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Relationships of p40^{phox} with p67^{phox} in the Activation and Expression of the Human Respiratory Burst NADPH Oxidase[1]

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p40^{phox} of the phagocyte NADPH oxidase forms a complex with p67^{phox} in cytosol, and coincidentally decreases in patients who lack p67^{phox}. Here we investigated the mode of translocation of p40^{phox} to the membrane, its cytoskeletal localization on activation of the NADPH oxidase, and the dependency of its expression relative to that of p67^{phox}. When human polymorphonuclear leukocytes (PMNs) were stimulated with phorbol myristate acetate (PMA), p40^{phox} was translocated to the membrane along with p67^{phox}, and not was released into the cytosol. Studies with resting PMNs using Triton X-100 revealed the exclusive localization of p67^{phox} in the cytoskeletal fraction. Unexpectedly, however, about half of p40^{phox}, which is deemed to be fully associated with p67^{phox}, was recovered in the non-cytoskeletal fraction. Unlike p47^{phox}, the association of p40^{phox} with cytoskeleton was not induced by the PMA-stimulation. These results indicate not only that p40^{phox} associates with cytoskeleton *via* a molecule of p67^{phox}, but also that there are distinct states of p40^{phox} that can be manipulated with Triton X-100. Lastly, Western-blot analysis of hematopoietic cells revealed no correlation between p40^{phox} and p67^{phox} in their protein expressions during cell differentiation, and also that p40^{phox} can be stably present alone in cells, unless in the case of mature PMNs. In this regard, definitive proof was obtained with Epstein-Barr virus–transformed B cells of a p67^{phox}-deficient patient, in which p40^{phox} was normally expressed.

Key words: NADPH oxidase, neutrophils, p40^{phox}, p67^{phox}, superoxide anion.

The respiratory burst of phagocytes yields superoxide anion (O_2^-), a precursor of toxic reactive oxygens, which constitute a major non-specific host defense against an array of bacterial and fungal pathogens. Activation of the O_2^--generating NADPH oxidase requires the assembly of membrane-integrated cytochrome b_{558}, composed of gp91^{phox} and p22^{phox} subunits, and cytosolic p47^{phox} and p67^{phox}, under the control of Rac. Defects in the components, excluding Rac, are responsible for chronic granulomatous disease (CGD), a syndrome characterized by recurrent infections and formation of granuloma (for reviews, see Refs. *1–6*).

We and others found that the p40^{phox} molecule associated with porcine p63^{phox} (*7*), guinea pig p63^{phox} (*8*), or human p67^{phox} (*9*, *10*). Although coincident depletion of p40^{phox} in patients with CGD who lack p67^{phox} suggested the importance of p40^{phox} in the NADPH oxidase system (*9*, *10*), earlier findings that p40^{phox} was dispensable in a minimal oxidase reconstitution system composed of cytochrome b_{558}, p47^{phox}, p67^{phox}, and Rac had argued against its participation (*11*, *12*). We settled this issue using anti-p40^{phox} antibodies and demonstrated that p40^{phox} is practically involved in the NADPH oxidase system (*13*). The interacting sites of p40^{phox} and p67^{phox} are now specified to be in the C-terminal tail (aa 269–339) of p40^{phox} and in the inter-Src homology 3 (SH3) domain (aa 301–460) of p67^{phox} (*14*, *15*).

The role of SH3 domains in the NADPH oxidase function has been the subject of intensive investigation (see references in Refs. *4*, *5*, *14*, *15*). p40^{phox}, p47^{phox}, and p67^{phox} contain respectively one, two, and two SH3 domains, which mediate multiple associations with polyproline targets within the NADPH oxidase complex. In resting cells, the C-terminal polyproline region (aa 338–390) of p47^{phox} is forming intramolecular binding with its N-terminal SH3 domain (aa 151–214) (*16*, *17*). The crucial event for the activation of the NADPH oxidase is the breakage of this SH3/polyproline binding and subsequent intermolecular binding with the C-terminal SH3 domain (aa 458–526) of p67^{phox}. It is generally accepted that this direct association between p47^{phox} and p67^{phox} leads the cytosolic complex to translocate to cytochrome b_{558} *via* a cytoskeletal scaffold and recognize it (*1–6*).

Recently, the down-regulation of the NADPH oxidase activity through the SH3 domain of p40^{phox} was confirmed

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Abbreviations: CGD, chronic granulomatous disease; DFP, diisopropyl fluorophosphate; EBV, Epstein-Barr virus; FCS, fetal calf serum; HRP, horseradish peroxidase; PAGE, polyacrylamide gel electrophoresis; PBSG, Ca^{2+}/Mg^{2+}-free phosphate-buffered saline containing 5 mM glucose, pH 7.4; PMA, phorbol 12-myristate 13-acetate; PMN, polymorphonuclear leukocyte; PNS, postnuclear supernatant; PSL, photostimulative luminescence; PVDF, polyvinylidene difluoride; SH3, Src homology 3.

by both a cell-free oxidase assay and cotransfection of $p40^{phox}$ into K-562 cells (*15*). The SH3 domain of $p40^{phox}$ is closely similar to the C-terminal SH3 of $p67^{phox}$ (*10*). Because of their high homology, these two SH3 domains in $p40^{phox}$ and $p67^{phox}$ compete with each other in binding to the C-terminal polyproline region of $p47^{phox}$ to modulate the NADPH oxidase activity. However, despite detailed work on the interactions among oxidase components, other features of $p40^{phox}$ remain to be settled. In the present communication, we focused first on the mode of $p40^{phox}$ translocation to the membrane, examining whether it is complexed with or released from $p67^{phox}$, and on its cytoskeletal localization upon activation of the NADPH oxidase. Next, to determine whether $p40^{phox}$ can stably exist alone, its expression at the protein level was examined in hematopoietic cells in relation to that of $p67^{phox}$.

MATERIALS AND METHODS

Isolation of PMNs and T-Cell Subsets—Polymorphonuclear leukocytes (PMNs) were obtained from healthy donors as previously described (*18*). The isolated PMNs were finally suspended in cold Ca^{2+}/Mg^{2+}-free phosphate-buffered saline containing 5 mM glucose (PBSG, pH 7.4). For larger scale preparation, PMNs were also prepared from buffy coat residues. For T-cell subset preparation, mononuclear cells isolated by Ficoll-Paque sedimentation were washed three times with RPMI 1640 containing 10% (v/v) fetal calf serum (10% FCS-RPMI 1640). The cells were incubated in a 175-ml culture flask for 1.5 h at 37°C in a 5% CO_2 atmosphere, and nonadherent cells were collected. This step was repeated twice. The $CD4^+$ and $CD8^+$ T cells were immunomagnetically isolated at 4°C using Dynabeads M-450 CD4 and M-450 CD8, respectively, basically according to the manufacturer's protocol (Dynal A.S., Oslo, Norway). Detachment of Dynabeads from rosetted cells was carried out at 4°C with DETACHaBEAD. The cells were then washed three times and collected in cold PBSG. Both the isolated T-cell subsets were almost all viable and more than 95% pure.

Preparation of EBV-Transformed B Cells—Mononuclear cells were isolated from either normal donors or CGD patients after informed consent was obtained. Transformation of mononuclear cells (1×10^6) was carried out in 1 ml of 20% FCS-RPMI 1640 containing 200 ng/ml of cyclosporin A, in a 24-well flat-bottomed plate, after infection with 0.2 ml of Epstein-Barr virus (EBV)–containing supernatant from B95-8 marmoset cell line. Medium exchange of a half portion (0.5 ml) was started on day 4 after infection, and continued twice a week for approximately 1 month until transformed cells appeared. Cells were then grown in a large scale.

Cell Culture—Hematopoietic cell lines used to examine the expression of $p40^{phox}$ protein were generous gifts from the Fujisaki Cell Center (Hayashibara Biochemical Laboratories, Okayama): B-cell lines (NALM-6, DAUDI, NK-9, and RIVA), T-cell lines (P30/OHKUBO, HPB-ALL, MOLT-4, JURKAT, and SALT-3), and non-T/B cell lines (KG-1, DAMI, MEG-01, HEL, KU-812, and K-562). All cell lines including HL-60 cells (see below) and EBV-transformed B cells were first treated with 0.5 μg/ml of MC-210 (Dainippon Pharmaceutical Co., Osaka) for a week to eliminate mycoplasma. Subsequently, cells were grown in stationary suspension culture in 10% FCS-RPMI 1640 at 37°C in a 5% CO_2 atmosphere.

Differentiation of HL-60 Cells—PMN-like differentiation of HL-60 cells was induced with 0–1.25% (v/v) Me_2SO. The cultivation was started at a concentration of 1×10^5 cells/ml for Me_2SO-dependent differentiation and 0.25×10^5 cells/ml for undifferentiated control cells (0% Me_2SO) in 10% FCS-RPMI 1640. Cells were harvested after 6 d of culture, when they showed the highest O_2^--generating activity.

Subcellular Fractionation—All kinds of cells were first treated with 2 mM diisopropyl fluorophosphate (DFP) for 15 min on ice, washed three times with cold PBSG, and finally suspended in buffer A [100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, and 10 mM PIPES (pH 7.3) containing 10 μM leupeptin and 1 mM phenylmethylsulfonyl fluoride]. Cells were then disrupted by sonication and spun at 500 $\times g$ for 5 min at 4°C to get their postnuclear supernatants (PNS). The PNS fractions of EBV-transformed B cells were further spun at 200,000 $\times g$ for 20 min to obtain cytosol fractions.

For translocation studies of cytosolic components to the membrane, PMNs were stimulated for periods up to 10 min at 37°C with 100 ng of phorbol 12-myristate 13-acetate (PMA)/2×10^6 cells/ml of PBSG containing 1.2 mM $MgCl_2$ and 2 mM NaN_3. Cells were diluted with cold PBSG, spun at 1,500 $\times g$ for 5 min, suspended in buffer A, and finally sonicated. Their PNS fractions were then separated into membrane and cytosol at 200,000 $\times g$ for 20 min at 4°C, of which membranes were resuspended in an initial volume of buffer A. Protein concentration was determined with BCA reagents (Pierce, Rockford, IL), using bovine serum albumin as a standard.

Preparation of PMN Cytoskeleton—Cytoskeletal localization of cytosolic components was examined basically as reported elsewhere (*19*). PMNs were stimulated with 1 μg of PMA/10^7 cells/ml of PBSG containing 1.2 mM $MgCl_2$ and 2 mM NaN_3 for either 3 or 7 min at 37°C. These cells were spun at 1,500 $\times g$ for 5 min at 4°C, resuspended at $5–10 \times 10^7$ cells/100 μl of Triton X-100 lysis buffer [1% (w/v) Triton X-100 in 100 mM Tris-HCl, pH 7.4 containing 5 mM EGTA, 0.25 mM leupeptin, and 1 mM DFP], and then sonicated for 10 s at 70-W output. After standing on ice for 10 min, the sonicates were loaded on top of 0.3 ml of 6% (w/v) sucrose in Triton X-100 lysis buffer and centrifuged at 200,000 $\times g$ for 30 min at 4°C (Beckman TLA 100.2). The top 100 μl and the pellet, which was resuspended in an initial volume (0.4 ml) of Triton X-100 lysis buffer, were used later as Triton X-100 soluble (Sol) and insoluble (Skl) fractions, respectively.

Immunochemical Studies—Antisera against the C- and N-terminal polypeptides of the NADPH oxidase components were raised in rabbits (*13*, *18*). For immunoprecipitation, cytosol (1×10^7 cell equivalents) from EBV-transformed B cells was incubated with the rabbit antiserum (10 μl) against the C- or N-terminus of $p40^{phox}$ for 90 min on ice in 0.5 ml of immunoprecipitation buffer (150 mM NaCl, 10 mM EDTA, 1% (w/v) deoxycholate, and 1% (w/v) Nonidet P-40 in 10 mM Tris-HCl, pH 7.4). $p40^{phox}$ was then precipitated with 4 mg of Protein A–Sepharose CL-4B, as previously reported (*7*, *9*, *13*).

For immunoblot analysis, membrane, cytosol, cytoskeleton, and non-cytoskeleton fractions were all subjected to SDS–polyacrylamide gel electrophoresis (PAGE), and pro-

teins were transferred to polyvinylidene difluoride (PVDF) sheets. The transferred proteins were then probed with the primary antisera (1:1,000 dilution). Immunoreactive bands were visualized with a secondary horseradish peroxidase (HRP)–conjugated swine anti–rabbit IgG (1:1,000 dilution) (Dakopatts, Glostrup, Denmark) *plus* *o*-dianisidine or a donkey ^{125}I-labeled F(ab')$_2$ fragment against rabbit Ig (1:1,000 dilution) (Amersham, Arlington, Heights, IL). For quantification of cytosolic components, PVDF sheets were analyzed by a pdi 420 oe™ scanner (Arcus II, pdi, NY) or a bio-image analyzer (Fuji BAS 2000, Fuji Photofilm, Tokyo), as previously described (*13*, *20*).

Superoxide Generation—O_2^--generation was determined by the rate of superoxide dismutase-inhibitable ferricytochrome *c* reduction. Its generation from HL-60 cells stimulated with PMA (200 ng of PMA/2 × 10^6 cells/ml) was measured in a reaction mixture consisting of 1.2 mM $MgCl_2$, 2 mM NaN_3, and 30 μM ferricytochrome *c* in PBSG. For membrane fractions of PMNs, this reaction mixture was supplemented with 0.1 mM EGTA and 225 U/ml catalase in saline–20 mM HEPES buffer (pH 7.4), and the reaction was begun by adding 0.2 mM NADPH, as previously reported (*7*). At maximal velocity, 200 U/ml of superoxide dismutase was added to determine the net ferricytochrome *c* reduction by O_2^-.

Materials—Catalase, cyclosporin A, deoxycholate, DFP, *o*-dianisidine, EDTA, EGTA, ferricytochrome *c*, HEPES, Me_2SO, Nonidet P-40, PIPES, PMA, protease inhibitors, Protein A–Sepharose CL-4B, superoxide dismutase, and Triton X-100 were purchased from Sigma (St. Louis, MO). NADPH and SDS were from Wako (Tokyo). Molecular markers for SDS-PAGE and PVDF sheets for Western blotting were obtained from Bio-Rad Lab. (Hercules, CA) and Millipore Corp. (Bedford, MA), respectively. All other reagents were of analytical grade.

RESULTS AND DISCUSSION

Associated Translocation of p40^{phox} with p67^{phox} in PMA-Stimulated PMNs—The three cytosolic phox-proteins, p40^{phox}, p67^{phox}, and p47^{phox} reside as a complex in resting cytosol, in which p40^{phox} interposes between p67^{phox} and p47^{phox} (*14*, *15*, *21*, *22*). In an earlier report (*13*), we demonstrated that SDS and myristic acid, *in vitro* stimulants of the NADPH oxidase, were both unable to disrupt a p40^{phox}-p67^{phox} complex. However, the observations that p40^{phox} down-regulated the NADPH oxidase activity *in vivo* as well as *in vitro* (*15*), and a recent cell-free study that Rac1-GTP disrupted the existing recombinant p40^{phox}-p67^{phox} complex (*23*), make us imagine that p67^{phox} may release a suppressive molecule of p40^{phox} into the cytosol on cell stimulation, and then move to cytochrome b_{558} to activate the NADPH oxidase (*24*).

Here, we assessed quantitatively the translocation of p40^{phox} in relation to that of p67^{phox}. Although there are a few qualitative reports on the translocation of p40^{phox} upon cell stimulation (*10*, *25*), quantitative information is not available. On stimulation of PMNs with PMA, all the three cytosolic components translocated to membranes (Fig. 1). Quantitative analysis revealed that 34.4 ± 7.5% of p40^{phox} and 37.7 ± 2.5% of p67^{phox} became membrane-associated with an increase in O_2^--generating activity after a 10-min stimulation (Table I). The close similarity of these rates of translocation suggests that p40^{phox} migrates together with p67^{phox} to the membrane as a complexed unit, without its release to the cytosol, or that even if dissociated from p67^{phox}, it still remains attached to the cytoskeleton.

Stability of p40^{phox}—The expression of p40^{phox} protein was greatly reduced in PMNs from patients with CGD who lack p67^{phox}, as we and others previously reported (*9*, *10*, *13*). On the basis of this observation, we investigated the correlation of p40^{phox} with p67^{phox} in their protein expressions during the differentiation of HL-60 cells, a human premyelocytic leukemia cell line. Figure 2A shows the O_2^--generation by PMA-stimulated HL-60 cells 6 d after induction of differentiation with various concentrations of Me_2SO. As reported previously (*26*), 1.25% (v/v) Me_2SO conferred good O_2^--generating activity on HL-60 cells.

Immunoblot analysis indicated independent regulation of the expression of each cytosolic component (Fig. 2B). p40^{phox} and p47^{phox} were both expressed in earlier undifferentiated HL-60 cells. In contrast to the negligible p67^{phox} in these cells, a considerable amount of p40^{phox} was expressed, whereas that in PMNs from p67^{phox}-deficient CGD patients was shown to be only on a trace level (*9*, *10*, *13*). Induction of HL-60 cell differentiation with Me_2SO greatly enhanced the expression of p47^{phox} in a dose-dependent manner. Expression of p67^{phox} became striking only over 0.625% (v/v) Me_2SO (Fig. 2B), with the concomitant appearance of O_2^--generating activity (Fig. 2A). This agrees with the idea that

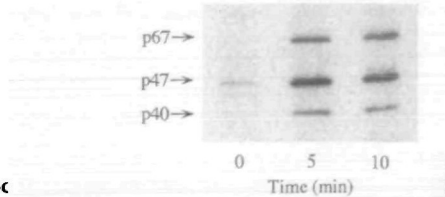


Fig. 1. **Assoc** **in PMA-stimulated PMNs.** Membranes were fractionated from resting (at 0 min, treated with 0.02% Me_2SO) and PMA-stimulated cells (100 ng of PMA/2 × 10^6 cells/ml) for the indicated periods. The membrane fractions (2 × 10^6 cell equivalents) were subjected to SDS-PAGE (10% acrylamide). Proteins were transferred onto a PVDF sheet and immunoblotted with a mixture of primary antisera raised in rabbits against the C-terminal polypeptides of p67^{phox}, p47^{phox}, and p40^{phox}. Cytosolic components were then revealed by a bio-image analyzer, with a secondary ^{125}I-labeled F(ab')$_2$ fragment against rabbit Ig.

TABLE I. **Quantitative correlation between p40^{phox} and p67^{phox} in translocation to the membrane.** Membranes and the corresponding cytosol were fractionated from resting and PMA-stimulated PMNs, respectively. The protocol for immunoblot was the same as that in Fig. 1. The radioactivity stored in an imaging plate was measured as photostimulative luminescence (PSL) counts. Finally, percent translocation was expressed as the PSL count in the membrane fraction divided by the sum of counts in both the membrane and cytosol fractions. O_2^--generation was initiated by adding 0.2 mM NADPH to 50 μg of membranes.

Stimulation (min)	O_2^--generation[a] (nmol/min/mg prot.)	Translocation[b] (%)		
		(p40)	(p67)	(p47)
0	0.1 ± 0.3	5.2 ± 1.8	4.0 ± 4.1	6.0 ± 2.2
5	36.2 ± 1.4	34.1 ± 4.1	33.7 ± 0.8	30.2 ± 0.4
10	36.4 ± 5.8	34.4 ± 7.5	37.7 ± 2.5	29.9 ± 3.1

[a]Data are means ± SD (n = 4), representative of four experiments.
[b]Data are means ± SD (n = 2), representative of four experiments.

the limiting factor for activation of the NADPH oxidase is a $p67^{phox}$ molecule. In fact, it was found that pronounced activation of the NADPH oxidase could be achieved in a cell-free system, by exposing cytochrome b_{558} to $p67^{phox}$ and Rac in the total absence of $p47^{phox}$ (*27*, *28*). In contrast to $p67^{phox}$, the amount of $p40^{phox}$ at 0.625% (v/v) Me_2SO lay similar to that in the undifferentiated HL-60 cells. At a concentration of 1.25% (v/v) Me_2SO, optimal for O_2^--generation, the expression of $p67^{phox}$ was further enhanced with a slight increment of $p40^{phox}$. We assume that this increase is not due to an enhanced level of $p40^{phox}$ mRNA, but to a lowering of $p40^{phox}$ protein turnover rate by its stabilization through complexing with $p67^{phox}$. These observations suggest that $p40^{phox}$ is able to exist alone in the undifferentiated HL-60 cells, and that $p67^{phox}$ synthesized along cell differentiation is captured by free $p40^{phox}$ ready to form a stable $p40^{phox}$-$p67^{phox}$ complex.

Although $p40^{phox}$ was apparently able to exist *per se* in undifferentiated HL-60 cells, it was still unclear whether the $p40^{phox}$ molecule is fully able to exist alone. In this respect, definitive proof was obtained with EBV-transformed B cells of a $p67^{phox}$-deficient CGD patient. Figure 3 shows the results of immunoprecipitation of cytosol with antiserum against the N- or C-terminal polypeptide of $p40^{phox}$. Contrary to the earlier observations with $p67^{phox}$-deficient PMNs (*9*, *10*, *13*), both the antisera immunoprecipitated $p40^{phox}$ in spite of the total absence of $p67^{phox}$ in the EBV-transformed $p67^{phox}$-deficient B cells, to a similar extent as seen in EBV-transformed normal or $p47^{phox}$-deficient B cells. Furthermore, anti-$p40^{phox}$C serum completely disrupted the $p40^{phox}$-$p67^{phox}$ complex, so that it failed to coimmunoprecipitate $p67^{phox}$ along with $p40^{phox}$, whereas anti-$p40^{phox}$N serum did cause coprecipitation, as we previously demonstrated using PMNs (*13*). These results demonstrate that $p40^{phox}$ can also exist alone in B cells, but developed proteolysis systems and a poor protein synthesis due to the degeneration of endoplasmic reticulum in mature PMNs predispose $p67^{phox}$-lacking PMNs toward the coincident lowering of $p40^{phox}$.

Expression of $p40^{phox}$ in Hematopoietic Cells—The membrane component $gp91^{phox}$, and cytosolic components $p67^{phox}$ and $p47^{phox}$ are expressed nearly exclusively in phagocytic cells. In contrast, $p22^{phox}$, which stabilizes $gp91^{phox}$, is expressed in a variety of cells (see Refs. *1–6*). By analogy with this, we studied here by Western-blot analysis the expression of $p40^{phox}$ protein in relation to that of $p67^{phox}$, using hematopoietic cells (Fig. 4). $p40^{phox}$ could be present in all B cell lines, even if $p67^{phox}$ is not expressed. As pre-B cells (lane 1: NALM-6) progressed to the mature states, B-blast I (2: DAUDI), B-blast II (3: NK-9), and B-blast III (4: RIVA), the expression of $p47^{phox}$ significantly increased, whereas the amount of $p40^{phox}$ did not (Fig. 4, top panel). EBV-transformed normal B cells showed a full set of cytosolic components (data not shown). It is known that neither $p47^{phox}$ nor $p67^{phox}$ is expressed in peripheral blood T cells. However, it was recently reported that the T-cell line (EL4) expressed the mRNA of $p40^{phox}$ (*29*). In the present study, T-blast I (5: P30/OHKUBO) and T-blast V (9: SALT-3) also showed $p40^{phox}$ along with $p47^{phox}$ and $p67^{phox}$, respectively (Fig. 4, middle panel). Intermediate stages, T-blast II (6: HPB-ALL) and T-blast III (7: MOLT-4 and 8: JURKAT), lacked all of these cytosolic components. P30/OHKUBO cells might not be differentiated enough to T-type cells. However, we could not find an answer to the expression of $p67^{phox}$ in SALT-3 cells. Thus, in an attempt to ascertain the expression of $p40^{phox}$ in normal T cells, we isolated their $CD4^+$ and $CD8^+$ subsets from healthy blood. However neither showed any detectable $p40^{phox}$ (Fig. 4, middle panel). Of non-T/B cell lines, only the myeloblast cell line (10: KG-1), which is an immature precursor of promyelocytes and further granulocytes, was found to carry all the three cytosolic components. Megakaryocyte cell lines (11: DAMI and 12: MEG-01) and

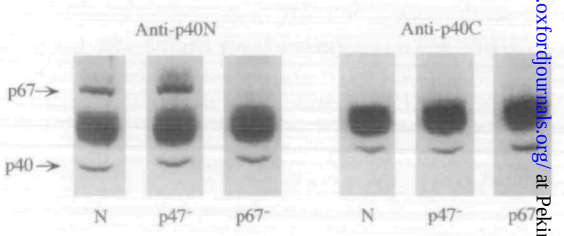


Fig. 3. **Expression of $p40^{phox}$ in $p67^{phox}$-deficient EBV-transformed B cells.** Cytosol (1×10^7 cell equivalents) of EBV-transformed B cells from CGD patients lacking either $p67^{phox}$ ($p67^-$) or $p47^{phox}$ ($p47^-$) as well as from a normal subject (N) was immunoreacted with antiserum (10 μl) against the C- or N-terminus of $p40^{phox}$ in 0.5 ml of immunoprecipitation buffer, followed by precipitation with 4 mg of Protein A–Sepharose CL-4B. The presence of $p40^{phox}$ and $p67^{phox}$ in immunoprecipitates was determined by HRP-immunoblot analysis. Broad bands are due to the heavy chains of the primary rabbit IgGs. Results are representative of two reproducible experiments.

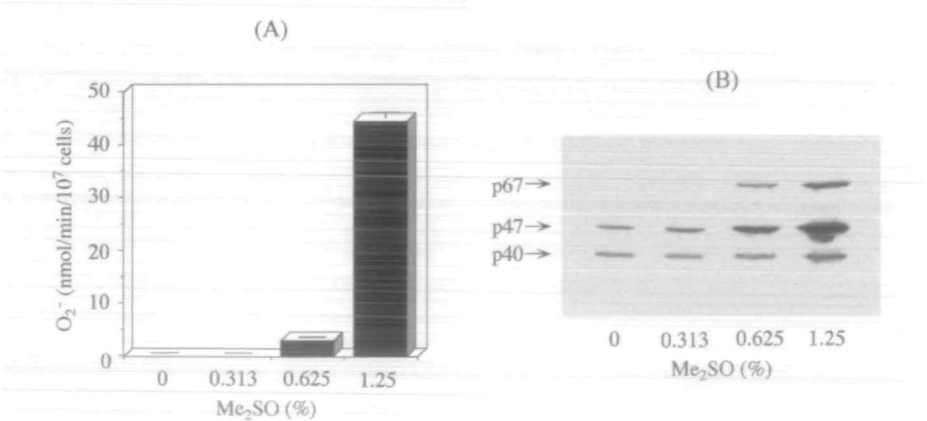


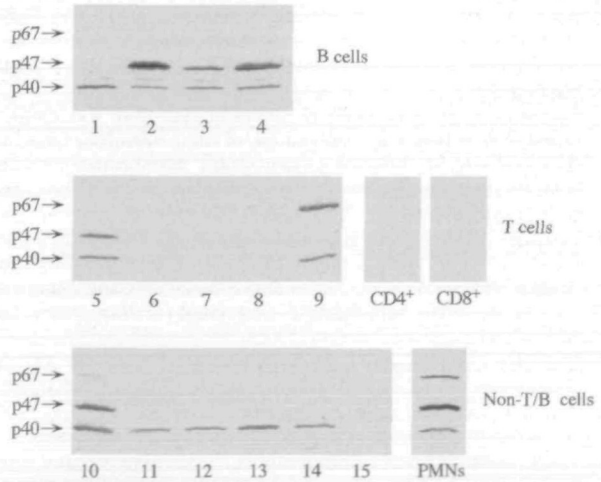
Fig. 2. **NADPH oxidase activity and expression of cytosolic components in HL-60 cells during differentiation.** Differentiation of HL-60 cells to PMN-like cells was induced with the indicated concentrations of Me_2SO under the conditions described in "MATERIALS AND METHODS." Cells were collected on day 6 of culture and their O_2^--generating activities (A) were determined by stimulation with PMA (200 ng of PMA/2×10^6 cells/ml). The expression of cytosolic components (B) was examined in PNS fractions of resting cells (50 μg) by HRP-immunoblot analysis. Data are representative of three experiments performed in duplicate (A) or in single (B).

other cell lines that can potentially differentiate into megakaryocytes (13: HEL, differentiation into megakaryocytes and 14: KU-812, triphenotypic differentiation into megakaryocytes, basophils, and erythrocytes) all expressed p40^{phox} alone. The K-562 cell line (lane 15), which has the potential to differentiate into erythroid cells, showed none of the three cytosolic components. The expression pattern of p40^{phox} protein in hematopoietic cells was basically in agreement with that of its mRNA shown by Northern-blot analysis (*30*). As concluded above in the section "Stability of p40^{phox}", the results obtained here bear out as well the notion that p40^{phox} can be stably present without the assistance of p67^{phox}, except in the case of mature PMNs. Furthermore, this conclusion raises the possibility that p40^{phox} interacts with not only p67^{phox} but also other molecules, as it was recently found to do with ubiquitous proteins such as coronin (*31*) and thioredoxin (*32*).

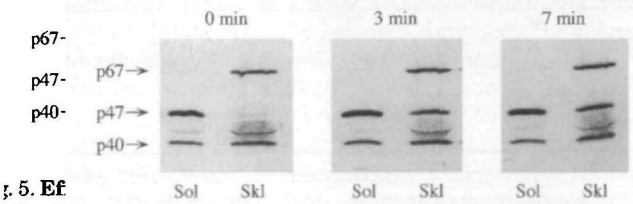
p40^{phox} Associates with Cytoskeleton via a Molecule of p67^{phox}—Earlier works suggested a close association between the NADPH oxidase and the PMN cytoskeleton (*19*, *33*, *34*). The O_2^--generating activity of stimulated PMNs was restricted to the cytoskeletal fraction. In resting cytosol, p47^{phox} was entirely free from the cytoskeleton, being incorporated into this location following the phosphorylation of its C-terminal polyproline region (aa 338–390) upon cell stimulation. In contrast, almost all p67^{phox} was shown to be associated with the cytoskeleton in the resting state. However, these studies did not take into account the presence of p40^{phox}. This was because that p40^{phox} had not yet been discovered, and since its discovery, doubt has been thrown on its participation in the NADPH oxidase system until recent confirmative evidence was obtained (*13–15*, *21*).

It still remains to be settled which molecule of the p40^{phox}-p67^{phox} complex directly associates with cytoskeleton. We and others suggested, as a result respectively of coimmunoprecipitation (*13*) and anti-p67^{phox} affinity column (*22*) studies using isolated resting cytosol from sonicated PMNs, that almost all of p40^{phox} exists in a complexed form with p67^{phox}. Accordingly, we treated PMNs with Triton X-100, expecting that all p40^{phox} could be recovered in a Triton X-100 insoluble fraction, generally equated with the cytoskeleton. Resting PMNs treated with Triton X-100 showed results, consistent with earlier studies (*19*, *33*, *34*), that nearly 100% of p47^{phox} and p67^{phox} located in non-cytoskeletal (Sol) and cytoskeletal (Skl) fractions, respectively. Unexpectedly, however, p40^{phox} was equally distributed between the non-cytoskeletal (45.6%) and cytoskeletal fractions (54.4%) (Fig. 5 and Table II). This observation shows that the treatment of resting PMNs with 1% (w/v) Triton X-100 dissociated ~50% of p40^{phox} from the p40^{phox}-p67^{phox} complex in the cytoskeleton. Moreover, it indicates not only that the p40^{phox}-p67^{phox} complex in cytosol associates with cytoskeleton *via* a molecule of p67^{phox}, but also that there are at least two subpopulations of p40^{phox} with differing modes of interaction with p67^{phox} in the cytoskeleton. This notion is supported by the recent finding (*31*) that coronin, an actin-binding protein, attached to the C-terminal half of p40^{phox}, which is also a binding partner of p67^{phox}. Most recently, during our investigation, El Benna *et al.* (*35*) reported that almost all of p40^{phox} was recovered in the Triton X-100 insoluble fraction. Presently, we can not find a plausible explanation for the discrepancy.

Although the amount of p40^{phox} in the non-cytoskeletal



F
Cells were sonicated to prepare PMN fractions. A total of 50 μg of protein was subjected to SDS-PAGE *plus* HRP-immunoblot analysis. B-cell lines (1: NALM-6, 2: DAUDI, 3: NK-9, and 4: RIVA), T-cell lines (5: P30/OHKUBO, 6: HPB-ALL, 7: MOLT-4, 8: JURKAT, and 9: SALT-3), non-T/B cell lines (10: KG-1, 11: DAMI, 12: MEG-01, 13: HEL, 14: KU-812, and 15: K-562), and PMNs are shown. The CD4$^+$ and CD8$^+$ T-cell subsets were immunomagnetically isolated using Dynabeads as described in "MATERIALS AND METHODS." Results are representative of three reproducible experiments.



g. 5. Ef
the Triton X-100 soluble and insoluble fractions. PMNs re incubated with PMA (1 μg of PMA/10^7 cells/ml) at 37°C for the licated times as well as with Me_2SO vehicle (0.33%, control at 0 n). After washing with cold PBSG, 6×10^7 cells were suspended in) μl of Triton X-100 lysis buffer and sonicated for 10 s on ice. Tri- X-100 soluble (Sol) and insoluble (Skl) fractions were then iso- ed as described in "MATERIALS AND METHODS." Equivalent ounts (1.25×10^6 cell equivalents) of both fractions were sub- ted to SDS-PAGE *plus* HRP-immunoblot analysis. Results are presentative of four reproducible experiments.

BLE II. **Quantitative distribution of p40^{phox} and p67^{phox} to e Triton X-100 soluble and insoluble fractions.** The HRP- munoblots of Triton X-100 soluble (Sol) and insoluble (Skl) frac- ns in Fig. 5 were quantitatively analyzed by a pdi 420 oe™ scan- r. Percent localization was expressed as the reflection density in e respective fraction divided by the sum of values in both the fractions.

Stimulation (min)	Localization (%)[a]					
	p40		p67		p47	
	(Sol)	(Skl)	(Sol)	(Skl)	(Sol)	(Skl)
0	45.6	54.4	5.7	94.3	93.5	6.5
3	44.2	55.8	2.7	97.3	73.7	26.3
7	39.0	61.0	0.0	100.0	59.9	40.1

[a]Data are representative of four reproducible experiments.

fraction of resting PMNs was appreciable, their stimulation with PMA did not cause $p40^{phox}$ to migrate to the cytoskeletal fraction even after a 7-min stimulation (Fig. 5 and Table II). In contrast, soluble $p47^{phox}$ moved to the cytoskeletal fraction (40.1%) with PMA-stimulation, but leaving 59.9% in the non-cytoskeletal fraction.

In conclusion, the present study demonstrated that $p40^{phox}$ associates with the cytoskeleton *via* a molecule of $p67^{phox}$ and translocates to the membrane as a complexed unit with $p67^{phox}$, rather than being released to the cytosol, using a cytoskeleton scaffold for the activation of the NADPH oxidase. Currently, the partners of the N-terminal SH3 domain (aa 241–304) of $p67^{phox}$ and the N-terminal polyproline motif of $p47^{phox}$ (aa 70–83), remain to be determined. Since an SH3 domain is also present in a variety of proteins that associate with cytoskeleton (*36*), the N-terminal SH3 domain of $p67^{phox}$, which was shown to be important for the NADPH oxidase activation in a whole-cell system (*37*), might be responsible for its association with cytoskeleton. In addition, further studies that take coronin into account should help clarify the role of $p40^{phox}$ in the relationship between the cytoskeleton and NADPH oxidase, and such studies have recently started (*31*, *38*).

We also found that $p40^{phox}$ can be present alone in undifferentiated HL-60 cells. As they differentiate to phagocytic cells, $p67^{phox}$ is synthesized and captured by pre-existing $p40^{phox}$. However, free $p40^{phox}$ can not exist stably in differentiated phagocytes without the assistance of $p67^{phox}$, as previously demonstrated by mature PMNs from $p67^{phox}$-deficient CGD patients (*9*, *10*, *13*). Probably, only the tertiary structure of the $p40^{phox}$-$p67^{phox}$ complex resists attacks by a number of proteases in PMNs. This complex formation will endow PMNs with two benefits: (i) stabilization of $p67^{phox}$, which is known to be a very fragile molecule; and (ii) prohibition of spontaneous O_2^--generation, by means of interposition of $p40^{phox}$ between $p47^{phox}$ and $p67^{phox}$. The ternary complex including $p47^{phox}$ present in resting cells points to this possibility, which avoids extremely dangerous effects of reactive oxygens on the host. Thus, $p40^{phox}$ is to be a molecule with bifunction, stabilization of $p67^{phox}$ and modulation of the NADPH oxidase activity.

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