The neutrophil NADPH oxidase produces superoxide anions in response to infection. This reaction is activated by association of cytosolic factors, p47\textsuperscript{phox} and p67\textsuperscript{phox}, and a small G protein, Rac with the membrane flavocytochrome b\textsubscript{558}. Another cytosolic factor, p40\textsuperscript{phox}, is associated to the complex and is reported to play regulatory roles. Initiation of the NADPH oxidase activation cascade has been reported as consecutive to phosphorylation on serines 359/370 and 379 of the p47\textsuperscript{phox} C terminus. These serines surround a polyproline motif that can interact with the Src homology 3 (SH3) module of p40\textsuperscript{phox} (SH3\textsuperscript{p40}) or the C-terminal SH3 of p67\textsuperscript{phox} (C-SH3\textsuperscript{p67}). The latter one presents a higher affinity in the resting state for p47\textsuperscript{phox}. A change in SH3 binding preference following phosphorylation has been postulated earlier. Here we report the crystal structures of SH3\textsuperscript{p40} alone or in complex with a 12-residue proline-rich region of p47\textsuperscript{phox} at 1.46 Å resolution. Using intrinsic tryptophan fluorescence measurements, we compared the affinity of the strict polyproline motif and the whole C terminus peptide with both SH3\textsuperscript{p40} and C-SH3\textsuperscript{p67}. These data reveal that SH3\textsuperscript{p40} can interact with a consensus polyproline motif but also with a noncanonical motif of the p47\textsuperscript{phox} C terminus. The electrostatic surfaces of both SH3 are very different, and therefore the binding preference for C-SH3\textsuperscript{p67} can be attributed to the polyproline motif recognition and particularly to the Arg-368-G\textsuperscript{p47} binding mode. The noncanonical motif contributes equally to interaction with both SH3. The influence of serine phosphorylation on residues 359/370 and 379 on the affinity for both SH3 domains has been checked. We conclude that contrarily to previous suggestions, phosphorylation of Ser-359/370 does not modify the SH3 binding affinity for both SH3, whereas phosphorylation of Ser-379 has a destabilizing effect on both interactions. Other mechanisms than a phosphorylation induced switch between the two SH3 must therefore take place for NADPH oxidase activation cascade to start.

The neutrophil NADPH oxidase is a central component of the nonspecific host resistance against microbial infection. The critical role of this enzyme is illustrated by a genetic disease, chronic granulomatous disease (CGD),\textsuperscript{1} in which mutations in genes coding for NADPH oxidase components impair the ability of the patient to fight against infection. The NADPH oxidase is composed of several proteins, a membrane-bound heterodimeric flavocytochrome b (p91\textsuperscript{phox} and p22\textsuperscript{phox}), cytosolic proteins (p40\textsuperscript{phox}, p47\textsuperscript{phox}, p67\textsuperscript{phox}), and a small GTP-binding protein (Rac1 or Rac2) (for review, see Ref. 1). Upon exposure to specific stimuli, activation of the enzyme takes place through multiple phosphorylation events of the cytosolic components (2–5). Phosphorylations rearrange the protein-protein interactions among the cytosolic proteins ending with their translocation, simultaneously with Rac-GTP, to the membrane embedded flavocytochrome b. The resulting activated complex catalyzes the electron transfer from NADPH to O\textsubscript{2}, leading to the production of superoxide anions, thus generating highly reactive oxygen species (ROS) in the phagocytic vacuole. For many years these ROS have been designated as the main actors in the neutrophil killing activity. Recent work suggests that in addition to its role in ROS formation, O\textsubscript{2} generation leads to phagosomal Cathepsin G and Elastase mobilization in the microbicidal process (6).

The three cytosolic proteins p47\textsuperscript{phox}, p67\textsuperscript{phox}, and p40\textsuperscript{phox} are modular proteins formed by various domains, such as SH3, PX, PB1, and TPR, usually found in signal transduction cascades. Numerous possible interactions within these proteins have been reported and are summarized in Fig. 1A. p47\textsuperscript{phox} and p67\textsuperscript{phox} are known to be essential for activation. In CGD patient lacking p47\textsuperscript{phox}, p67\textsuperscript{phox} is not translocated to the membrane. In contrast p67\textsuperscript{phox} is not essential to p47\textsuperscript{phox} translocation (7). In the activation process, p47\textsuperscript{phox} is the sensor of the activation signal through multisite phosphorylations and secondly the scaffolding protein conducting translocation of the other cytosolic factors to the membrane component of the oxidase. p67\textsuperscript{phox} is an activating factor in the electron transfer reaction (8). In contrast to p47\textsuperscript{phox} and p67\textsuperscript{phox}, nowadays no CGD related to p40\textsuperscript{phox} is known. p40\textsuperscript{phox} was first identified as a p67\textsuperscript{phox}-associated protein in resting neutrophils (9–11). p40\textsuperscript{phox} translocates to the membrane upon stimulation in a p47\textsuperscript{phox}-dependent manner (12). The protein comprises the fol-
lowing domains, PX, SH3, and PB1 (Fig. 1A) (13). These modules confer to p40phox the ability to interact respectively with phosphatidylinositol 3-phosphate (14–16), p47phox C terminus (17–19), and p67phox PB1 domain (20). The physiological role of p40phox is still a matter of debate. It has been postulated to modulate superoxide production, either positively (21–23) or negatively (18, 24–26). In the last couple of years, an increasing body of structural and biochemical data shed light on the organization of the cytosolic complex in the resting state and on some events consecutive to p47phox phosphorylation. In the resting state p47phox, p67phox, and p40phox are thought to exist as a 1:1:1 ternary complex (27) as shown with purified proteins. Although p40phox and p67phox are both able to interact with the C-terminal polyproline region of p47phox, the p47phox/p67phox complex is favored (19, 27). A ternary complex can be achieved by the association of p40phox with p67phox (28) through their respective PB1 domain (20, 27–29) (Fig. 1B). However, it has been recently shown (30) that, in vivo, p47phox is dissociated from the p67phox/p40phox complex, as suggested previously (31).

Activation initiated by the binding of chaotropic substances to neutrophil receptors leads, through signaling pathways, to primary phosphorylation of Ser-359 and/or Ser-370 of p47phox (5). Additional phosphorylations can occur among the seven other C-terminal serines of p47phox. Among them, Ser-303, Ser-328, and Ser-379 are thought to exist through their respective PB1 domain (28) through their C-terminal polyproline region of p47phox (27). At the present stage, several structures of individual domains or of complexes made by two domains are available (16, 29, 34–36, 39–41). It has been proposed that the phosphorylation of p47phox could modulate its interaction with p67phox or p40phox, respectively (17, 24, 27). To understand the structural rearrangements occurring upon activation, structural information on the effect of nonphosphorylated and phosphorylated serines is needed. We addressed this question, characterizing at a molecular level the interaction of the SH3 domain of p40phox (SH3390) with the polyproline region of p47phox.

We report the three-dimensional structure of SH3390 with and without the C-terminal polyproline of p47phox at 1.46 and 2 Å resolution, respectively. Comparison with the structure of the C-terminal SH3 domain of p67phox (SH3270) complexed to the p47phox C terminus (41) highlights a strong difference between both SH3 domains. We compare the dissociation constants of both SH3390 and C-SH3270 with polyproline peptides by fluorometric techniques. The effect of phosphorylation on Ser-359, Ser-370, and Ser-379 on the binding of both SH3 toward p47phox is also investigated.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**

SH3390—A DNA fragment encoding the p40phox SH3 module, from Arg-174 to Lys-228, was amplified by PCR using the full-length p40phox sequence (31) as a template. The PCR fragment was cloned into the pCRscript Amp SK(+) cloning vector according to the manufacturer’s protocol (Stratagene). The resulting pCRscript-SH3phox plasmid was cleaved with NdeI and XhoI and the 168-base fragment containing the SH3phox sequence was subcloned in pIVEX 2.4 (Roche Applied Science). The resulting vector named pIVEX-SH3phox was transformed and amplified in Escherichia coli BL21(DE3) strain. The sequence of the construct was checked by sequencing.

The His-SH3390 was overexpressed as described for p40phox (19). After 3 h, cells were harvested and resuspended in chilled lysis buffer (20 mM Hepes, pH 7.5, 0.4 M NaCl supplemented with 40 mM imidazole and 5% glycerol). All of following operations were carried out at 4 °C. Cells were disrupted by sonication and centrifuged at 40,000 rpm for 40 min in a Beckman 45 Ti rotor. The supernatant was loaded at 2 ml/min on 4 ml of Ni2+–nitrilotriacetic acid column equilibrated in the previous buffer. Proteins were eluted with a 50-ml linear imidazole gradient (40–300 mM) at 2 ml/min. Fractions containing His-SH3390 were pooled, concentrated to 6 mg/ml, and subjected to overnight digestion at 20 °C with 2.4 units of factor Xa/mg of protein in the presence of 2.5 mM CaCl2. Digestion products were loaded at a flow rate of 1 ml/min onto the Hilo 16/60 Superdex 75 gel filtration column (Amersham Biosciences) equilibrated in 20 mM Hepes, pH 7.5, 150 mM NaCl. The
resulting protein fractions containing SH3p40 were concentrated to 14 mg/ml. The final recombinant protein comprised 5 N-terminus residues (LIKHM) from the previous linker, as determined by N-terminal sequencing.

C-SH3p40-The sequence encoding residues 455–516, the C-terminal SH3 domain of p67phox (C-SH3p67), was amplified by PCR and cloned into pET15b (Novagen). The sequence of the resulting vector named, pET-CSH3p67, was checked by sequencing. The protein was expressed in E. coli BL21(DE3) strain and first enriched by affinity chromatography on a Ni2+-nitrilotriacetic acid column equilibrated in 20 mM Hepes, pH 7.5, 150 mM NaCl, 20 mM imidazole and eluted with a linear gradient of imidazole (20–300 mM). The His tag was cleaved with thrombin (Sigma) overnight at 20 °C with 2.4 units thrombin/mg of protein, and further purification was carried out by gel filtration on Superdex 75 (Amersham Biosciences) equilibrated with 150 mM NaCl, 20 mM Hepes, pH 7.5, 2 mM dithiothreitol.

Proline-rich region of p47phox

The peptide KPQAPVPRPPSD (residues 360–372 of p47phox) was synthesized using conventional technology. The peptide was purified by HPLC using a C18 column and a linear gradient of 5–60% CH3CN in water with 0.1% trifluoroacetic acid. The peptide was lyophilized prior to resuspension in water (50 mg/ml) for crystallization. Peptide concentration was determined by amino acids analysis. The six other peptides were synthesized by Neosystem (Table II). They were repurified by HPLC using a C18 column and a linear gradient of 5–60% CH3CN in water with 0.1% trifluoroacetic acid. The purity of the peptides was higher than 95%.

Tryptophan Fluorescence Spectroscopy

Fluorescence measurements were performed at 20 °C in a Fluoromax (Jobin-Yvon) spectrofluorometer. The excitation wavelength was set at 290 nm, and the fluorescence emission was recorded between 295 and 450 nm. Increasing amounts of peptide were added to a fluorescence cuvette containing 100 μl of a SH3p40 solution. The resulting shift of maximal emission wavelength (Δλ) was monitored over a large range of p47phox peptide concentration. The equilibrium dissociation constant (Kd value) characterizing the SH3/polyproline interaction was determined according to Equation 1 under the assumption that the fluorescence shift Δλ was proportional to the concentration of the SH3/polyproline complex.

\[ K_d = \left( \frac{\Delta \lambda}{\Delta \lambda_{\text{max}}} - 1 \right) \left( \frac{\Delta \lambda_{\text{max}}}{\lambda_{\text{max}}} - 1 \right) \]  

(1)

where [SH3] = total SH3p40 on the surface, [pept] = total peptide concentration, [cpx] = complex concentration, \( \Delta \lambda = \lambda_{\text{SH3}} - \lambda_{\text{pept}} \) and \( \Delta \lambda_{\text{max}} = \lambda_{\text{SH3}} - \lambda_{\text{pept}} \).

Crystallization

Crystals were grown by hanging-drop vapor diffusion by mixing equal volumes of protein and reservoir solutions. The best diffracting crystals of free SH3p40 were obtained at 14 °C from a protein solution at 14 mg/ml in 20 mM Hepes, pH 7.5, 150 mM NaCl, and a reservoir solution containing 200 mM sodium acetate, pH 4.6, 100 mM ammonium sulfate, 40% polyethylene glycol monomethyl ether 2000. Crystals grew up within 2 months. Crystals of SH3p40/polyPro47 were grown at 20 °C with a reservoir containing 100 mM sodium citrate/citric acid, pH 5, 2.4 mM ammonium sulfate. To obtain the complex, SH3p40 at 14 mg/ml in 20 mM Hepes, pH 7.5, and 150 mM NaCl was mixed at 1:5 molar ratio to the polyproline peptide solution (50 mg/ml in water). This ratio was determined from the dissociation constant extrapolated from fluorescence measurements and corresponds to the presence of roughly 90% complexed SH3p40/polyPro47. Crystals were obtained within two or 3 weeks. Their size and quality were improved by macro-seeding with a reservoir solution containing 100 mM citrate/citric acid, pH 5, and 2.2 mM ammonium sulfate to a final size of 200 × 50 × 20 μm.

Prior to x-ray diffraction experiments crystals were flash frozen in liquid nitrogen. Free SH3p40 crystals were frozen directly from the mother liquor and SH3p40/polyPro47 crystals were soaked in a cryo-buffer (reservoir solution containing 15% glycerol) prior to freezing.

Data Collection, Structure Determination, and Refinement

Free SH3p40-Free SH3p40 crystals cooled at 100 K diffracted to 2 Å on a rotating anode (Nonius) using a MARCCD detector (Mar 345, X-ray Research). Crystals diffracted anisotropically and were highly mosaic; several crystals were screened to obtain the best diffracting crystal. Data were processed with DENZO and SCALEPACK (42) and reduced with the CCP4 package (43). Statistics are shown in Table I. The space group is pseudo-orthorhombic and P2₁2₁2₁ values are 11.5 and 10.8% in C222, or P2₁, space groups, respectively. The structure of SH3p40 was solved by molecular replacement using the program AMORE (44). The search model was a superposition of eight SH3 structures: SEM (PDB code ISEM), spectrin (PDB code 1SHG), tyrosine kinase Abl (PDB code 1ABQ), tyrosine kinase Fyn (PDB code 1FYN), Ephs8 (PDB code 1I0C), Crk (PDB code 1B07), amphiphysin 2 (PDB code 1BB9), Hck (PDB code 1BU1), restricted to main chain atoms. These SH3 domains share 38, 33, 15, 20, 22, 24, 22, and 18% sequence identity with SH3p40, respectively. Molecular replacement using each structure individually as a starting model failed. The model of SH3p40 was refined using CNS (45), and 5% of the data (randomly selected reflections) was excluded from refinement as free R-flagged reflections. The refinement was initially tried in both space groups C222, and P2₁; the Rfree values clearly excluded the orthorhombic form. The asymmetric unit contains two SH3p40 domains, which were refined by constraining the core of the monomers (i.e. β-strands excluding the loops) with a 2-fold symmetry. SH3p40/PolyPro47-The crystals flash frozen to 100 K diffracted poorly only showing elongated spots to 3.5 Å and a very high mosaicity.
that prevented data integration, despite the fact that no crystalline ice was formed in the liquid surrounding the crystal. To improve the diffraction, the crystal temperature was raised by blocking the cryostream for a few seconds and rapidly dropped back to 100 K. After a few cycles of annealing, crystals cooled to 100 K diffraeted to high resolution. A complete data set to 1.46 Å was collected on beamline EM30A, European Synchrotron Radiation Facility-Grenoble, using a Mar CCD detector and a wavelength of 0.98 Å. The same programs as for the free SH3p40 were used for data treatment; statistics are shown in Table I. The space group is $P2_12_12_1$ with a unit cell of $a = 39.6$ Å, $b = 50.5$ Å, $c = 68.2$ Å. Free SH3p40 was used as a search model to solve the structure of SH3p40/polyProp47, similar with root mean square deviations on main chain atoms of 0.06 and 0.3 Å for SH3p40 and SH3p40/polyProp47, respectively.

The free SH3p40 structure comprises residues 174–228 for both monomers present in the asymmetric unit and 61 water molecules. The SH3p40/polyPro47 structure consists of residues 169–228 for both SH3p40 (chains A and B) and of residues 360 to 372 (chain C) and 360–369 (chain D) for the polyPro47 peptides associated to monomers A and B, respectively. PolyPro47 peptides are acetylated in their N-terminal end. In addition to the protein, four sulfate ions, two trifluoroacetic acid molecules and 227 water molecules per asymmetric unit were located from the electron density maps and refined. The two SH3p40 structures, free or in complex with polyPro47, are very similar even in the peptide binding site. Because of the much higher resolution of SH3p40/polyPro47 crystals, this structure is discussed below.

The general topology of SH3p40 is very similar to that of other SH3 domains (50–53) and consists of five $\beta$-strands arranged in a $\beta$-barrel (Fig. 2A). The binding site located at the surface of the barrel is flanked by the RT and n-Src loops. These loops are variable among SH3 domains and contribute to the specificity of polyproline binding.

**Polyproline Binding Site**—Residues 360–369 of polyPro47 (360p47 to 369p47) are well determined in both molecules present in the asymmetric unit. They adopt a polyproline helix of type II (PPII) conformation with three residues per turn. The peptide is located in a groove formed by hydrophobic residues highly conserved among SH3 domains and negative electrostatic patches on the SH3p40 surface (Figs. 2A and 3A). Residues 360p47 to 369p47 interact with SH3p40 mainly via van der Waals contacts involving Phe-179p40, Asn-184p40, Asp-206p40, Trp-207p40, Pro-220p40, and Phe-223p40 located in the hydrophobic pocket of SH3p40 (Fig. 4). A few hydrogen bonds are also involved, in particular between the indole nitrogen of...
FIG. 3. Comparison of SH3p40/polyPro47 and C-SH3p67/p47phox-Cter. The C-SH3p67/p47phox-Cter structure shown in the figure is the most representative NMR structure (PDB code 1k4u). In A and B the electrostatic potential surfaces are compared. Positive and negative surfaces are shown in blue and red, respectively. A, SH3p40. The surface shows a negative patch in the vicinity of Glu-188p40 interacting with Arg-368p47.
Trp-207p40 and the carbonyl oxygen of Pro-367p47 or between the N/H of Gln-362p47 and the carbonyl oxygen of Asp-180p40. Arg-368p47 seems to play a key structural role in the complex as it is involved in several interactions: an electrostatic bonding with Glu-188p40 located in the RT loop of SH3p40 and van der Waals contacts involving the aliphatic side chain of Arg-368p47 and Trp-207p40 (Fig. 2B). Two water molecules are also involved in the peptide binding and bridge Ser-222p40 to Ala-364p47 and Asn-204p40 to Pro-367p47. As a result of the complex formation, Trp-207p40 is buried and hidden from the solvent in SH3p40/polyProp47 in contrast to the free SH3p40.

Comparison of SH3p40/PolyProp47 and C-SH3p67/p47phox-Cter—SH3p40 shares 44% sequence identity with C-SH3p67 (Fig. 3E), and the structures superimpose with root mean square deviations on main chain atoms of less then 1 Å. The structure of C-SH3p67 was solved by NMR in complex with the C-terminal end of p47 (residues 359–390, PDB code 1k4u) (41). Fig. 3 compares the electrostatic surfaces of both SH3s showing the large negative surface for C-SH3p67 due to the presence of many acidic residues. In our structure the side chain of Arg-368p47 has a low B-factor indicative of a very well defined position in the complex, in line with a strong electrostatic interaction with Glu-188p40. Negatively charged surfaces are limited to Glu-188p40 located in the SH3p40 RT loop (Fig. 3, A and C). On the contrary, in C-SH3p67/p47phox-Cter (41), the side chain of Arg-368p47 has multiple conformations as seen from the various NMR structures. In contrast, other residues in the vicinity of Arg-368p47 presented only one side chain conformation. The C-SH3p67 RT loop contains up to five acidic amino acids responsible for a strong negatively charged surface and allowing multiple positioning of the Arg-368p47 side chain (Fig. 3, B and D). The superposition of both SH3 structures also highlights possible interactions of SH3p40 with the C-terminal end of p47phox beyond polyProp47 residues present in our structure. Previously some residues of C-SH3p67 were identified as being particularly important in the interaction (gray boxes in Figs. 3E and Fig. 5).

Several serine residues in p47phox-Cter have been shown to be critical in NADPH oxidase activation. Among the serine C-SH3p67. The surface is highly negative. The difference of Arg-368p47/polyProp47 in contrast to the free SH3p40.

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Several serine residues in p47phox-Cter have been shown to be critical in NADPH oxidase activation. Among the serine residues of C-SH3p67 involved in the p47phox-Cter interaction (41) are boxed. Moreover, gray boxes underline residues Ile-505p67 and Val-490p67 directly in contact with non-PXXP motif of p47phox-Cter. Residues are referred as identical (*), strongly similar (:), or weakly similar (.). Surfaces A and B were drawn with GRASP (66). C and D were drawn with PyMol.
residues studied herein, only Ser-370p47 is present in our structure. Its side chain points toward the solvent and is clearly not involved in the interaction with SH3p40. From 144u coordinates, the same observation is made for C-SH3p67. Ser-359p47 and Ser-379p47 are not present in our peptide, their interaction is extrapolated from the superposition of 144u coordinates with our structure. Ser-359p47 is located at the N-terminal end of the peptide, its location is close to Glu-480p47 equivalent to Ala-193p67. Ser-379p47 interacts with Glu-496p67 (conserved in SH3p67) and from our extrapolated structure is likely to interact also with SH3p40.

Interaction between p47phox and p40phox or p67phox Characterized by Fluorescence Measurements—We have measured the dissociation constants of a series of peptides corresponding to the PXXP motif (358–372) or the whole p47phox-Cter (358–390) with SH3p40 or the C-SH3p67 (Fig. 6 and Table II). Both types of peptides (short or long) are almost identical to the peptides used for the structural studies and therefore they will be denoted in the following text polyPro47 and p47phox-Cter, respectively. The entire proteins, p47phox and p40phox, interact with an estimated $K_d$ of 5 nM (19, 27). This value is 40 times lower than the $K_d$ between SH3p40 and polyPro47, suggesting that the interaction between the two proteins involves other regions in addition to the SH3/polyproline couple. The $K_d$ of p47phox-Cter toward SH3p40 is 380 times lower than that of PolyPro47 alone. Thus, our measurements show that the binding between SH3p40 and p47phox-Cter implicates both the PXXP motif and a non-PXXP region of p47phox-Cter. Interestingly, it corresponds to the same interacting regions for p47phox with C-SH3p67 as identified by Kami et al. (41). In addition, our $K_d$ value for the C-SH3p67/p47phox-Cter interaction (Table II) is consistent with their results. Although the same regions of p47phox seem to be involved in the interaction with both SH3, it is clear from Table II that the complex with C-SH3p67 is favored ($K_d$ ratio of 11, line 4 in Table II). The preference for C-SH3p67 is already observed for the short polyPro47 peptide ($K_d$ ratio of 20, line 1 in Table II), and the additional residues of p47phox-Cter do not modify significantly the difference in affinity toward SH3p40 and SH3p67 (comparison of line 1 and line 4 in Table II). Therefore, the contribution of the strict PXXP motif by itself induces the preference for p67phox. The difference in affinities of p47phox-Cter for both SH3 domains obtained from our fluorescence measurements is consistent with our structural data. Indeed, the comparison of the structures highlights a strong difference in the electrostatic potential surface between the two SH3 along the polyPro binding site that enhances the binding of polyPro47 to C-SH3p67, whereas only few differences are present among the interactions with the extra C-terminal residues.

Effect of Ser-359/Ser-370 or Ser-379 Phosphorylation on the Binding of p47phox to SH3p40 and C-SH3p67—Table III summarizes data from the literature on the specific role of each serine phosphorylation. We have investigated the role of the phospholysylatable serines surrounding polyPro47 on SH3 recognition. Various peptides have been synthesized, in which Ser-359p47 and Ser-370p47 are either substituted by aspartates or phosphorylated (Table II). Both modifications (mutation or phosphorylation), in either short or long peptide, do not affect significantly the binding to SH3p40. Therefore, as a first approximation, our observations validate the Ser-to-Asp mutation approach which has been widely used to mimic serine phosphorylations (4, 5, 32). The affinity of C-SH3p67 for short polyPro47 peptides is moderately reduced by aspartate mutations and slightly more by phosphorylations. Even in the absence of a direct interaction of Ser-359p47 and Ser-370p47 with C-SH3p67, the higher electronegative character of additional phosphates compared with Asp residues, added to the strong electronenegative surface of C-SH3p67, might explain this effect. However, even if phosphorylation reduces the $K_d$ ratios toward both SH3 from 20 to 5 (comparison of lines 1 and 3 in Table II), C-SH3p67 still has a stronger affinity than SH3p40. With p47phox-Cter, in a native form or with Ser-to-Asp mutations, no major differences are observed, probably because the increase of binding interface with the addition of the non-PXXP region of p47phox masks the small differences in affinity observed among the short peptides.

To address the question of the role of Ser-379p47 during activation, binding properties of p47phox-Cter, with Ser-379p47 phosphorylated or not, have been investigated. Upon phosphorylation, the $K_d$ ratios of the two peptides toward both SH3s increase by a factor of 5 and 30 for SH3p67 and SH3p40, respectively (comparison of lines 4 and 6 in Table II), denoting an important modification of the interaction between p47phox-Cter and both SH3.

We show herein that phosphorylations of Ser-359p47 and Ser-370p47 have no significant impact neither on the binding of p47phox-Cter to both SH3s nor in modifying the binding preference between SH3p67 and SH3p40. The side chain orientation of the two serines in the structures explains the low effect of the phosphorylation of Ser-359p47 and Ser-370p47; both side chains point away from the interaction. In contrast, the phosphorylation of Ser-379p47 destabilizes SH3p67/p47phox-Cter and C-SH3p67/p47phox-Cter with a stronger effect for SH3p40.

DISCUSSION

Structural Determinants of the Interaction between p47phox-Cter and SH3p40

Two possible SH3 interacting partners have been identified for the polyproline motif of the p47phox C terminus: SH3p40 and C-SH3p67 (Fig. 1A). Various models for the cytosolic complex in the resting state have been proposed, arguing for polyPro47 complex with either SH3p40 or C-SH3p67 (10, 12, 31). Several studies based on the respective affinities of both p40phox and p67phox for p47phox (19, 27, 54, 55) led to the accepted model of a trimeric organization (Fig. 1B) in which C-SH3p67 interacts with polyPro47, while p40phox and p67phox are held together via a PB1/PB1 interaction (27). Despite the importance of SH3-polyproline interactions in the NADPH oxidase complex, no high resolution structural information is available to explain the favored interactions occurring within the p47phox/p67phox/p40phox complex. Previous NMR-based structure of C-SH3p67/polyPro47 highlighted the contribution of the whole p47phox.
**TABLE II**

Dissociation constants comparison of p47\(^{phox}\) C-terminal peptides towards SH3\(^{p47}\) and C-SH3\(^{p40}\)

The dissociation constants of the various p47\(^{phox}\) C-terminal peptides were determined by tryptophan fluorescence as described under "Experimental Procedures". Standard deviations were calculated from fits to individual data sets.

| p47\(^{phox}\) peptides | SH3\(^{p47}\) | C-SH3\(^{p40}\) | K\(_d^{p47}/K_{d^{p40}}\) | p40\(^{phox}\) | p67\(^{phox}\) |
|--------------------------|--------------|---------------|------------------------|-------------|-----------|
| p47\(^{phox}\) (1–390)   |              |              |                        |             |           |
| PolyProp\(^{a}\) (358–372) |              |              |                        |             |           |
| 1. RSKKQPAYPRPSAD         | 200 ± 20     | 10 ± 0.7      | 20                     |             |           |
| 2. RDKKQPAYPRPSAD         | 270 ± 27     | 31 ± 1.9      | 8.7                    |             |           |
| 3. RSFKPOH\(_L\)KPQAPVPRPSAD | 268 ± 13   | 61 ± 4.3      | 4.4                    |             |           |
| p47\(^{phox}\)-Cter (358–390) |              |              |                        |             |           |
| 4. RSKKQPAYPRPSADLILRNCDESTKRKLASAV | 0.52 ± 0.11  | 0.047 ± 0.030 | 11                     |             |           |
| 5. RDKKQPAYPRPSADLILRNCDESTKRKLASAV | 1.17 ± 0.28  | 0.060 ± 0.030 | 20                     |             |           |
| 6. RSKKQPAYPRPSADLILRNCOSTKRKLASAV | 14.4 ± 0.60  | 0.290 ± 0.037 | 50                     |             |           |

\(^a\) From Ref. 19.  
\(^b\) From Ref. 27.

**TABLE III**

Review of effects of some mutations on p47\(^{phox}\) serines susceptible to phosphorylations

All results reported in this table come from *in vivo*, whole cell systems, using EBV transform p47\(^{phox}\)-deficient B cells expressing different forms of p47\(^{phox}\) except for Ref. 32 where K562 cell systems have been used with the same results.

| Serines | Mutants | Phosphorylations on other serines | Membrane transfer | NADPH oxidase activity | Ref. |
|---------|---------|----------------------------------|-------------------|------------------------|-----|
| Ser-303/304 | S303A/S304A | +                               | +                 | +                      | 4, 32 |
|          | S303E/S304E | +                               | +                 | +                      | 4   |
|          | S303D/S304D | +                               | +                 | +                      | 4   |
| Ser-326  | S228A    | ND\(^a\)                         | ND\(^a\)          | +                      | 3, 32 |
| Ser-359/370| S359A/S370A | –                               | –                 | –                      | 5, 3 |
|          | S359K/S370K | –                               | –                 | –                      | 5   |
|          | S359D/S370D | –                               | –                 | –                      | 5   |
|          | S359E/S370E | –                               | –                 | –                      | 5   |
|          | S379A    | +                               | +                 | +                      | 5   |
|          | S379E    | +/-                              | –                 | –                      | 33  |

\(^a\) ND, not determined.

C-terminal sequence, downstream the PXXP motif (41). Indeed, this additional non-PXXP sequence showed an extra interaction that results in high affinity and specificity. This suggested that the non-PXXP region can be responsible for stronger interactions with C-SH3\(^{p40}\) rather than SH3\(^{p47}\). To discuss the role of the non-PXXP region of p47\(^{phox}\) in SH3\(^{p47}\)/p47\(^{phox}\), we compared the binding properties of p47\(^{phox}\)-Cter to the SH3 modules of p67\(^{phox}\) and p40\(^{phox}\), based on fluorometric results and three-dimensional structure analysis. Fluorescence measurements reveal three major points: 1) as for C-SH3\(^{p67}\), SH3\(^{p40}\) is able to establish an additional interaction with the non-PXXP motif of p47\(^{phox}\)-Cter, 2) the stronger affinity for C-SH3\(^{p40}\) than for SH3\(^{p47}\) is due to the PXXP motif itself and more precisely the Arg-368p47 binding mode, and 3) the isolated SH3\(^{p40}\) module presents a higher affinity for p47\(^{phox}\)-Cter than the whole p40\(^{phox}\) protein for p47\(^{phox}\).

*p40\(^{phox}\) Interacts with a PXXP and a Non-PXXP Motif of p47\(^{phox}\)-Cter—SH3\(^{p40}\) exhibits a K\(_d\) of 0.52 \(\mu\)M for p47\(^{phox}\)-Cter, which is a high affinity compared with 10–50 \(\mu\)M for most SH3-peptide interactions. Using p47\(^{phox}\) peptides corresponding to the PXXP motif (residues 358–372) or corresponding to p47\(^{phox}\)-Cter (residues 358–390), we show that the C-terminal non-PXXP region is not responsible for the difference in affinities toward the two SH3 modules. Despite the fact that the C-terminal region allows in each case a much stronger affinity than with the PXXP motif alone, the specificity resides mainly within the PXXP motif recognition. Interaction of both SH3 with the non-PXXP motif of p47\(^{phox}\)-Cter seems to be identical, the residues of C-SH3\(^{p40}\) identified as part of the binding surface for this noncanonical interaction have been previously identified using NMR cross-saturation experiments (Fig. 3E) (41).

Stronger Affinity of p47\(^{phox}\)-Cter toward SH3\(^{p40}\) Is Explained by the Arg-368p47 Binding Mode—Interactions of polyPro\(^{p47}\) with both SH3 are mainly hydrophobic except for Arg-368p47. A strong difference in the electrostatic potential surface of the two SH3 may be responsible for the stronger binding of PXXP motif to C-SH3\(^{p40}\). Indeed, Arg-368p47 interacts only with Glu-188p40 in our structure, whereas it has multiple interacting mode with the RT loop of the C-SH3\(^{p40}\). Previous experiments have focused on the importance of Arg-368p47 in the binding strength (41): (i) replacement of this arginine by an alanine decreases affinity for C-SH3\(^{p40}\) by a factor of 625 and (ii) binding of the isolated non-PXXP motif of p47\(^{phox}\) (residue 369\(^{p47}\) to 390\(^{p40}\)) is observed only when Arg-368\(^{p47}\) is added to the peptide. A much stronger negatively charged RT loop in C-SH3\(^{p40}\) than in SH3\(^{p47}\) can account for a higher association of the two binding partners and/or a stronger efficiency in keeping the protein complexed.

*Modules Flanking SH3\(^{p40}\) Modulate Its Affinity for p47\(^{phox}\)—* Our fluorescence measurements reveal that the affinity between p47\(^{phox}\) and p67\(^{phox}\) is identical to the affinity between p47\(^{phox}\) peptides and C-SH3\(^{p40}\) domain (Table II). This confirms that the p47\(^{phox}\)/p67\(^{phox}\) complex is completely determined by C-SH3\(^{p40}\) and p47\(^{phox}\)-Cter sequences. On the contrary, strong difference in affinity between p47\(^{phox}\) and p40\(^{phox}\) on one side and between p47\(^{phox}\)-Cter and SH3\(^{p40}\) on the other side is observed (Table II). The presence of PX and PB1 domains on each side of SH3\(^{p40}\) lowers the affinity toward p47\(^{phox}\) by 1 order of magnitude. Thus, in p40\(^{phox}\), the binding properties of SH3\(^{p40}\) are tuned down by the flanking modules. Interestingly, Lopes et al. (26) have shown recently that phosphorylation of p40\(^{phox}\) on threonine 154 changes the conformation of the protein thus resulting in an inhibitory effect on NADPH oxidase. Thr-154\(^{p40}\) is located between the PX and the SH3 domains (26), and the authors proposed that, after its phosphorylation, SH3\(^{p40}\) could be exposed and compete with p67\(^{phox}\) for p47\(^{phox}\) binding. Moreover, Sathyamoorthy et al. (24) reported that...
that transient expression of the SH3domain in vitro inhibits more efficiently the NADPH oxidase than the full-length p40phox. Although the role of p40phox (inhibitory or stimulatory) is still a matter of debate, these observations are in line with our results of an increased affinity toward p47phox of SH3phox alone with respect to the whole p40phox. However, such an increase in affinity due to a possible exposure of SH3phox, is not sufficient to compete with the strength of the p67phox/p47phox interaction (Table II). Additional effects have to occur that might be related to additional phosphorylations on p67phox (56, 57), p40phox (58), and particularly p47phox, which is polyphosphorylated (2, 3).

**Phosphorylation on p47phox C Terminus; Consequences toward the SH3/PolyPro Interaction between Cytosolic Factors**

Phosphorylation on Ser-359/370 of p47phox Does Not Promote a Switch of p47phox Binding from C-SH3p67 to SH3p40.— Data from numerous studies delineating the phosphorylation of p47phox serine residues during the activation cascade are summarized in Table III. Globally they suggest (5) that Ser-359/370 are phosphorylated first, allowing subsequent phosphorylation of Ser-379 for an efficient membrane translocation and finally of Ser-303/304 to induce the activity of the complex. The effect of Ser-303/304 phosphorylation, in the activation mechanism, has been clearly unraveled and confirmed (4, 32, 36) and involves the phosphorylation of Ser-328 (32). Mutation of serines 359 and 370 to an alanines abrogated completely the subsequent steps in the activation mechanism from consecutive phosphorylation on other serines to NADPH oxidase activation. In a cellular context, replacement of serines by glutamate or aspartate residues restored the phosphorylation events on other serines of p47phox as well as membrane translocation of the cytosolic factors but not NADPH oxidase activity. Therefore prior phosphorylation of Ser-359/370 is necessary to allow subsequent phosphorylations and the activation cascade to take place (5). The presence of two phosphorylation sites, Ser-359/370, surrounding a polyproline motif crucial for the organization of the ternary complex suggested that phosphorylation may modify the interactions among the cytosolic factors during activation. Several groups suggested a possible switch from one SH3 to the other (24) during the phosphorylation process (17, 27, 58). At the molecular level, very little is known on the evolution of the interaction network between the cytosolic factors following phosphorylation. Until now studies have mainly focused on the phosphorylation consequences in the intramolecular organization of p47phox itself. In our work, Kd measurements of p47phox peptides, phosphorylated or not, reveal no significant modifications in the difference in affinity toward the isolated SH3 domains of p67phox and p40phox. Thus, the switch hypothesis of binding of p47phox from C-SH3p67 to SH3p40, as a direct effect of phosphorylations on SH3 affinities, can now be ruled out.

Recent work (30) has shown that in the resting state before any priming event, p47phox was dissociated from the ternary complex in the cytoplasm. The ternary complex was seen only in phorbol 12-myristate 13-acetate-activated neutrophils. These results suggest that in a cellular context, despite a possible strong interaction of p47phox with the p67phox/p40phox heterodimer as observed in vitro (27), phosphorylations are required for ternary complex formation. Here we show that such phosphorylations are fully compatible with the formation of p47phox/C-SH3phox complex, since they do not affect the dissociation constant. Thus, Ser-359/370 phosphorylation might promote the initial ternary complex formation in the neutrophil where the C terminus of p47phox may not be accessible due to the cellular environment in contrast to recombinant cytosolic factors in solution.

**Phosphorylation on Ser-379p47 Weakens Cytosolic Complex in Vitro**—Finally we investigated consequences of phosphorylation on Ser-379p47 previously reported as a potential site of kinase action for NADPH oxidase regulation (2, 33). The C-SH3phox/p47phox-Cter structure solved by Kami et al. (41) shows that Ser-379p47 is accessible and can contribute to the binding of the non-PXXP motif. Depending on the various NMR structures, Ser-379p47 can establish an hydrogen bond with Glu-496p67, one of the residues identified in the interaction interface (Figs. 3E and 5). As seen in Table II, phosphorylation of Ser-379p47 impairs the affinity of p47phox-Cter for both SH3. This can be interpreted as a steric effect but more probably as a repulsive charge effect between the phosphorylated Ser-379p47 and the neighboring residues Glu-496p67 or Glu-209p40. The consequence of this phosphate addition in p47phox-Cter is not SH3 specific and weakens the cytosolic factor complex. Ser-379p47 was the first Ser whose mutation to Ala, reported in Table II, impaired oxidase activation and membrane translocation (33). The loss of affinity may be surprising for a translocation mechanism needing a tightly associated complex to co-migrate as a whole to the membrane for NADPH oxidase activation. However, upon Ser-379p47 phosphorylation the modification of affinity toward SH3p67 is moderate, contrary to the change of affinity toward SH3p40. The only conclusion that can be drawn from our study is that Ser-379p47 phosphorylation will not promote SH3p40 binding. The clarification of the cellular role of this serine phosphorylation will have to await further studies.

In summary, the p47phox binding preference toward p67phox rather than p40phox is attributable to a negative contribution of SH3p40 neighboring modules and the difference in the p47phox polyPro binding properties is probably due to a strong difference in the negative net charge of the SH3 RT loops (Fig. 3). However, we show herein that SH3p40 is competent for p47phox binding with a strong affinity and specificity involving both PXXP and the non-PXXP motif of p47phox-Cter. The existence of a physiologic situation in the activation or regulation mechanism where a SH3p40/p47phox interaction takes place has not been evidenced so far. However, in light with the binding specificity of SH3p40 toward p47-Cter, the reported roles of p40phox in oxidase activity regulation could arise from a possible p40phox/p47phox interaction occurring during the various steps of the NADPH oxidase activation/deactivation mechanism. Moreover, we have shown clearly that the phosphorylation event on Ser-359/370 cannot initiate the activation cascade through modification of the SH3 binding properties and therefore the molecular mechanism involved here has still to be deciphered. Finally, we have shown that a phosphate on Ser-379 has a deleterious effect on the strength of interaction of p47phox with SH3p40 and C-SH3p67.

In addition to a better understanding of the molecular basis of the polyproline-SH3 interactions within the cytosolic phox complex, and a clarification of the phosphorylation consequence in the complexes formation and their stabilities, this work provides the high resolution structure of the last p40phox module not yet reported. Indeed, the crystal structures of PX and PB1 domains have been previously reported isolated (16) or in complex with p67phox PB1 (29). Thus, the whole structure of p40phox is now known as individual pieces. Deeper understanding in the structure-function relationship of p40phox will have to await three-dimensional positioning of the different modules with respect to each others. Moreover, a clear and definitive view of the kinetics and order of the phosphorylation events on p47phox but also on p40phox and p67phox will be es-
sential to solve the complete molecular puzzle of the activation cascade.

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