RESEARCH COMMUNICATION

p40^{phox}, a third cytosolic component of the activation complex of the NADPH oxidase to contain *src* homology 3 domains

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The NADPH oxidase generates superoxide in phagocytic cells. It is important for immunity and its deficiency leads to chronic granulomatous disease (CGD). It consists of a membrane-bound flavocytochrome *b* that lies dormant until activated by the translocation to the plasma membrane of cytosolic proteins, p47^{phox} (*phox* for *ph*agocyte *ox*idase), p67^{phox} and p21^{rac}, a small GTP-binding protein. We show here that a novel component, p40^{phox}, forms an activation complex with p47^{phox} and p67^{phox} with which it translocates to the membrane to associate with the flavocytochrome *b*. cDNA cloning and amino acid analysis revealed that p40^{phox} has an *src* homology 3 (SH3) domain and a large region of sequence similarity with the N-terminus of p47^{phox}. The primary association of p40^{phox} appears to be with p67^{phox}, and it is present in reduced amounts in patients with CGD lacking p67^{phox}.

INTRODUCTION

The efficient killing of microbes within the endocytic vacuole of the 'professional' phagocytes, neutrophils, monocytes, macrophages and eosinophils, is dependent upon the generation of large amounts of superoxide within this compartment. Electron transport from the substrate NADPH in the cytoplasm to oxygen is accomplished by a flavocytochrome *b* in the plasma membrane that is incorporated into the wall of the phagocytic vacuole (Morel et al., 1991; Segal and Abo, 1993). Because of the toxicity of its products and the requirement for their compartmentalization within the vacuole, this flavocytochrome, a member of the FNR family of reductases (Segal et al., 1992), lies dormant until stimulated by one of a number of agonists. Activation is absolutely dependent upon cytosolic proteins which translocate to associate with the flavocytochrome *b* in the membrane.

Deficiencies of the specific components of this oxidase led to chronic granulomatous disease (CGD) and hence to their discovery. The commonest cause of this syndrome is an X-linked inheritance resulting in the absence or abnormality of the large β subunit of flavocytochrome *b*. p67^{phox} and p47^{phox} are cytosolic proteins, deficiencies of which lead to CGD with an autosomal recessive inheritance (Segal et al., 1985; Nunoi et al., 1988; Volpp et al., 1988). They each contain two *src* homology 3 (SH3) domains (Rodaway et al., 1990; Leto et al., 1990), which are indicative of sites of protein–protein interaction (Musacchio et al., 1992), but no other structural clues as to their mode of action. Upon activation of the oxidase they move to the plasma membrane to associate with the flavocytochrome *b* (Heyworth et al., 1989, 1991; Clark et al., 1990).

It was found that electron transport could be induced in a mixture of unstimulated membranes and cytosol by the addition of amphiphiles like arachidonic acid or SDS, a system known as the 'cell-free' assay. Fractionation of the cytosol added to this system revealed the requirement for a factor other than p67^{phox} and p47^{phox}, which was subsequently shown to be a complex between the small GTP-binding protein p21^{rac} and rhoGDI (Abo et al., 1991). We have now shown that upon activation, p21^{rac} dissociates from rhoGDI and also moves to the plasma membrane (Abo et al., 1994).

Although the oxidase could be completely reconstituted in the cell-free assay from recombinant p21^{rac}, p67^{phox} and p47^{phox} together with pure flavocytochrome *b* (Abo et al., 1992), there were indications that other components might be involved. On gel-filtration chromatography, p67^{phox} separates with a mass of about 250 kDa (Park et al., 1992). p47^{phox} appears to contribute to this complex because a proportion of this protein separates together with p67^{phox}, although some of it appears to be smaller (Park et al., 1992). In the present study we have identified a 40 kDa protein, which we have called p40^{phox}, as a component of this complex. It contains an SH3 domain similar to one of the SH3 domains on p67^{phox}, and in addition it has a region sharing great similarity with the N-terminal part of p47^{phox}. Although not required for activity of the oxidase in the cell-free system, it is likely to be important in the regulation of the activation complex of the cytosolic oxidase components in the intact cell.

EXPERIMENTAL

Preparation and fractionation of neutrophils

Neutrophils were obtained from the blood of healthy donors or CGD patients as described (Segal and Jones, 1980). The cells were broken by sonication as described (Segal et al., 1992) in the presence of protease inhibitors (1 mM di-isopropyl fluorophosphate, 1 mM phenylmethanesulphonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin and 1 μg/ml tosyl-lysylchloromethane). Cytosol and membranes were obtained from the homogenate by centrifugation on discontinuous sucrose gradients as described (Segal et al., 1992).

For translocation studies, 1×10^8 neutrophils in 1 ml of PBS containing 5 mM glucose were activated with 1 μg/ml phorbol myristate acetate (PMA) at 37 °C for 3 min. Ice-cold PBS (15 ml) was then added and the cells centrifuged at 400 *g* at 4 °C for 5 min. Control cells were treated similarly, but without PMA. After cell fractionation, membranes were aspirated from the 15/34% sucrose interface, diluted 1:2 with lysis buffer, and recentrifuged for 15 min at 200000 *g* ($r_{av.}$ 5.9 cm) in a Beckman TLX centrifuge with a TLS 55 rotor. Membranes were solubilized in sample buffer and half of each sample was electrophoresed and Western blotted.

Abbreviations used: CGD, chronic granulomatous disease; SH3, *src* homology 3; PMA, phorbol myristate acetate.

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Fractionation of cytosol

Cytosol (500 μl from 1×10^9 neutrophils, or 2×10^8 in the case of the p67^{phox}-deficient patient) was prepared as described above and chromatographed at 1.5 ml/min on a 1.6 cm × 50 cm Superose 12 column (Pharmacia) equilibrated with 10 mM Pipes/100 mM KCl/3 mM NaCl/3.5 mM $MgCl_2$ (pH 7.0) containing the protease inhibitors mentioned earlier. Fractions (1.5 ml) were collected, 15 μl of every second fraction precipitated with trichloroacetic acid and 5 μl of cytosol analysed by Western blotting. The fractions containing all three proteins were pooled and chromatographed on a MonoQ HR 5/5 column (Pharmacia), equilibrated in the same buffer, at a flow rate of 0.5 ml/min and were eluted with a linear gradient of NaCl into 1-ml fractions. The fractions were precipitated with trichloroacetic acid and Western blotted as described above.

Immunochemistry

The antisera used for immunoprecipitation and blotting were raised against recombinant p47^{phox} and p67^{phox} (Abo et al., 1992) in rabbits and were affinity purified on the recombinant proteins coupled to *N*-hydroxysuccinimide-activated Sepharose (Hitrap column; Pharmacia). Antibodies to p40^{phox} were raised in mice by injecting synthetic peptides corresponding to the N- and C-terminal 15 amino acids (Alta Bioscience) coupled to haemocyanin as carrier protein (Gullick, 1988). Both of the anti-peptide antisera recognized p40^{phox} on immunoblots and normally a mixture of both was used. Cytosol used for immunoprecipitation or affinity chromatography was recentrifuged before use for 10 min at 125000 *g* in a Beckman TLX centrifuge with a TLA 100.4 rotor ($r_{av.}$ 3.7 cm). Immunoprecipitation was performed by the incubation of 2 μg of IgG with 250 μl of cytosol (approx. 1 mg of protein) at 4 °C for 1 h followed by incubation with Protein A–Sepharose (Sigma) for 1 h at 4 °C with rotation. The beads were washed successively with Tris/EDTA buffer (10 mM Tris/1 mM EDTA, pH 7.5) first containing 1% Triton X-100, then with 0.5 M NaCl and finally without additions; they were then boiled in SDS sample buffer and run on PAGE [10% (w/v) polyacrylamide] and stained with Coomassie Brilliant Blue. For immuno-affinity chromatography, anti-p67^{phox} IgG (0.4 mg) was bound to a Hitrap column. Cytosol (20 mg of protein in 4 ml) was passed over the column (flow rate 0.2 ml/min) and the column was washed extensively with 0.5 M NaCl in Tris/EDTA buffer. Immuno-absorbed proteins were eluted with 0.1 M glycine, pH 2.5, and the eluate was neutralized with 1 M Tris, pH 8.0. The eluate was run on SDS/PAGE and either stained with Coomassie Brilliant Blue or Western blotted. Immunoblots were probed with antibodies to p47^{phox}, p67^{phox} and p40^{phox}. The blots were developed with the appropriate second antibody conjugated to alkaline phosphatase (Promega).

Microsequencing and cloning

The eluate from the anti-p67^{phox} column was dissolved in SDS sample buffer and applied to SDS/PAGE. The p40 protein was visualized by staining with Coomassie Brilliant Blue G-250, excised and digested with detergents in the gel slice using either trypsin or *Achromobacter lyticus* lysyl endopeptidase (J. J. Hsuan, unpublished work). Peptides were separated by h.p.l.c. using Aquapore AX-300 (30 cm × 2.1 mm) and OD-300 (100 cm × 2.1 mm) columns in series on a Hewlett–Packard 1090M with diode-array detection. The columns were developed with a linear acetonitrile gradient in 0.08% trifluoroacetic acid. Fractions were applied directly to an Applied Biosystems 477A sequencer fitted with a miniature reaction chamber as described (Totty et al., 1992). Due to the specificity of the protease, a lysine residue was assumed to exist before the N-terminus of each of the peptides produced by lysyl endopeptidase digestion. Degenerate oligonucleotides were made corresponding to the beginning and end of the longest peptide (two overlapping sequences, amino acids 192–213 in the sequence are shown in Figure 3); the 5′ primer was AARGCYGGNGAYGTSATCTTYCT and the 3′ primer GTCCAYTCNAGCCARTCYTTRTT. These oligonucleotides were used to prime a PCR reaction on cDNA from induced HL60 cells. A product of the expected size was purified, subcloned in the *Sma*I site of the Bluescript phagemid (Stratagene) and, after sequencing to confirm the right sequence, the amplified insert was used to screen a retinoic acid-induced HL60 cDNA library in λgt10. The cDNA inserts of several of the positive clones were subcloned in Bluescript and sequenced with the dideoxy-chain-termination method (Sanger et al., 1977) using Sequenase version 2 from United States Biochemical (Cleveland, OH, U.S.A.). Searches for homology were performed with the FASTA program of the sequence-analysis software package of the Genetics Computer Group (GCG, Madison, WI, U.S.A.). This program uses the method of Pearson and Lipman (1988) to find similarities between a sequence and a group of sequences. The SwissProtein database (Release 25.0) was searched with the amino acid sequence and the EMBL database (Release 34.0) with the nucleotide sequence. Besides similarity with p47^{phox}, the FASTA program also found similarities with a number of SH3 domains. These were aligned using the PILEUP program (Feng and Doolittle, 1987) of the same package to generate a multiple-sequence alignment.

RESULTS

p40^{phox} is present as a complex with p67^{phox} and p47^{phox} in neutrophil cytosol

In an attempt to identify proteins associated with the cytosolic oxidase factors, we immunoprecipitated p67^{phox} from neutrophil cytosol and discovered that it had two other proteins complexed to it, p47^{phox} and a protein with an apparent molecular mass of about 40 kDa, subsequently referred to as p40^{phox} (Figure 1). p40^{phox} and p67^{phox} seemed to be present in the complex in roughly equimolar quantities. The presence of p47^{phox} in the immunoprecipitates was obscured by the heavy chain of IgG, but purification of the complex by affinity chromatography on a column containing anti-p67^{phox} IgG showed p47^{phox} to be present in the complex, although the amounts recovered varied greatly (cf. Figure 1, lanes 8 and 9). This probably reflects a relatively weak binding of p47^{phox} to the other proteins in the complex, as a result of which it was partially eluted off during the high-salt wash (see also below). The primary association seemed to be between p67^{phox} and p40^{phox} because both these proteins were precipitated by an antibody to p67^{phox} in p47^{phox}-deficient cytosol, whereas p40^{phox} was not precipitated by an antibody to p47^{phox} from p67^{phox}-deficient cytosol; however, this might be because of the reduced levels of p40^{phox} seen in these patients (see below, Figure 4). In addition, the association between p67^{phox} and p40^{phox} appears to be more stable than that between either protein and p47^{phox} on the basis of chromatography of the complex. Partial purification of the cytosolic oxidase components with gel-filtration and anion-exchange chromatography (Figure 2) confirmed the presence of p40^{phox} in the material which was eluted at about 250 kDa, and showed that the three proteins remain complexed during separation by anion-exchange chromatography, although some p47^{phox} appeared in the unbound fraction of the anion-exchange column.

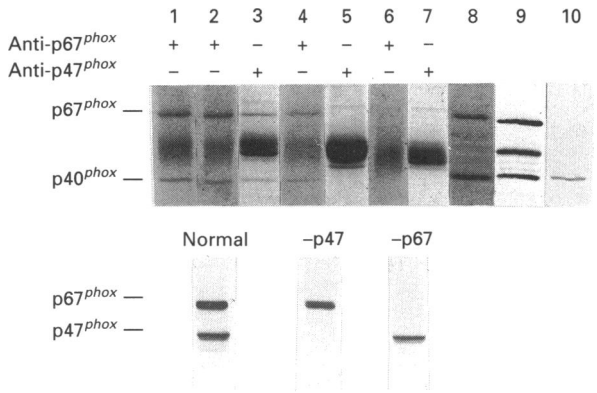

Figure 1 Association of p67^{phox}, p47^{phox} and p40^{phox} in neutrophil cytosol

Lanes 1–7 are immunoprecipitates of normal and CGD-patient neutrophil cytosols with antisera to p67^{phox} and p47^{phox} analysed by SDS/PAGE. The cytosols were prepared from resting cells, except in the case of lane 1 which was from PMA-activated cells. Lanes 1–3, cytosol from normal cells; lanes 4 and 5, cytosol from p47^{phox}-deficient cells; lanes 6 and 7, cytosol from p67^{phox}-deficient cells. The immunoprecipitates were obtained with anti-p67^{phox} (lanes 1, 2, 4 and 6) and anti-p47^{phox} (lanes 3, 5 and 7). Immunoblots showing the presence or absence of p47^{phox} and p67^{phox} in the different cytosols are shown below the Coomassie Blue-stained tracks. Lanes 8 and 9 show eluates obtained by immunoaffinity chromatography of normal cytosol on an anti-p67^{phox} column as analysed by SDS/PAGE and Coomassie Blue staining (lane 8) and immunoblotting against antisera to all three proteins (lane 9). Lane 10 is an immunoblot of cytosol against the anti-(p40^{phox} peptide) antisera.

Cloning of the cDNA for p40^{phox} and the identification of similarities with SH3 domains and with a region of p47^{phox}

To obtain an amino acid sequence from p40^{phox}, it was isolated by electrophoresis after immunoabsorption and cleaved with trypsin or lysyl endopeptidase; the peptides were then separated and sequenced. Amino acid sequences of 14 different peptides from p40^{phox}, ranging from 7 to 17 amino acids in length, were obtained (underlined sequences in Figure 3a). Database searches with these sequences identified p40^{phox} as a novel protein. Screening of an HL60 cDNA with a PCR product corresponding to the longest peptide resulted in the isolation of several clones. Figure 3 shows the sequence of one of these clones in which 130 non-transcribed nucleotides are followed by an open reading frame of 1017 nucleotides, predicting a molecular mass of 37 kDa, which is in reasonably good agreement with the observed value. The presumed start codon is preceded by a consensus sequence found for mRNAs (Kozak, 1987). Searches of the SwissProtein database with the amino acid sequence and the EMBL database with the nucleotide sequence confirmed the novelty of the protein. Interestingly, the greatest similarities were with p47^{phox} and p67^{phox} (Figures 3b and 3c). That with p47^{phox} extended from the N-termini of both proteins over a large part of their amino acid sequence, with 22% identity over 245 amino acids. Taking conservative substitutions into account, the two proteins are 67% similar over the N-terminal 70% of their sequence (Figure 3b).

p40^{phox} also contained a region having similarity with SH3 domains of a number of proteins and the strongest of these similarities was with the C-terminal SH3 domain of p67^{phox} (Figure 3c). Northern-blot analysis of cell lines and tissues to examine the pattern of expression of p40^{phox} indicated that, like p47^{phox} and p67^{phox}, its tissue expression is predominantly in bone marrow and neutrophils, and in cultured cells it is most abundant in myeloid cells, with lower levels in B-cell lines (results not shown).

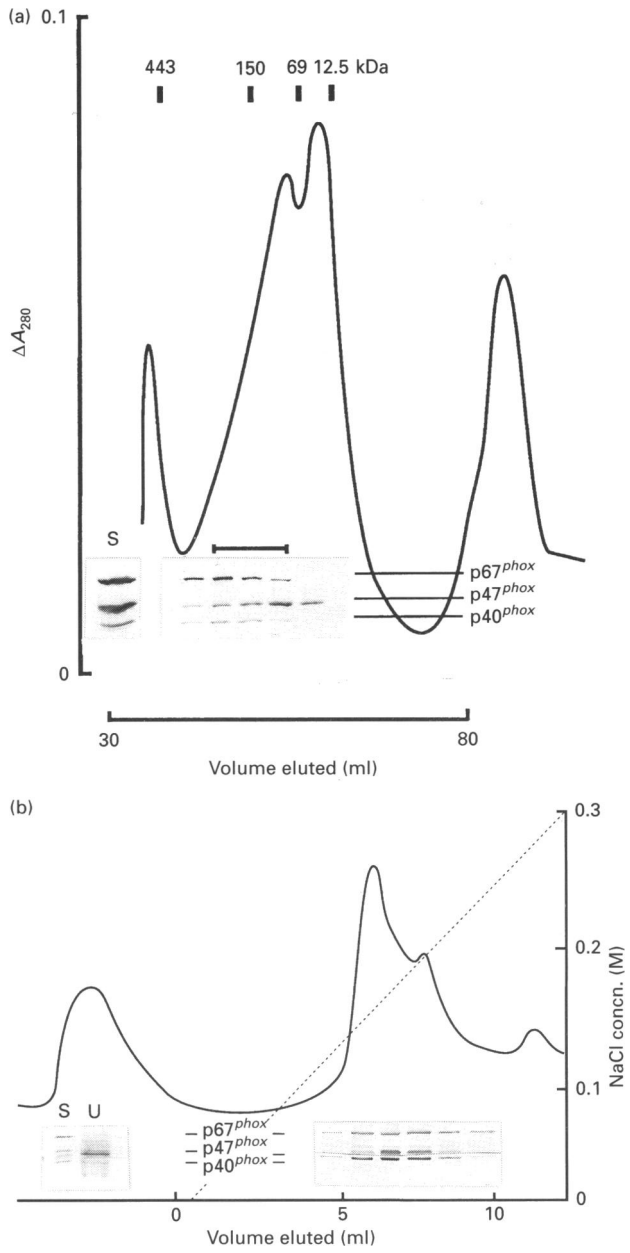

Figure 2 p67^{phox}, p47^{phox} and p40^{phox} chromatograph as a high-molecular-mass complex

(**a**) Elution profile of gel-filtration chromatography of normal neutrophil cytosol with Western blots of fractions against antibodies to p67^{phox}, p47^{phox} and p40^{phox} shown below the profile. p67^{phox} and p40^{phox} separate with a similar size distribution, indicative of involvement in a complex of between 200 and 400 kDa. A portion of p47^{phox} also appears to separate with a high molecular mass, whereas about half of it is clearly smaller. S, Western blot of starting material. (**b**) The fractions which were eluted with high molecular masses from the gel-filtration column in (**a**) were pooled (horizontal bar in **a**) and subjected to anion-exchange chromatography on a MonoQ column. The fractions were analysed by Western blotting as in (**a**). S, pooled gel-filtration samples. Some p47^{phox} failed to bind to the MonoQ column and was observed in the unbound fraction (U). The three proteins were eluted with a similar profile, although there was slight tailing of p67^{phox}.

p40^{phox} is depleted during p67^{phox} deficiency and translocates to the membranes upon activation of the cells

Western-blot analysis of cytosols of CGD patients showed that p40^{phox} is present, but markedly reduced, in cytosols deficient in p67^{phox} (Figure 4), but not in those lacking p47^{phox} or in X-linked

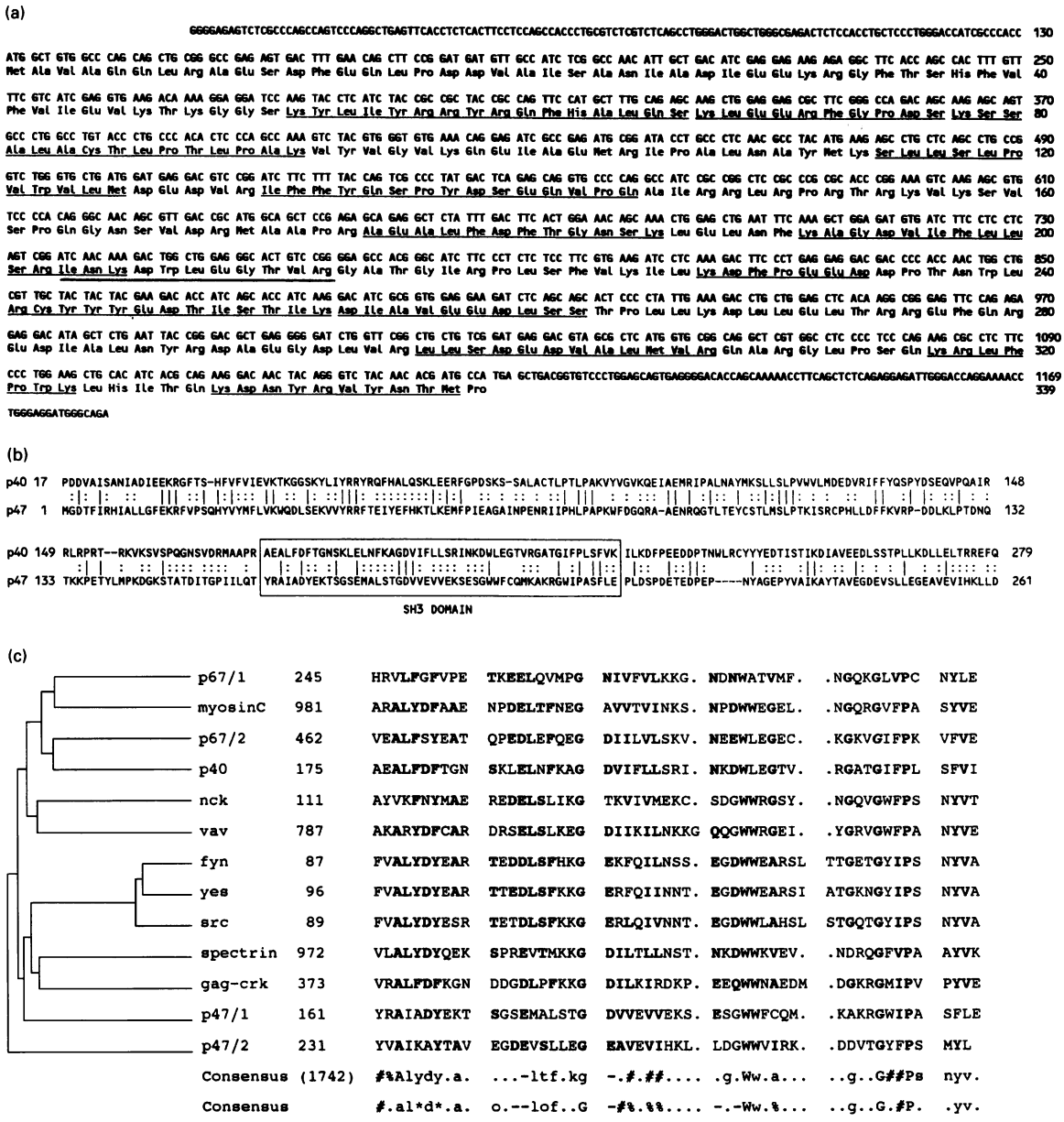

Figure 3 The sequence of p40^{phox} and its similarities

(**a**) The DNA sequence as found in one of the λgt10 clones which hybridized with the 60-bp PCR product (see the text for description). The sequence contains an open reading frame beginning at position 131. The predicted amino acid sequence of the open reading frame is shown underneath the nucleotide sequence with the peptide sequences obtained by direct sequencing of the p40 protein underlined. (**b**) and (**c**) The one-letter code for amino acids is used in the sequence comparisons. In (**b**) the alignment of p40^{phox} and p47^{phox} is shown as found by the FASTA program of the GCG package. Identical amino acids are indicated by a vertical line and similar amino acids by a colon. The SH3 domain is boxed. (**c**) Alignment of the 13 SH3 domains most similar to the one in p40^{phox}. On the left, a dendrogram of the alignment is shown, illustrating the close relationship of the p40 SH3 domain and the C-terminal one of p67^{phox}. The SH3-domain consensus sequence described by Musacchio et al. (1992) is shown where % and # are hydrophobic residues (> 50% and > 80% respectively) and —, predominantly E + D. Our consensus is shown below with the same symbols and in addition: —, acidic amino acids; *, aromatic amino acids and o, predominantly S and T.

CGD (not shown), a further indication of the close association and primary interaction between p67^{phox} and p40^{phox}. It is possible that in some cases of p67^{phox} deficiency the primary molecular lesion affects p40^{phox}, possibly affecting its binding to, and hence producing instability of, p67^{phox}.

On activation with PMA, all three of these cytosolic proteins translocated to the membranes, and none of the proteins did so in X-linked CGD patients in which the flavocytochrome *b* is missing (Figure 4).

DISCUSSION

The complex of cytosolic proteins required for the activation of electron transport across flavocytochrome *b* in the membrane of the phagocytic vacuole clearly involves at least three proteins, p47^{phox}, p67^{phox} and that identified in this study, p40^{phox}. All three proteins contain SH3 domains, p47^{phox} and p67^{phox} having two each, providing a wide variety of possible protein–protein interactions.

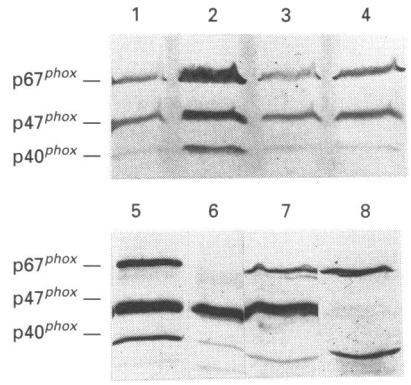

Figure 4 $p40^{phox}$ translocates to the plasma membrane in normal, but not X-linked CGD-activated neutrophils, and is depleted in the cytosol of $p67^{phox}$-deficient patients

Membranes and cytosol were prepared by fractionating sonicated neutrophils as described in the Experimental section. One half of the final membrane preparations were applied on SDS/PAGE and immunoblotted against antibodies to $p67^{phox}$, $p47^{phox}$ and $p40^{phox}$ (lanes 1–4); 20 μg of cytosol was run (lanes 5–8) and immunoblotted. Translocation of $p67^{phox}$, $p47^{phox}$ and $p40^{phox}$ to the membranes was observed in PMA-activated as compared with resting normal cells (lanes 2 and 1 respectively). Translocation of all three proteins to membranes was not seen in X-linked CGD cells lacking the flavocytochrome *b* (lane 3, resting and lane 4, activated cells). $p40^{phox}$ was present, but at reduced levels, in a patient with complete deficiency (lane 6) and one with partial deficiency (lane 7) of $p67^{phox}$ as compared with normal cytosol (lane 5) and that from a patient lacking $p47^{phox}$ (lane 8).

SH3 domains appear to bind to proline-rich sequences on polypeptides (Ren et al., 1993), and $p67^{phox}$ has a proline-rich region, PPPRPKTP (amino acids 227–234), that could represent an SH3-binding domain, possibly providing a binding site to its own SH3 domains or those on one of the other two proteins.

Electron transport by the flavocytochrome *b* in the artificial cell-free assay does not require $p40^{phox}$, while at the same time being absolutely dependent upon $p47^{phox}$ and $p67^{phox}$. This suggests that $p40^{phox}$ plays a regulatory role that is replaced or circumvented by the SDS or arachidonic acid used to activate the cell-free system. The similarity between $p40^{phox}$ and $p47^{phox}$ provides an interesting clue to the possible interactions of these proteins. It seems possible that both proteins share a binding site on $p67^{phox}$, and that the one displaces the other upon activation, thereby changing the conformation of the complex as a whole. However, all three proteins translocate to the plasma membrane upon activation of the oxidase, indicating that $p47^{phox}$ does not simply displace $p40^{phox}$ from its binding site on $p67^{phox}$, and that a more subtle rearrangement might occur. Clearly all three of these cytosolic proteins require the presence of flavocytochrome *b*, or some associated molecule, to act as a docking site because these proteins fail to translocate to the membranes in X-linked CGD upon activation, as has been described previously for $p47^{phox}$ and $p67^{phox}$ (Heyworth et al., 1989, 1991). The C-terminus of the α subunit of flavocytochrome *b* is very proline-rich and includes the sequence PPSNPPPRPP (amino acids 150–159), a possible binding site for the SH3 domain on one of the cytosolic proteins. This interaction may be made possible by the exposure of one, or both, domains upon activation of the oxidase, possibly by a change in confirmation mediated by phosphorylation of flavocytochrome *b* (Garcia and Segal, 1988) or $p47^{phox}$ (Heyworth et al., 1989).

The interaction of these three SH3-domain-containing cytosolic proteins, their involvement with small GTP-binding proteins (Abo et al., 1991) and their translocation to the membranes upon activation has some similarity to the involvement of Sos and Grb in tyrosine kinase and Ras-receptor-mediated signal transduction (McCormick, 1993; Egan et al., 1993). The elucidation of the mechanism of interaction of the activation complex components of the oxidase, the binding of $p21^{rac}$ to these proteins, and the mechanism by which it induces activation of the complex and its translocation to the membranes, culminating in electron transport, will provide important additional insight into our understanding of protein–protein interactions and signal-transduction mechanisms.

Note added in proof (Received 29 October 1993)

Someya et al. (1993) observed co-purification of a 39 kDa protein with the guinea pig equivalent of $p67^{phox}$ and $p47^{phox}$. Peptide sequences confirmed this as being homologous with $p40^{phox}$.

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