# Assembly of the neutrophil respiratory burst oxidase: A direct interaction between p67$^{PHOX}$ and cytochrome $b_{558}$ II

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**Activation of the phagocyte NADPH oxidase complex requires assembly of the cytosolic factors p47$^{PHOX}$, p67$^{PHOX}$, p40$^{PHOX}$, and Rac with the membrane-bound cytochrome $b_{558}$. We recently established a direct interaction between p67$^{PHOX}$ and cytochrome $b_{558}$. In the present study, we show that removal of the C-terminal domain of p67$^{PHOX}$ increased its binding to cytochrome $b_{558}$. Whereas phosphorylated p40$^{PHOX}$ alone did not bind to cytochrome $b_{558}$, phosphorylated p47$^{PHOX}$ did, and, moreover, it allowed the binding of p40$^{PHOX}$ to the cytochrome. Furthermore, both increased the binding of p67$^{PHOX}$ to the cytochrome. Phosphorylated p47$^{PHOX}$ thus appears to increase the binding of p67$^{PHOX}$ to cytochrome $b_{558}$ by serving as an adapter, bringing p67$^{PHOX}$ into proximity with cytochrome $b_{558}$, whereas phosphorylated p40$^{PHOX}$ may increase the binding by inducing a conformational change that allows p67$^{PHOX}$ to interact fully with cytochrome $b_{558}$.**

The NADPH oxidase of phagocytes plays a pivotal role in host defense against microbial infection. It catalyzes the reduction of oxygen to $O_2^-$ at the expense of NADPH. The $O_2^-$ is the precursor of potent oxidants used to kill the invading microorganisms (1–4).

The NADPH oxidase is composed of a number of cytosolic and membrane-bound proteins—in the cytosol, p67$^{PHOX}$, p47$^{PHOX}$, p40$^{PHOX}$, and Rac1/Rac2; in the membrane, gp91$^{PHOX}$ and p22$^{PHOX}$—which, together, comprise cytochrome $b_{558}$. When activation takes place, the cytosolic components migrate to the membranes, where they associate with the membrane-bound components to assemble the catalytically active oxidase (5). Cytochrome $b_{558}$, which contains both flavin and heme groups (6), likely is responsible for electron transfer from NADPH to oxygen upon activation of the cells, although it was demonstrated that p67$^{PHOX}$ also possesses an NADPH-binding site (7). P47$^{PHOX}$, which is phosphorylated extensively during the activation of the oxidase (8), probably initiates the assembly of the enzyme, whereas the function of p67$^{PHOX}$ appears to be to activate catalysis by cytochrome $b_{558}$, although the mechanism of activation is obscure (9). Rac appears to promote the interaction between p67$^{PHOX}$ and cytochrome $b_{558}$ (10).

During the NADPH oxidase activation process, a number of defined protein–protein binding interactions occur (11). For example, p47$^{PHOX}$ and p67$^{PHOX}$ each contain two SH3 domains, the C-terminal SH3 domain of p67$^{PHOX}$ interacts with p47$^{PHOX}$, and the N-terminal SH3 domain of p47$^{PHOX}$ is able to interact with p22$^{PHOX}$ (12).

In the present study, we established a direct interaction between p67$^{PHOX}$ and cytochrome $b_{558}$ (10) and showed that this interaction increased when the C-terminal region of the protein was removed and also when the whole protein was incubated in the presence of phosphorylated p47$^{PHOX}$ or phosphorylated p40$^{PHOX}$.

## Experimental Procedures

**Expression and Purification of Recombinant Proteins in *Escherichia coli*.** P67$^{PHOX}$ and its truncated forms were expressed in *E. coli* as glutathione *S*-transferase (GST) fusion proteins and purified with glutathione-Sepharose beads (13). The proteins then were separated from GST while on the beads by cleavage with PreScission protease (Amersham Pharmacia) or eluted from the beads by incubating for 20 min at 4°C with 1 ml of 50 mM Tris·HCl, pH 8/5 mM glutathione/150 mM NaCl. P47$^{PHOX}$ was expressed in *E. coli* as GST fusion proteins and purified with glutathione-Sepharose beads (Amersham Pharmacia) followed by thrombin cleavage when needed. P40$^{PHOX}$ cDNA in pET-32a plasmid (a kind gift of A. Fuchs, Centre National de la Recherche Scientifique, Grenoble, France) was transformed in BL21-DE3 (pLysS) *E. coli* strain and expressed as follows. An overnight culture was diluted 10-fold in fresh Terrific Broth medium containing 100 $\mu$g/ml ampicillin and grown for an additional hour at 37°C. The culture then was induced with 0.5 mM isopropyl $\beta$-D-thiogalactoside for 4 h at 25°C. Bacteria were harvested by centrifugation and lysed by sonication in 20 mM Hepes, pH 7.9/0.5 M NaCl/10 mM imidazole in the presence of 0.2 mM PMSF/100 $\mu$g/ml leupeptin/100 $\mu$g/ml pepstatin/0.5 mM diisopropyl fluorophosphate. The homogenate then was centrifuged at 150,000 $\times$ *g* for 15 min, and the supernatant was incubated with Probond resin (Invitrogen) for 1 h at 4°C. The resin then was packed into a gravity column, washed twice in 20 mM Hepes, pH 7.9/0.5 M NaCl/20 mM imidazole, and then again washed twice in 20 mM Hepes, pH 7.9/0.5 M NaCl/30 mM imidazole. The protein then was eluted with 150 mM imidazole and dialyzed against 20 mM Hepes, pH 7.9/0.1 mM DTT/2 mM EGTA. Protein concentrations were determined with the Bio-Rad Assay Kit with BSA as a standard.

**Contruction of Truncated p67$^{phox}$ Forms.** P67$^{PHOX}$ truncated forms were constructed as described by Dang *et al.* (13). Briefly, p67$^{PHOX}$ (1–243), p67$^{PHOX}$ (1–210), p67$^{PHOX}$ (1–199), and p67$^{PHOX}$ (244–526) were obtained by PCR with Pfu polymerase (Stratagene) and wild-type p67$^{PHOX}$ cDNA cloned into the vector pBK-CMV. The PCR products were ligated into the *Sma*I site of pGEX-6P1 for p67$^{PHOX}$ (1–243), *Bam*HI and *Eco*RI of pGEX-6P1 for p67$^{PHOX}$ (1–210) and p67$^{PHOX}$ (1–199), and *Eco*RI and *Xho*I sites of pGEX-6P3 for p67$^{PHOX}$ (244–526) and transformed into DH5$\alpha$ for expression of the proteins. The sequences all were confirmed by DNA sequencing in the Scripps Research Institute molecular biology facility.

**Purification of Cytochrome $b_{558}$.** Cytochrome $b_{558}$ was purified from human neutrophil plasma membranes by heparin and hydroxy-

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Abbreviation: GST, glutathione *S*-transferase.

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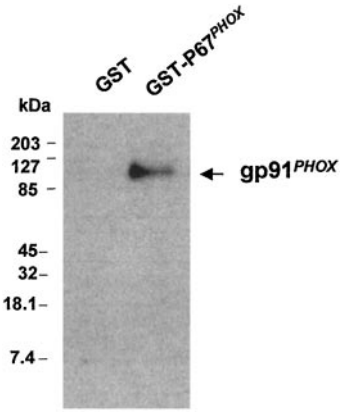



**Fig. 1.** Affinity precipitation of cytochrome $b_{558}$ with GST-p67$^{PHOX}$. Eighty pmol of GST or full-length GST-p67$^{PHOX}$ was incubated in the presence of 4.3 pmol of cytochrome $b_{558}$ in 200 $\mu$l of 20 mM Hepes, pH 7.5/1% Nonidet P-40/10 mM NaCl/1 mM EGTA for 2 h at room temperature. Then, 40 $\mu$l of glutathione-Sepharose 4B beads (Pharmacia) was added, followed by incubation for 2 h at 4°C. After washing five times with the same buffer, the beads were pelleted, resuspended in 40 $\mu$l of Laemmli sample buffer (19), and boiled for 5 min. Proteins were resolved by SDS/12% PAGE and transferred onto nitrocellulose. The proteins then were detected with a monoclonal anti-gp91$^{PHOX}$ antibody.

apatite chromatography and subsequently relipidated and reflavinated as described by Cross *et al.* (14).

**Affinity Precipitation.** For affinity precipitation, 80 pmol of GST, full-length GST-p67$^{PHOX}$ or truncated GST-p67$^{PHOX}$ (1–243), GST-p67$^{PHOX}$ (1–210), GST-p67$^{PHOX}$ (1–199), or GST-P67$^{PHOX}$ (244–526) was incubated in the presence of 4.3 pmol of cytochrome $b_{558}$ in 200 $\mu$l of 20 mM Hepes, pH 7.5/1% Nonidet P-40/10 mM NaCl/1 mM EGTA for 2 h at room temperature. In some assays, phosphorylated GST-p67$^{PHOX}$ was used instead. Then, 40 $\mu$l of glutathione-Sepharose 4B beads (Amersham Pharmacia) then was added followed by incubation for 2 h at 4°C. After washing five times with the same buffer, the beads were pelleted, resuspended in 40 $\mu$l of Laemmli sample buffer, and boiled for 5 min. Proteins were separated by SDS/PAGE on a 12% Tris-glycine gel (Bio-Rad) and transferred onto nitrocellulose. The cytochrome $b_{558}$ on the nitrocellulose then was visualized by using an anti-gp91$^{PHOX}$ mAb (15). To determine the effect of p47$^{PHOX}$ and p40$^{PHOX}$ on p67$^{PHOX}$ binding to gp91$^{PHOX}$, 80 pmol of phosphorylated p47$^{PHOX}$ or unphosphorylated p47$^{PHOX}$, phosphorylated p40$^{PHOX}$ or unphosphorylated p40$^{PHOX}$ was added to the assay at the same time as p67$^{PHOX}$.

Affinity precipitation of cytochrome *b* by p47$^{PHOX}$ was carried out as described above except that GST-p47$^{PHOX}$ was used instead of GST-p67$^{PHOX}$.

For affinity precipitation of cytochrome *b* by p40$^{PHOX}$, 40 pmol of His-tagged p40$^{PHOX}$ or phosphorylated His-tagged p40$^{PHOX}$ was incubated in the presence of 2.15 pmol of cytochrome $b_{558}$ in the presence of 100 $\mu$l of 20 mM Hepes, pH 7.5/1% Nonidet P-40/10 mM NaCl/1 mM EGTA/40 mM imidazole for 2 h at room temperature and then precipitated with nickel-chelating beads.

**Western Blotting.** Nitrocellulose membranes were blocked with 5% nonfat dry milk in borate-buffered saline, pH 8.4 (100 mM boric acid/25 mM borax/75 mM NaCl) for 1 h at room temperature and then incubated with 1:1,000 mouse monoclonal anti-gp91$^{PHOX}$ antibody overnight. The membranes then were washed extensively and incubated with horseradish peroxidase-conjugated 1:5,000 anti-mouse IgG for 1 h at room temperature.

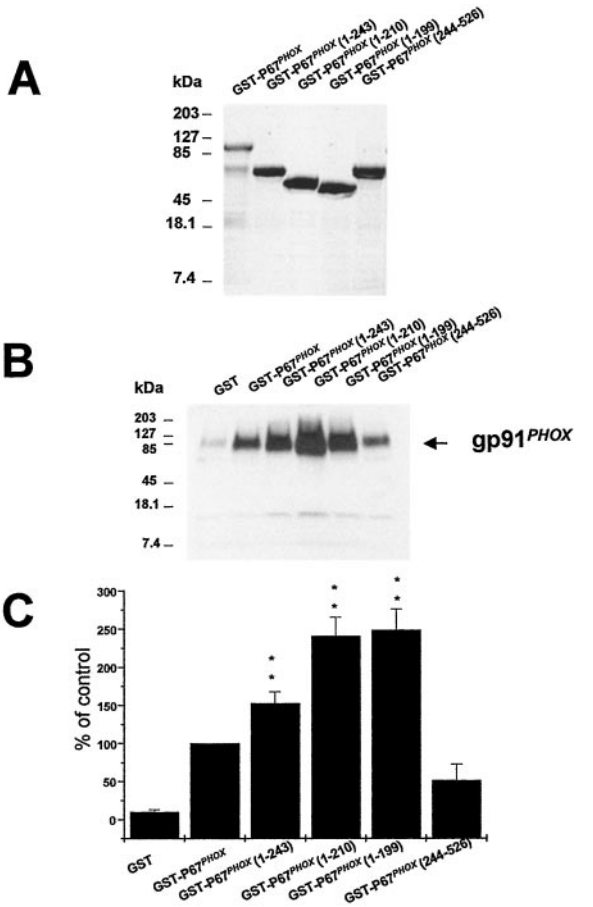



**Fig. 2.** Binding of various truncated forms of p67$^{PHOX}$ to cytochrome $b_{558}$. (*A*) SDS/PAGE of full-length and truncated GST-p67$^{PHOX}$ forms expressed and purified as described under *Experimental Procedures*. The samples were visualized by Coomassie blue staining. (*B*) Affinity precipitation of cytochrome $b_{558}$ by full-length GST-p67 $^{PHOX}$ or truncated GST-p67$^{PHOX}$ (1–243), GST-p67$^{PHOX}$ (1–210), GST-p67$^{PHOX}$ (1–199), or GST-p67$^{PHOX}$ (244–526). The precipitation was performed as described in Fig. 1. Quantification of binding was determined by scanning densitometry. The data are expressed as percentage of full-length GST-p67$^{PHOX}$ and are the means ± SE of four experiments.

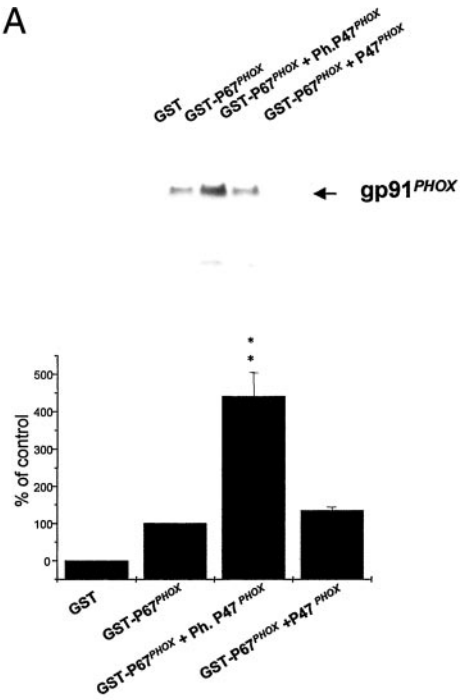
Blots were visualized by using enhanced chemiluminescence Western blotting reagents (Amersham Pharmacia).

**Phosphorylation.** Phosphorylation of p67$^{PHOX}$ by mitogen-activated protein kinase and protein kinase C (Calbiochem) in combination was performed by incubating 80 pmol of p67$^{PHOX}$ in a reaction mixture containing 40 mM Hepes (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 0.6 mM $CaCl_2$, 5 $\mu$g/ml 1,2-dioleoyl-*sn*-glycerol (Sigma), 50 $\mu$g/ml L-$\alpha$-phosphatidylserine (Sigma), the indicated kinase, and 50 $\mu$M ATP in a total volume of 40 $\mu$l for 30 min at 30°C. Phosphorylation of p40$^{PHOX}$ and p47$^{PHOX}$ by protein kinase C was performed the same way. The reactions were stopped by adding 5 $\mu$M GF109203X, an inhibitor of conventional and novel protein kinase C.

**Statistical Analysis.** All of the experiments were repeated a minimum of three times. Results are expressed as means ± SE. Statistical analysis was performed with one-way ANOVA. $P < 0.05$ was considered to be statistically significant.

## Results

**P67$^{PHOX}$ Interacts Directly with Cytochrome $b_{558}$.** The direct binding of p67$^{PHOX}$ to cytochrome $b_{558}$ was established by affinity-



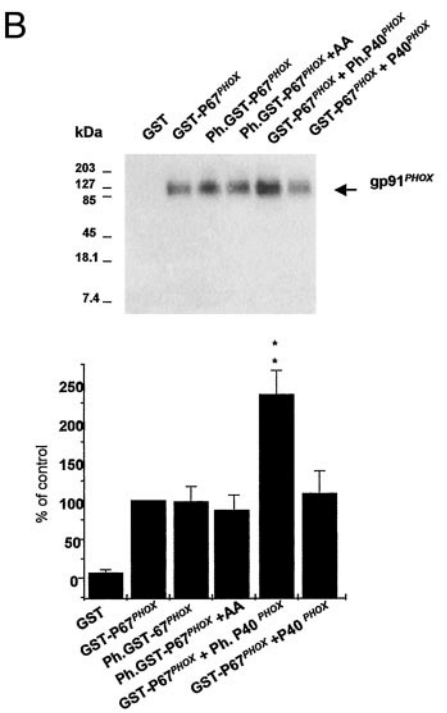




**Fig. 3.** Phosphorylated p47$^{PHOX}$ and phosphorylated p40$^{PHOX}$ increase the binding of p67$^{PHOX}$ to cytochrome $b_{558}$. (*A*) Effect of p47$^{PHOX}$. (*Upper*) Affinity precipitation was performed as described in Fig. 1, except that in some assays 80 pmol of phosphorylated p47$^{PHOX}$ (lane 3) or unphosphorylated p47$^{PHOX}$ (lane 4) was added. (*Lower*) Quantification of the binding was determined by scanning densitometry. The data are expressed as the percentage of full-length GST-p67$^{PHOX}$ and are the means ± SE of three experiments. (*B*) Effect of phosphorylated p67$^{PHOX}$, p40$^{PHOX}$. (*Upper*) Affinity precipitation was performed as described in Fig. 1. Lanes: 3, phosphorylated GST-p67$^{PHOX}$ was used instead of GST-p67$^{PHOX}$; 4, phosphorylated GST-p67$^{PHOX}$ and 10 $\mu$M arachidonic acid were added; 5, phosphorylated p40$^{PHOX}$ (80 pmol) was added to the assay; 6, unphosphorylated p40$^{PHOX}$ (80 pmol) was added to the assay. (*Lower*) Quantification of the binding was determined by scanning densitometry. The data are expressed as percentage of full-length GST-p67$^{PHOX}$ and are the means ± SE of three experiments.

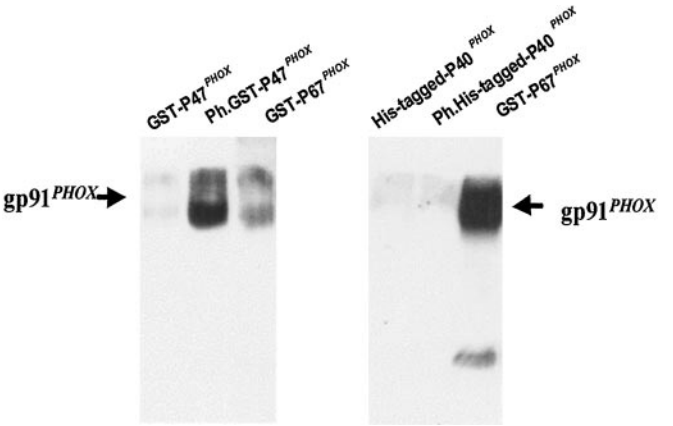



**Fig. 4.** Phosphorylated p47$^{PHOX}$ binds to cytochrome *b* whereas phosphorylated p40$^{PHOX}$ does not. (*Left*) Precipitation of cytochrome *b* by p47$^{PHOX}$. Affinity precipitation was performed as described in *Experimental Procedures* in the presence of unphosphorylated GST-p47$^{PHOX}$ (lane 1) or phosphorylated GST-p47$^{PHOX}$ (lane 2). (*Right*) Precipitation of cytochrome *b* by p40$^{PHOX}$. Affinity precipitation was performed as described in *Experimental Procedures* in the presence of unphosphorylated His-tagged p40$^{PHOX}$ (lane 1) or phosphorylated His-tagged p40$^{PHOX}$ (lane 2). His-tagged P40$^{PHOX}$ was precipitated with nickel-chelating beads.

precipitation experiments. Cytochrome $b_{558}$ was incubated in the presence of GST-p67$^{PHOX}$ or GST. Fig. 1 shows that cytochrome $b_{558}$ was precipitated with GST-p67$^{PHOX}$ coupled to glutathione-Sepharose beads but not with GST coupled to glutathione-Sepharose beads. The results demonstrate a direct interaction between cytochrome $b_{558}$ and p67$^{PHOX}$.

**Interaction of Truncated Forms of p67$^{PHOX}$ with Cytochrome $b_{558}$.** In an attempt to localize the region of p67$^{PHOX}$ responsible for binding to cytochrome $b_{558}$, a series of truncated GST-p67$^{PHOX}$ forms were generated and expressed (Fig. 2*A*). Full-length GST-p67$^{PHOX}$ or truncated GST-p67$^{PHOX}$ was used in affinity-precipitation experiments. As shown in Fig. 2 *B* and *C*, affinity precipitation of cytochrome $b_{558}$ was increased when the C-terminal GST truncations of p67$^{PHOX}$ were used. This increase is especially dramatic for GST-p67$^{PHOX}$ (1–210) and GST-p67$^{PHOX}$ (1–199). By quantitation, the increase was 153 ± 15% for GST-p67$^{PHOX}$ (1–243) and 241 ± 27% and 242 ± 24% for GST-p67$^{PHOX}$ (1–210) and GST-p67$^{PHOX}$ (1–199), respectively. The C-terminal fragment GST-p67$^{PHOX}$ (244–526) precipitated cytochrome $b_{558}$ much less effectively. Indeed, scanning of several experiments showed no significant difference compared with control GST. These results suggest that in the N-terminal forms of p67$^{PHOX}$, the binding site for cytochrome $b_{558}$ is unmasked, whereas in the full-length p67$^{PHOX}$, this site is masked partially by the C-terminal half of the molecule. When this region is removed, the interaction with cytochrome $b_{558}$ is increased.

P67$^{PHOX}$ (1–210) is active in the cell-free NADPH oxidase system, whereas p67$^{PHOX}$ (1–199) is inactive (16). It is interesting that the GST counterparts of those two p67$^{PHOX}$ fragments both are able to bind to cytochrome $b_{558}$. This result suggests that the binding of GST-p67$^{PHOX}$ (1–199) to cytochrome $b_{558}$ serves to hold the active portion of p67$^{PHOX}$ to the cytochrome.

**Effect of p67$^{PHOX}$ Phosphorylation, p47$^{PHOX}$, and p40$^{PHOX}$ on the Interaction of p67$^{PHOX}$ with Cytochrome $b_{558}$.** The above results indicate that a change in p67$^{PHOX}$ conformation is required for its interaction with cytochrome $b_{558}$. We next aimed to determine whether the phosphorylation of p67$^{PHOX}$ or the presence of other components of the NADPH oxidase complex could affect the association of cytochrome $b_{558}$ with GST-p67$^{PHOX}$. The phos-

phorylation of p67$^{PHOX}$ had little effect (Fig. 3*B*). Even arachidonic acid, which increases the interaction of p47$^{PHOX}$ with the cytosolic tail of p22$^{PHOX}$ (17), had no effect (Fig. 3*B*). Interestingly, when phosphorylated p47$^{PHOX}$ or phosphorylated p40$^{PHOX}$ was added to the assay, the coprecipitation of cytochrome $b_{558}$ by GST-p67$^{PHOX}$ was found to be increased (Fig. 3 *A* and *B*). On the other hand, unphosphorylated p47$^{PHOX}$ and unphosphorylated p40$^{PHOX}$ had no effect. These data indicate that phosphorylated p47$^{PHOX}$ and phosphorylated p40$^{PHOX}$ are able to increase the binding of p67$^{PHOX}$ to cytochrome $b_{558}$. Phosphorylated p47$^{PHOX}$ has been shown to interact directly with the cytoplasmic tail of p22$^{PHOX}$ (17), and we established here its interaction with cytochrome $b_{558}$. Indeed, phosphorylated GST-p47$^{PHOX}$ was able to affinity-precipitate cytochrome $b_{558}$ in the presence of glutathione-Sepharose beads (Fig. 4 *Left*), whereas unphosphorylated GST-p47$^{PHOX}$ was not capable of this interaction. Thus, phosphorylated p47$^{PHOX}$ might increase the binding of p67$^{PHOX}$ to cytochrome *b* by acting as an adapter protein, bringing p67$^{PHOX}$ into proximity with cytochrome $b_{558}$. In contrast, we were not able to find any interaction of phosphorylated p40$^{PHOX}$ with cytochrome $b_{558}$. Indeed, phosphorylated or unphosphorylated His-tagged-p40$^{PHOX}$ was not able to affinity-precipitate cytochrome *b* in the presence of nickel-chelating beads (Fig. 4 *Right*). Thus, it is probable that phosphorylated p40$^{PHOX}$ increases the binding of p67$^{PHOX}$ with cytochrome *b* by inducing a conformational change of p67$^{PHOX}$, allowing it to fully interact with cytochrome $b_{558}$.

## Discussion

In an earlier study, we demonstrated by several methods that p67$^{PHOX}$ is able to bind directly to cytochrome $b_{558}$ (10). A direct interaction between p67$^{PHOX}$ and cytochrome $b_{558}$ is in accord with the idea that p67$^{PHOX}$ regulates the transfer of electrons from NADPH to the flavin (18) because p67$^{PHOX}$ then would be in proximity to the flavin center, enabling it to perform a regulatory function in this part of the protein. Recently, one specific domain of p67$^{PHOX}$, called the activation domain, has been shown to be involved in this regulation (16). The activation domain, p67$^{PHOX}$ (1–210), binds more strongly to cytochrome $b_{558}$ than does the complete protein, but so does a domain of similar size, p67$^{PHOX}$ (1–199), which shows no activity in the cell-free NADPH oxidase system. These results suggest that the C truncation that yields p67$^{PHOX}$ (1–210) and p67$^{PHOX}$ (1–199) affects the binding of the fragments to cytochrome $b_{558}$ but has nothing to do with oxidase activity.

The effect of p47$^{PHOX}$ and p40$^{PHOX}$ on the binding of p67$^{PHOX}$ to cytochrome $b_{558}$ also was examined. Our results showed that the unphosphorylated proteins had no effect, but that phosphorylated p47$^{PHOX}$ and phosphorylated p40$^{PHOX}$ both increased the binding of p67$^{PHOX}$ to cytochrome $b_{558}$. Phosphorylated p47$^{PHOX}$ probably serves as an adapter protein, bringing full-length p67$^{PHOX}$ into proximity with cytochrome $b_{558}$. Phosphorylated p40$^{PHOX}$ appears to induce a conformational change in p67$^{PHOX}$, although the functional significance of this conformational change is unclear. In conclusion, our data establish a direct interaction between p67$^{PHOX}$ and cytochrome $b_{558,}$ as demonstrated previously. Furthermore, we have shown that full binding requires a conformation change that, in the intact cells, might be induced by phosphorylated p40$^{PHOX}$.

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1. Babior, B. M. (1999) *Blood* **93,** 1464–1476.
2. Segal, A. W. (1989) *J. Clin. Invest.* **83,** 1785–1793.
3. Malech, H. L. & Gallin, J. I. (1987) *N. Engl. J. Med.* **317,** 687–694.
4. Chanock, S. J., El Benna, J., Smith, R. M. & Babior, B. M. (1994) *J. Biol. Chem.* **269,** 24519–24522.
5. Clark, R. A., Volpp, B. D., Leidal, K. G. & Nauseef, W. M. (1990) *J. Clin. Invest.* **85,** 714–721.
6. Rotrosen, D., Yeung, C. L., Leto, T. L., Malech, H. L. & Kwong, C. H. (1992) *Science* **256,** 1459–1462.
7. Smith, R. M., Connor, J. A., Chen, L. M. & Babior, B. M. (1996) *J. Clin. Invest.* **98,** 977–983.
8. Hayakawa, T., Suzuki, K., Suzuki, S., Andrews, P. C. & Babior, B. M. (1986) *J. Biol. Chem.* **261,** 9109–9115.
9. Koshkin, V., Lotan, O. & Pick, E. (1996) *J. Biol. Chem.* **271,** 30326–30329.
10. Dang, P. M.-C., Cross, A. R. & Babior, B. M. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 3001–3005.
11. Deleo, F. R. & Quinn, M. T. (1996) *J. Leukocyte Biol.* **60,** 677–691.
12. Sumimoto, H., Kage, Y., Nunoi, H., Sasaki, H., Nose, T., Fukumaki, Y., Ohno, M., Minakami, S. & Takeshige, K. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 5345–5349.
13. Dang, P. M.-C., Johnson, J. L. & Babior, B. M. (2000) *Biochemistry* **39,** 3069–3075.
14. Cross, A. R., Erickson, R. W., Ellis, B. A. & Curnutte, J. T. (1999) *Biochem. J.* **338,** 229–233.
15. Burrit, J. B., Quinn, M. T., Jutila, M. A., Bond, C. W. & Jesaitis, A. J. (1995) *J. Biol. Chem*. **270,** 16974–16980.
16. Han, C.-H., Freeman, J. L. R., Lee, T., Motalebi, S. A. & Lambeth, J. D. (1998) *J. Biol. Chem.* **273,** 16663–16668.
17. Shiose, A. & Sumimoto, H. (2000) *J. Biol. Chem.* **275,** 13793–13801.
18. Nisimoto, Y., Motalebi, S., Han, C. H. & Lambeth, J. D. (1999) *J. Biol. Chem.* **274,** 22999–23005.
19. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.