Coregulation of NADPH oxidase activation and phosphorylation of a 48-kD protein(s) by a cytosolic factor defective in autosomal recessive chronic granulomatous disease.

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Research Article

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Coregulation of NADPH Oxidase Activation and Phosphorylation of a 48-kD Protein(s) by a Cytosolic Factor Defective in Autosomal Recessive Chronic Granulomatous Disease

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Abstract

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NADPH oxidase could be activated by SDS in reconstitution mixtures of cytosolic and membrane subcellular fractions from normal PMN, and SDS also enhanced phosphorylation of at least 16 cytosolic and 14 membrane-associated proteins. However, subcellular fractions from CGD PMN plus SDS expressed little NADPH oxidase activity, and phosphorylation of a 48-kD protein(s) was selectively defective. The membrane fraction from CGD cells could be activated for NADPH oxidase when mixed with normal cytosol and phosphorylation of the 48-kD protein(s) was restored. In contrast, the membrane fraction from normal cells expressed almost no NADPH oxidase activity when mixed with CGD cytosol, and phosphorylation of the 48-kD protein(s) was again markedly decreased. Protein kinase C (PKC) activity in PMN from the two patients appeared to be normal, suggesting that a deficiency of PKC is not the cause of the defective 48-kD protein phosphorylation and that the cytosolic factor is not PKC.

These results demonstrate that the cytosolic factor required for activation of NADPH oxidase also regulates phosphorylation of a specific protein, or family of proteins, at 48 kD. Although the nature of this protein(s) is still unknown, it may be related to the functional and phosphorylation defects present in CGD PMN and to the activation of NADPH oxidase in the cell-free system.

Introduction

Human neutrophils (PMN) play a critical role in host defense against microorganisms and in the inflammatory response (1). One of the primary mechanisms for effecting these reactions is the generation of toxic oxygen radicals by the PMN. The enzyme in PMN that triggers oxygen radical production is an activatable NADPH oxidase, whose mechanism of activation is still unknown. The active enzyme appears to be a multicomponent complex, most likely consisting of a flavoprotein and a unique low potential cytochrome, cytochrome b_{559} (2).

Until recently, activation of NADPH oxidase could only be achieved by stimulation of intact PMN with a wide variety of agents, including opsonized particles, phorbol esters, chemotactic peptides, and certain detergents (2). One characteristic these stimuli share is the ability to trigger activation of protein kinases inside the cell resulting in the phosphorylation of endogenous proteins (3–8). Protein kinase C (PKC)[1] has been of particular interest as an intermediate. Direct activators of PKC, such as phorbol esters and cell-permeable diacylglycerols (9), trigger oxidase activation in the intact cell by an apparent PKC-dependent mechanism (10–13). In addition, stimuli, such as concanavalin A and chemotactic peptides, induce the release of endogenous stores of diacylglycerol from membrane phospholipids and thus could activate PKC (14).

Further evidence supporting a role for protein phosphorylation in the activation of NADPH oxidase is given by recent reports of a phosphorylation defect in the PMN from several patients with chronic granulomatous disease (CGD), observed by stimulation of the intact cell (15–17). CGD is an inherited disease characterized by increased susceptibility to bacterial infection, in which the only known functional defect is the inability of their phagocytic cells to produce oxygen radicals in response to cell stimulation. Two modes of inheritance of the disease are observed, X-linked recessive and autosomal recessive (18, 19). Recently, the gene defect in the X-linked form has been described (20) and the gene product appears to be a subunit of the cytochrome b_{559} (21, 22). PMN from most patients with this form of the disease lack the characteristic spectrum of the cytochrome (23). The gene defect in the autosomal recessive form has yet to be identified. However, the phosphorylation defect in CGD has been attributed solely to the autosomal recessive form (15), although, this has been disputed (16, 24).

Recently, a cell-free system for activation of NADPH oxidase has been developed by ourselves (25) and others (26–28). Activation requires the apparent interaction of cytosolic and membrane cellular cofactors and can be achieved by the addition of an amphiphile, either arachidonic acid or SDS (29), to the isolated cell fractions. The identities of the cytosolic and membrane cofactors are unknown, although at least one nec-

1. *Abbreviations used in this paper:* CB, cytochalasin B; CGD, chronic granulomatous disease; DFP, diisopropylfluorophosphate; EP, extractable particulate fraction; NEP, nonextractable particulate fraction; PKC, protein kinase C; PMA, phorbol myristate acetate.

essary membrane component is cytochrome b_{559} (30). In addition, a recent abstract reports that the cytosolic factor activity is absent in a CGD patient with the autosomal recessive form (31).

It is not yet clear if a protein kinase is involved in oxidase activation in this system. Arachidonate can activate PKC (32), but inhibition or depletion of PKC does not block arachidonate-mediated activation of NADPH oxidase in the cell-free system (30, 33, 34). Contradictory reports as to the requirement for ATP in the system have appeared (30, 35). Finally, it has not yet been determined if arachidonate or SDS can induce phosphorylation of endogenous protein substrates in conjunction with activation of NADPH oxidase in the cell-free system.

In this report, the role of protein phosphorylation in the activation of NADPH oxidase in the cell-free system is examined. Using both normal PMN and PMN lacking cytosolic factor activity from patients with the autosomal recessive form of CGD, we have compared the ability of SDS to induce oxidase activation and phosphorylation of endogenous proteins in cell-free mixtures of cytosolic and membrane fractions. Results suggest that the cytosolic factor required for NADPH oxidase activation in the cell-free system is also specifically required for the SDS-dependent phosphorylation of a 48-kD protein, or family of proteins.

Methods

Isolation of cells. PMN (> 95% purity) were purified from human peripheral blood by dextran sedimentation, Ficoll-Hypaque centrifugation, and hypotonic lysis as described (36). Two patients with autosomal recessive CGD were studied, one of which (S.S.) is a 27-yr-old male reported previously (37). The other (S.H.) is a 25-yr-old female patient of Dr. Michael Cohen (University of North Carolina School of Medicine, Chapel Hill, NC) and was diagnosed based on clinical history and neutrophil oxidative functional studies (defective in vitro bactericidal activity and the absence of a luminol-dependent chemiluminescence response to phorbol myristate acetate, PMA, or opsonized zymosan).

Subcellular fractionation. Purified PMN were suspended at 5×10^7 PMN/ml in Hanks' balanced salt solution containing 4.2 mM sodium bicarbonate and 10 mM Hepes, pH 7.4 (HBSS). To inhibit proteolysis (38), cells were treated with 2 mM diisopropylfluorophosphate (DFP) for 5 min at 4°C, and then washed with a 15-fold volume of ice-cold HBSS. PMN were resuspended at 1×10^8/ml in a sonication buffer containing 0.34 M (11%, wt/vol) sucrose, 10 mM Hepes, 1 mM EGTA, 1 mM NaN_3, 130 mM NaCl, 100 mM NaF, and 10 mM sodium pyrophosphate. In some experiments, in which NADPH oxidase activity only was measured, the DFP treatment was omitted, 0.5 mM PMSF was included, and NaF and pyrophosphate were omitted. These alterations had no effect on the final oxidase activity obtained. 1-ml aliquots of cells were sonicated in 5-s bursts in a melting ice bath with a setting of 1.5 using a sonicator (model W-220; Heat Systems-Ultrasonics, Inc., Farmingdale, NY) equipped with a stepped microtip. Cell breakage of ~ 90% was achieved as monitored by phase microscopy.

Sonicates were centrifuged at 800 g for 10 min at 4°C to pellet unbroken cells and nuclei. Supernatants were fractionated by discontinuous sucrose density gradient centrifugation. In the first experiment, supernatants in the 11% sucrose buffer were layered over 40% (wt/vol) sucrose in a ratio of 2:1 (vol/vol), as described by Gabig et al. (39). In subsequent experiments, a 15% (wt/vol) sucrose interface layer was included to more easily separate membrane and cytosolic fractions (40), the final ratio being 2:1:1 (vol/vol) of 11, 15, and 40%, respectively. Gradients were centrifuged at 4°C (SW 60.1 rotor; Beckman Instruments, Inc., Palo Alto, CA) at 150,000 g for 30 min (39). After centrifugation, the top layer of each discontinuous gradient (crude cytosol) was collected and centrifuged again at 150,000 g in a Beckman Type 50 rotor for 60 min at 4°C to pellet any contaminating particulate material (cleared cytosol). The white band at the 11%/40% or the 15%/40% interface from each discontinuous gradient and most of the 40% sucrose layer was collected (membrane fraction). The discontinuous gradients also yielded granule fractions as pellets, which were resuspended in sonication buffer to the original volume layered onto the gradient. All fractions were either used immediately or stored at −70°C.

Gradient fractions were analyzed for purity by measuring the distribution of the following marker enzymes: lactic dehydrogenase (LDH; 41) for cytosol, alkaline phosphatase (42) for plasma membrane, vitamin B_{12}-binding protein (43) for specific granules, lysozyme (44) for both specific and azurophil granules, and myeloperoxidase (MPO; 45) for azurophil granules. Table I summarizes the distribution

Table I. Subcellular Marker Enzyme Distribution in Fractions Isolated by Discontinuous Sucrose Gradient Fractionation

Fraction assayed[‡]	% Distribution of enzyme[*]				
	LDH	Alkaline phosphatase	B_{12}-binding protein	Lysozyme	MPO
11%/40% gradient					
Cytosolic	95.9	0.9	ND	0	7.1
Membrane	2.5	93.8	ND	8.7	3.7
Granule	2.0	5.3	ND	91.3	89.2
11%/15%/40% gradient					
Cytosolic	86.7	1.1	2.4	5.3	2.9
15% layer	8.3	7.3	0.4	0.1	0.4
Membrane	1.4	83.2	12.1	8.2	3.7
Granule	0.6	5.6	85.1	86.3	92.7

[*] Marker enzyme levels were determined in subcellular fractions as described in Methods. Numbers given are the percentage of total activity recovered in each fraction for one experiment with the 11%/40% gradient and the mean of two experiments with the 11%/15%/40% gradient. In the 11%/15%/40% gradient, total recovery for each marker (mean of two experiments) was: LDH, 85.7%; alkaline phosphatase, 97.4%; vitamin B_{12}-binding protein, 79.9%; lysozyme, 102.9%; MPO, 68.2%; and protein (46), 87.3%. Distribution of protein in the 11%/40% gradient was: cytosolic, 50.4%; membrane, 7.5%; and granule, 42.2%. Protein distribution in the 11%/15%/40% gradient was: cytosolic, 43.8%; 15% layer, 5.3%; membrane, 7.8%; and granule, 41.4%. [‡] Normal PMN were sonicated and fractionated by either an 11%/40% or an 11%/15%/40% discontinuous sucrose gradient as described in Methods. Fractions were assayed immediately for LDH and stored at −70°C before the remainder of the marker enzyme assays.

of these markers. In one experiment with the 11%/40% gradient, the cleared cytosol fraction contained 95.9% of the recovered LDH, 0.9% of alkaline phosphatase, and a mean of 3.5% of lysozyme and MPO. The membrane fraction contained 93.8% of the recovered alkaline phosphatase, 2.5% of LDH, 8.7% of lysozyme, and 3.7% of MPO. In the 11%/15%/40% gradient procedure, the cleared cytosol contained 86.7% of the recovered LDH, 1.1% of alkaline phosphatase, 2.4% of B_{12}-binding protein, 5.3% of lysozyme, and 2.0% of MPO. The membrane fraction contained 83.2% of the recovered alkaline phosphatase, 1.4% of LDH, 12.1% of B_{12}-binding protein, 8.2% of lysozyme, and 3.7% of MPO. The granule fractions from both gradient procedures contained > 85% of the recovered granule enzyme markers. The discontinuous sucrose gradient fractionation procedure, therefore, was considered to yield cytosolic and membrane fractions of acceptable purity for use in the cell-free oxidase and phosphorylation systems.

NADPH oxidase assay in the cell-free system. Assays measured the SOD-inhibitable reduction of cytochrome *c*, and were performed as previously described (25, 36) with slight modifications. The assay mixture consisted of 0.048 mM potassium phosphate (pH 7.0), 0.076 mM cytochrome *c* (type III), 1.0 mM EGTA, 10.0 μM flavin adenine nucleotide (all from Sigma Chemical Co., St. Louis, MO), 7.5 mM MgCl$_2$, and either a 2:1 or 4:1 (vol/vol) ratio of cytosol to membrane fraction. Cell equivalence in the assay was 1.5×10^7/ml of cytosol and either 0.75 or 1.5×10^7/ml of membrane fraction. The final assay volume was 0.105 ml. Activation of NADPH oxidase was achieved by addition of 0 (H$_2$O control) to 175 μM SDS (Bio-Rad Laboratories, Richmond, CA) and incubation at room temperature for 4 min. The concentration of SDS yielding optimal activation of NADPH oxidase in reconstitution mixtures of control fractions varied from 120 to 175 μM and was determined in each experiment. Equal aliquots of assay mixture were placed into two cuvettes: the reference cuvette, containing 48 μg/ml SOD (Diagnostic Data, Inc., Mountain View, CA), and the sample cuvette, containing H$_2$O. NADPH oxidase activity was measured by addition of 0.19 mM NADPH to both cuvettes, and the change in absorbance at 550 nm recorded by a dual beam spectrophotometer (Cary 2390; Varian Instruments, Sunnyvale, CA). Initial rates were used for calculations and activity was expressed as nmol/min/mg membrane protein (46) using an extinction coefficient of 21 mM^{-1} cm^{-1} for cytochrome *c* (47). Assays were performed on both fresh and frozen cell fractions with no differences in results obtained.

Assay for cytochrome b_{559}. The method used was a modification of published procedures (48, 49). PMN from normal donors and the autosomal recessive CGD patients were sonicated and fractionated by discontinuous sucrose density gradient centrifugation with sodium pyrophosphate and NaF omitted from, and PMSF included in, the sonication buffer. The membrane and granule fractions were analyzed for cytochrome b_{559} content by difference spectroscopy after dithionite titration of an aerobic sample. Fractions were diluted 1:1 with 2X buffer, yielding final concentrations of 0.25 M potassium phosphate (pH 7.5), 0.12 mM sodium deoxycholate, 5.0 mM NaN$_3$, and 0.5 mM H$_2$O$_2$. The reduced minus oxidized spectrum of each diluted fraction was determined on a Cary 2390 spectrophotometer after complete reduction of the sample cuvette by 6–10 mM anaerobically prepared dithionite. Amounts of cytochrome b_{559} were expressed as pmol/10^8 cell equivalents using an extinction coefficient of 106 mM^{-1} cm^{-1} at the Soret peak (49).

Cell-free protein phosphorylation. Cell-free mixtures for phosphorylation studies consisted of 1.4 mM MgCl$_2$, 6.0×10^7 cell equivalents/ml each of cytosolic fraction and membrane fraction, and 51 μCi/ml [γ-^{32}P]ATP (12.88 Ci/mmol; New England Nuclear, Boston, MA). SDS at a final concentration of 460 μM or an equal volume of distilled water was added and the samples were incubated 5 min at room temperature. The higher concentration of SDS was used to keep the ratio of SDS to cellular material similar to that used in the procedure for activation of NADPH oxidase. Reactions were stopped by adding a two-fold volume of ice-cold sonication buffer, which also reduced the viscosity of the mixtures for subsequent centrifugation. Samples were then centrifuged at 150,000 *g* for 12 h at 4°C in a Beckman Type 50

rotor. Supernatants (cytosolic fractions) were reserved and pellets (membrane fractions) were resuspended by sonication in a small volume (< 1 ml) of sonication buffer. Part of each sample (200–300 μl) was solubilized in electrophoresis sample buffer (50), boiled for 2 min in a water bath, and stored for no more than 3 d at −70°C before analysis by SDS-polyacrylamide gel electrophoresis, as described below. The remainder of each sample was frozen at −70°C without solubilization; these nondenatured fractions were assayed for protein by Peterson's modification (51) of the method of Lowry et al. (46).

Endogenous protein phosphorylation in intact PMN. Cells at 1 $\times 10^8$/ml from a normal donor and a CGD patient (S.S.) were suspended in buffer (4) containing 6 mM Hepes/Tris (pH 7.4), 0.15 M NaCl, 10 mM glucose, 5 mM KCl, 1 mM MgCl$_2$, 0.25 mM CaCl$_2$, and ^{32}P$_i$ (1 mCi/1-2 $\times 10^8$ cells; New England Nuclear). PMN were incubated at 37°C for 30 min, washed, and resuspended at 5×10^7/ml in the Hepes/Tris buffer without ^{32}P$_i$. Cells were prewarmed for 5 min at 37°C in the presence of 10^{-5} M cytochalasin B (CB), then treated with either dimethylsulfoxide (DMSO) or 100 ng/ml PMA for 30 s. Reactions were terminated by addition of a 15-fold excess volume of ice-cold incubation buffer containing 1.0 M NaF and 10 mM EDTA. Cells were washed and then treated with DFP and sonicated as described above. Sonicates were centrifuged at 500 *g* for 10 min at 4°C, and postnuclear supernatants were centrifuged at 100,000 *g* for 1 h at 4°C in a fixed angle rotor. Supernatants (cytosolic fractions) were removed and stored at −70°C: one aliquot was solubilized in electrophoresis sample buffer as described above and the remainder analyzed for protein content (51).

SDS-polyacrylamide gel electrophoresis and autoradiography. Reduced, denatured samples (30–100 μg protein/lane) were electrophoresed on 8–15% gradient polyacrylamide slab gels ($1.5 \times 140 \times 320$ mm) using the discontinuous buffer system of Laemmli (50). Except where noted, within each gel, equal amounts of protein were loaded onto gel lanes. After electrophoresis, gels were silver stained by the method of Wray et al. (52), photographed, and then dried between two sheets of cellophane using a slab gel dryer (model 443; Bio-Rad). Each dried gel was exposed, typically for 1–3 d, to preflashed Kodak X-Omat x-ray film with a DuPont Cronex Lightning Plus intensifying screen at −70°C (53) to generate autoradiograms of the radioactive phosphoproteins. Autoradiograms were scanned with a laser densitometer (UltroScan XL) and scans were analyzed by the software package (GelScan XL; LKB-Produkter AB, Bromma, Sweden).

PKC assay. PKC activity and distribution in normal PMN and PMN from the patients with CGD were determined in crude subcellular fractions as previously described (10). Briefly, PMN at 5×10^7/ml in HBSS were exposed, in the presence of 10^{-5} M CB at 37°C, to either solvent alone (DMSO), 10^{-6} M fMLP, or 100 ng/ml PMA for 30 s and reactions were terminated by addition of a 10-fold excess of ice-cold HBSS. After centrifugation, PMN were resuspended in an extraction buffer containing 50 mM Tris-HCl (pH 7.5), 50 mM 2-mercaptoethanol, 1 mM PMSF, and 2 mM EGTA. PMN were sonicated as described above, centrifuged at 500 *g*, and postnuclear supernatants were centrifuged at 150,000 *g* for 90 min. Supernatants (cytosolic fractions) were collected; pellets were resuspended by sonication in extraction buffer containing 1.0% Triton X-100 (Sigma Chemical Co.) and centrifuged at 150,000 *g* for 90 min. Supernatants, the extractable particulate fractions (EPs), were saved and pellets, the non-extractable particulate fractions (NEPs), were resuspended in extraction buffer containing 0.1% Triton X-100 and saved. Fractions were stored at 0°C and assayed within 2 d of preparation.

Protein kinase assay mixtures (10) contained, in a final volume of 0.25 ml: 35 mM Tris-HCl (pH 7.5), 10 mM MgCl$_2$, 160 μg/ml histone type H1 (type III, Sigma), 50 μM ATP with 2×10^6 cpm [γ-^{32}P]ATP, 0.4 mM EGTA, 0.2 mM PMSF, 10 mM 2-mercaptoethanol, 0.01% Triton X-100, and 50 μl diluted subcellular fraction. Cytosolic fractions were diluted 10-fold, EP fractions 20-fold, and NEP fractions 2-fold. Assays were performed in the presence or absence of activators (0.6 mM added CaCl$_2$, 20 μg/ml phosphatidylserine, and 2 μg/ml diolein) at 30°C for either 5 min (cytosolic fractions), 60 min (EP

fractions), or 30 min (NEP fractions). Reactions were stopped with 20% (wt/vol) TCA, precipitates collected and counted, and activity was expressed as pmol ^{32}P incorporated/min per 10^7 cell equivalents. Previous studies in normal PMN (10, 54) had demonstrated that protein kinase activity in cytosolic and EP fractions was completely dependent on the presence of phosphatidylserine, with no activity above background in the presence of calcium alone. Activity in NEP fractions showed no dependence on added Ca^{2+} and lipids (10).

Results

Activation of NADPH oxidase in a cell-free system from normal PMN and PMN from patients with autosomal recessive CGD. Activation of NADPH oxidase in a cell-free system was measured using cell fractions from normal PMN and PMN from two patients with the autosomal recessive form of CGD. As shown in Table II, substantial activation of NADPH oxidase by SDS occurred using reconstitution mixtures of normal membrane and cytosolic fractions. In contrast, mixtures of CGD membrane and cytosolic fractions had very low levels of NADPH oxidase activity in the presence of SDS. If corrected for activity observed in the absence of SDS in reconstitution mixtures of normal cell fractions (2.5±0.7 nmol O_2^-/min per mg, mean±SEM, $n = 6$), oxidase activity induced in CGD reconstituted fractions was only 2.1% of the normal control. This defect in the ability to activate NADPH oxidase appeared to reside in the cytosolic fractions from the patients with CGD, since (*a*) reconstitution mixtures of CGD cytosol with normal membrane supported very little activation of the oxidase by SDS, and (*b*) the CGD membrane fraction in combination with normal cytosol did allow normal levels of NADPH oxidase activation to occur. The membrane fractions of the two patients showed equal ability to support NADPH oxidase activation in the presence of normal cytosol (S.H., 84% of activity seen with paired normal membrane, $n = 2$; S.S., 106% of

Table II. Deficient Cytosolic Factor for Activation of NADPH Oxidase in Two Patients with Autosomal Recessive CGD

Reconstitution mixture*	NADPH oxidase activity[‡]
	nmol O_2^-/min/mg membrane protein
Normal cytosol + normal membrane	159.0±52.7 (6)
CGD cytosol + CGD membrane	5.8±0.4 (3)
Normal cytosol + CGD membrane	153.4±62.4 (6)
CGD cytosol + normal membrane	8.2±0.8 (4)

* Cytosolic and membrane fractions were isolated from two patients (S.S. and S.H.) with the autosomal recessive form of chronic granulomatous disease and from six normal donors as described in Methods. One fractionation with CGD (S.S) and one with a normal donor utilized the 11%/40% (wt/vol) discontinuous sucrose gradient. The remaining fractionations (one with S.S., one with S.H., and five normal donors) utilized the 11%/15%/40% (wt/vol) gradient separation. ‡ NADPH oxidase activation was achieved by an optimal concentration of SDS (120–175 μM) and activity was measured as described in Methods. Data are given as the mean±SEM with the number of experiments performed, each done in triplicate on a separate day, in parentheses. At $n = 3$, two experiments were with S.S. fractions and one was with S.H. fractions. In all other instances involving CGD fractions, two experiments were performed with S.H. fractions and the remainder were with S.S. fractions.

normal, $n = 4$). In one experiment (not shown), an excess of CGD cytosol (5.6 × 10^7 cell equivalents/ml versus the usual 1.5 × 10^7/ml) was mixed with normal membrane fraction to test the possibility of a quantitative defect. The markedly decreased level of activation of NADPH oxidase was not enhanced, suggesting that the cytosolic factor is not present in decreased amounts, at least to the level of sensitivity of our detection system.

To further exclude an oxidase defect in the CGD membrane, membrane and granule fractions from both patients were examined for their content of cytochrome b_{559}. No cytochrome b was detected in cytosolic fractions from normal PMN. The level of cytochrome b in normal PMN was 495±80 pmol/10^8 cells (mean±SD, $n = 7$) and the cytochrome was distributed such that 32% was present in the membrane fraction and 68% in the granule fraction. Both patients had normal (S.H.) or slightly elevated (S.S.) levels of cytochrome b (S.H., 492 pmol/10^8 cells; S.S., 757±3 pmol/10^8 cells, mean±SD, $n = 2$) and a similar distribution as a paired normal control. Taken together, these results indicate that the PMN of the two patients with autosomal recessive CGD do not have a defect in cytochrome b_{559} and, instead, are defective in the cytosolic factor required for the activation of NADPH oxidase in the cell-free system.

Protein phosphorylation in the cell-free system for NADPH oxidase activation. The cell-free system for activation of NADPH oxidase was examined for protein phosphorylation changes that might correlate with oxidase activation. Reconstitution mixtures of membrane and cytosolic fractions isolated from normal PMN and the PMN of the two patients with autosomal recessive CGD were incubated with or without SDS in the presence of [γ-^{32}P]ATP and then subjected to ultracentrifugation to allow separate analysis of phosphoproteins in the two fractions. Representative results obtained with reseparated cytosolic fractions are shown in Fig. 1 and with reseparated membrane fractions in Fig. 2. The left panel of each figure shows the silver-stained protein pattern of the gel and the right panel shows the autoradiogram obtained from the same gel. No apparent differences in the pattern of silver-stained proteins was observed, comparing fractions obtained from the normal donor with those from the CGD patient.

As many as 24 phosphoproteins were detected in cytosolic fractions following reconstitution of normal fractions in the presence of SDS (Fig. 1 *B*, lane *1*), of which 16 were phosphorylated more intensely and 3 were dephosphorylated by the SDS treatment (compare to lane *2*). An increase in phosphorylation of a 48,000-D protein(s) (arrow) was observed in cytosolic fractions from the normal reconstitution mixture by SDS treatment (lanes *1* and *2*), but not in cytosolic fractions from the CGD reconstitution mixture (lanes *3* and *4*). This was the only obvious phosphorylation defect observed in the cytosolic fraction from the CGD cell-free reconstitution. Crossover mixing of normal cytosol with CGD membrane fraction (lanes *5* and *6*) restored the SDS-dependent phosphorylation of the 48-kD protein(s), suggesting that any CGD membrane components participating in the phosphorylation reaction are functional. In contrast, when the CGD cytosolic fraction was mixed with normal membrane fraction (lanes *7* and *8*), SDS induced no increase in background phosphorylation in the 48–50-kD region of the gel. These results indicate that a cytosolic factor necessary for phosphorylation of a 48-kD phosphoprotein(s) is either defective or absent in the

-200

-116

-92.5

-66

-45

-31

-21.5

-14

+ −   + −   + −   + −
Ctrl.−C CGD−C Ctrl.−C CGD−C
Ctrl.−M CGD−M CGD−M Ctrl.−M

- 200

- 116

- 92.5

- 66

- 45

- 31

- 21.5

- 14

+ −   + −   + −   + −
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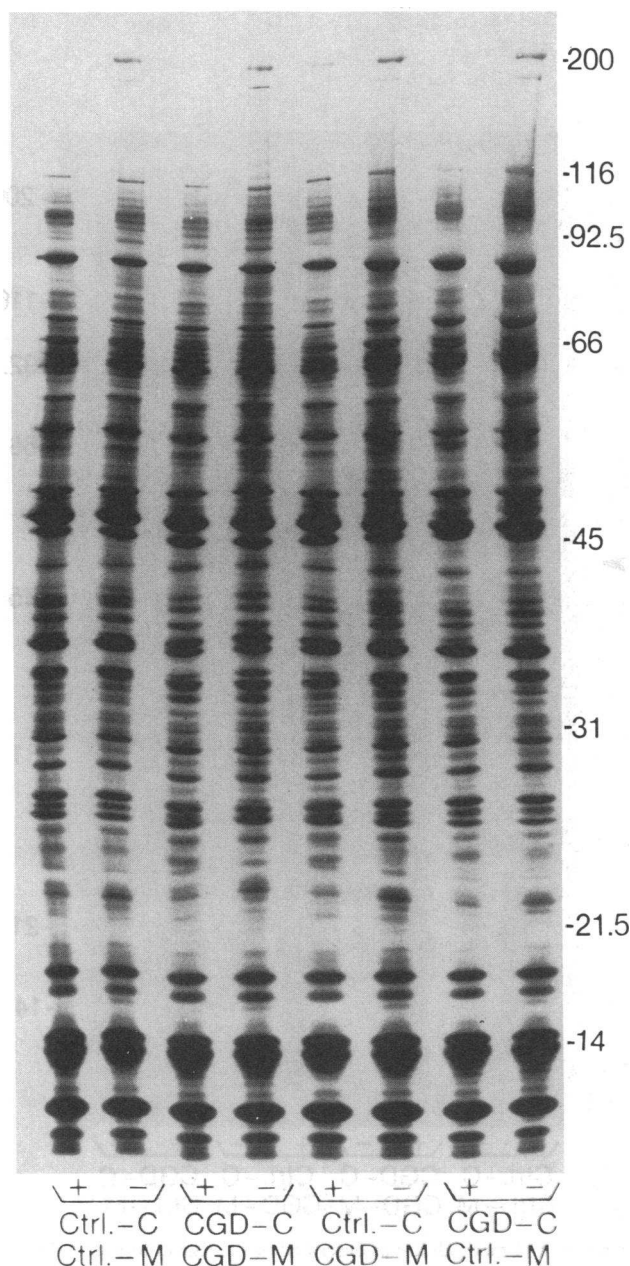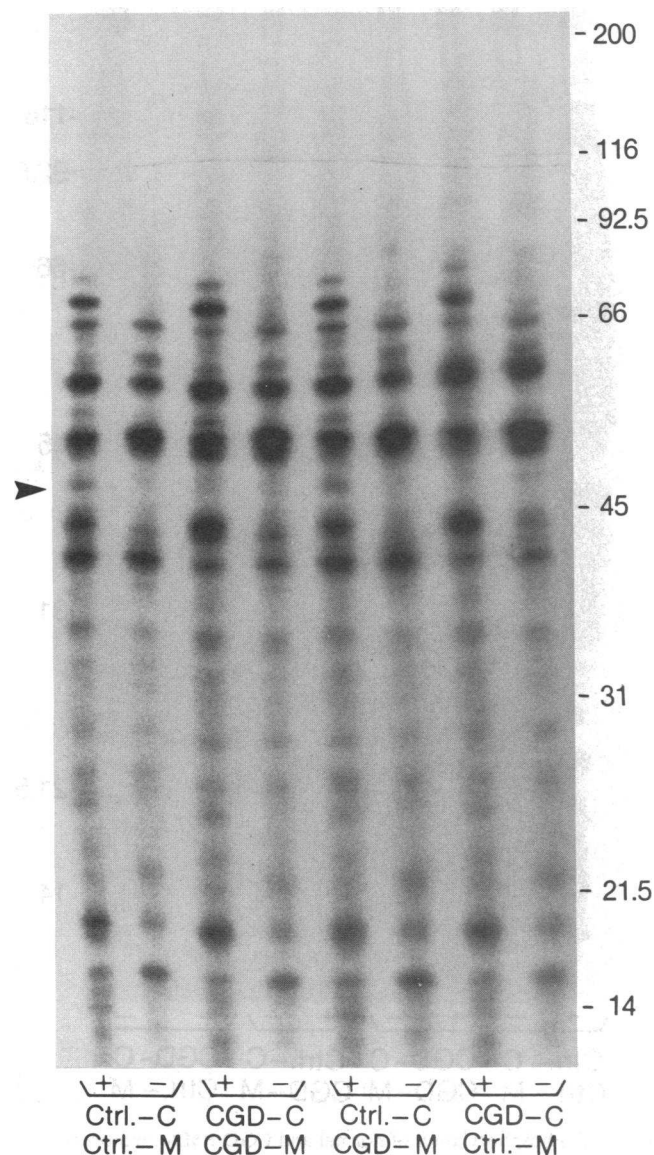Ctrl.−M CGD−M CGD−M Ctrl.−M

Figure 1. Phosphorylation of normal and CGD PMN cytosolic proteins in the cell-free system. PMN from a normal donor and a CGD patient (S.S.) were fractionated; cytosolic and membrane fractions were mixed with 50 μCi [γ-^{32}P]ATP in the presence of 460 μM SDS (+) or of H_2O as solvent control (−); and fractions were reseparated as described in Methods. Cytosolic fractions (31 μg protein) were analyzed by SDS-polyacrylamide gel electrophoresis, silver staining,

and indirect autoradiography. Ctrl.-C/Ctrl.-M, control cytosol + control membrane; CGD-C/CGD-M, CGD cytosol + CGD membrane; Ctrl.-C/CGD-M, control cytosol + CGD membrane; CGD-C/Ctrl.-M, CGD cytosol + control membrane. Arrow marks the position of the 48-kd phosphoprotein(s). Molecular weight markers are in kilodaltons. (*A*) Silver-stained polyacrylamide gel. (*B*) Corresponding autoradiogram.

CGD cytosolic fraction, or that the 48-kD phosphoprotein(s) is not present in the cytosolic fraction from the CGD PMN.

Analysis of membrane fractions, reisolated after reconstitution of normal membrane and cytosolic fractions in the presence of SDS, by SDS-polyacrylamide gel electrophoresis and autoradiography showed that ∼ 32 phosphoproteins were present (Fig. 2 *B*, lane *1*). Of these phosphoproteins, 14 had

increased phosphorylation and three had decreased phosphorylation compared with the same fraction isolated from a reconstitution mixture incubated in the absence of SDS (lane *1* vs lane *2*). Analogous to the results with the cytosolic fractions, phosphorylation of a 48-kD protein(s) (arrow) was markedly enhanced by SDS treatment in the mixture of normal cytosolic and membrane fractions (lanes *1* and *2*), but not during re-
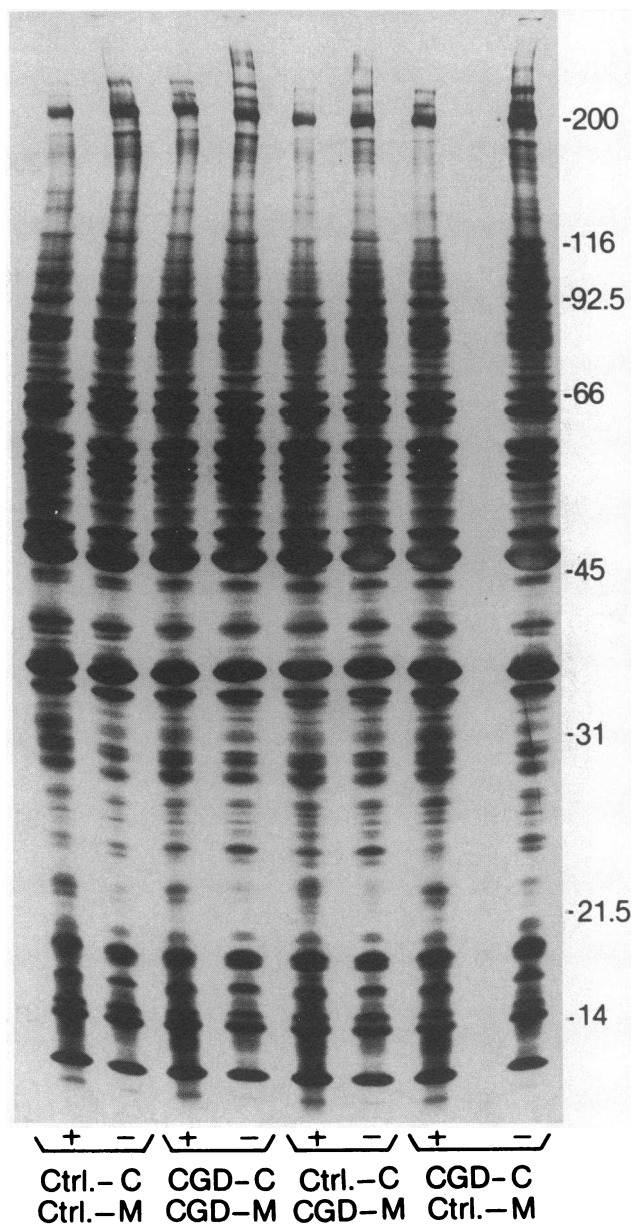
A. Membrane Fractions - Silver Stain

-200
-116
-92.5
-66
-45
-31
-21.5
-14

+	−	+	−	+	−	+	−
Ctrl.− C		CGD− C		Ctrl.−C		CGD− C	
Ctrl.−M		CGD−M		CGD−M		Ctrl.−M	

B. Membrane Fractions - Autoradiogram

- 200
-116
- 92.5
-66
- 45
-31
- 21.5
-14

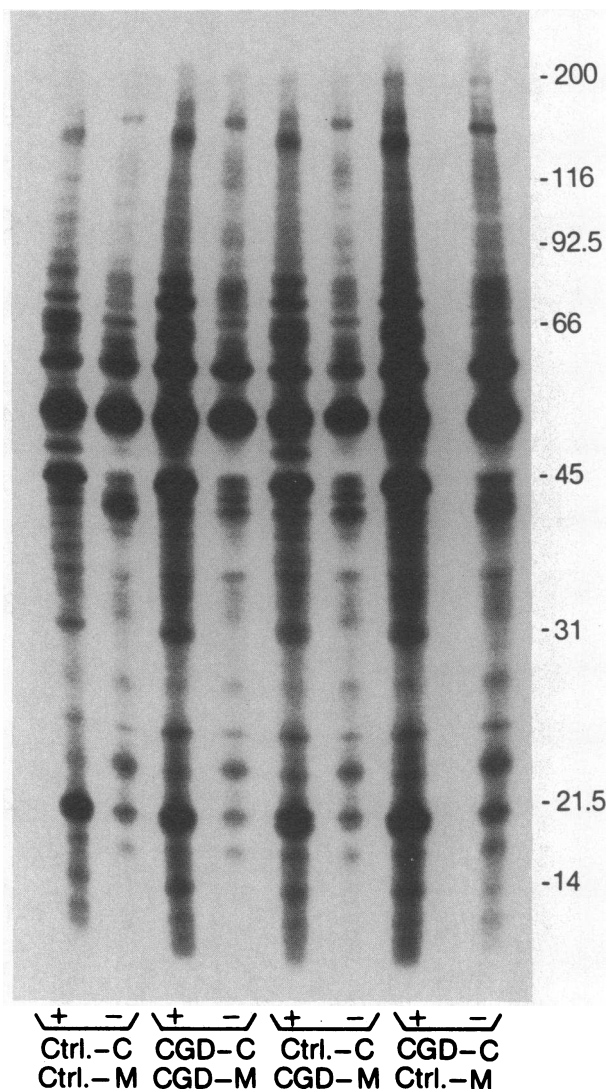| + | − | + | − | + | − | + | − |
| Ctrl.−C | | CGD−C | | Ctrl.−C | | CGD−C | |
| Ctrl.−M | | CGD−M | | CGD−M | | Ctrl.−M | |

Figure 2. Phosphorylation of normal and CGD PMN membrane proteins in the cell-free system. Membrane fractions (70.3 μg protein) reseparated from cytosolic fractions in the reconstitution experiment described in Fig. 1 were analyzed by SDS-polyacrylamide gel electrophoresis, silver staining, and indirect autoradiography. Abbre-viations and methods are as described in the legend to Fig. 1. The arrow marks the position of the 48-kd phosphoprotein(s). Molecular weight markers are in kilodaltons. (*A*) Silver-stained polyacrylamide gel. (*B*) Corresponding autoradiogram.

constitution of the CGD cytosol and membrane fraction (lanes *3* and *4*). Reconstitution of CGD membrane fractions with normal cytosol fully restored SDS-dependent phosphorylation of the 48-kD phosphoprotein(s) (lanes *5* and *6*). In contrast, minimal phosphorylation of the 48-kD phosphoprotein(s) by SDS occurred when the CGD cytosolic fraction was reconstituted with normal membrane fraction (lanes *7* and *8*), further indicating the requirement for a cytosolic component that is not functional in autosomal recessive CGD PMN.

Autoradiograms of membrane fractions from two additional cell-free reconstitution experiments, one from each pa-tient having autosomal recessive CGD, were subjected to densitometric analysis. Phosphoproteins in the region of 48 kD (arrows) are shown in Fig. 3. In the presence of SDS, phosphorylation of a 48-kD protein(s) as a result of reconstitution of normal cytosol with normal membrane fractions was observed. No phosphorylation of that protein(s) was observed during reconstitution of CGD cytosol with CGD membrane fractions in the presence of SDS. Reconstitution of normal cytosol with CGD membrane fractions and SDS restored phosphorylation of the 48-kD protein(s). In contrast, reconstitution of CGD cytosol with normal membrane fractions and
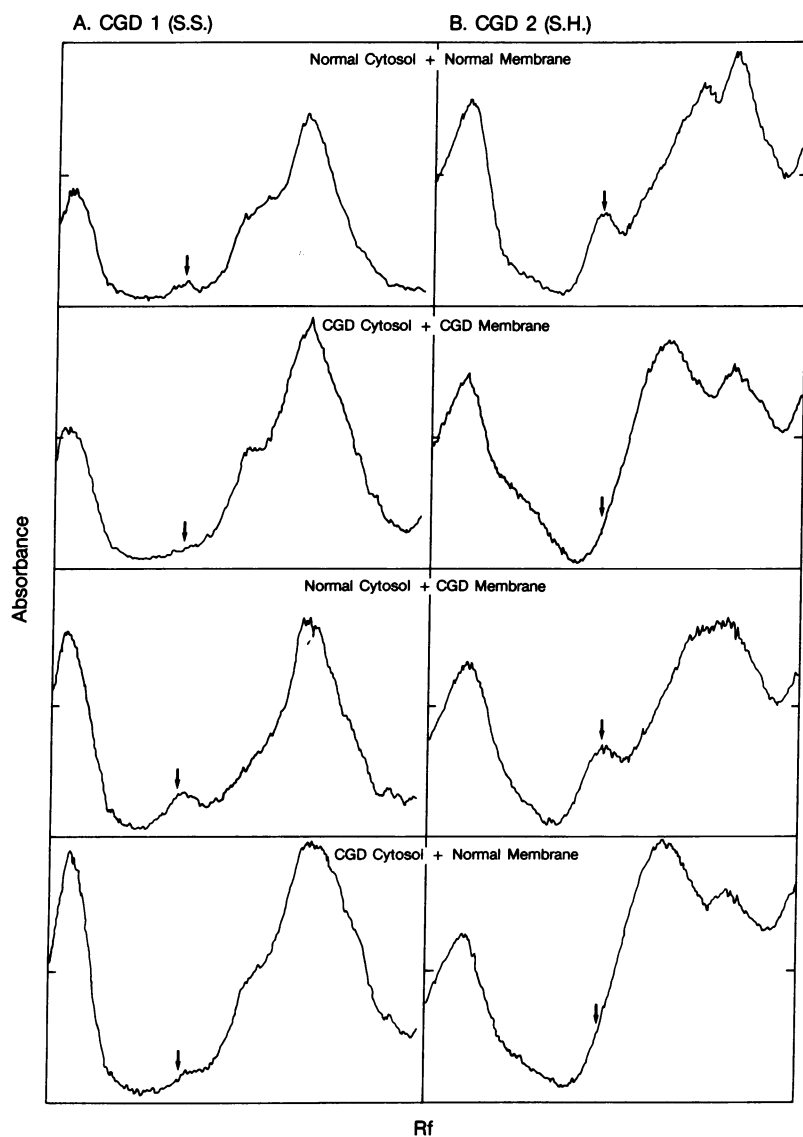
A. CGD 1 (S.S.)   B. CGD 2 (S.H.)

Normal Cytosol + Normal Membrane

CGD Cytosol + CGD Membrane

Normal Cytosol + CGD Membrane

CGD Cytosol + Normal Membrane

Absorbance

Rf

Figure 3. Densitometry scans of autoradiograms of membrane fractions from normal and CGD PMN following reconstitution with SDS in the cell-free system. Unstimulated PMN from two normal donors and two patients with autosomal recessive CGD (S.S. and S.H.) were fractionated; cytosolic and membrane fractions were reconstituted in the presence of $[\gamma^{-32}P]$-ATP and 460 μM SDS and reseparated as described in Methods. The experiment with S.S. is separate from the one described in Fig. 1. Membrane fractions (S.S., 100 μg protein; S.H., 41 μg protein) were subjected to SDS-polyacrylamide gel electrophoresis, silver-staining, and autoradiography. Autoradiograms were scanned by a soft laser densitometer in the region of the 48 kD phosphoprotein(s) (arrows). In all scans the bottom of the gel is to the left. (*A*) CGD 1 (S.S.). (*B*) CGD 2 (S.H.). (*Top panels*) Normal cytosol + normal membrane; (*second panels*) CGD cytosol + CGD membrane; (*third panels*) normal cytosol + CGD membrane; (*bottom panels*) CGD cytosol + normal membrane.

SDS resulted in minimal, or no, phosphorylation in that region of the gel.

The area under the curve at 48 kD on the scans shown in Fig. 3 and on scans of the lanes containing fractions isolated from SDS-containing mixtures in the autoradiogram shown in Fig. 2 was determined. If the area for the reconstitution mixture of normal cytosol and CGD membrane is set at 100%, the summarized data were as follows: normal cytosol + normal membrane = 75±22; CGD cytosol + CGD membrane = 1.5±1.5; CGD cytosol + normal membrane = 14±8 (percent, mean±SEM, n = 3). The inability of the CGD cytosol and membrane to phosphorylate the membrane-associated 48-kd protein(s), and the markedly decreased ability of the CGD cytosol to support phosphorylation when mixed with normal membrane thus was observed in three separate experiments. Taken together, these results indicate that a cytosolic factor activity necessary for SDS-dependent activation of NADPH oxidase and defective in two patients with autosomal recessive CGD, is also necessary to support SDS-dependent phosphorylation of a 48-kD phosphoprotein(s).

Since phosphorylation of a 48-kD protein(s) by SDS in

reconstitution mixtures of normal cell fractions and the lack of phosphorylation in mixtures of CGD cell fractions was observed in both cytosolic and membrane fractions, it is possible that the same protein(s) is distributed between both fractions. Cytosolic and membrane fractions reisolated from the normal and CGD reconstitution mixtures described in Figs. 1 and 2 were analyzed on the same gel to test this possibility. The results are shown in Fig. 4. The position of the 48-kD phosphoprotein(s) can be identified by the increase in phosphorylation induced by SDS in the normal cell fractions and the absence of that increase in the fractions from the CGD PMN. The 48-kD phosphoprotein(s) (arrow) in both cytosolic and membrane fractions comigrated (lanes *1* and *2* from left), supporting the possibility that they are the same protein or family of proteins.

It has been reported previously that neutrophils from patients with the autosomal recessive form of CGD demonstrate a defect in phosphorylation of a protein(s) in the 47–48-kD region when intact cells are exposed to a stimulus (15, 17). It is thus possible that the 48-kD protein(s) observed in our cell-free system is the same as that in the intact cell system. We tested
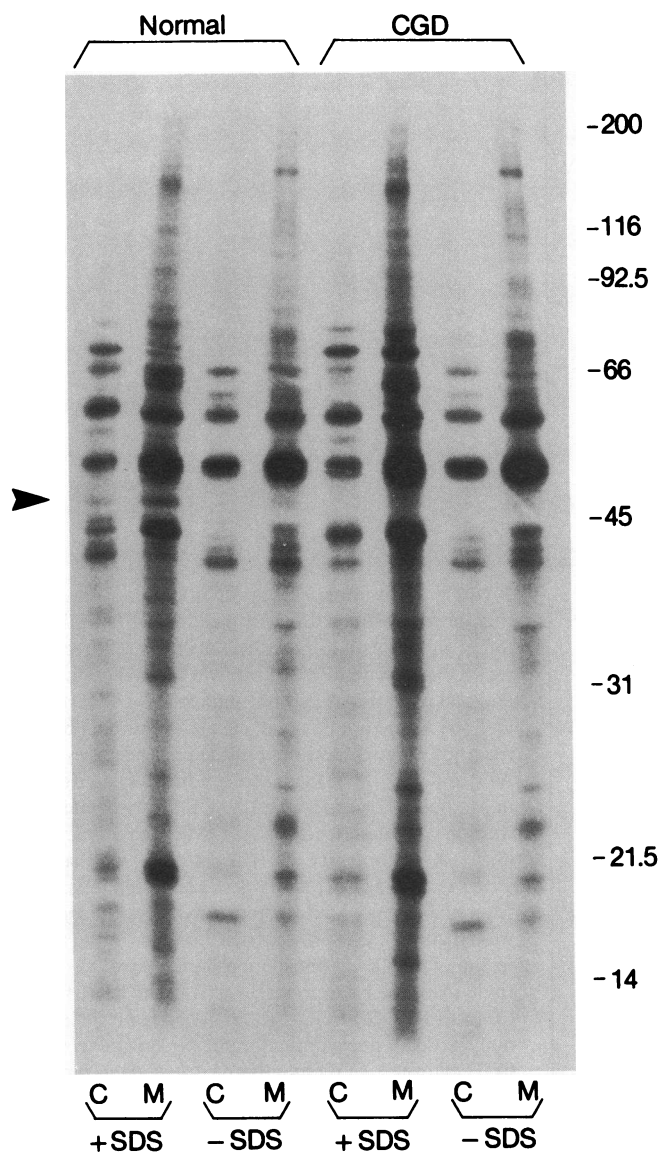
Normal   CGD

−200

−116

−92.5

−66

−45

−31

−21.5

−14

C M C M C M C M
+SDS −SDS +SDS −SDS

Normal   CGD

−200

−116
−92.5

−66

−45

−31

−21.5

−14

SDS H₂O DMSO PMA SDS H₂O DMSO PMA
Recon. Intact Recon. Intact

Figure 4. Comigration in SDS-polyacrylamide gel of the 48 kd phosphoprotein(s) in both cytosolic and membrane fractions following reconstitution with SDS in the cell-free system. Cytosolic (C, 31 μg protein) and membrane (M, 70 μg protein) fractions from the experiment described in Fig. 1 were electrophoresed on the same polyacrylamide gel and autoradiography was performed. +SDS, fractions reconstituted in the presence of 460 μM SDS; −SDS, fractions reconstituted in the presence of H₂O (solvent control); Normal, PMN fractions from a normal donor; CGD, PMN fractions from a patient with autosomal recessive CGD (S.S.). Molecular weight markers are in kilodaltons. The arrow indicates the 48-kD position.

Figure 5. Comparative phosphorylation patterns in cytosolic fractions from cell-free reconstitution mixtures and from intact normal and CGD PMN. Cytosolic fractions from the reconstitution experiment described in Fig. 1 (31 μg protein; Recon.) and from intact normal or CGD PMN that had been loaded with ³²Pᵢ and exposed to either DMSO or 100 ng/ml PMA for 30 s (64 μg protein; Intact) were analyzed in the same SDS-polyacrylamide gel and subjected to autoradiography. SDS, +460 μM SDS; H₂O, solvent control; Normal, fractions isolated from normal PMN; CGD, fractions isolated from a patient (S.S.) with autosomal recessive CGD. Molecular weight markers are in kilodaltons. Arrow marks the position of the 48-kD phosphoprotein(s).

that possibility by analyzing, in the same gel, phosphoprotein patterns of cytosolic fractions prepared from cell-free reconstitution mixtures incubated with [γ-³²P]ATP and cytosolic fractions prepared from resting or PMA-stimulated ³²Pᵢ-labeled intact cells (Fig. 5). Interestingly, the phosphoprotein patterns of the cell-free versus the intact cell cytosolic fractions were quite dissimilar. However, a defect in both SDS-dependent phosphorylation of a 48-kD protein(s) (arrow) in reconstitution mixtures and in PMA-stimulated phosphorylation of a
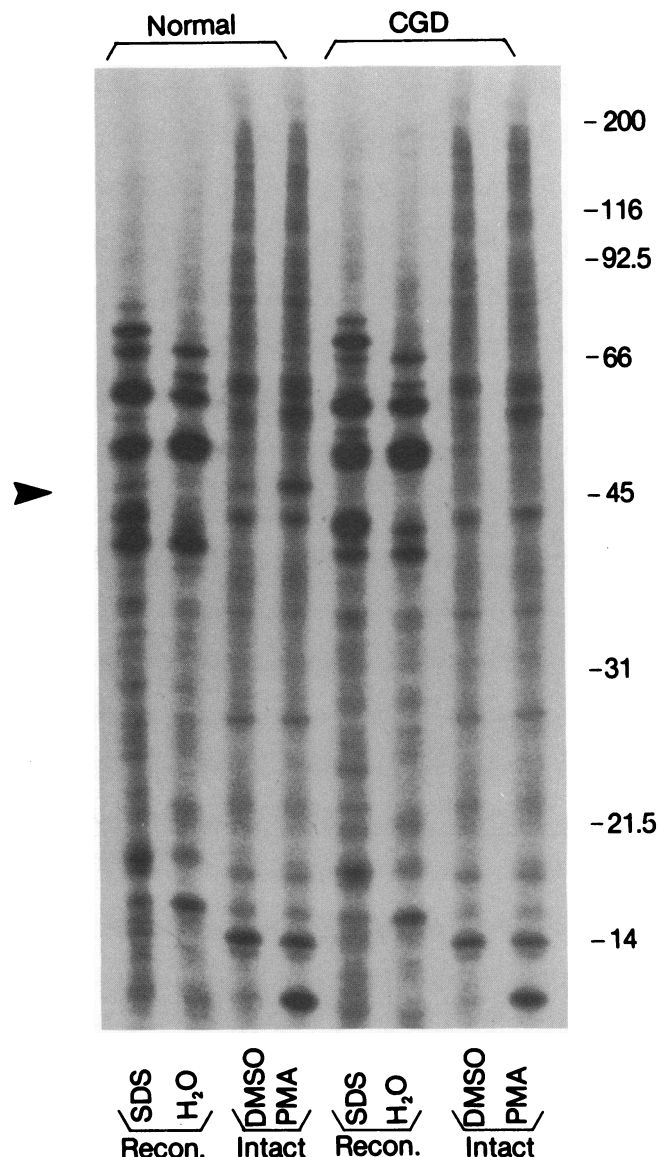
48-kD protein(s) was observed in cytosolic fractions from CGD cells. In addition, the 48-kD phosphoprotein(s) in cytosolic fractions from normal reconstitution mixtures and from normal intact cells appeared to comigrate, supporting the possibility that they are the same protein(s).

PKC activity in autosomal recessive CGD PMN. It was possible that the lack of phosphorylation of the 48-kD phosphoprotein(s) and the absence of NADPH oxidase activity were due to deficient levels of PKC. The activity and distribu-

tion of PKC were determined in crude subcellular fractions from unstimulated and stimulated PMN of normal controls and the two patients with autosomal recessive CGD. As shown in Fig. 6, the level and distribution of PKC activity in cytosolic and particulate EP and NEP fractions was similar in normal and CGD PMN, under all conditions examined. PMA stimulation of PMN from both patients with CGD induced the redistribution and loss of PKC activity similar to that observed in each paired normal control (Fig. 6, B). The effect of fMLP stimulation was examined in one patient with CGD and the normal increase in particulate-associated (EP and NEP) kinase activity was observed. These studies suggest that PKC is not defective in the two patients with CGD.

Discussion

The mechanisms that regulate NADPH oxidase, the respiratory burst enzyme in human PMN, are not yet well understood; although, progress in elucidating the nature of the en-

Figure 6. PKC activity and distribution in subcellular fractions from normal and CGD PMN. PKC activity was measured in subcellular fractions isolated from normal or CGD PMN that had been treated with either DMSO (A), 100 ng/ml PMA (B) or 10^{-6} M fMLP (C) for 30 s at 37°C as described in Methods. Fractions assayed were the cytosolic (□, Cytosol), the detergent extract of the particulate fraction (▨, EP), and the resuspended residual particulate pellet from the detergent extract (■, NEP). Activity shown is the mean of triplicate determinations on each fraction and is given as net activity (the activity in the presence of calcium and lipids minus the activity in the absence of calcium and lipids) for the cytosolic and EP fractions and as activity in the absence of calcium and lipids for the NEP fractions (see Methods). Activity in the absence of calcium and lipids in the EP and cytosolic fractions was not different in the fractions from normal and CGD PMN and was < 91 pmol/min per 10^7 PMN in cytosolic and 17 pmol/min per 10^7 PMN in EP fractions. Two paired experiments, each with PMN from a different normal and a different patient with autosomal recessive CGD, were performed: CGD 1, S.S.; CGD 2, S.H.

zyme complex at the molecular level is being made (20–22). Recently, a cell-free system for activation of NADPH oxidase was developed by us (25) and others (26–28) and can now be utilized to more directly study the mechanisms involved. Of particular interest is the role of a newly discovered cytosolic cellular cofactor required for activation of the oxidase in this system (25–28). Protein phosphorylation has been implicated as a mechanism of oxidase activation (3–17) and phosphorylation has not been directly studied in the cell-free system. Therefore, we have examined whether or not phosphorylation of endogenous proteins correlated with the cell-free activation of NADPH oxidase and have investigated the role of the cytosolic factor in the regulation of the observed phosphorylation events.

Activation of NADPH oxidase in the cell-free system correlated specifically with phosphorylation of a 48-kD protein(s). NADPH oxidase activation was achieved by the addition of SDS to a reconstituted mixture of cytosolic and membrane fractions from unstimulated normal PMN, in agreement with previous reports in guinea pig macrophages (29) and human PMN (33), and similar to results obtained with arachidonic acid instead of SDS as the activator (25–28). SDS also induced the phosphorylation or dephosphorylation of at least 19 cytosolic and 17 membrane-associated proteins, including a 48-kD protein(s) seen in both fractions, out of ~ 56 phosphoproteins observed in the reconstituted cell-free system. Thus, SDS appears capable of activating a protein kinase(s), as well as either activating a phosphatase(s) or inhibiting a protein kinase(s). Although the identity of the kinase or kinases affected by SDS is not yet known, we have found that SDS can activate PKC in cytosolic fractions from human PMN as measured by exogenous histone phosphorylation (data not shown). Therefore, at least some of the phosphorylation reactions observed may be catalyzed by PKC.

Phosphorylation of the 48-kD protein(s) by SDS was markedly decreased in cell-free reconstitution mixtures of cytosolic and membrane fractions from PMN of two patients with autosomal recessive CGD. This was the only phosphorylation defect noted and it correlated with the dramatic decrease in the ability of SDS to activate NADPH oxidase in the same fractions. The defects in activation of NADPH oxidase and in phosphorylation of the 48-kD protein(s) could both be attributed to a defect in the cytosolic fraction from the CGD PMN. The membrane fraction from CGD PMN appeared to be normal, in that it supported normal levels of both phosphorylation of the membrane-associated 48-kD protein(s) and activation of NADPH oxidase when reconstituted with a normal cytosolic fraction. In addition, reconstitution of CGD cytosolic fraction with normal membrane fraction mimicked the defects in phosphorylation of the 48-kD protein and in activation of NADPH oxidase seen with the reconstituted CGD fractions. Thus, the cytosolic factor required for activation of NADPH oxidase in the cell-free system is defective in PMN of two patients with the autosomal recessive form of CGD, as originally suggested by Curnutte et al. (31), and this defect correlates with defective phosphorylation of a 48-kD protein(s).

These results clearly indicate that the cytosolic factor required for activation of NADPH oxidase also is required for phosphorylation of the 48-kD protein(s). Our results do not differentiate between two possible explanations for the relationship between the cytosolic factor and the 48-kD protein(s).
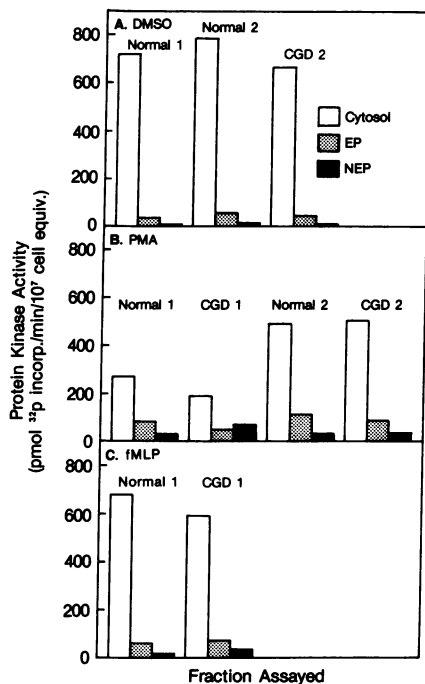
The cytosolic factor could be the 48-kD phosphoprotein(s) and its presence in the membrane due to partial translocation induced by SDS. If so, then the presence of the 48-kD phosphoprotein in the CGD membrane fraction could be caused by translocation from the normal cytosolic fraction during reconstitution. In fact, the 48-kD protein(s) in normal membrane fractions comigrated with the 48-kD protein(s) in normal cytosolic fractions, supporting the possibility that they are the same protein(s) and could either have dual localization or be translocated by SDS treatment. Observations consistent with the possibility that translocation of the cytosolic factor occurs are that, under conditions that prime intact normal PMN or macrophages, NADPH oxidase in membrane fractions can be activated in the cell-free system without the addition of cytosolic fractions (25, 29).

It is also possible that the cytosolic factor regulates phosphorylation of the 48-kD protein(s) because the factor is itself a kinase or it activates a kinase. Our results suggest that the cytosolic factor is not PKC, since PKC activity appeared to be present at normal levels and to respond normally to stimulation by either PMA or fMLP in CGD PMN. However, we cannot exclude the possibility of a more subtle defect in PKC activity or regulation not detected by our assay procedure. That the cytosolic factor is not PKC is also supported by others using inhibitors (33, 34, 55) or column fractionation (30) to dissociate PKC activity from the oxidase activating factor in normal PMN cytosol. It is interesting that 46–48-kD proteins in guinea pig (56) and human (57) PMN have been reported to be substrates for PKC or its catalytic subunit, although it is not yet known if these and the protein(s) observed here are the same. Further understanding of the relationship between the 48-kD protein, the cytosolic factor required for NADPH oxidase activation, and PKC or other kinases will necessitate purification of the components and more rigorous physical characterization of the 48-kD protein or proteins.

A defect in phosphorylation of a 47–48-kD protein, or family of proteins, in PMN from patients with CGD has been observed previously by stimulation of intact cells (15–17). Segal et al. (15), using one-dimensional polyacrylamide gel electrophoretic separation, reported that the defect was restricted to patients with the autosomal recessive form of the disease. However, Hayakawa et al. (16) and Okamura et al. (17), using two-dimensional gel electrophoresis, found that both forms of the disease expressed defects in phosphorylation of a 48-kD family of proteins and suggested that a more pronounced defect is present in the autosomal recessive form. A third group (24), using two-dimensional gel electrophoresis, reported no defect in protein phosphorylation in six patients with CGD (presumably representing both forms of inheritance). Our results, using one-dimensional gel electrophoresis, indicate, for the first time, that a defect in phosphorylation of a 48-kD protein(s) can be observed in a cell-free system from PMN of patients with the autosomal recessive form of CGD and relate the defect to a deficient cytosolic factor required for activation of NADPH oxidase. The 48-kD protein(s) observed in our cell-free system by SDS treatment comigrated with a 48-kD protein observed by stimulating intact PMN with PMA, consistent with the possibility that the phosphorylation defect in intact CGD PMN is the same as in the cell-free system. However, further analysis by two-dimensional gel electrophoresis, peptide mapping, and phosphoamino acid determination will be necessary to fully identify and characterize the

48-kd protein(s) observed by the various groups and to determine if all groups are studying the same family of proteins.

The relationship of the phosphorylation of the 48-kD protein(s) to the activation of NADPH oxidase in the intact cell or by SDS in the cell-free system is still not clear. Phosphorylation of a protein or proteins at 46–48 kD has been correlated with activation of the respiratory burst in intact cells by several laboratories (8, 56–58). However, other data dissociates phosphorylation of a 48-kD protein(s) from stimulation of oxidative metabolism (59–61). With the exception of Ohtsuka et al. (56), these studies have utilized one-dimensional gel separation systems and, if the 48-kD region contains a family of proteins (16, 17, 24), such discrepancies might be expected.

A requirement for a protein kinase activity in the SDS-mediated activation of NADPH oxidase in the cell-free system has not yet been demonstrated nor conclusively eliminated. Oxidase activation by SDS or arachidonate does not require exogenously added ATP (30, 35) and depletion of endogenous ATP has been reported by two groups to inhibit (35, 55) and by another to have no effect (30) on oxidase activation. PKC has been shown to activate NADPH oxidase in a cell-free system (62); however, the mechanism utilized by PKC appears to be different than that utilized by SDS and arachidonate (33) and PKC does not seem to be required for the latter compounds to exert their activating effect (30, 33, 34). These results and our own suggest that if a kinase is involved in the SDS-activated cell-free system, it is not PKC.

Based on the present state of knowledge and the work reported here, at least three possibilities for the role of the 48-kD protein(s) in the activation of NADPH oxidase are apparent. The 48-kD protein(s) may be unrelated to oxidase activity in PMN, but its phosphorylation may depend upon a normal cytosolic factor activity. Alternatively, the 48-kD protein(s) may be a component(s) of NADPH oxidase; a protein of that molecular weight appears, albeit variably, in preparations of the purified oxidase flavoprotein (63). Finally, the 48-kD protein(s) could be a regulatory molecule(s), influencing one or more of several pathways (2, 11, 18, 19, 33, 36) of activation of oxidative metabolism. Further efforts will be made to distinguish between these possibilities and to resolve the current discrepancies in the literature by fully characterizing the 48-kD protein(s).

Acknowledgments

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