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Cytosolic *phox* proteins interact with and regulate the assembly of coronin in neutrophils

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SUMMARY

The NADPH oxidase generates microbicidal superoxide in phagocytes, and when defective it leads to chronic granulomatous disease (CGD). Oxidase specific proteins in the cytosol, p47*phox* and p67*phox*, as well as the small GTP binding protein p21*rac* are important for activation of superoxide production. Because the activity of this oxidase is normally tightly restricted to the phagocytic vacuole, and its temporal and spatial organisation might be regulated by cytoskeletal proteins, we examined the cytosolic *phox* proteins for interactions with cytoskeletal elements. p67*phox* copurified with a 57 kDa protein, identified as coronin, an actin binding protein that is important for movement and phagocytosis in *Dictyostelium*. Binding studies revealed that coronin attaches to the C-terminal half of p40*phox*, a binding partner of p67*phox*. The *phox* proteins and coronin had a similar distribution in the cell, and both accumulated around the phagocytic vacuole. PMA activation of adherent neutrophils resulted in a major rearrangement of these proteins, and of actin, which were lost from the periphery of the cell and condensed around the nucleus. The rearrangement of F-actin and coronin in adherent cells, were absent, or markedly diminished, in cells from patients lacking p47*phox* or p67*phox* in which an abnormally large proportion of the coronin was present as part of a large complex. The cytosolic *phox* proteins might play a regulatory role in the reorganisation of the cytoskeleton accompanying superoxide generation.

Key words: Neutrophil, NADPH oxidase, Coronin, Cytoskeleton, Actin binding protein, Chronic granulomatous disease, *Dictyostelium*, p47*phox*, p67*phox*

INTRODUCTION

Specialised phagocytic cells, such as the neutrophil, act by engulfing microbes, which they encapsulate in a membrane vacuole and then destroy by releasing superoxide (Babior et al., 1973; Briggs et al., 1975b) and the contents of cytoplasmic granules (Segal et al., 1980) into this compartment. The respiratory burst of superoxide can also be provoked by extraneous agonists, such as the chemotactic peptide fMLP (McPhail et al., 1981) and the protein kinase C activator, PMA. The superoxide is generated by a membrane-associated flavocytochrome b (Segal et al., 1992; Taylor et al., 1993), which acts as a NADPH oxidase. To develop activity the enzyme must first bind p47*phox* (Segal et al., 1985; Volpp et al., 1989; Lomax et al., 1989) and p67*phox* (Leto et al., 1990), cytosolic proteins known as *phox* proteins (from *ph*agocyte *ox*idase). Absence or dysfunction, of the flavocytochrome, p47*phox* or p67*phox* results in impaired production of superoxide and the clinical condition of CGD, characterised by a severe, often fatal, incapacity to combat bacterial or fungal infections (Thrasher et al., 1994). p40*phox* co-immunoprecipitated with p67*phox* and cellular levels were markedly reduced in cells lacking p67*phox* (Wientjes et al., 1993), but primary lesions of p40*phox* have not yet been implicated as the cause of CGD. In vitro experiments suggest that p40*phox* might down regulate the oxidase (Sathyamoorthy et al., 1997).

The formation of the phagocytic vacuole is a highly localised event, in which the membrane-bound cytoskeleton (Valerius et al., 1981) plays an important role. This cytoskeleton has also been surmised to participate in the NADPH oxidase. The evidence is circumstantial; thus, fMLP and PMA have both been shown to engender marked changes in the shape of the cell (Howard and Wang, 1987; Wymann et al., 1989) and also in the polymerisation state of actin (Howard and Wang, 1987; Al Mohanna and Hallett, 1990; Sheterline et al., 1986), and p47*phox* and p67*phox* have been localised to the detergent insoluble Acytoskeleton@ (Nauseef et al., 1991; Woodman et al., 1991). Further, the small GTP-binding protein, p21*rac*, which initiates such processes as membrane ruffling in fibroblasts (Ridley et al., 1992), is essential for oxidase in the ‘cell free’ assay of NADPH oxidase activity (Abo et al., 1991; Knaus et al., 1992).

We present here evidence that extensive cytoskeletal rearrangements accompany activation; we identify coronin, a protein previously shown to bind to actin, as a protein that is associated with the cytosolic components of this oxidase, and we demonstrate the malfunction of this cytoskeletal rearrangement in different genetic forms of CGD.

MATERIALS AND METHODS

Purification of neutrophils and measurement of superoxide generation

Neutrophils were purified from fresh human blood by dextran sedimentation and centrifugation through Ficoll/Hypaque as described previously (Segal and Jones, 1980). Superoxide generation was measured kinetically by superoxide dismutase inhibitable cytochrome C reduction (Mandell et al., 1987) in a dual beam spectrophotometer (Kontron, Uvicon 860).

Electrophoresis and western blotting

Samples were run on 10% SDS-PAGE minigels and transferred to nitrocellulose membranes with a semidry blotter for 1 hour at 1.4 mA/cm^2. Membranes were stained with Ponceau Red (Sigma), destained and probed with antibodies from rabbit polyclonal antisera raised to recombinant p67^{phox}, p47^{phox}, p40^{phox} and coronin. Affinity purified antibodies were prepared as described previously (Wientjes et al., 1993).

Horseradish peroxidase-conjugated goat anti-rabbit antibody was used to detect reactive bands by ECL (Amersham). In some experiments these ECL bands were quantitated by densitometry (Alpha imager 2000, Alpha Innotech Corporation), in others ^{125}I Protein A (ICN) was added and bound radioactivity measured on a phosphoimager (Fuji). Recombinant coronin, p47^{phox} and p67^{phox} were used as standards.

Chromatography of cytosol

Neutrophils (1×10^{10}) in 10 ml PBS containing glucose (5 mM) were incubated at 37°C for 5 minutes, stimulated with PMA (1 μg/ml) for 3 minutes, diluted to 60 ml with ice-cold PBS, and centrifuged at 200 ***g*** for 5 minutes at 4°C. The cell pellet was sonicated for 3× 5 seconds in 'break' buffer (KCl (10 mM), NaCl (3 mM), $MgCl_2$ (4 mM), Pipes (10 mM), pH 7.0, containing protease inhibitors: leupeptin (1 μg/ml); pepstatin (1 μg/ml); tosyl-lysine-chloromethyl ketone (1 μg/ml); phenyl methyl sulphonyl fluoride (0.5 mM); and phosphatase inhibitors: microcystin (0.1 μM); sodium pyrophosphate (5 mM); sodium fluoride (25 mM)) and centrifuged at 120,000 ***g*** for 45 minutes at 4°C. The supernatant cytosol (approximately 2 ml of 25 mg/ml protein) was chromatographed successively on the following columns equilibrated in break buffer; Superose 12 (75 × 2.6 cm, 2 ml/minute), Q Sepharose (15 × 1.6 cm, 1 ml/minute) and heparin agarose (5 × 1 cm, 0.5 ml/minute). Elution from the latter two columns was with a linear gradient of 0-1.0 M NaCl in break buffer with inhibitors. Relevant fractions from the Q Sepharose column were diluted with two volumes of break buffer before application to the heparin agarose column. Gel filtration markers were apoferritin (440 kDa), alcohol dehydrogenase (160 kDa) and bovine serum albumin (67 kDa).

Amino acid sequencing

Proteins were separated by SDS-PAGE, stained with Coomassie Blue and digested in excised gel pieces with either endoproteinase lys-C (Wako) or alkylated trypsin (Promega). Peptides were recovered by sonication and applied directly to a Reliasil C 18 column (150 × 1 mm) fitted with an upstream Aquapore AX-300 trap column (2 × 0.5 mm) on a Michrom Ultra Fast Protein Analyser HPLC system. The columns were developed with a linear acetonitrile gradient in 0.1% trifluoroacetic acid at a flow rate of 50 μl/minute. Fractions were collected and sequenced using a high sensitivity Applied Biosystems Procise system employing capillary HPLC (250 × 0.8 mm C18 column). Initial yields were in the range 0.5 to 5 pmole.

Recombinant proteins and binding studies

Recombinant p47^{phox}, p67^{phox} and p40^{phox} were made as glutathione transferase fusion proteins in the pGEX system (Pharmacia) and were purified from the bacterial lysates as described (Abo et al., 1992; Wientjes et al., 1996). The N and C termini of p40^{phox} were made by amplification of the relevant regions of the gene by PCR. The complete products were cloned into pGEX. p40^{phox} lacking the SH3 domain was constructed by site directed mutagenesis using the USE method (Pharmacia). Recombinant p40^{phox} containing a His.Tag was produced in the pET23d plasmid (Novagen) and purified over a nickel chelate column (Invitrogen). Coronin was expressed in insect cells (Sf9) using the baculovirus system. The vector and virus used were pVL1393 and baculogold, respectively (Pharmingen). The protein (8-10 mg) was purified from insect cells which were washed, pelleted and frozen (for at least 4 hours at −20°C) in relaxation buffer (Pipes (10 mM), KCl (100 mM), NaCl (3 mM), $MgCl_2$ (3.5 mM)). They were then resuspended in relaxation buffer plus Triton X-100 (1%) and protease inhibitors, sonicated (3× 10 seconds, centrifuged at 15,000 ***g*** for 15 minutes at 4°C, and the supernatant chromatographed by anion-exchange on Source Q (30 × 1.6 cm 1 ml/minute). Coronin eluted at between NaCl in 0.1 and 0.2 M in relaxation buffer. Peak fractions were further purified on phenyl Sepharose (30 × 1.6 cm, 1 ml/minute) eluted with a linear gradient of $(NH_4)_2SO_4$ of 1.0-0 M in water, the protein eluting at between 0.4 and 0.5 M.

Recombinant p67^{phox}, p47^{phox}, p40^{phox}, p40^{phox} mutants or human serum albumin (300 nmol) were coupled onto 1 ml of beads from NHS activated HiTrap columns (Pharmacia) and the beads were then blocked with 1% gelatin (Sigma) in Tris buffered saline (TBS) and 0.05% Tween-20 at 37°C for 2 hours. An aliquot of cytosol (500 μl) or recombinant coronin (150 μg) was added to 100 μl of the beads and incubated at 25°C for 15 minutes. The beads were washed and bound proteins eluted as described (Wientjes et al., 1996).

Subcellular fractionation of neutrophils and sedimentation of proteins

Neutrophils (1×10^8) in PBS glucose were either unstimulated or stimulated with l μg/ml PMA for 3 minutes, cooled with 10 volumes of ice-cold PBS, pelleted, resuspended in break buffer (750 μl) containing protease inhibitors, sonicated for 3× 5 seconds and centrifuged at 200 ***g*** for 10 minutes at 4°C. An aliquot of this post nuclear supernatant (PNS, 500 μl) was centrifuged at 70,000 ***g*** for 10 minutes at 4°C in a Beckman TLS100.2 head through 10% (300 μl) and 34% sucrose (300 μl) (w/w in break buffer). Membranes at the 10%/34% interface were diluted 1:1 with water and pelleted at 250,000 ***g*** for 15 minutes in a TLA head. The 70,000 ***g*** supernatant was layered onto 10% sucrose (1 ml) and centrifuged at 250,000 ***g*** for 30 minutes to separate a pellet of polymerised proteins and cytosol. Proteins in these different fractions were separated by SDS-PAGE and western blotted.

In some experiments the PNS was directly centrifuged at 250,000 ***g*** to produce the cytosol.

Immunohistochemistry and electron microscopy

Adherent neutrophils were produced by placing the cells in PBS on glass coverslips after which they were incubated at 25°C for 5 minutes and then at 37°C for 5 minutes, and then for a further 10 minutes at 37°C in the presence or absence of PMA (1 μg/ml).

To examine phagocytosing cells they were exposed to opsonised fluorescently labelled zymosan. Zymosan particles (10 mg/ml; Sigma) were boiled in PBS for 10 minutes washed once in PBS and washed and resuspended to the original volume in $NaHCO_3$ (100 mM, pH 9.0). Rhodamine isothiocyanate (Sigma, 100:l of 10 mg/ml in DMSO)

was incubated with 1 ml of zymosan for 1 hour at room temperature. The reaction was terminated with 100 μl of hydroxylamine (1.5 M, pH 8.5) and the zymosan washed, resuspended in PBS and opsonised with fresh human serum (1 ml to 2.5 mg for 1 hour at 37°C and washed 3 times with PBS. Phagocytosis was carried out with 2 mg of zymosan per 5×10^7 neutrophils with agitation at 37°C for 7 minutes. Cells were then adhered to glass coverslips for 2 minutes.

Subsequent steps were at room temperature. Cells were washed in PBS and fixed for 10 minutes in 4% paraformaldehyde, washed in PBS and permeabilised with 0.2% Triton X-100 in PBS for 10 minutes, and blocked with $NaBH_4$ (10 mM) in H_2O for 1 hour, washed in PBS and incubated with rhodamine phalloidin (1 μg/ml in PBS, Sigma) for 30 minutes or affinity purified antibodies for 2 hours, followed by fluorescein labelled goat anti-rabbit IgG antibody (Stratech Scientific Ltd). Control samples were exposed to crude preimmune serum, non-specific rabbit IgG or goat anti-rabbit IgG antisera alone.

For electron microscopy, neutrophils attached to coverslips, were unroofed (Hartwig et al., 1989) in the presence of 5 μM phallicidin. The cells were fixed with glutaraldehyde and labelled with rabbit anti-coronin and goat anti-p67*phox*. Secondary antibodies (chicken anti-rabbit and swine anti-goat) were coupled to 8 and 15 nm gold particles, respectively. After immunogold labelling, the samples were snap-frozen, freeze-dried and rotary-coated using a Cressington freeze-fracture apparatus. Replicas were viewed with a JEOL 1200-EX electron microscope.

Patients

In all patients with CGD the complete absence of superoxide production after stimulation with PMA was assessed by superoxide dismutase inhibitable reduction of cytochrome C.

All the X-linked patients had a classical X-linked pattern of inheritance, the absence of the flavocytochrome b on spectral analysis and western blotting, and a lesion in the gene coding for gp91*phox* demonstrated by nucleotide sequencing (Roos et al., 1996). p47*phox* and p67*phox* deficient patients were completely deficient in the relevant protein on western blotting.

RESULTS

p40phox and p67phox are associated with coronin in the cytosol

It is well recognised that in gel filtration of the cytosol, p67*phox* migrates in a single zone of apparent relative molecular mass of about 250 kDa, and that a portion of the p47*phox* separates with this peak, with the remainder eluting at a much lower molecular mass, compatible with that of the monomeric protein (Curnutte et al., 1987; Someya et al., 1993). To examine p40*phox*, p47*phox* and p67*phox* for an interaction with cytoskeletal elements, the cytosol was chromatographed in an attempt to identify associated cytoskeletal or linker proteins. Fig. 1 shows that after permeation chromatography through Superose 12, p67*phox* and p40*phox* eluted together with a molecular mass of about 250 kDa, with about half the p47*phox* associated with these proteins and the remainder coming off with a much lower apparent molecular mass. This pattern reflects the tighter binding of p67*phox* to p40*phox* than to p47*phox* (Wientjes et al., 1996).

The peak fractions containing the p67*phox* and p40*phox* and some of the p47*phox* were then separated by anion exchange chromatography on Q Sepharose. p40*phox* and p67*phox* bound to this resin and eluted together at a NaCl concentration of about 0.5 M, whereas p47*phox* remained in the unbound fraction. Upon subsequent fractionation over heparin agarose p40*phox* and p67*phox* again coeluted, and staining of the SDS-PAGE for protein showed that these two proteins were visible, and that they coeluted with one major protein band with a relative molecular mass of 57 kDa (Fig. 1Cii).

The protein of molecular mass 57 kDa was eluted from the gel and proteolytic fragments were subjected to N-terminal sequencing. The sequences (D), identified it as a human homologue of coronin, an actin-binding protein implicated in phagocytosis and movement in *Dictyostelium* (de Hostos et al., 1991). Four of the five peptides were identical and the fifth (in which the quality of the sequence was less adequate), very similar, to the human homologue found in Hl-60 cells (Suzuki et al., 1995). Prior to the publication of this cDNA sequence we had searched a combined GenBank/EMBL nucleotide data base for DNA sequence encoding protein sequence homologous to the peptides we obtained. Two homologous cDNA human infant brain clones (ATCC85437 and ATCC85531, EST06940 (Adams et al., 1993)) were identified. ATCC85531 was fully sequenced and the nucleotide sequence we obtained corresponded exactly with that of the Hl-60 cDNA (Suzuki et al., 1995).

The coronin was cloned, sequenced and expressed in insect cells. A polyclonal antibody was prepared by immunising rabbits and was used to follow the distribution of coronin through the purification described above, and for immunofluorescence and immuno-electron microscopy. As shown in Fig. 1A, the bulk of the coronin eluted from the gel filtration column together with the p67*phox* and p40*phox* containing complex, although about a third of the total was of a much higher relative molecular mass. The elution profiles of the three proteins were similar on the Q Sepharose and heparin agarose.

Quantitative western blotting with recombinant proteins as standard indicated that coronin accounted for about 4% of the total cellular protein as compared with 0.1% for p47*phox* and 0.05 for p67*phox*, very similar to the levels previously recorded for these two proteins (Leto et al., 1991).

In *Dictyostelium*, coronin is located in the cytoplasm and in the submembranous cytoskeleton (de Hostos et al., 1991). A small proportion of the cytosolic *phox* proteins and coronin were associated with neutrophil membranes (shown in Figs 5 and 6) and we therefore examined the distribution of these proteins on the submembranous cytoskeleton by immunohistochemistry on deroofed, adherent neutrophils. Immunogold labelling revealed that p67*phox* and coronin co-localised in clusters on filaments of the meshwork of the submembranous cytoskeleton (Fig. 1E).

Coronin binds to p40phox

To determine whether coronin was binding to p40*phox* or p67*phox*, studies were performed with these purified proteins, as well as with p47*phox* immobilised on beads. Albumin was used as control. Fig. 2A shows the Coomassie stained gel of proteins eluted from the beads after they were incubated with neutrophil cytosol. The results of the western blots are shown in (B). Samples of whole cytosol are shown in lanes marked (S). Coronin attached exclusively to p40*phox* under these conditions. The p40*phox* beads also strongly bound p67*phox*, some p47*phox* and a small amount of p21*rac*, possibly through its attachment to bound p67*phox*, as the p67*phox* beads themselves extracted a relatively large amount of p21*rac* from the cytosol.

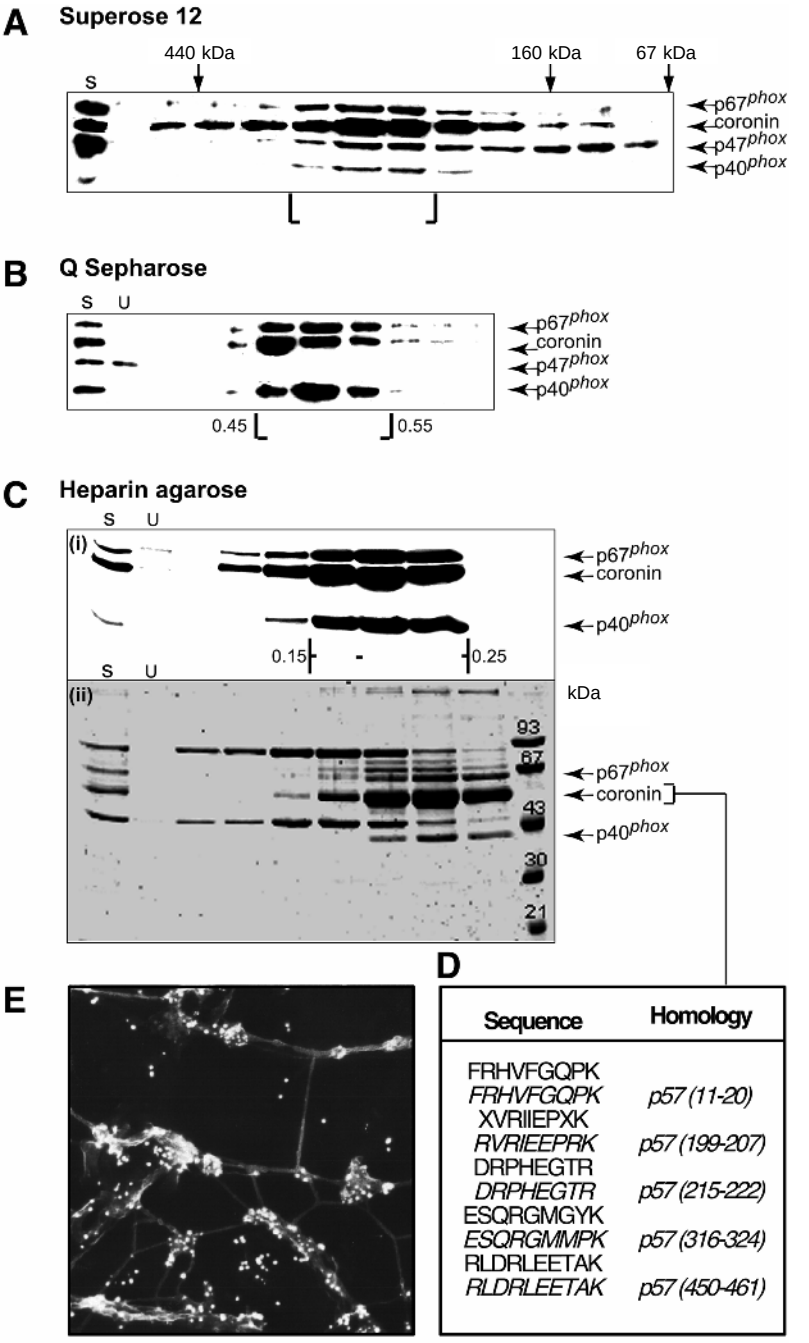

Fig. 1. p40*phox* and p67*phox* are associated with coronin. Neutrophil cytosol was chromatographed successively by gel filtration on Superose 12 (A), anion exchange on Q Sepharose (B) and then an affinity heparin agarose column (C). After each separation, those fractions containing the highest concentrations of p67*phox* and p40*phox* were pooled and applied to the next column. Subsequently, after the association between these proteins and coronin had been identified, antibodies were raised to coronin and the blots were reprobed to demonstrate its elution profile. The images are of western blots for the designated proteins except for Cii which shows a Coomassie Blue stained gel of the fractions eluted from the heparin agarose column. The 57 kDa protein that copurified with p40*phox* and p67*phox* was identified by amino acid sequencing of peptides separated from a digest of the protein eluted from the gel. As shown in D, the sequences of these peptides were homologous with the amino acid sequence of the human homologue of coronin (Suzuki et al., 1995) shown below in italics. In each case S and U represent the start and unbound material for that particular column. In each case ⌊ ⌋ indicate the fractions taken through to the next separation and, where appropriate, the concentration of the linear NaCl gradient over which those fractions eluted. (E) An electron micrograph of a deroofed neutrophil (Hartwig et al., 1989) showing the cytoskeletal network on the cytosolic surface of the plasma membrane. Immunostaining with gold-labelled antibodies to coronin (15 nm particles) and p67*phox* (8 nm particles) colocalises these two proteins on filaments of this network.

Both the p40*phox* and p67*phox* beads bound some p47*phox*. Although the p67*phox* bound p40*phox*, this did not in turn bind enough coronin to be detected. Some of these proteins can be identified on the Coomassie stained gel as indicated. In addition, a protein at about 55 kDa bound to all the beads in differing amounts. It was identified as tubulin by amino acid microsequencing. Another at roughly 75 kDa, that eluted from the p40*phox* beads, was identified as a bacterial contaminant.

Further investigation of the coronin/p40*phox* interaction, using p40*phox* constructs immobilised on beads, verified the binding of coronin from cytosol to the full length p40*phox* and more specifically to the C terminus (Fig. 2D). p67*phox* also bound to the same region of p40*phox*, as has been demonstrated previously (Wientjes et al., 1996). It would appear that the binding of p67*phox* does not impair the binding of coronin to p40*phox*, as these three proteins are associated in the cytosol.

The absence of p47phox or p67phox is associated with excessive assembly of coronin as part of a large complex

Having demonstrated that coronin copurifies with p40*phox* and p67*phox*, and that it binds to p40*phox* on affinity columns, we wished to determine whether the lack of these cytosolic *phox* proteins had any effect upon the behaviour of coronin in cells. We first looked at its subcellular distribution. The results of one such study are shown in Fig. 3A. Cytoskeletal proteins are dis-

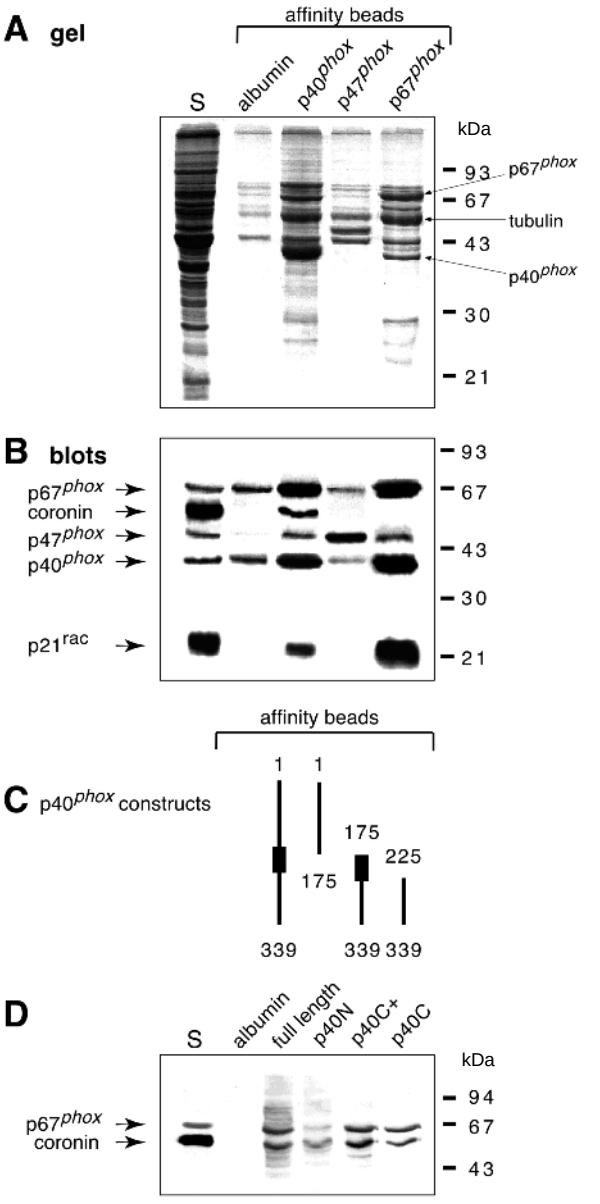

Fig. 2. Coronin binds to p40^{phox}. Binding studies were performed with neutrophil cytosol passed over equimolar amounts of p40^{phox},p47^{phox}, p67^{phox} or human albumin, as control, immobilised on beads. The eluates were electrophoresed and the Coomassie Blue dyed gel (A) and corresponding western blot (B) with the designated antibodies are shown. (C and D) Binding of coronin from cytosol to different regions of p40^{phox} . The constructs from which the mutant proteins containing the N terminus or the C terminus, with and without the SH3 domain, were made are shown schematically in C. Coronin and p67^{phox} that were attached to the immobilised proteins are shown in the western blot (D).

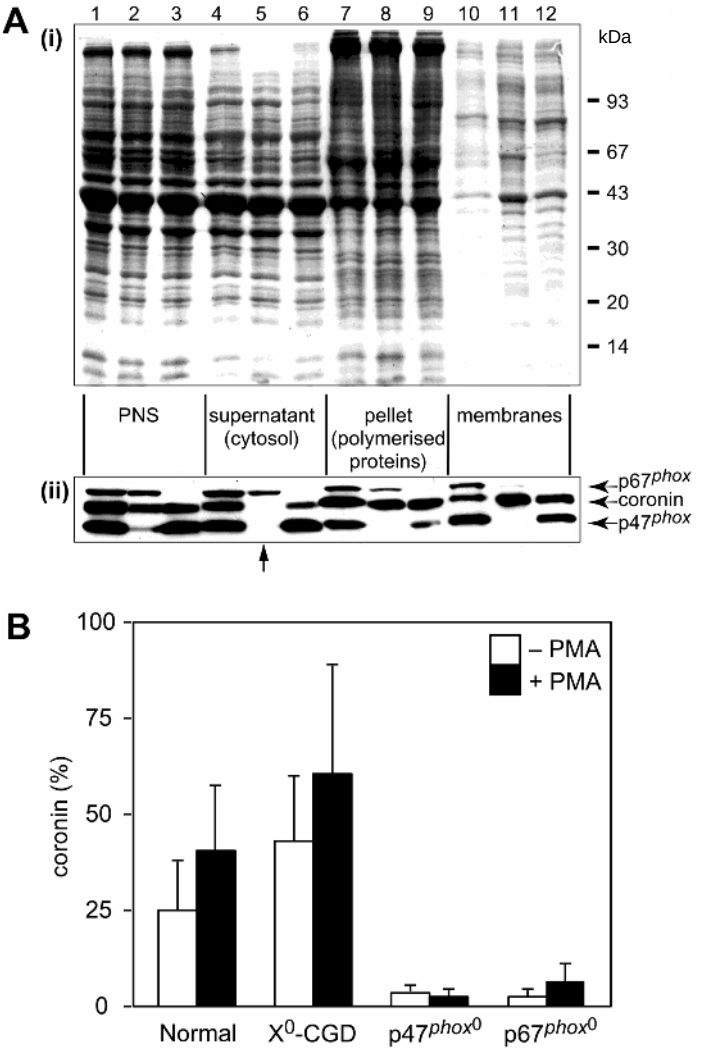

Fig. 3. The solubility of coronin is diminished in neutrophils deficient in p47^{phox} or p67^{phox}. The subcellular distribution of coronin (A) was determined in PMA activated cells from normal neutrophils (lanes 1, 4, 7 and 10) and those from patients lacking p47^{phox} (lanes 2, 5, 8 and 11) or p67^{phox} (lanes 3, 6, 9 and 12). The striking abnormality was the marked depletion in coronin in the high speed supernatant of the cytosol from the p47^{phox} deficient cells (lane 5, arrow) and to a lesser extent p67^{phox} (lane 6). Postnuclear supernatant (PNS, 140 μg protein), cytosol (110 μg), polymerised protein pellet (90 μg) and membranes (6, 15 and 12 μg in lanes 10, 11 and 12) were separated by SDS-PAGE and Coomassie Blue stained (i) or western blotted (ii) with antibodies to p47^{phox}, p67^{phox} and to coronin. (B) The percentage of coronin (mean ± s.e.m.) remaining in the cytosol was determined in neutrophils from 4 normal individuals, patients with CGD lacking the flavocytochrome b (X^{O}-CGD), p47^{phox} or p67^{phox} before (open bars) and after (filled bars) stimulation with PMA (4 subjects in each group apart from three measurements on two p67^{phox} deficient patients).

tributed between three main pools*:* an assembled cytoskeleton that is insoluble and sediments when centrifuged at low speed; a labile pool of polymerised proteins that are released from detergent solubilised cells but consist of assemblies of proteins that are large enough to pellet fairly rapidly at high centrifugal force; and a pool of soluble proteins which remain in the high speed supernatant cytosol (Hinshaw et al., 1993). In normal cells (lanes 1, 4, 7 and 10) coronin was found in the membrane fraction, the high-speed pellet and the cytosol (3Aii). In cells lacking p47^{phox} (lanes 2, 5, 8 and 11) or p67^{phox} (lanes 3, 6, 9 and 12) there was a striking decrease of coronin in the cytosol (lanes 5 and 6). The overall F-actin concentration and its subcellular distribution were undisturbed in the patients as shown in the Coomassie Blue stained gel (Fig. 3Ai). The failure of p67^{phox} to translocate to the membranes of p47^{phox} deficient

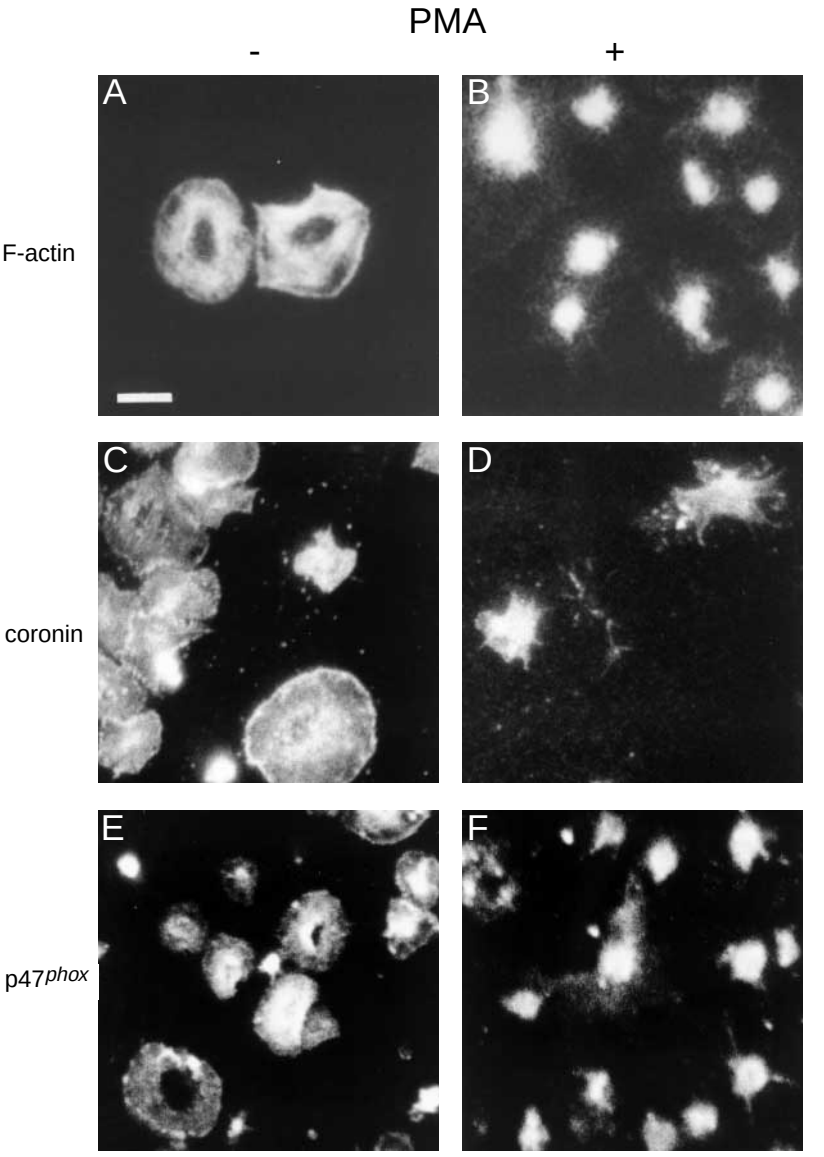

Fig. 4. The distribution of F-actin, coronin and p47^{phox} in spread neutrophils before and after stimulation with PMA. The distribution of F-actin (A and B), coronin (C and D) and p47^{phox} (E and F) in neutrophils adherent to glass slides, before (A,C,E) and after (B,D,F) stimulation with PMA. The distribution of these proteins was predominantly perinuclear and around the margin of the cell in unstimulated cells, and after stimulation they condensed around the nucleus. Bar, 10 μm.

cells upon activation (lane 11) is well recognised (Heyworth et al., 1991).

Fig. 3B shows the results of a study to extend these observations. Cells from all 4 of the patients lacking p47^{phox} and both those deficient in p67^{phox} had markedly reduced levels of coronin in the cytosol of both unstimulated and PMA activated cells, when compared with those from 4 normal subjects.

The solubility of coronin can be separated from activity of the NADPH oxidase by examining it in cells from patients with X-linked CGD. In these cells superoxide generation is also completely lacking as a result of an absence of the flavocytochrome b_{558}, and levels of p47^{phox} and p67^{phox} are normal. In cells from 4 patients with X-linked CGD release of coronin was at normal, or supranormal, levels. An isolated absence of p40^{phox} has not been described, although it is reduced in cells lacking p67^{phox} (Wientjes et al., 1993) so it has not been possible to determine the functional consequences of a selective absence of this protein.

In all these cases the normal control cells were taken at the same time as, and processed in parallel with, the patient samples.

The actin and coronin cytoskeleton of adherent neutrophils undergo a major reorganisation after activation of the oxidase with PMA, a change that is not observed in p47^{phox} and p67^{phox} deficient cells

We used fluorescence microscopy with TRITC-phalloidin to localise F-actin, and antibodies to coronin, p40^{phox}, p47^{phox} and p67^{phox} to follow the distribution of these proteins in resting and phagocytosing cells and to determine the effects of stimulation with PMA.

The cells were examined under three different conditions: cells that had attached to the glass coverslips and spread; cells that had attached but had not spread, maintaining a rounded shape; and cells that had phagocytosed zymosan and had attached to the glass but not spread.

In the spread cells (Fig. 4), F-actin (4A and B) coronin (4C and D), and the *phox* proteins had a similar distribution, being located predominantly around the margins of the cell and in

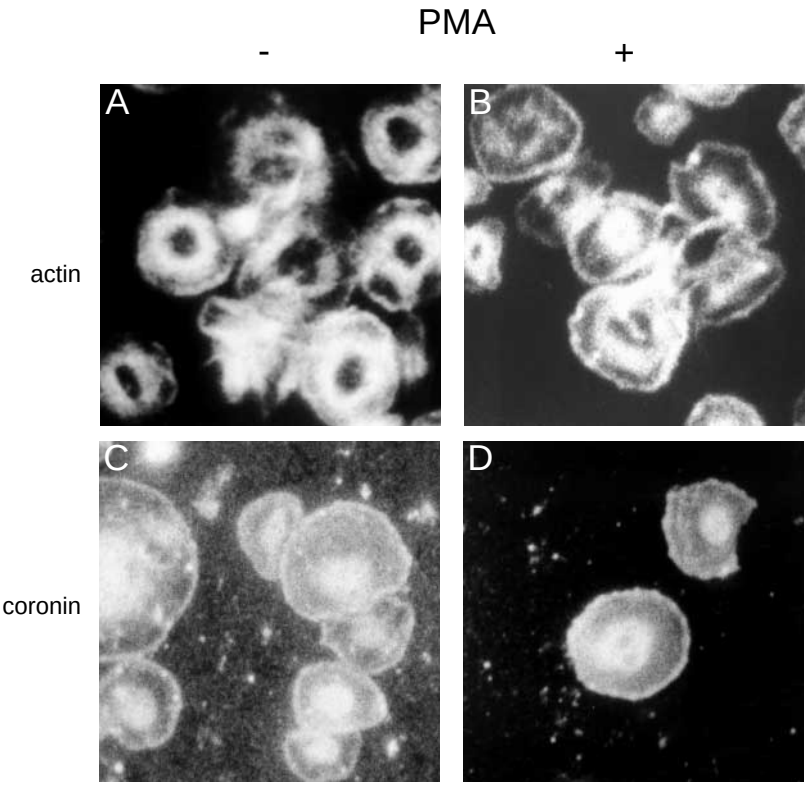

Fig. 5. The distribution of F-actin and coronin in p47*phox* deficient spread neutrophils before and after stimulation with PMA. The distribution of F-actin (A and B) and coronin (C and D) in neutrophils adherent to glass slides, before (A,C), and after (B,D), stimulation with PMA. In p47*phox* or p67*phox* deficient cells the F-actin and coronin had a normal distribution which did not change upon stimulation.

the perinuclear region (p47*phox* is shown in E and F and had a analogous appearance to p40*phox* and p67*phox*). Activation gave rise to an extensive structural upheaval in which all these proteins condensed around the nucleus (shown in Fig. 4B,D,F). That this is the result of the loosening of the submembraneous cytoskeleton is strongly implied by the further observation that cytochalasin B produced similar effects (not shown).

Because of the demonstrated association of the *phox* proteins with, and their influence on the solubility of, coronin, we examined the distribution of coronin and of F-actin in cells with the CGD phenotype lacking the different components of the NADPH oxidase complex. In cells lacking p47*phox* or p67*phox* the F-actin (Fig. 5A) and coronin (Fig. 5B) had a normal distribution in unstimulated adherent cells. The striking anomaly observed in cells deficient in p47*phox* or p67*phox* (Fig. 5B and D are representative) was the failure of the redistribution of coronin, F-actin or the complementary cytosolic *phox* protein after stimulation with PMA. This abnormality was restricted to cells from these types of CGD as cells from X-linked patients responded normally.

In adherent rounded cells, the coronin and *phox* proteins were fairly homogeneously distributed throughout the cytoplasm, with a slightly increased density at the periphery. After stimulation with PMA these cells became irregular in shape, and there was a redistribution of a proportion of the coronin and of all three *phox* proteins, which moved to form aggregates under the plasma membrane, with a lower concentration in the cytoplasm which appeared slightly reticulate (not shown).

Phagocytosing neutrophils

When the adherent neutrophils phagocytosed zymosan we found that the coronin and cytosolic *phox* proteins accumulated at the plasma membrane at the point of contact with the zymosan particle, and then surrounded the phagocytic vacuole as it invaginated into the cytoplasm (Fig. 6A-D).

In normal cells, p47*phox* (Fig. 6C), p67*phox* (Fig. 6B and D) and coronin (Fig. 6A) showed a similar distribution in relationship to the phagocytic vacuole. They accumulate around the phagocytic cup as it develops, and continue to surround the vacuole after phagocytosis is complete and it has moved into the interior of the cell. Fig. 6E and F show a neutrophil from a p47*phox* deficient CGD patient with two zymosan particles, one completely ingested, and another soon after attachment, demonstrating the early stages of the formation of a phagocytic cup. The cell has been probed with antibodies to both coronin (Fig. 6E) and to p67*phox*. Fig. 6F shows rhodamine fluorescence of the zymosan particles and the labelling of p67*phox*. In Fig. 6E the rhodamine fluorescence of the zymosan and antibodies to p67*phox* have been subtracted revealing the distribution of the antibodies to coronin labelled with fluorescein. The coronin is seen forming dense deposits around the phagocytic cup, and it also surrounds the internalised particle. p67*phox* showed a similar distribution around the phagocytic cup, although in this preparation the fluorescence from p67*phox* around the vacuole could not be distinguished from the intense fluorescence of the zymosan.

DISCUSSION

An association between the NADPH oxidase and the cytoskeleton could provide a mechanism by which the superoxide generation could be restricted to the phagocytic vacuole. Previous

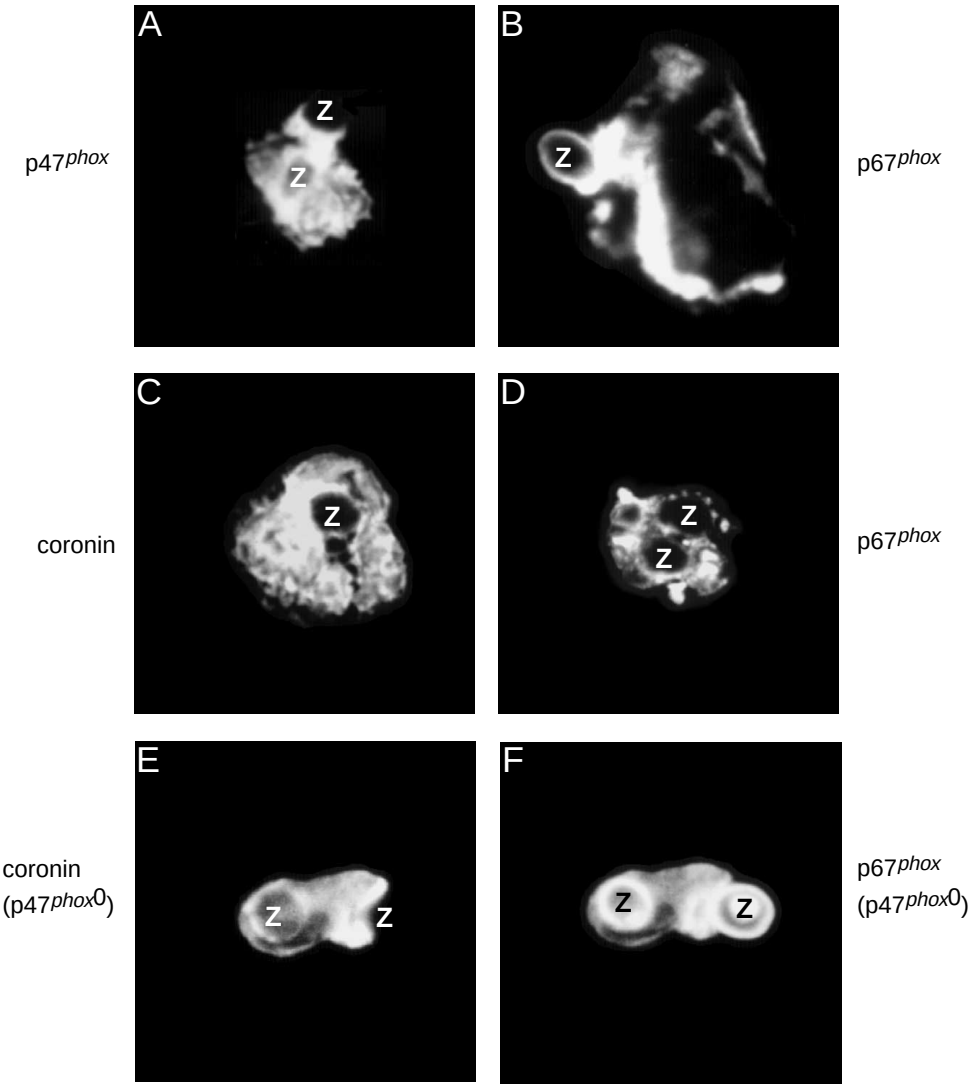

Fig. 6. Distribution of coronin (C and E), p47*phox* (A) and p67*phox* (B,D,F) in neutrophils phagocytosing serum opsonised zymosan. All three of these proteins accumulated around the phagocytic cup and around the endocytosed particles (Z) in both normal and p47*phox* deficient cells. (A,B,C,D) Normal phagocytosing cells labelled with FITC antibodies to coronin (A), p47*phox* (C) and p67*phox* (B and D). (E and F) A cell from a patient deficient in p47*phox*, labelled with FITC antibodies to coronin (E). (F) Both the zymosan (Z) and anti-p67*phox* antibodies were labelled with TRITC.

studies have given some indication of an association between the cytosolic *phox* proteins and the cytoskeleton based largely upon their insolubility after detergents were added to whole cells (Nauseef et al., 1991; Woodman et al., 1991) or to membranes (El Benna et al., 1994).

The most interesting novel observation in the present study was that in the cytosol p40*phox* and p67*phox* are bound to coronin (de Hostos et al., 1991), which provides intriguing clues to an additional layer of organisation of the interaction between the NADPH oxidase and the cytoskeleton.

Coronin derived its name from its association with crown-shaped projections from growth-phase *Dictyostelium discoideum* (de Hostos et al., 1991), from which all the functional information we have, is derived. It is a cytoplasmic actin-associated protein that accumulates at the cortical sites of moving cells and contributes to the dynamics of the actin system. It is a member of the WD-repeat (de Hostos et al., 1991), and leucine zipper (Suzuki et al., 1995), families of proteins, and is known to interact with actin-myosin complexes. In coronin null mutants, cell locomotion is slowed and cytokinesis is impaired (Gerisch et al., 1995). A coronin-GFP fusion protein was used to demonstrate that coronin is recruited from the cytoplasm and is incorporated into the actin network of a nascent leading edge of the cell where it participates in the reorganisation of the cytoskeleton (Gerisch et al., 1995). Coronin is also important for phagocytosis (Maniak et al., 1995) which is depressed in null mutants. It accumulates in phagocytic cups and is then released from the wall of the vacuole after it is formed.

The coronin is solubilised when neutrophils are stimulated with fMLP or PMA and the oxidase activated. This solubilisation was selective and did not affect the other cytoskeletal proteins examined, actin, α-actinin or gelsolin, or the cytosolic *phox* proteins (data not shown). At first sight this discrepancy between the solubilisation of coronin and of p47*phox* and p67*phox* might appear somewhat surprising, as these proteins are associated in the cytosol, but the coronin is present in an approximately fortyfold excess, and clearly the *phox* proteins would only be bound to a minor component. Most interestingly, p47*phox* and p67*phox* played an important part in the reorganisation of the cytoskeleton induced by activators of the oxidase, providing the most convincing linkage between the oxidase and cytoskeleton. In the absence of these proteins the coronin was very sparingly solubilised, and the perinuclear

condensation of F-actin and coronin in adherent cells was not seen. This was not simply a result of the failure of superoxide generation in general because normal release was observed in X-CGD cells lacking the flavocytochrome b. Taking into account the stoichiometry discussed above, the *phox* proteins are likely to be playing a regulatory role, possibly through interactions with other regulatory proteins, such as p21*rac* which binds to p67^{phox} (Diekmann et al., 1994) and/or PAK which in turn binds to p21*rac* (Prigmore et al., 1995; Martin et al., 1995). p21*rac* (Diekmann et al., 1994) is important for activity of the NADPH oxidase in the cell free assay (Abo et al., 1992). It also induces membrane ruffling when injected into Swiss 3T3 cells (Ridley et al., 1992) and activates uncapping of F-actin in permeabilised platelets (Hartwig et al., 1995). These cytosolic *phox* proteins could play a more general role in the organisation of the cytoskeleton in other cells, for example in B lymphocytes, in which they are much more abundant (Chetty et al., 1995) than would be expected if their only requirement was for the relatively low oxidase activity produced by these cells (Cohen Tanugi et al., 1991).

Despite the obvious effects of the lack of p47^{phox} or p67^{phox} on coronin solubility and cytoskeletal morphology, the lack of these proteins did not appear to grossly influence functions of the cell other than that of the NADPH oxidase, which was completely absent. Neutrophil motility and chemotaxis was found to be normal in cells lacking p47^{phox} or p67^{phox}, as well as those with X-linked CGD (Zicha et al., 1997). Although this might at first appear surprising, it has been found that *Dictyostelium* defective in ABP-120 (Brink et al., 1990) or in α-actinin (Wallraff et al., 1986) show essentially normal chemotaxis, whereas the complete absence of coronin (de Hostos et al., 1993) only leads to partial reductions in motility and chemotaxis.

Under natural conditions the NADPH oxidase is dormant until activated by contact of the cells with, and phagocytosis of, opsonised microbes. This activation is tightly restricted to that part of the plasma membrane comprising the phagocytic vacuole (Briggs et al., 1975a) and normally only occurs after the vacuole has closed (Segal and Meshulam, 1979) because it is important to prevent the release of toxic free radicals and enzymes to the extracellular environment. Involvement of the cytoskeleton could provide the means of co-ordinating this process in time and place. For electron transport to take place the cytosolic *phox* proteins have to move to the membrane where they attach to the flavocytochrome. Coronin clearly accumulates around the phagocytic cup in neutrophils as was observed in *Dictyostelium* (Gerisch et al., 1995; Maniak et al., 1995), but unlike the findings in amoebae where it then disperses from around the endocytosed vacuole, it persists in this situation in neutrophils. In this location it is ideally situated to participate in events associated with the cytosolic surface of the vacuole, and in particular to the recruitment of cytosolic oxidase components. p40^{phox} and p67^{phox} might associate with coronin because it is concentrated around the phagocytic cup, thereby translocating these oxidase components to their site of action.

The effect of stimulating protein kinase C with PMA upon the organisation of the actin cytoskeleton of neutrophils has previously been examined in cells in suspension in which it induced F-actin accumulation and membrane ruffling (Downey et al., 1992; Sheterline et al., 1986). We found here that in adherent cells the F-actin cytoskeleton, and coronin, underwent a major reorganisation upon activation of adherent cells with PMA. This reorganisation, in which the F-actin and coronin appear to detach from the cell margin and to condense around the nucleus, could occur as a result of the disruption of the connection between the central network and the peripheral submembranous cytoskeleton, as a fairly similar pattern was observed if the cells were treated with cytochalasin B. This perinuclear condensation of F-actin has also been seen in mast cells activated with GTP-γ-S (Norman et al., 1994) and has a similar appearance to the condensation of F-actin seen in porcine aortic endothelial cells microinjected with the WASP protein (Symons et al., 1996).

Neutrophils and *Dictyostelium discoideum* are very similar in size, appearance and function as they are both highly motile and engulf and digest bacteria. It is therefore not surprising that they have similar cytoskeletal structures to accommodate their common physiological requirements, including the role of coronin in phagocytosis. The work undertaken in this study indicates that the cytosolic *phox* proteins are involved in the regulation of this specialised cytoskeleton in neutrophils, and that they provide a connection between it and regulation of the NADPH oxidase.

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