Atypical Membrane-embedded Phosphatidylinositol 3,4-Bisphosphate (PI(3,4)P2)-binding Site on p47phox Phox Homology (PX) Domain Revealed by NMR

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Background: The p47phox PX domain binds PI(3,4)P2 during assembly and activation of NADPH oxidase.

Results: The PI(3,4)P2-binding site on the p47phox PX domain was identified by NMR.

Conclusion: The position of the phosphoinositide-binding site on p47phox PX is unique.

Significance: Our results provide insight into a particular role of p47phox PX in the assembly and activation of phagocyte NADPH oxidase.

The phagocyte NADPH oxidase is a multicomponent enzyme that produces reactive oxygen species as a defense against invading microorganisms. Its importance for innate immunity is exemplified by chronic granulomatous disease, a genetic impairment of NADPH oxidase that leads to recurrent infections and death (1, 2). The NADPH oxidase consists of cytosolic (p47phox, p40phox, p67phox, and small GTPase Rac1 or Rac2) and membrane-integrated components (gp91phox and p22phox). The enzyme switches from an inactive state to an active state and is capable of producing reactive oxygen species when the cytosolic components are recruited to the membrane and interact with the membrane-integrated components to form a functional complex (3–6).

p47phox and p40phox contain PX2 domains, which are crucial for the recruitment of cytosolic components (7–10). In addition, the PX domain of p47phox is important for the activation of the NADPH oxidase, based on the observation that in stimulated cells the presence of a PX-truncated p47phox mutant led to decreased superoxide production (10).

The PX domain is a functional module that targets membranes through specific interactions with phosphoinositides. The p47phox PX domain preferably binds phosphatidylinositol 3,4-bisphosphate (PI(3,4)P2) and plays a pivotal role in the assembly of phagocyte NADPH oxidase. We describe the PI(3,4)P2 binding mode of the p47phox PX domain as identified by a transferred cross-saturation experiment. The identified PI(3,4)P2-binding site, which includes the residues of helices α1 and α1′ and the following loop up to the distorted left-handed PPII helix, is located at a unique position, as compared with the phosphoinositide-binding sites of all other PX domains characterized thus far. Mutational analyses corroborated the results of the transferred cross-saturation experiments. Moreover, experiments with intact cells demonstrated the importance of this unique binding site for the function of the NADPH oxidase. The low affinity and selectivity of the atypical phosphoinositide-binding site on the p47phox PX domain suggest that different types of phosphoinositides sequentially bind to the p47phox PX domain, allowing the regulation of the multiple events that characterize the assembly and activation of phagocyte NADPH oxidase.

The Phox homology (PX) domain is a functional module that targets membranes through specific interactions with phosphoinositides. The p47phox PX domain preferentially binds phosphatidylinositol 3,4-bisphosphate (PI(3,4)P2) and plays a pivotal role in the assembly of phagocyte NADPH oxidase. We describe the PI(3,4)P2 binding mode of the p47phox PX domain as identified by a transferred cross-saturation experiment. The identified PI(3,4)P2-binding site, which includes the residues of helices α1 and α1′ and the following loop up to the distorted left-handed PPII helix, is located at a unique position, as compared with the phosphoinositide-binding sites of all other PX domains characterized thus far. Mutational analyses corroborated the results of the transferred cross-saturation experiments. Moreover, experiments with intact cells demonstrated the importance of this unique binding site for the function of the NADPH oxidase. The low affinity and selectivity of the atypical phosphoinositide-binding site on the p47phox PX domain suggest that different types of phosphoinositides sequentially bind to the p47phox PX domain, allowing the regulation of the multiple events that characterize the assembly and activation of phagocyte NADPH oxidase.

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p47phox and p40phox contain PX2 domains, which are crucial for the recruitment of cytosolic components (7–10). In addition, the PX domain of p47phox is important for the activation of the NADPH oxidase, based on the observation that in stimulated cells the presence of a PX-truncated p47phox mutant led to decreased superoxide production (10).

The PX domain is a functional module that targets membranes through a specific interaction with membrane-embedded phosphoinositides (11, 12). Although the p40phox PX domain specifically binds to PI3P, the p47phox PX domain reportedly binds to PI(3,4)P2, PA, and PtdSer.

The crystal structure of p40phox in complex with C4-Ptd3P has been solved. In the structure, Arg-60 interacts with the 3-phosphate (through its backbone) and the inositol 2-hydroxyl and 1-phosphate groups (through its side chain) of the bound PI3P. However, Lys-92 interacts strongly with the 1-phosphate group of the bound PI3P. The three-dimensional structures of p47phox PX alone have been determined by solution NMR (13) and x-ray crystallography (14). However, the structure of p47phox PX in complex with its ligand has not been reported. The crystal structure of its free form revealed two sulfate molecules bound in two distinct sites (14). One of the sulfates is hydrogen-bonded to Arg-43 and...

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† This article contains supplemental Figs. S1–S10 and additional references.

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2 The abbreviations used are: PX, Phox homology; TCS, transferred cross-saturation; PI(3,4)P2, phosphatidylinositol 3,4-bisphosphate; CPM, 7-diethylamino-3-(4-maleimidophenyl)-4-methylcoumarin; IAEDANS, 5-(((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid; PA, phosphatidic acid; PtdSer, phosphatidylserine; POPC, 1-palmitoyl-2-oleoyl-sn-phosphatidylcholine; HSCQ, heteroleuqar single quantum coherence; POPE, 1-palmitoyl-2-oleoyl-phosphatidylethanolamine; PI3P, phosphatidylinositol 3-phosphate.

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Thr-45 on the C-terminal end of the β3 strand, and it coincides with the 3-phosphate of the bound PI3P in the crystal structure of p40phox PX. The second sulfate is hydrogen-bonded to His-51 and Lys-55 on the α1 helix and to Arg-70 on the adjacent loop that leads to the distorted left-handed PP₁ helix. Based on these results and surface plasmon resonance-based mutational analyses, the first sulfate-binding site was proposed to be the PI(3,4)P₂-binding site, and the second sulfate-binding site was considered to be the PA-binding site. In the crystal structure of the CISK PX domain, however, one of the two bound sulfate groups was found in a completely unexpected area, which probably represents a nonspecific binding site (15). Therefore, it is unclear whether these sulfate-binding sites of p47phox PX are the actual ligand-binding sites, and thus the mechanism of the broad ligand specificity of p47phox PX is still unclear.

Under physiological conditions, phosphoinositides interact with effector proteins in the context of lipid bilayers. In some cases, the consideration of membrane-mimicking conditions can greatly affect the results of in vitro studies. For instance, the ability of the actin regulatory protein N-WASP to sense phosphatidylinositol 4,5-bisphosphate in the membrane is lost when soluble phosphatidylinositol 4,5-bisphosphate or inositol trisphosphate headgroups are used instead (16). As another example, the actual affinity of the vacuole protein sorting HOPS for PI3P can be revealed only by a liposome assay (17).

NMR is a useful technique for investigations of biological systems within a certain size limit (currently in the area of 800 kDa) (18). To overcome this inherent size limitation in NMR, appropriate NMR methodologies and sample preparations are necessary. This is particularly true in cases involving biomembranes, because a protein bound on a lipid bilayer is a huge biological structure that cannot be observed easily by NMR. Transferred cross-saturation (TCS) is an NMR method that enables the identification of the residues of protein ligands in close proximity to huge (>100 kDa) complexes (19–21). Successful applications of TCS for huge and/or inhomogeneous systems have previously been reported (22–29).

In this study, we employed TCS experiments to identify the PI(3,4)P₂-containing membrane-interacting interface on the p47phox PX domain, using liposomes as a membrane-mimicking environment. Mutational analyses verified the results of TCS and revealed that Lys-55 is important for PI(3,4)P₂ recognition. Moreover, we demonstrate that Lys-55 is also important for the proper function of phagocyte NADPH oxidase. Altogether, our data indicate that the p47phox PX domain binds PI(3,4)P₂ in a unique area, as compared with the phosphoinositide-binding sites of all of the other PX domains characterized thus far (30–35).

**EXPERIMENTAL PROCEDURES**

**Sample Preparation**—The human p47phox PX domain (residues 1–128), encoded on the pGEX-2T vector (GE Healthcare), was expressed and purified as reported (13). The human p40phox PX domain (residues 1–167) on the pGEX-2T vector was expressed and purified similarly. The DNA encoding the Vam7p PX domain (residues 1–122) was prepared by total synthesis using the ligation-independent cloning method. The Vam7p PX on the pET43a vector was expressed as an untagged protein and purified by a combination of anion and cation exchange chromatography. Mutants were prepared according to the QuikChange (Stratagene) protocol, and their expression and purification were performed similarly. Their correct folding was confirmed by an inspection of their HSQC spectra. The uniformly double-labeled 2H-15N PX domain was prepared by growing cells in 99% D₂O minimal medium, with 1 g liter⁻¹ ¹⁵NH₄Cl and 3 g liter⁻¹ 97% [²H]glucose. The cells were grown at 37 °C to an A₆₀₀ of 1.0 and then were induced by 1 mM isopropyl-1-thio-β-D-galactopyranoside and incubated overnight at 25 °C. The cells were harvested and lysed by sonication, and the supernatant was applied to glutathione-Sepharose 4B beads (GE Healthcare). The GST tag was removed by thrombin, and the isolated ²H-¹⁵N PX domain was purified according to the reported procedure (13). Large unilamellar vesicles were prepared by extrusion. Briefly, the required amounts of POPC (Avanti), POPE (Avanti), and di-C₁₆P(3,4)P₂ (Echelon) or di-C₁₆P13P (Echelon) were mixed in CHCl₃, dried over a N₂ stream, and placed under vacuum overnight. The lipid mixture was resuspended in 5 mM ¹⁵NMES, pH 5.5, 90% D₂O, or 5 mM MES, pH 5.5, 100% H₂O at 37 °C. Hydrated lipids were subjected to extrusion using an Avanti mini-extruder with a 100-nm polycarbonate filter. Phospholipid concentrations were determined by a phosphate assay (36). The size of the prepared liposomes was estimated by Dynamic Light Scattering experiments, using a DynaPro instrument at 25 °C with 10% laser intensity. The phosphoinositide-containing and control liposomes gave an average radius of 60.4 and 65.9 nm, respectively, with high homogeneity (low % polydispersity, 100% intensity, and 100% mass). The lamellarity of prepared liposomes was calculated as reported previously (37), and for the phosphoinositide-containing and control liposomes, the lamellarities were estimated to be 0.8 and 0.72, respectively. The stability of the liposomes during the interactions with proteins was evaluated by Dynamic Light Scattering experiments, in which the size of the liposomes was recorded over time. No significant change was observed in the size of the liposomes. The stability of the liposomes was further evaluated by fluorescence measurements of liposomes loaded with carboxyfluorescein. No significant change in self-quenching of carboxyfluorescein was observed over time. For titration experiments, stock solutions of di-C₁₆P(3,4)P₂ (Echelon), di-C₁₆PA (Avanti), and di-C₁₆PtdSer (Avanti) were prepared in 5 mM MES, 5 mM DTT, pH 5.5. Phosphates (Wako) were mixed to produce a phosphate buffer at pH 5.5. **Fluorescence Assay**—Cys-less p47phox PX (Cys-98 and Cys-111 were substituted with Ala and Ser, respectively) was labeled at position 21 (CPM-(21)p47phox PX) or alternatively at position 65 (CPM-(65)p47phox PX) with the fluorescent probe CPM, after the introduction of the required Cys in the labeling site. For labeling, a 10-fold molar excess of CPM was used, in 50 mM Tris, pH 7.4, and 100 mM NaCl, at 4 °C overnight. The reaction mixture was cleared by centrifugation, and the excess CPM was removed by passage through a PD-10 column. The purity of CPM-labeled PX was assessed by comparison of a Coomassie Brilliant Blue-stained gel and a UV-transilluminated SDS-polyacrylamide gel for the same sample. The labeling efficiency was...
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calculated from a molar extinction coefficient of 33,000 (M⁻¹ cm⁻¹) for CPM, and it was >90%. The correct folding of CPM-labeled p47phox PX and its ability to interact with C₄₋ P(3,4)P₂ were confirmed by an inspection of its HSQC spectra with and without ligand. For the 1,5-IAEDANS labeling of Cys-less p47phox PX at position 21 or position 80, a 20-fold molar excess of 1,5-IAEDANS was used in 20 mM Tris, 100 mM KCl, pH 7.5, at 20 °C, overnight and gave >90% labeled product. The stock solution of lipid vesicles and the sample solution of labeled p47phox PX were prepared in 5 mM MES, pH 5.5. Measurements were conducted on an RF-5300PC Shimadzu spectrofluorophotometer. The CPM-labeled sample was excited at 384 nm, and the emission was detected at 470 nm at 22 °C. The IAEDANS-labeled sample was excited at 336 nm, and the emission was detected at 490 nm at 22 °C.

NMR Spectroscopy—HSQC spectra were recorded on a Bruker Avance 500 or Bruker Avance 600 spectrometer equipped with a cryo-probe. Assignment data were provided by Dr. Hidekazu Hiroaki, Kobe University. We reconfirmed them by a combination of three-dimensional NMR spectra and selection was detected at 490 nm at 22 °C. HI10032

Activation System for the Phagocyte NADPH Oxidase—The cDNA for human wild type (WT) p47phox was prepared as described previously (39). A mutation leading to the K55A substitution in p47phox was introduced by PCR-mediated site-directed mutageneses. The CDNs were ligated to a pEF-BOS-based vector for expression of p47phox (WT) and p47phox (K55A) as HA-tagged protein in mammalian cells (39). All the constructs were sequenced for confirmation of their identities. The K562 cells stably expressing gp91phox and p67phox (K562-gp91phox/p67phox) cells were prepared as described previously (40). The cells (5 × 10⁶ cells/ml) were electroporated in the presence of 60 μg of pEF-BOS-HA-p47phox (WT) or pEF-BOS-HA-p47phox (K55A) at 170 V and 1,070 microfarads using a GenePulser (Bio-Rad). After culture for 48 h, the superoxide-producing activity of these transfected cells was determined by superoxide dismutase-inhibitable chemiluminescence with an enhancer-containing luminol-based detection system (DIOGENES, National Diagnostics), as described previously (39, 41). After the addition of DIOGENES, cells (1.0 × 10⁶ cells/ml) were preincubated for 5 min at 37 °C and subsequently stimulated with 200 ng/ml phorbol 12-myristate 13-acetate. The chemiluminescence was monitored at 37 °C using a luminometer (Auto Lumat LB953; EG&G Berthold). For estimation of protein levels of HA-p47phox, p67phox, and p22phox, cell lysates of K562 cells were subjected to SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Millipore), and probed with an anti-HA monoclonal antibody (Covance Research Products), an anti-p67phox monoclonal antibody (BD Biosciences), or an anti-p22phox polyclonal antibodies (Santa Cruz Biotechnology). The blots were developed using ECL-prime (GE Healthcare) for visualization of the antibodies, as described previously (41).

RESULTS

Determination of Affinity of the Complex between the p47phox PX Domain and Insoluble Long Lipid Chain, C₁₆₋ P(3,4)P₂ Embedded in Liposomes—For the quantitative analyses of the affinity of the p47phox PX domain for insoluble long lipid chains, C₁₆₋ P(3,4)P₂ embedded in liposomes, a 7-diethylamino-3-(4’-maleimidylphenyl)-4-methylcoumarin (CPM)-based fluorescence assay for Kₐ estimation (binding isothers obtained by titrating lipid vesicles [large unilamellar vesicles] composed of POPC/POPE/C₂₀₋ P(3,4)P₂ = 79.5:20.0:0.5 (A) or control vesicles composed of POPC/POPE = 79.5:20 (B), into a solution of 0.3 μM CPM-(21)-p47phox PX in 5 mM MES, pH 5.5. The sample was excited at 384 nm, and the emission was detected at 470 nm at 22 °C. Data were fit by the nonlinear least squares methods with the Microcal Origin software, using the reported equation (53). In the titration with control vesicles, the x axis corresponds to the lipid concentration (in μM), as measured for a hypothetical 0.5% component of control liposomes. The error bars in A represent average ± 1 S.D. calculated from three independent experiments.

FIGURE 1. CPM-based fluorescence assay for Kₐ estimation. Binding isothers obtained by titrating lipid vesicles (large unilamellar vesicles) composed of POPC/POPE/C₂₀₋ P(3,4)P₂ = 79.5:20.0:0.5 (A) or control vesicles composed of POPC/POPE = 79.5:20 (B), into a solution of 0.3 μM CPM-(21)-p47phox PX in 5 mM MES, pH 5.5. The sample was excited at 384 nm, and the emission was detected at 470 nm at 22 °C. Data were fit by the nonlinear least squares methods with the Microcal Origin software, using the reported equation (53). In the titration with control vesicles, the x axis corresponds to the lipid concentration (in μM), as measured for a hypothetical 0.5% component of control liposomes.
was obtained, which was similar to the value previously reported from surface plasmon resonance analyses, using liposomes composed of POPC/POPE/PtdIns(3,4)P2/H11005 (79.5:20:0.5) (see Table 3 of Ref. 14) or liposomes of increased percentage of PtdIns(3,4)P2 (43).

Interaction of p47phox PX Domain with an Insoluble Long Lipid Chain, C16-PI(3,4)P2, Embedded in Liposomes—In this study, we performed TCS experiments to investigate the interactions of C16-PI(3,4)P2-containing liposomes with the p47phox PX domain to identify the C16-PI(3,4)P2-containing membrane-interacting interface on the p47phox PX domain. An overview of the experiment is shown in Fig. 2A. The TCS experiments are performed with an excess amount of [U-2H,15N]p47phox PX domain, relative to the C16-PI(3,4)P2 embedded in the liposomes. In the TCS experiment, the complex is irradiated at a frequency centered at the aliphatic proton resonances, which exclusively affects the liposomes, because almost no aliphatic protons exist in the deuterated PX domain. The saturation in the liposomes is transferred to all of the hydrogen atoms of the liposomes, through intramolecular cross-relaxation (spin diffusion). The saturation is not limited within the liposomes but is transferred to the residues at the interface of the PX domain bound to the liposomes, through intermolecular cross-relaxation (cross-saturation). If the complexes have a sufficiently fast exchange rate between the free and bound states, then the saturation in the interface residues is effectively transferred to the free state of the PX domain. As a result, the irradiation causes a reduction in the intensities of the resonances from the interface residues of the PX domain in the free state.

The efficiency of the TCS effects depends on various experimental conditions, such as the binding constant between ligands and receptors, the molar ratio of the receptors to the ligands, and the molecular weight of the receptors. We investi-
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FIGURE 3. Interaction of WT-p47phox PX with C₄₆-PI(3,4)P₂. A, chemical shift perturbation profile of the p47phox PX domain caused by C₄₆-PI(3,4)P₂. Normalized chemical shift changes were calculated based on the equation \( n = 25(\Delta \delta H^2 + (\Delta \delta N/5)^2)^{1/2} \) (49). Residues that showed strong (\( N > 5 \) ppm) and moderate (3 ppm < \( N < 5 \) ppm) normalized chemical shift changes are colored red and orange, respectively. The error bars represent 25(\( \Delta \delta H^2 + (\Delta \delta N/5)^2 \))/5, where \( \Delta \delta H \) and \( \Delta \delta N \) are the digital resolutions in ppm in the \(^1H\) and \(^15N\) dimensions, respectively. The inset shows superimposed portions of the \(^1H\)-\(^15N\) HSQC spectra of [U-\(^15N\)]p47phox PX with (red) and without C₄₆-PI(3,4)P₂ (black). The full spectrum is shown in supplemental Fig. S4. B, mapping of residues displaying strong chemical shift perturbations onto a ribbon representation of the crystal structure of p47phox PX. The residues are color-coded as in A. C, chemical shift changes for the Arg-\( \epsilon N \) signals of p47phox PX upon interaction with C₄₆-PI(3,4)P₂; p47phox PX (0.2 mM) was incubated with no C₄₆-PI(3,4)P₂ (red) and with 0.2 mM C₄₆-PI(3,4)P₂ (blue). Peaks were assigned through mutations.

gated the effects of the physical parameters on TCS by a simulation (44), which indicated that the TCS effects would be sufficiently observed under the present experimental condition (supplemental Fig. S1).

To investigate whether the TCS method can accurately identify the membrane-interacting interface on a peripheral membrane protein, the method was first employed on a model protein, the Vam7p PX domain. The location of the phosphoinositide-binding site on this PX domain is well established. The results of the TCS experiment for the interaction of the Vam7p PX domain with C₁₆⁻PI₃P-containing liposomes are shown in supplemental Fig. S2. Significant intensity reductions were selectively observed in its reported PI₃P binding pocket.

Subsequently, we applied the TCS experiments to identify the C₁₆⁻PI(3,4)P₂-containing membrane-interacting interface on the p47phox PX domain. The spectra with and without irradiation are shown in Fig. 2B. Several residues, including Glu-49, Lys-55, and Met-57, are affected by the irradiation, although others are not. As shown in Fig. 2 and D, residues Glu-49 and Phe-58 of helix \( \alpha_1 \) and Ile-71 of the following loop displayed intensity losses within the 0.3–0.5 range. Moderate 0.25–0.3 intensity losses were exhibited by Tyr-48, Lys-55, and Met-57 in helix \( \alpha_1 \), as well as by Gly-63 in the following loop. All of these residues form a well-defined area on the surface of the p47phox PX domain, outlined by helix \( \alpha_1 \), helix \( \alpha_1' \), and the following loop up to the distorted left-handed PP₁ helix. The ectopic intensity losses shown by Met-27, which is located far away from the main affected area, might be the result of enhanced spin diffusion, due to the large molecular weight of the liposomes.

To investigate the intramolecular saturation effects induced by the residual protons in the [U-\(^2H\),\(^15N\)]p47phox PX domain, an identical TCS experiment, using the \(^2H\),\(^15N\) PX domain alone and without liposomes, was performed (supplemental Fig. S3, A and B). As a result, no selective intensity reductions were observed for the residues of the p47 phox PX domain. To examine the effect of the nonspecific binding between the p47phox PX domain and POPC and/or POPE, we performed the TCS experiments, using liposomes without PI(3,4)P₂ (supplemental Fig. S3), the resonances from the residues in the PI₃P binding pocket of the p40phox PX and Vam7p PX domain were selectively shifted upon the addition of soluble C₈⁻PI₃P.

Based on the above experiments, we conclude that the C₁₆⁻PI(3,4)P₂-containing membrane-interacting interface on the p47phox PX domain is the area on the surface of the protein, formed by helix \( \alpha_1 \), helix \( \alpha_1' \), and the following loop up to the PP₁ helix. Hereafter, this area and the site that correspond to the PI3P-binding site of p40phox PX are referred to as “atypical binding site” and “typical binding site,” respectively.

**Interaction of p47phox PX with Soluble C₄₆-PI(3,4)P₂**—To investigate the interaction between the p47phox PX domain and the polar headgroup of PI(3,4)P₂, NMR titration experiments with soluble phosphoinositides were performed. Chemical shifts of main chain NH and arginine NeH groups, which were observed in these experiments, are affected by both the direct binding effects and the conformational change in each residue (45). As shown in Fig. 3A and supplemental Fig. S4, soluble C₄₆-PI(3,4)P₂ induced chemical shift perturbations for specific signals on the HSQC spectrum of the wild type p47phox PX. Most of these perturbed signals were derived from the residues in the loop between helix \( \alpha_1 \) and the PP₁ helix (Fig. 3B), which is located inside the membrane-interacting interface identified by the TCS experiments (Fig. 2D), although several residues that are outside the membrane-interacting interface and within the site that corresponds to the PI3P-binding site of p40phox PX were also perturbed. The eNH of Arg-70, which coordinates the sulfate within the identified membrane-interacting interface, displayed a strong chemical shift perturbation (Fig. 3C). From the titration curve, a \( K_d \) value of 38.7 \( \mu M \) was obtained. Further structural analyses using intermolecular NOEs were hampered by the low signal intensities of the complex, which are probably due to exchange broadening.

For the comparison of the binding modes, the titration experiment was also performed with the model proteins the p40phox PX and Vam7p PX domains. As shown in supplemental Fig. S5, the resonances from the residues in the PI3P binding pocket of the p40phox PX and Vam7p PX domain were selectively shifted upon the addition of soluble C₈⁻PI₃P.
Interaction of p47phox PX with Various Soluble Ligands—To clarify the ligand specificity of the p47phox PX domain, we further investigated the interaction between the p47phox PX domain and other soluble phosphoinositides. Although the p47phox PX shows a preference for PI(3,4)P₂, it can also bind to other types of phosphoinositides, such as PI3P and PI(4,5)P₂, with lower affinity. They induced similar patterns of chemical shift perturbation on p47phox PX and therefore are probably accommodated in the same area as the PI(3,4)P₂ (supplemental Fig. S6).

PA and PtdSer have been reported as putative interacting partners of the p47phox PX domain (14). We assessed the binding of soluble C₄₆-PtdSer or C₆₆-PA to the p47phox PX domain by NMR chemical shift perturbation experiments. The addition of 0.5 mM PtdSer did not induce any chemical shift perturbations of the resonances from the p47phox PX domain (Fig. 4A). However, the NMR signals from the p47phox PX domain were perturbed upon the addition of 0.5 mM C₆₆-PA (Fig. 4A). To examine whether C₆₆-PA binds to the PI(3,4)P₂-interacting area of p47phox PX, determined by the TCS experiments, competition experiments were performed for the PA-p47phox PX domain and PI(3,4)P₂-p47phox PX domain interactions. As a result, the chemical shifts of the resonances from the 0.2 mM p47phox PX domain with both 0.3 mM C₄₆-PI(3,4)P₂ and 0.6 mM C₆₆-PA were similar to those with 0.3 mM C₄₆-PI(3,4)P₂ (Fig. 4B) but different from those with 0.6 mM C₆₆-PA (Fig. 4E), suggesting that PA and soluble C₄₆-PI(3,4)P₂ competitively bind to the p47phox PX domain.

To obtain further evidence, the p47phox PX was titrated with phosphate buffer. At a low concentration, the phosphates induced chemical shift changes in the same area on the surface of p47phox PX as C₄₆-PI(3,4)P₂. In contrast, Thr-45, a residue that coordinates the sulfate outside the identified phosphoinositide-interacting interface, showed a strong chemical shift change only at high phosphate concentrations, suggesting the lack of a specific interaction (supplemental Fig. S7).

Mutational Analyses to Reveal the Residues Important for Phosphoinositide Binding—To confirm the results of the NMR experiments, a series of p47phox PX mutants were prepared, and their respective affinities for soluble C₄₆-PI(3,4)P₂ were assessed by NMR titration experiments (Fig. 5 and Table 1). The obtained spectra and titration curves of the alanine mutants of Arg-43 and Thr-45, which formed hydrogen bonds with the sulfate outside the identified phosphoinositide interacting area of p47phox PX in the crystal structure (Fig. 5A), were similar to those of the wild type (Fig. 5B, supplemental Fig. S4B, and Table 1). In contrast, the mutations inside the phosphoinositide interacting area, as determined by the TCS experiments (R70K, H51Q, and H74A), reduced the affinity. Among them, the resonances from the K55A mutant were not perturbed upon the addition of soluble C₄₆-PI(3,4)P₂ (Fig. 5C and Table 1).

Moreover, to examine whether the K55A mutant also abolishes PI(3,4)P₂ binding in the presence of a membrane, a CPM-based fluorescence assay was conducted. Although the fluorescence of the CPM-labeled T45A mutant was increased upon the addition of C₁₆-PI(3,4)P₂-containing liposomes (gray down triangle in supplemental Fig. S8), the K55A mutant was not perturbed (gray diamonds in supplemental Fig. S8). The fluorescence of another mutant of Lys-55, K55E, was also not affected by the addition of C₁₆-PI(3,4)P₂-containing liposomes (Table 2). The fluorescence intensities of K55A and K55E were also not perturbed upon the addition of liposomes containing C₁₆-PI3P, C₁₆-PI(4,5)P₂, and C₁₆-PI(3,5)P₂ (data not shown).

Role for Lys-55 of p47phox PX Domain in Phagocyte Oxidase Activation—We examined the role of Lys-55 in the activation of the NADPH oxidase in intact cells (Fig. 6). K562-gp91phox/p67phox cells, which lack p47phox but contain the other essential components of the phagocyte oxidase (40), were transfected with cDNA for the wild type and the K55A mutant of the HA-tagged p47phox and stimulated with phorbol 12-myristate 13-acetate. As a result, the amount of superoxide produced in cells that express the K55A mutant was ~40% less than that in cells that express the wild type. We confirmed that the expression level of p47phox, p67phox, and p22phox are similar in these cells. Thus, Lys-55 would be important for the oxidase activation.

T45R/P78K Mutations Restore the PI3P-binding Site of p40phox PX on p47phox PX—We showed that the Lys-55 mutant of p47phox PX is unable to interact with phosphoinositides. Guided by the structural similarity between p47phox PX and
p40phox, we attempted to establish a p47phox mutant that mimics the p40phox PX-like ligand-binding site and ligand specificity. In the crystal structure of p40phox PX in complex with

### TABLE 1

Values of the dissociation constants for the interaction of soluble C_{4-PI(3,4)P_2} with WT and mutants of p47phox PX domain, obtained from NMR titration curves

| Protein   | K\textsubscript{d} (\mu M) | \chi^2 |
|-----------|---------------------------|--------|
| WT        | 39 ± 12                   | 0.044  |
| T45A      | 37 ± 8                    | 0.073  |
| R43A      | 30 ± 10                   | 0.033  |
| H51Q      | 141 ± 27                  | 0.012  |
| R70K      | 85 ± 1                    | 0.002  |
| H74A      | 72 ± 15                   | 0.017  |
| K55A      | ND                        |        |

ND indicates not detectable.

### FIGURE 5

Mutational analysis of the interaction between p47phox and C\_4-PI(3,4)P_2. A, position of inserted mutations relative to bound sulfates in the crystal structure of p47phox PX. Arg-43 and Thr-45, which are in the typical binding site, are colored green, and His-51, His-55, Arg-70, and His-74, which are in the atypical binding site, are colored blue. Thr-45 and Arg-43 were hydrogen-bonded to the sulfate residing outside the PI(3,4)P_2-containing membrane-interacting interface. B and C, C\_4-PI(3,4)P_2-induced chemical shift perturbation on mutants of p47phox PX. Superimposed portions of 1H-15N HSQC spectra of [U-15N]p47phox PX with (red) and without C\_4-PI(3,4)P_2 (black) for R43A (B) and K55A (C) are shown. Arg-43 is located outside and Lys-55 is inside the PI(3,4)P_2-containing membrane-interacting interface. The full spectrum of R43A is shown in supplemental Fig. S4 B.

### FIGURE 6

Role for Lys-55 of p47phox in phagocyte oxidase activation. K562-gp91phox/p67phox cells were transfected with the cDNA for HA-tagged p47phox (WT) or p47phox (K55A). A, transfected cells (1.0 \times 10^6 cells/ml) were preincubated for 5 min at 37°C and stimulated with phorbol 12-myristate 13-acetate (PMA) (200 ng/ml). Chemiluminescence change by superoxide produced was continuously monitored with DIOGENED, as described under “Experimental Procedures.” The superoxide scavenger superoxide dismutase (SOD) (50 \mu g/ml) was added at the indicated time. B, each graph represents superoxide production estimated by chemiluminescence change (the average ± S.D.) from four independent transfections. Protein levels of HA-p47phox, p67phox, and p22phox were analyzed by immunoblot with the anti-HA, anti-p67phox, and anti-p22phox antibodies, respectively, as described under “Experimental Procedures.”

### TABLE 2

Values of dissociation constants for the interaction of liposome-embedded C\_16-PI(3,4)P_2 with WT and mutants of p47phox PX, obtained from the CPM-based fluorescence assay

| CPM-labeled protein | K\textsubscript{d} (\mu M) | \chi^2 |
|---------------------|---------------------------|--------|
| CPM-(21)            | 25 ± 3                    | 7.2 \times 10^{-4} |
| CPM-(21)T45A        | 38 ± 6                    | 22.9 \times 10^{-4} |
| CPM-(21)W80A        | 71 ± 11                   | 22.3 \times 10^{-4} |
| CPM-(21)K55E        | ND                        |        |
| CPM-(21)R90L        | 42 ± 6                    | 25.6 \times 10^{-4} |
| CPM-(65)            | 86 ± 30                   | 14.6 \times 10^{-4} |
| CPM-(65)T45A        | 66 ± 21                   | 42.1 \times 10^{-4} |
| CPM-(65)K55A        | ND                        |        |

ND indicates not detectable.
C₄⁺PI₃P, Arg-60 and Lys-92 of p40phox PX interact with the phosphates at position 3, 1, and 1 of C₄⁺PI₃P, respectively, and these residues are not conserved in p47phox PX. Therefore, we further mutated the K55E p47phox PX at residues Thr-45 (T45R) and Pro-78 (P78K). The T45R mutation aims to restore the role of Arg-60 in p40phox PX, and the P78K mutation aims to restore that of Lys-92 in p40phox PX (Fig. 7). The ligand-binding site and ligand specificity of the mutant were examined by NMR titration experiments with soluble C4-PI(3,4)P2 and C8-PI3P. As shown in Fig. 8, the resonances from a site that corresponds to the PI3P-binding site of p40phox PX were affected by the addition of C8-PI3P, whereas those corresponding to the phosphoinositide-binding site of the wild type p47phox PX, determined by the TCS experiments (Fig. 2D), were not affected. These results suggest that the T45R and P78K mutations restored the PI3P-binding site of p40phox PX on p47phox PX. Importantly, most of these resonances were also affected by the addition of C4-PI(3,4)P2 (Fig. 9 and supplementary Fig. S9), suggesting that the mutation could not restore the absolute PI3P specificity of p40phox PX.

**DISCUSSION**

NMR spectra provide structural information about proteins at an atomic resolution. The position of the NMR signal, chemical shift, is directly affected by the microenvironment. In NMR titration experiments with soluble ligands, the binding of a ligand causes the change of the microenvironment in the binding site. In addition, the ligand binding can induce conformational changes in the area located away from the binding site. Therefore, the chemical shift perturbation of the protein signals is useful for the detection of the binding site for the ligands and the site with the induced conformation change. The intensity reduction of the signal in the TCS experiment here indicates the membrane-interacting interface of the protein. Based on the results of the TCS experiment, the C₄⁺PI(3,4)P₂-containing membrane-interacting interface on the p47phox PX domain includes the residues from helix α1, helix α1', and the following loop up to the distorted left-handed PP₁ helix (Fig. 2, C and D). The mutational analyses verified the results of the TCS experiments and revealed that Lys-55 is indispensable for the phosphoinositide recognition (Fig. 5). Lys-55 is located inside the herein identified membrane-interacting interface and directly contacts the sulfate molecule in the crystal structure of p47phox PX.

**FIGURE 7. T45R/P78K/K55E mutant of p47phox PX.** Solution structure of free p47phox PX (left) and crystal structure of p40phox PX with bound PI3P (right) are shown. The structure of p47phox PX, only one of the two bound sulfates is shown. The Pro-78 and Thr-45 residues are shown in stick representations. Thr-45 and Pro-78 of p47phox PX correspond to Arg-60 and Lys-92 of p40phox PX. They were mutated to Lys-78 and Arg-45, respectively, in an attempt to restore the Lys-92 and Arg-58 of p40phox PX.

**FIGURE 8. Interaction of the T45R/P78K/K55E mutant of p47phox PX with C₄⁺PI3P.** A, HSQC spectrum of T45R/P78K/K55E mutant of p47phox PX before (black) and after (red) the addition of C₄⁺PI3P. Representative residues from the typical and atypical binding site are colored green and blue, respectively. B, chemical shift perturbation profile of the T45R/P78K/K55E mutant of p47phox PX caused by C₄⁺PI3P. Normalized chemical shift changes and their errors were calculated with the same formula as in Fig. 3, but the color code is different. Residues that showed strong (|Δδ| >1.2 ppm) normalized chemical shift changes are colored orange, and the broadened out residues are colored red. Note that the scale of y axis is different. The chemical shift perturbation profile is similar at critical points with a reported one for the Vam7p PX (49) in conformity with the presence of a typical binding site in both proteins. C, affected signals are mapped on the crystal structure of p47phox PX. Signals broadened beyond detection and broadened and shifted signals are colored red and orange, respectively.
Binding of Liposome-embedded PI(3,4)P$_2$ on p47$^{\text{phox}}$ PX Domain

The determined interface differs from those of all of the PX domains elucidated so far, including the p40$^{\text{phox}}$ PX domain. Whereas the loss of the phosphoinositide binding activity of p47$^{\text{phox}}$ PX by the K55E and R70K mutations (Fig. 5 and Tables 1 and 2) suggests that Lys-55 and Arg-70 are the key residues for phosphoinositide binding, these residues are not conserved in the other PX domains. Therefore, Lys-55 and Arg-70 would be responsible for the unique phosphoinositide-binding site of p47$^{\text{phox}}$ PX.

The chemical shift perturbation area on p47$^{\text{phox}}$ PX, caused by the addition of C$_4$-PI(3,4)P$_2$, is larger than the phosphoinositide-binding site determined by the TCS experiments and includes residues in the site that corresponds to the PI3P-binding site of p40$^{\text{phox}}$ PX (Fig. 3, A and B). Considering that chemical shift perturbation is induced by both the direct binding effects and the conformational change (45), the perturbation of the residues in the site that corresponds to the PI3P-binding site of p40$^{\text{phox}}$ PX would be a secondary effect, due to a conformational change. The absence of the perturbation on the segment of the $\beta$ sheet comprising the C terminus of $\beta$1 and the N terminus of the $\beta$2 strands, which also corresponds to the PI3P-binding site of p40$^{\text{phox}}$ PX and was perturbed in the titration experiments on p40$^{\text{phox}}$ and Vam7p (supplemental Fig. S5), supports the conclusion. The phosphoinositide-binding site and the site that corresponds to the PI3P-binding site of p40$^{\text{phox}}$ PX are adjacent, when projected not only on the tertiary structure but also on the secondary structure of p47$^{\text{phox}}$ PX, because they are partly formed by the same structural features, including the $\alpha$1 helix and the long loop that connects helices $\alpha$1 and $\alpha$2. Therefore, a conformational change would easily propagate from one site to the other.

However, in the T45R/P78K/K55E mutant of p47$^{\text{phox}}$ PX, the residues that correspond to the phosphoinositide-binding site on p47$^{\text{phox}}$ PX were not perturbed, and the segment of the $\beta$ sheet composed of the C terminus of $\beta$1 and the N terminus of the $\beta$2 strands was strongly perturbed (Figs. 8 and 9 and supplemental Fig. S9). Therefore, the T45R/P78K/K55E mutant of p47$^{\text{phox}}$ PX would assume the p40$^{\text{phox}}$-like phosphoinositide-binding site, whereas the phosphoinositide-binding site of the wild type p47$^{\text{phox}}$ PX differs from that of p40$^{\text{phox}}$ PX.

These results suggest that p47$^{\text{phox}}$ PX is unable to accommodate a phosphoinositide in the area corresponding to that in p40$^{\text{phox}}$ PX, because the critical residues, such as Arg-60 and Lys-92 of p40$^{\text{phox}}$ PX, which correspond to Thr-45 and Pro-78 of p47$^{\text{phox}}$ PX, are absent (Fig. 7). We should point out that the P78K mutation not only restored the essential role of Lys-92 in p40$^{\text{phox}}$ PX but also removed the residue Pro-78 that occupies valuable space inside the ligand binding pocket. This might contribute considerably to restoring binding capability (Figs. 8 and 9 and supplemental Fig. S9). It could also explain why the adjacent residue Lys-79, which in the solution structure of p47$^{\text{phox}}$ PX points away from the binding pocket (Fig. 7), cannot assume the role of Lys-92 in p40$^{\text{phox}}$ PX. Therefore, such small but critical variations inside and outside the typical phosphoinositide binding pocket may cause the differences in the ligand-binding sites of these two PX domains.

Interestingly, our results can provide a rationale for the in vivo behaviors of two mutants, those of Pro-73 and Arg-70, that are within the presently identified C$_{16}$-PI(3,4)P$_2$-containing membrane-interacting interface. It has been reported that the mutation Pro-73 abrogates phosphoinositide recognition and interferes with the in vitro activity of NADPH oxidase (46), while the mutation Arg-70 prevents membrane translocation in HEK293 cells (47).

Our experiments revealed that PA and soluble C$_4$-PI(3,4)P$_2$ competively bind to the p47$^{\text{phox}}$ domain (Fig. 4). Therefore, the atypical ligand-binding site on p47$^{\text{phox}}$ recognizes both PA and PI(3,4)P$_2$. This low specificity is in stark contrast with the stringent PI3P specificity of the typical binding site on p40$^{\text{phox}}$. 
Although at this point the physiological meaning of an atypical phosphoinositide-binding site on the p47\textsuperscript{phox} PX domain is unclear, the role of this atypical binding site should be important for the function of phagocyte NAPDH oxidase because mutation of Lys-55, a key residue of this binding site, significantly impedes normal production of superoxides (Fig. 6). In the same biological system, the phagocyte NAPDH oxidase, the two PX domains, p47\textsuperscript{phox} and p40\textsuperscript{phox} PXs, differ in the locations of the binding sites and in the affinities and selectivities toward phosphoinositides. These different structural properties may hint at distinct functional roles. It has been shown that in the assembled active NAPDH oxidase complex, p40\textsuperscript{phox} PX remains continually bound on membrane-embedded PI3P during reactive oxygen species production (48). We speculate that p47\textsuperscript{phox} PX is not. As the phagosome matures and PI(3,4)P\textsubscript{2} gives space to PI3P, p47\textsuperscript{phox} PX may be released from the membrane and reorient itself, relative to the other components of the active enzymatic complex. In this context, the shallowness of the atypical binding site, with its few binding determinants, may be a key structural feature that enables p47\textsuperscript{phox} PX to interact with multiple phosphoinositide signals, depending on the stage of phagocyte NAPDH oxidase assembly and/or activation.

It is worth mentioning that residues corresponding to His-51, Lys-55, and Arg-70 (that contribute to the formation of the atypical binding site) and to Thr-45 and Pro-78 that contribute to the collapse of the typical binding site of human p47\textsuperscript{phox} PX domain are highly conserved among different species. They are identical among organisms like human, bovine, pig, dog, and mouse. Thus, the atypical phosphoinositide-binding site does not appear to be an exclusive characteristic of the human p47\textsuperscript{phox} PX domain.

Based on the dodecyl-phosphocholine titration experiments of the Vam7 PX domain, Cheever et al. (49) proposed that the long loop between helices α1 and α2 functions as a membrane insertion loop. Importantly, the determined binding interface of p47\textsuperscript{phox} PX does not include the speculated membrane insertion loop in the 77–86-residue region (14). Because the membrane insertion loop can affect the binding affinity, its existence was examined further. It has been shown that the fluorescence of IAEDANS, covalently attached to membrane insertion loops, increases upon membrane insertion (50). The residues at positions 21 and 80, which are located outside and inside the speculated membrane insertion loop region, respectively, were substituted with cysteines and labeled with IAEDANS. Both of the IAEDANS-labeled p47\textsuperscript{phox} PX domains showed similar and only weak fluorescence increases upon the addition of the PI(3,4)P\textsubscript{2}-containing liposomes, and the magnitude of the fluorescence increase was similar to that induced by control liposomes without PI(3,4)P\textsubscript{2} (supplemental Fig. S10). In addition, the dodecyl-phosphocholine titration experiments with and without soluble C\textsubscript{3}-PI(3,4)P\textsubscript{2} did not reveal an increased propensity of the above region for membrane interactions (data not shown). Based on these results, we conclude that the 77–86-residue region of p47\textsuperscript{phox} PX is not a membrane insertion loop. The 77–86-residue region of p47\textsuperscript{phox} PX is much shorter and protrudes less than the membrane insertion loop on the Vam7p PX domain (Protein Data Bank code 1KMD). This area of p47\textsuperscript{phox} PX may nonspecifically contact with the lipid bilayer, as the protein tries to accommodate the polar head of its ligand, but it does not function as a specific enhancer of membrane binding. Herein, the K\textsubscript{s} value for the interaction of the p47\textsuperscript{phox} PX domain with liposome-embedded PI(3,4)P\textsubscript{2} was estimated to be 24 μM (fluorescence assay), which is comparable with the K\textsubscript{s} of 38.7 μM (Table 1) for the soluble C\textsubscript{3}-PI(3,4)P\textsubscript{2} (NMR titration assay). The similarity of the above values is in agreement with the absence of a membrane insertion loop that could enhance the membrane docking. Glu-49 and Tyr-48, which were affected by the irradiation in the present TCS experiments, are spatially separated from the other affected residues (Fig. 2D). These residues might be important for a productive interaction with the lipid bilayer.

The weak affinity of p47\textsuperscript{phox} PX for membrane-embedded PI(3,4)P\textsubscript{2} is not necessarily incompatible with accurate membrane targeting. The combination of phosphoinositide recognition by the PX domain and protein-protein interactions involving other domains (such as the interaction of the two tandem SH3 domains with the proline-rich area of the integral membrane protein p22\textsuperscript{phox}) can provide for accurate membrane targeting of the full-length p47\textsuperscript{phox}. Such cooperation between phosphoinositide recognition and protein-protein interactions for membrane targeting has already been demonstrated for other proteins, including Vam7p (51). In the case of p47\textsuperscript{phox}, it appears that protein-protein interactions are the primary mechanism of membrane targeting, because PX-truncated p47\textsuperscript{phox} is still able to find its way to the phagosomal membrane (10). However, membrane targeting of isolated p47\textsuperscript{phox} PX domain in intact cells has not been reported and based on our unpublished observations, isolated p47\textsuperscript{phox} PX domain invariably fails in membrane localization. This conforms to its herein demonstrated low affinity for phosphoinositides.

The different patterns of phosphoinositide recognition between p47\textsuperscript{phox} PX and p40\textsuperscript{phox} PX, despite their similar overall structures, underscore how the substitution of the key residues on the surface of the PX domain can significantly alter its function. The functional alterations can be dramatic. The case of the PX domains from phospholipase D1 and D2, which were identified as a GAP protein and an interacting partner for Dynamin (52), is one of the first reports that challenged the conventional role of PX as merely a membrane interacting domain.

In conclusion, we performed TCS experiments that led to the correct identification of the interacting interface of membrane-embedded PI(3,4)P\textsubscript{2} on the p47\textsuperscript{phox} PX domain. In similar applications, the TCS experiments could provide insights into the phosphoinositide recognition modes of various phosphoinositide-effector proteins, such as the PX, pleckstrin homology, ENTH, and C2 domains.

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