis-tris-HCl, pH 6.5 at 293 K. The structure was solved by molecular replacement with MolRep (Vagin and Teplyakov, 1997) and the CNS program suite (Brunger *et al*, 1998) using the p40^{phox} PX domain coordinates from the complex with PtdIns(3)P (PDB code 1H6H), the p40^{phox} SH3 domain coordinates (PDB code 1W6X), and the p40^{phox} PB1 domain coordinates from the complex with the p67^{phox} PB1 domain (PDB code 1OEY chain J) as a search model. The correct orientations of the two PX and single PB1 domains were found by MolRep and CNS, respectively, but those of other domains were not found. Electron density map was thus calculated using phase information from two PX and one PB1 domains, and other domains were manually fitted into the density by using program O (Jones *et al*, 1991).

Initial refinement was performed by the torsion angle molecular dynamic-simulated annealing method and bulk-solvent correction against the maximum-likelihood amplitude target using CNS. For each cycle, the model was rebuilt manually using program O and Coot (Emsley and Cowtan, 2004) in several steps alternated with cycles of automated refinement using data to 3.15 Å resolution with $R = 26.3\%$ and $R_{\text{free}} = 30.2\%$, respectively. There are no residues that lie in disallowed regions in a Ramachandran analysis. The refinement statistics are summarized in Table I. All the structure figures were prepared using PyMOL (<http://pymol.sourceforge.net>).

SAXS data analysis and shape restoration

SAXS data were acquired at the BL-10C beamline at the Photon Factory in Tsukuba, Japan. Data on p40^{phox} were collected at several concentrations from 2.2 to 13 mg ml⁻¹ in the Q range 0.017 to 0.443 Å⁻¹ to estimate the possible effects of protein concentration on the determination of structural parameters. The wavelength of the X-ray was 1.488 Å. The sample cell had a volume of 50 µl and a 1-mm path length with quartz windows. The data acquisition time was 600 s for each measurement. The identical buffer solution to the sample was recorded to measure solvent scattering. Protein scattering was obtained by subtracting the solvent scattering as the background trace. The values of R_g from the Guinier analysis were stable from the lowest (2 mg ml⁻¹) to highest (13 mg ml⁻¹) concentration. The distance distribution function, $P(r)$, was calculated with the program GNOM (Svergun, 1992) with a Q -value of

$0.0328 < Q < 0.2508 \text{ Å}^{-1}$ at the protein concentration of 13 mg ml⁻¹. The *ab initio* shape determination program DAMMIN (Svergun, 1999) was used to generate a low-resolution model of p40^{phox} from the SAXS data. DAMMIN calculates a volume of a protein filled with densely packed spheres (dummy atoms) to fit the experimental scattering data by a simulated annealing minimization procedure. Ten independent fits were carried out and superimposed using the program SUPCOMB (Kozin and Svergun, 2001) and averaged by the program DAMAVER (Volkov and Svergun, 2003), highlighting common structural features.

Immunoprecipitation analysis

HeLa cells were transfected with the cDNAs encoding GFP-fused p40^{phox} and HA-tagged p67^{phox}, and cultured for 24 h. The cells were lysed at 4°C with a lysis buffer (150 mM NaCl, 0.4 mM EDTA, 10% glycerol, 0.5% Triton X-100, and 50 mM Tris, pH 7.5) containing 1 mM dithiothreitol and Protease inhibitor cocktail (Sigma). The lysates were centrifuged for 30 min at 20 000 g, and the resultant supernatant was precipitated with an anti-GFP monoclonal antibody (nacalai tesque) or mouse control IgG (DakoCytomation) coupled to protein G-Sepharose (GE Healthcare Bio-Sciences). The precipitates were washed three times with a buffer (150 mM NaCl, 0.05% Tween-20, and 20 mM Tris, pH 7.4) containing 1 mM dithiothreitol and Protease inhibitor cocktail, and applied to SDS-PAGE, followed by immunoblot analysis with an anti-HA monoclonal antibody (Covance) or anti-GFP polyclonal antibodies (MBL). The blots were developed using ECL-plus agents (GE Healthcare Bio-Sciences) for visualization of the antibodies.

In vitro pull-down binding assay

Purified GST-p40^{phox} (10 µg) and His-tagged p67^{phox} (10 µg) were incubated in 500 µl of PBS with or without liposomes containing PI or PtdIns(3)P at the indicated concentration. Proteins were pulled down with glutathione-Sepharose-4B (GE Healthcare Bio-Sciences), and precipitated proteins were eluted with 10 mM glutathione. The eluates were analyzed by immunoblot with an anti-GST monoclonal antibody (Upstate Biotechnology) or a Penta-His monoclonal antibody (QIAGEN).

Accession code

The coordinates and structure factors of the p40^{phox} have been deposited in the Protein Data Bank with accession code 2DYB.

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