Cell-Free Translocation of Recombinant p47-phox, a Component of the Neutrophil NADPH Oxidase: Effects of Guanosine 5′-*O*-(3-Thiotriphosphate), Diacylglycerol, and an Anionic Amphiphile†

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Received July 26, 1991; Revised Manuscript Received December 20, 1991

ABSTRACT: We reported previously that diacylglycerol (diC8) and GTPγS synergize with an anionic amphiphile such as sodium dodecyl sulfate (SDS) to produce high rates of superoxide generation in a cell-free system consisting of neutrophil plasma membrane plus cytosol [Burnham, D. N., Uhlinger, D. J., & Lambeth, J. D. (1990) *J. Biol. Chem. 265*, 17550–17559]. Here we investigate the effects of these activating factors on the plasma membrane association in an in vitro translated radiolabeled recombinant p47-phox protein. Apparent translocation, assayed by cosedimentation with plasma membranes, required the presence of excess cytosol and an anionic amphiphile, was enhanced by both GTPγS and diC8, and was inhibited by high salt, correlating qualitatively with activation; up to 70% cosedimentation was observed with the combination of activators (compared with <20% in their absence). Similar results were obtained using heat-inactivated cytosol, wherein another oxidase component, p67-phox, has been inactivated. Unexpectedly, from 50 to 80% of the apparent translocation occurred in the absence of membranes, indicating that protein aggregation accounted for a significant part of the observed translocation. Nevertheless, the percent translocation was increased in all cases by the presence of membranes, indicating some degree of protein–membrane interaction. While a control in vitro translated protein failed to translocate, cosedimentation of p47-phox occurred equally well when red blood cell or neutrophil plasma membranes lacking cytochrome b_{558} were used. Also, the peptide RGVHFIF, which is contained within the C-terminus of the large subunit of cytochrome b_{558}, failed to inhibit translocation/aggregation of p47-phox, despite its ability to inhibit cell-free activation of the oxidase. The data are consistent with the following: (a) SDS, diC8, and GTPγS all act on cytosolic components to alter protein–protein and/or protein–membrane associations, and these changes are necessary (but not sufficient) for activation; (b) these altered asociations are likely to function by increasing the local concentration of p47-phox and other components at the plasma membrane; (c) a high background of nonspecific associations in the cell-free activation system is likely to obscure any specific, functionally relevant associations (e.g., with cytochrome b_{558}); and (d) the mechanism of translocation in the cell-free system differs from that seen in intact neutrophils.

The neutrophil [polymorphonuclear leukocyte (PMN)][1] provides the primary host defense against invading microorganisms, and can play a pathological role in a variety of inflammatory conditions (Rotrosen & Gallin, 1987; Malech & Gallin, 1987). One of its antimicrobial mechanisms involves the activation of the respiratory burst, which utilizes molecular oxygen to generate superoxide; the latter secondarily produces other cytotoxic species including H_2O_2, HOCl, and hydroxyl radical. The pivotal importance of the respiratory burst in combating infectious disease is illustrated by the inherited condition chronic granulomatous disease (CGD) wherein the neutrophils fail to generate oxidants, and afflicted individuals suffer frequent and severe infections (Smith & Curnutte, 1991). Oxidant generation is initiated by the activation of the superoxide-generating respiratory burst oxidase or NADPH oxidase (Bellavite, 1988). The oxidase, dormant in unstimulated cells, can be activated in cells by a variety of stimuli, including opsonized particles (e.g., bacteria, zymosan), protein kinase C activators such as diacylglycerol and phorbol esters, and chemoattractants such as formylmethionylleucylphenylalanine (fMLP) [e.g., see Lambeth (1988)].

Although the activated oxidase obtained from stimulated cells has been difficult to study because of its lability and low abundance [e.g., see Tamura et al. (1989)], a cell-free system obtained from nonactivated phagocytic cells has been developed (Curnutte, 1985; McPhail et al., 1985; Bromberg & Pick, 1984) and has allowed recent advances in understanding the protein components and regulatory factors which participate in the respiratory burst. The system consists of cytosol, plasma membranes, and an anionic amphiphile such as sodium dodecyl sulfate (SDS) or arachidonate. The use of molecular cloning approaches, together with studies using cell fractions from CGD neutrophils in the cell-free system, has led to the defi-

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[1] Abbreviations: phox (as used in p47-phox and p67-phox), *ph*agocyte *ox*idase; CGD, chronic granulomatous disease; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; diC8, 1,2-dioctanoylglycerol; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); PMN, polymorphonuclear leukocyte; DFP, diisopropyl fluorophosphate; PMSF, phenylmethanesulfonyl fluoride; PBS, phosphate-buffered saline; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester; BSA, bovine serum albumin; ABTS, 2,2′-azinobis(3-ethylbenzothiazolinesulfonic acid); PCR, polymerase chain reaction; PMA, phorbol 12-myristate 13-acetate.

0006-2960/92/0431-2765$03.00/0

nitive identification of several components of the respiratory burst oxidase. The plasma membrane associated cytochrome b_{558} consists of two subunits of apparent molecular weight by SDS–PAGE of 92 000 and 22 000, both of which have been cloned and sequenced (Royer-Pokora et al., 1986; Parkos et al., 1988). The cytochrome is generally assumed to be the terminal oxidase which univalently reduces oxygen to generate superoxide [e.g., see Cross et al. (1985)]. cDNAs encoding two cytosolic components, p47-phox and p67-phox, have been cloned and sequenced (Lomax et al., 1989a,b; Volpp et al., 1989; Leto et al., 1990), but the functions of these proteins remain unclear. Specifically, they do not show sequence homology to known electron transport or nucleotide binding proteins. In addition, there are a number of suspected components and/or activators including an NADPH binding species and/or a flavoprotein (Gabig, 1983; Babior & Kipnes, 1977).

The cell-free system has also permitted an investigation of the requirements for nonprotein activators and cofactors. Magnesium and an anionic amphiphile are absolutely required (Clark et al., 1987; Curnutte, 1985; McPhail et al., 1985; Babior et al., 1988; Bromberg & Pick, 1984), but the biological relevance of the latter is not clear, since free fatty acid levels are not known to correlate with cell activation; the anionic amphiphile may mimic another regulatory molecule such as phosphatidic acid, which is known to be produced upon phospholipase D activation [e.g., see Pai et al. (1988) and Rossi et al. (1990)]. We recently reported (Burnham et al., 1990) that added diacylglycerol synergizes with the anionic amphiphile to augment superoxide generation 2.5–7-fold over the rate with SDS alone. Since diacylglycerols are generated under a variety of activation conditions (Honeycutt & Niedel, 1986; Rider & Niedel, 1987; Lambeth et al., 1988; Tyagi et al., 1988, 1989; Burnham et al., 1989; Dougherty et al., 1989), we suggested that these cooperate with other activating factor(s) to elicit or augment the respiratory burst. The stimulation by diacylglycerol in the cell-free system is independent of ATP and protein phosphorylation (Uhlinger et al., 1991), indicating that the effect is not mediated by protein kinase C. In addition, GTPγS stimulates the anionic amphiphile-dependent superoxide generation 2–3-fold (Seifert & Schultz, 1987; Ligiti et al., 1988; Burnham et al., 1990; Uhlinger et al., 1991), suggesting that a guanine nucleotide regulatory protein participates in activation.

Although the mechanism of oxidase activation is not yet clear, the concept has begun to emerge that activation involves the assembly of protein components to form an active complex. In experiments using intact cells, PMA caused the cytosolic proteins p47-phox and p67-phox to become associated with the plasma membrane (Okamura et al., 1990; Clark et al., 1990; Ohtsuka et al., 1990). Because this failed to occur in CGD cells which lack cytochrome b_{558} (Heyworth et al., 1991), the cytosolic components may bind to the cytochrome or an associated component. Phosphorylation of p47-phox by protein kinase C and/or other kinases occurs at multiple sites upon activation in intact cells (Hayakawa et al., 1986; Rotrosen & Leto, 1990), and this has also been observed in the cell-free system (Ohtsuka et al., 1990; Burnham et al., 1990; Uhlinger et al., 1991). However, phosphorylation does not seem to be obligatory for activation, either in intact cells (Badwey et al., 1989) or in the cell-free system (Uhlinger et al., 1991). In intact cells, the presence of p47-phox is required for p67-phox translocation, since in CGD cells lacking p47-phox membrane association of p67-phox does not occur (Heyworth et al., 1991). While other interpretations are possible, it has been suggested that p47-phox forms the initial complex with the cytochrome and is required for the assembly of other components. Translocation of the components to the membrane has also been noted in the arachidonate-stimulated cell-free system (Clark et al., 1990; Ohtsuka et al., 1990). Also in the cell-free system, a peptide from the C-terminus of the large subunit of cytochrome b_{558}, RGVHFIF, blocked activation of superoxide generation, and experiments using complementing CGD cytosols suggested an effect involving early assembly of p47-phox (Rotrosen et al., 1990; Kleinberg et al., 1990).

The present studies were initiated to investigate the possible effect of the stimulatory factors diacylglycerol and GTPγS in facilitating translocation of p47-phox to the plasma membranes. Currently, two analytical methods are in use to assay p47-phox in subcellular fractions: (i) SDS–PAGE followed by immunoblotting (Nauseef et al., 1990); (ii) in vitro phosphorylation of subcellular fractions using partially purified protein kinase C and [γ-^{32}P]ATP, followed by SDS–PAGE and quantification by autoradiography (Strum et al., 1990). In preliminary studies (D. Uhlinger and D. Burnham, unpublished observations), we utilized antibodies raised against a predicted C-terminal peptide of the p47-phox protein to visualize this component by immunoblotting methods. However, quantification proved to be both cumbersome and difficult to reproduce, and we now believe that the latter difficulty was due to phosphorylation in the C-terminus of p47-phox which may affect recognition by the antibodies (D. Uhlinger, unpublished results), as well as to inherent difficulties in quantifying from immunoblots. In the present studies, we have utilized recombinant methodology and an in vitro transcription/translation system to generate a radiolabeled p47-phox protein, which was then used to assess the distribution of p47-phox between cytosol and membrane fractions. Using this novel and convenient methodology, we have investigated the cell-free translocation process and the effects of activating factors and the inhibitor peptide.

Experimental Procedures

Materials. Hespan (6.2% hetastarch in 0.9% NaCl) was obtained from American Hospital Supply Corp. Lymphocyte separation medium (6.2% Ficol/9.4% sodium diatrizoate) was purchased from Bionetics Laboratory Products. 1,2-Dioctanoylglycerol (diC8) was purchased from Avanti Polar Lipids (Birmingham, AL). Cytochrome *c* (type IV, horse heart), NADPH, diisopropyl fluorophosphate (DFP), phenylmethanesulfonyl fluoride (PMSF), leupeptin, pepstatin, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), keyhole limpet hemocyanin, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS), bovine serum albumin (BSA), reduced glutathione, 5,5′-dithiobis(2-nitrobenzoic acid), 2,2′-azinobis(3-ethylbenzothiazolinesulfonic acid) (ABTS), and peroxidase-linked goat anti-rabbit IgG antibodies were purchased from Sigma (St. Louis, MO). GTPγS was obtained from Boehringer Mannheim (Indianapolis, IN). Sodium dodecyl sulfate (SDS) was purchased from Bio-Rad (Richmond, CA). Restriction enzymes, T4 DNA ligase, and T4 DNA polymerase were purchased from BRL (Gaithersburg, MD) unless indicated otherwise. L-[^{35}S]Methionine (specific activity more than 1000 Ci/mmol) was purchased from NEN–Dupont (Wilmington, DE). Standard reagents such as salts and buffers were obtained from Sigma or Fisher. Neutrophil cytosol lacking p47-phox protein was the kind gift of Dr. John Curnutte.

Peptide Synthesis. Synthetic peptides were made by solid-phase *t*-Boc peptide synthesis at the Emory University

Microchemical Facility. Peptides corresponding to amino acids 457–463 (RGVHFIF) of the heavy subunit of cytochrome b_{558} and the carboxy-terminal 13 residues 378–390 (CSESTKRKLASAV) of p47-phox were produced. The peptides were purified and desalted by HPLC following HF cleavage.

Antigen and Antibody Production. The C-terminal 13-mer from p47-phox was coupled to keyhole limpet hemocyanin, through its N-terminal cysteine, by MBS according to Lerner et al. (1981), except that the reaction was performed in 100 mM borate buffer, pH 9.0, to augment the solubility of the hemocyanin. The coupled hemocyanin–peptide antigen was concentrated and desalted by centrifugation through Centricon-30 filtration units. Completeness of the coupling reaction was determined by thiol assays performed on the eluate with Ellman's reagent (Habeeb, 1972). Polyclonal antibodies were prepared by Pel-Freeze Biologicals (Rogers, AK). Adult female New Zealand white rabbits (2–5 kg) were immunized with hemocyanin–peptide according to standard protocols. Using a horseradish peroxidase enzyme-linked immunosorbant assay, the sera were titered against the peptide itself and against the BSA-conjugated peptide. The antisera recognized both the synthetic peptide, the native p47-phox protein in neutrophil cytosol, and recombinant p47-phox expressed in bacteria, as demonstrated by immunoblotting. A serum dilution of 1:5000 was used for immunoblotting methods, and the serum was used undiluted for immunoprecipitation studies.

Construction of the pCITE-p47 Plasmid. The cDNA clone 8a which encodes the full-length human p47-phox protein as well as an N-terminal 3-kDa segment of β-galactosidase (Lomax et al., 1989a,b) was a gift from Drs. Harry L. Malech and Thomas L. Leto (National Institute of Allergy and Infectious Disease). The pCITE-p47 plasmid was constructed by ligating the large *Nco*I–*Xba*I fragment from the pCITE-1 vector (Novagen, Madison, WI) with a segment of the pBluescript SK⁻ plasmid containing the 8a cDNA encoding the p47-phox protein (Lomax et al., 1989a,b), as illustrated in Figure 1. The region encompassing the open reading frame of the p47-phox protein (excluding the 5′ β-galactosidase sequence) and the 3′ end of the cDNA was recovered from the recombinant pBluescript plasmid using polymerase chain reaction (PCR) techniques. The N-terminus amplimer (GGAC↓CATGGGGGACACCTTCATCCGT) was designed to anneal to the p47-phox sequence and to introduce an *Nco*I site [boldface, with (↓) designating the cleavage site of *Nco*I which contains the ATG initiation codon (underlined)]. The C-terminus amplimer (GACT↓**CTAGA**CCCCCCTCGAGGTCGACGGTATC) was designed to anneal to the pBluescript SK⁻ vector sequence and to introduce an *Xba*I site [boldface, with (↓) designating the *Xba*I cleavage site]. The PCR reactions were carried out using reagents from an AmpliTaq kit (Perkin-Elmer Cetus, Norwalk, CT), Taq polymerase (Stratagene, La Jolla, CA), and a thermal cycler (Perkin-Elmer Cetus); the incubation temperatures and times were 94 °C for 1 min (denaturation), 65 °C for 1 min (annealing), and 72 °C for 2.5 min (extension), repeated for 30 cycles. The products from three separate 100-μL reactions were pooled and fractionated by agarose gel electrophoresis. The predominant 1.3-kb fragment was excised from the gel and electroeluted, and blunt ends were generated using T4 DNA polymerase and dNTP. Because *Xba*I fails to cleave restriction sites located at the ends of DNA (Jung et al., 1990), the blunt-ended fragments were self-ligated using ATP and T4 DNA ligase to generate concatamers, which were then cleaved using *Nco*I and *Xba*I. The resulting 1.3-kb fragments were gel-purified, ligated with the gel-purified

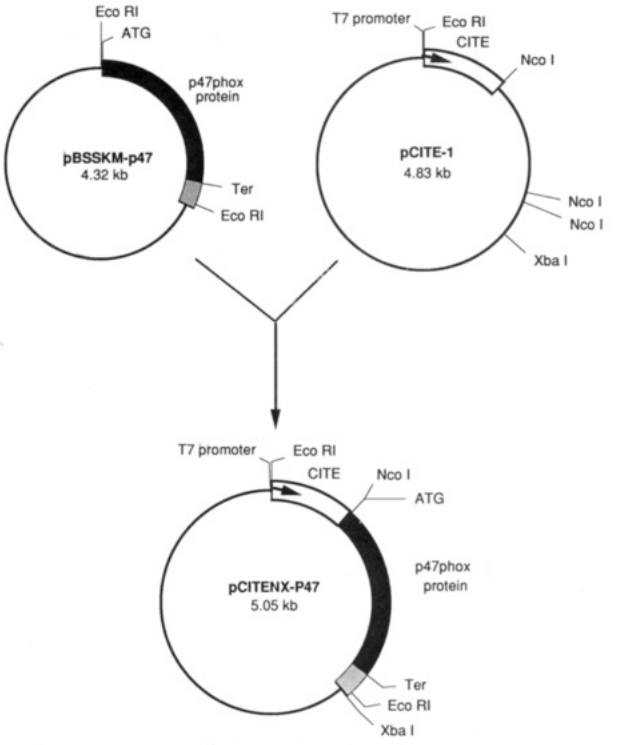

FIGURE 1: Schematic of the construction of the pCITE-p47-phox plasmid. As detailed under Experimental Procedures, the region coding for the translated sequence (black) and the 3′-untranslated region (stippled) of the p47-phox cDNA were selectively amplified while simultaneously introducing *Nco*I and *Xba*I sites by employing the polymerase chain reaction; the amplified cDNA was inserted into the pCITE vector between the *Nco*I and *Xba*I sites. The filled arrowhead refers to the T7 promoter.

*Nco*I–*Xba*I fragment of the pCITE vector, and cloned in *Escherichia coli* DH5α F′ cells (BRL). The recombinant pCITE-p47 clones were identified by colony hybridization and restriction analysis of DNA from mini-lysates (Grunstein & Hogness, 1975; Ausubel et al., 1989).

In Vitro Transcription and Translation. Recombinant pCITE-p47 plasmid DNA was isolated from bacteria grown in a 1-L culture. The plasmid DNA was purified by CsCl density gradient centrifugation, dialysis against Tris/EDTA buffer, phenol/chloroform extraction, and ethanol precipitation as described in Ausubel et al. (1989) and Sambrook et al. (1989). Residual RNA was removed by digestion with DNase-free RNase (Boehringer Mannheim). The pCITE-p47 plasmid DNA was linearized with *Xba*I, extracted with phenol/chloroform, precipitated with ethanol, and resuspended in RNase-free water. This DNA template was then transcribed in vitro using T7 RNA polymerase (Stratagene) according to the manufacturers' instructions (Novagen & Stratagene); the RNase inhibitor RNasin (Promega, Madison, WI) was included. Initially, 2 μg of linearized DNA template was employed in 40-μL transcription reactions, and diluted 5-fold to 200 μL with RNase-free water. Both DNA and RNA templates in these dilutions were analyzed by agarose gel electrophoresis. Subsequently, transcription reactions were scaled up accordingly; in some cases, RNase-free DNase was added after transcription. Diluted transcription reactions were either stored at −70 °C in 10-μL aliquots or used immediately to program in vitro translation reactions employing 10 μL of rabbit reticulocyte lysates (Novagen) and [^{35}S]methionine (15–20 μCi, NEN) per incubation following the protocol provided by Novagen. The radioactive proteins generated in the in vitro translation reaction were analyzed by SDS–PAGE (Laemmli, 1970) followed by autoradiography of the dried

gels. Unincorporated [^{35}S]methionine was removed from the translated protein by dialysis for 36 h against five changes of 500 mL of assay buffer (100 mM KCl, 3 mM NaCl, 4 mM $MgCl_2$, and 10 mM PIPES, pH 7.0) containing 0.5 mM phenylmethanesulfonyl fluoride (PMSF). Neutrophil cytosol (160 μL, 15 μg of protein/μL) was added to the translated protein (100 μL) to avoid its adsorptive loss during dialysis. The final concentration of protein and radioactivity were 10 mg/mL and 15.3 μCi/mL, respectively. For some experiments, excess [^{35}S]methionine was removed from the translated protein without the addition of carrier protein by FPLC gel filtration using a Superose 12 HR column (Pharmacia).

Immunoprecipitation. To test for the generation of the correct C-terminus, the [^{35}S]-labeled p47-phox was immunoprecipitated as described in Ausubel et al. (1989) with rabbit antibody specific for the 13 C-terminal residues. Briefly, approximately 60000 cpm of the radioactive protein were added to 500 μL of incubation buffer, and this was divided into two aliquots. After addition of 1 μL of either the undiluted peptide-specific antiserum or the preimmune serum, the samples were incubated with rotation on an orbital shaker at 4 °C overnight. Forty microliters of a 1:1 slurry of incubation buffer and protein A–Sepharose (Sigma) was added to each sample, and the shaking was continued for another 2 h. After resuspension in incubation buffer (500 μL) and centrifugation for 1 min at 200*g*, repeated twice, samples were centrifuged and resuspended in incubation buffer (50 μL). Aliquots (5 μL) were directly counted in a scintillation counter, and 15-μL aliquots were analyzed by SDS–PAGE followed by autoradiography.

Isolation of Human Neutrophils. Human neutrophils were isolated from peripheral blood from healthy adult donors as described previously (Pember et al., 1983a,b; Lambeth et al., 1988). Informed consent was obtained from all donors. Isolated cells were resuspended in PBS (2.6 mM KCl, 1.5 mM KH_2PO_4, 0.5 mM $MgCl_2$, 136 mM NaCl, 8 mM Na_2HPO_4, and 0.6 mM $CaCl_2$) containing 5.5 mM glucose and were used immediately.

Isolation of Plasma Membranes and Cytosol from Human Neutrophils. Neutrophils [(1–1.5) × 10^9 cells] were suspended in 10 mL of PBS/glucose and incubated on ice with 4 mM DFP for 25 min. The cells were pelleted at 600*g* for 7 min in incubation buffer containing 1 mM EGTA, 2 μM leupeptin, 2 μM pepstatin, and 0.5 mM PMSF. The cells (1 × 10^8/mL) were disrupted by nitrogen cavitation after being pressurized at 450 psi for 25 min at 4 °C. Briefly, the cavitate was centrifuged at 600*g* for 7 min, and the supernatant was layered onto a sucrose step gradient and centrifuged as in Burnham et al. (1990). The plasma membrane was recovered from the interface between the sucrose layers, pelleted by centrifugation, and resuspended in half-strength assay buffer containing 0.34 M sucrose and 1 mM EGTA. The cytosol fraction, recovered from the top of the gradient, was centrifuged at 230000*g* for 1 h at 4 °C to remove any residual membrane or particulate material. The resulting cytosol was concentrated to 15–25 mg/mL using a Centricon (Amicon) filtration device. Plasma membrane and cytosol fractions were recovered and stored in aliquots of 50–100 μL at −80 °C until used (Burnham et al., 1990). In some cases, heat-inactivated cytosols was prepared by incubating at 46 °C for 7.5 min (Curnutte et al., 1990). Red blood cell plasma membranes were prepared by hypotonic lysis (Hanahan & Ekholm, 1974).

Translocation/Cosedimentation Experiments. A typical 50-μL translocation reaction included incubation buffer, 100 μg of cytosol, 20 μg of plasma membrane, and 10000 cpm of the [^{35}S]methionine-labeled p47-phox protein. These reagents were combined at 4 °C in Beckman microcentrifuge tubes before addition of various agonists, alone or in combination, to the final concentrations indicated: SDS (250 μM), diC8 (200 μM), or GTPγS (10 μM). Unless otherwise indicated, the reaction mixture was incubated at 25 °C for 5 min and centrifuged immediately at 4 °C at 80000 rpm (279000*g*) for 5 min using a Beckman TL100 ultracentrifuge. The supernatant (50 μL) was removed, and the pellet was resuspended in 50 μL of incubation buffer by brief sonication using a water bath sonicator. Radioactivity in both fractions was assayed by liquid scintillation counting.

When translocation was to be monitored by SDS–PAGE and autoradiography, 6 μL (120000 cpm) of the [^{35}S]-labeled protein was used in the 50-μL reaction. Aliquots (5 μL) were used for counting, and the remainder (45 μL) was processed for SDS–PAGE by addition of 22.5 μL of 3 × SDS sample buffer (Studier et al., 1990) followed by 3-min boiling; 15 μL of this was loaded on a lane of a 12.5% SDS–polyacrylamide gel.

Polyacrylamide Gel Electrophoresis. Samples were subjected to electrophoresis on minigels (8 × 10 cm × 1 mm) according to Laemmli (1970). After the gel was stained with Coomassie blue, the gels were destained, dried, and subjected to autoradiography on Kodak X-Omat AR film overnight at room temperature with an intensifying screen.

Superoxide Generation and Incubation Conditions. NADPH-dependent superoxide generation was assayed by monitoring the superoxide dismutase inhibitable reduction of cytochrome *c* as described previously (Burnham et al., 1990), using a Thermomax Kinetic Microplate reader (Molecular Devices, Menlo Park, California, CA). Briefly, reaction mixtures contained 20 μg of plasma membrane proteins, 100 μg of cytosolic proteins, and varying amounts of the agonists (GTPγS, SDS, and diC8) in a total volume of 50 μL adjusted with incubation buffer. Four 10-μL aliquots of each reaction mixture were transferred to 96-well flat-bottom assay plates (Corning, New York, NY) and preincubated for 5 min at 25 °C. At the end of the preincubation period, 240 μL of a reaction mixture containing incubation buffer, 200 μM NADPH, and 80 μM cytochrome *c* was added to initiate superoxide generation. An extinction coefficient at 550 nm of 21 mM^{-1} cm^{-1} was used to calculate the quantity of cytochrome *c* reduced (Van Gelder & Slater, 1962). For each preparation of cytosol and membranes, the optimal concentration of SDS for activation was determined prior to carrying out experiments, and this concentration was used for each experiment.

Results

Generation of Recombinant p47-phox Protein. The pCITE-1 vector system was used to generate a radioactive recombinant p47-phox protein. The pCITE-1 plasmid contains a *C*ap-*I*ndependent *T*ranslation *E*nhancer between the T7 RNA polymerase promoter and the *Nco*I cloning site which also encodes the ATG initiation codon (see Figure 1). In an in vitro translation system, the CITE sequence facilitates the binding of ribosomes to RNA and thereby enhances the translation process (Parks et al., 1986; Elroy-Stein et al., 1989). The radioactive p47-phox protein obtained by in vitro transcription and translation shows the correct migration on SDS–PAGE as indicated in Figure 2. Because the pCITE-1 vector encodes an additional ATG codon 5′ to the *Nco*I site, it is likely that this construct gives rise to a fusion protein which contains four additional N-terminal amino acids (Met-Ala-Thr-Thr). The much larger N-terminal fusion protein (+3

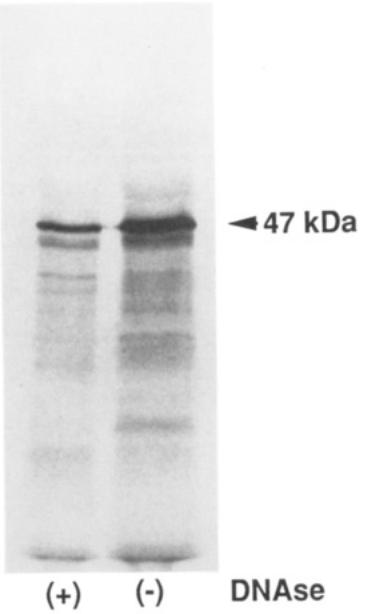

FIGURE 2: Autoradiograph of the [^{35}S]-labeled recombinant p47-phox protein resolved by SDS–PAGE. The radioactive protein was generated in vitro in a rabbit reticulocyte lysate to which RNA obtained by in vitro transcription of the linearized pCITE-p47 plasmid had been added, as described under Experimental Procedures. In a parallel incubation (+), the template DNA was removed from the transcribed RNA by adding RNase-free DNase prior to carrying out the translation reaction.

kDa) expressed by the pBluescript p47 clone in *E. coli* is active in reconstituting activity (Lomax et al., 1989a; Volpp et al., 1989), suggesting that this much smaller modification is unlikely to impair function. To test directly for biological activity, we investigated whether the pCITE recombinant material could restore function to CGD cytosol lacking p47-phox. In the absence of added recombinant p47-phox (in the presence of diC8, SDS, and GTPγS, as described under Experimental Procedures), the rate was 50 ± 3 nmol of cytochrome *c* reduced min^{-1} (mg of membrane protein)$^{-1}$ (n = 8). Although the actual mass of protein translated in the reticulocyte lysate was vanishingly small, added pCITE-translated material increased the rate to 60.5 and 68.5 nmol/min when 10 and 20 μL of the material was added to the assay system, respectively (n = 8 each). As a positive control, excess recombinant p47-phox expressed using a baculovirus expression system (Uhlinger, unpublished results) was added to the assay and resulted in a rate of 594 ± 10 nmol/min (n = 8). Thus, although low amounts of material were present, the pCITE expression system resulted in the expression of active protein.

Increasing the translation time to 2 h (data not shown) or treating the in vitro transcription product with DNase (Figure 2) did not further increase the yield or purity of the translated product. The translated product could be specifically immunoprecipitated with an antiserum raised against the 13 C-terminal amino acids predicted by the cDNA sequence, indicating that the full-length protein was produced (data not shown).

Translocation of p47-phox. In the cell-free activation system comprised of cytosol and plasma membrane, the combination of an anionic amphiphile (in this case SDS), diacylglycerol, and GTPγS produces the highest rate of superoxide generation (Burnham et al., 1990). This combination was therefore chosen to investigate the ability of activating stimuli to promote association with the plasma membrane. Radioactive recombinant p47-phox was initially mixed with cytosol, which was then combined with plasma membrane.

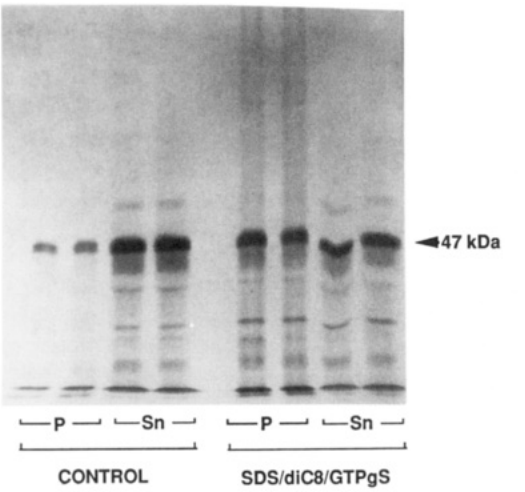

FIGURE 3: Autoradiograph of an SDS–polyacrylamide gel showing the effects of various agonists on the translocation of the radiolabeled p47-phox. Treatment conditions are as described under Experimental Procedures. Abbreviations: P, pellet; Sn, supernatant (cytosol). Samples from duplicate translocation experiments are shown.

The membrane pellet (P) was then reisolated by centrifugation, and both the pellet and the supernatant (Sn) were analyzed by SDS–PAGE followed by autoradiography. As shown in Figure 3, in the absence of agonists, most of the p47-phox remained soluble. However, with the combined agonists, more than half of the recombinant protein sedimented with the membrane fraction. Significant proteolysis was not detected during the course of the incubation as judged by the failure to observe new smaller molecular weight bands, either without or with agonists (compare Figures 2 and 3). Minor smaller molecular weight proteins which were present in the initial preparation also appeared to associate with the plasma membrane. Since these were not produced in a control experiment in which no RNA was added to the in vitro translation reaction, their presence seems specific for the p47-phox RNA. It therefore seems likely that these represent smaller molecular weight variants of the p47-phox protein, which could be due to aberrant initiation at internal AUG codons and/or proteolysis. In a control experiment, an unrelated protein, the human mitochondrial adenine nucleotide translocator (Neckelmann et al., 1987), was also transcribed and translated in vitro; using the same translocation and analytical protocol described above, about 50% of this 31-kDa protein occurred in a soluble form in the absence of agonists, and its association with the plasma membrane was not affected by the agonists (data not shown), thus suggesting some degree of specificity for the p47-phox protein.

Effect of Single and Combined Agonists on Superoxide Generation and Translocation of p47-phox to the Plasma Membrane. Because the p47-phox protein represents more than 90% of the radioactivity in this preparation, and since proteolysis was not a problem during the incubation, the translated protein was used without purification. ^{35}S counts in the fractions following centrifugation were thus used as a measure of the relative distribution of p47-phox. As shown in Table I (data column 3), SDS but not GTPγS or diC8 alone stimulated the production of superoxide in the cell-free system. However, the combination of either diC8 or GTPγS with SDS resulted in about a 2-fold augmentation of activity over that seen with SDS alone, and the combination of all three agonists resulted in more than 3-fold higher rates. Translocation of p47-phox was monitored under identical conditions to test whether cosedimentation of p47-phox with the plasma membrane correlated with activation (Table I, data column 1).

Table I: Effect of Various Agonists on Translocation of the Radiolabeled p47-phox and on Superoxide Generation in the Cell-Free System[a]

group	translocation (% of total counts in pellet)		superoxide generation [nmol of cyt *c* reduced min^{-1} (mg of protein)$^{-1}$]
	PM + cytosol	cytosol only	
control	19.2 ± 0.7 (0)	12.7 ± 2.5 (0)	48 ± 2
SDS	48.1 ± 0.5 (28.9)	29.0 ± 1.2 (16.3)	348 ± 8
diC8	19.3 ± 1.0 (0.1)	11.4 ± 0.4 (−1.3)	44 ± 2
GTPγS	20.8 ± 2.3 (1.6)	10.9 ± 0.5 (−1.8)	67 ± 4
SDS/diC8	67.4 ± 0.5 (48.2)	55.1 ± 1.0 (42.4)	736 ± 20
diC8/GTPγS	23.3 ± 0.5 (4.1)	10.3 ± 0.5 (−2.4)	75 ± 2
SDS/GTPγS	54.3 ± 0.2 (35.1)	38.5 ± 0.0 (25.8)	630 ± 18
SDS/diC8/GTPγS	69.6 ± 0.4 (50.4)	55.5 ± 1.5 (42.8)	1180 ± 42

[a] Treatment conditions were as described under Experimental Procedures. These results represent the mean ± standard error of three determinations from a single preparation, and the experiment was repeated 2 more times using plasma membranes (PM) and cytosol preparations from separate donors. The numbers in parentheses represent the percent translocation with the control (no agonist) value subtracted out.

Table II: Translocation of the Radiolabeled p47-phox in the Cell-Free System Using BSA or Heat-Inactivated Cytosol in Place in Native Cytosol[a]

group	% of total counts in pellet		
	cytosol	BSA	heated cytosol
control	16.2 ± 1.3	20.7 ± 0.5	20.8 ± 2.5
SDS		21.8 ± 0.6	31.4 ± 3.6
diC8		20.2 ± 1.2	24.8 ± 4.1
GTPγS		20.3 ± 0.7	20.4 ± 2.5
SDS/diC8		24.1 ± 2.0	40.7 ± 0.9
diC8/GTPγS		17.1 ± 0.3	20.2 ± 0.5
SDS/GTPγS		20.0 ± 0.5	31.0 ± 0.3
SDS/diC8/GTPγS	47.7 ± 1.1	24.4 ± 0.2	41.8 ± 2.1

[a] Treatment conditions were as in Table I, all in the presence of plasma membrane. These results represent the mean ± error of three determinations from two experiments. Heat-inactivated cytosol was prepared as described under Experimental Procedures.

Although a small amount of apparent translocation was seen in the absence of agonists, enhanced translocation was seen under all conditions in which activation was observed. As reported previously using arachidonate as the agonist (Clark et al., 1990; Ohtsuka et al., 1990), anionic amphiphile increased the apparent membrane association of p47-phox. Increased association was absolutely dependent on the presence of the anionic amphiphile, and was enhanced further by the presence of either diacylglycerol or GTPγS. Neither of the latter agents alone affected association. Interestingly, the small degree of enhanced translocation in the presence of all three agonists (compared with SDS plus either of the other agonists) was not strictly in proportion to the larger degree of activation. For example, GTPγS increased translocation only by 3% in the presence of the other two activators, while activity was enhanced by 60%. Thus, while translocation or cosedimentation seems to be necessary for activity, it does not appear to be the sole determinant of activity.

To test whether the appearance of label in the pellet required the presence of membrane, the same incubation and centrifugation procedure was carried out, except that plasma membranes were omitted. Results are shown in data column 2 of Table I. Surprisingly, although the magnitude was less than when membranes were present, appreciable pelleting of the p47-phox occurred in the absence of membranes, in particular in the presence of multiple activating factors. The results were qualitatively similar to those seen when membrane was present. Enhanced precipitation was seen only when SDS was present, and GTPγS and diC8 both increased the effect, with diC8 being the more potent of the two. If one subtracts control values for translocation from each group (see numbers in parentheses, Table I), it can be seen that precipitation in the absence of membranes accounted for all but around 10% (on average) of the apparent translocation, depending on activation conditions. Nevertheless, in all cases, translocation was enhanced in the presence of membranes, and these membrane-dependent differences were statistically significant with p values less than 0.05. Thus, while protein precipitation occurs in the absence of membranes, increased sedimentation in the presence of membranes indicates activator-enhanced association of p47-phox with the plasma membranes.

We also wished to determine whether the translocation/precipitation was dependent on the presence of added cytosol or required specific cytosolic components. In Table II, data column 2, the translocation experiment was carried out as above, using the same treatment conditions except that bovine serum albumin (BSA) was included in place of added cytosol, using the same protein concentration. The basal translocation was comparable to that seen in the presence of cytosol, but activating factors failed to produce a quantitatively or statistically significant degree of translocation/cosedimentation. The same experiment was also carried out in the absence of plasma membranes (data not shown), with quantitatively and qualitatively similar results. We also carried out the same experiment as in data column 2, using heat-treated cytosol to test whether the translocation was dependent on any heat-labile factors. Heat treatment inactivates cytosol, and this is partially restored using recombinant p67-phox, indicating that this component becomes heat-inactivated (D. Uhlinger, unpublished data). Maximal translocation using this preparation of cytosol and recombinant p47-phox was less than that seen in Table I (48% maximal using all three agonists when native cytosol was used). Using heat-inactivated cytosol, the maximal translocation was 42%, and results with specific activators alone or in combination were qualitatively similar to those using native cytosol (compare Tables I and II). Thus, the translocation/cosedimentation appears to require one or more heat-stable cytosolic components.

Effect of a Cytochrome b_{558} C-Terminal Peptide on Activation and Translocation. The peptide RGVHFIF, corresponding to residues 457–463 near the C-terminus of the heavy subunit of cytochrome b_{558}, inhibits arachidonate–activated superoxide generation in the cell-free activation system when added prior to the activator (Rotrosen et al., 1990). Studies using CGD cytosols which lacked either p47-phox or p67-phox suggested that the peptide inhibited the formation of an activation intermediate involving p47-phox (Kleinberg et al., 1990). To test whether its effect was to block the translocation of p47-phox, we investigated the effect of the peptide on activity and translocation. As reported, RGVHFIF inhibited oxidase activation by the anionic amphiphile (Table III), although the required inhibitory concentration was 2-fold higher than that reported previously. The peptide was also inhibitory in the presence of the other activating factors diacylglycerol and/or GTPγS, although the percent inhibition was reduced somewhat when all three activators were combined. In contrast, there was no statistically significant effect on RGVHFIF on the translocation using any of the activators. Thus, the inhibitory effect of RGVHFIF appears to involve a mechanism other than inhibition of aggregation/translocation.

Translocation of p47-phox to PMN Plasma Membranes Lacking Cytochrome b_{558} and Red Blood Cell Membranes. To determine whether cytochrome b_{558} is necessary for a portion of the apparent translocation of p47-phox in the cell-free system, we used CGD plasma membranes known to

Table III: Effect of Various Agonists in the Presence and Absence of the Cytochrome b_{558} Peptide (RGVHFIF) on the Translocation of Radiolabeled p47-phox and on Superoxide Generation in the Cell-Free System[a]

group	translocation (% of total counts in pellet)		superoxide generation [nmol of cyt c reduced min^{-1} (mg of protein)$^{-1}$]	
	-RGVHFIF	+RGVHFIF	-RGVHFIF	+RGVHFIF
control	17 ± 1	20 ± 1	23 ± 2	23 ± 4
SDS	35 ± 1	39 ± 1	253 ± 2	83 ± 4 (67%)
SDS/diC8	54 ± 2	51 ± 1	631 ± 2	106 ± 16 (83%)
SDS/GTPγS	62 ± 1	59 ± 1	662 ± 34	212 ± 16 (68%)
SDS/diC8/GTPγS	62 ± 1	57 ± 3	1554 ± 90	772 ± 42 (50%)

[a] Treatment conditions were as described under Experimental Procedures, and experiments were carried out in the absence or presence of 200 μM RGVHFIF peptide. Translocation and superoxide generation assays were performed in triplicate. These results represent the mean ± standard error of three determinations from a single preparation, and the experiment was repeated 2 more times with similar results using cytosol and membrane preparations from different donors.

Table IV: Translocation of Radiolabeled p47-phox in CGD and Normal Neutrophil and Normal Red Blood Cell Plasma Membranes[a]

plasma membrane source	translocation (% of total counts in pellet)	
	no agonist	SDS/diC8/GTPγS
CGD-A (~22 kDa)*	17.8 ± 0.4	70.6 ± 1.6
CGD-X (~91 kDa)*	17.2 ± 0.2	73.4 ± 0.1
normal*	17.8 ± 0.4	75.3 ± 0.5
normal*	18.8 ± 1.3	74.2 ± 1.6
normal	15.7 ± 0.7	60.4 ± 3.6
normal	16.8 ± 1.1	63.9 ± 1.1
red blood cell	15.6 ± 0.4	61.4 ± 3.1

[a] Translocation conditions were as described under Experimental Procedures except using plasma membranes from various sources as indicated. These data represent the mean ± standard error of these determinations from a single preparation, and the experiments were repeated 2 more times using the same cytosol and plasma membranes. To control for any laboratory-to-laboratory variations, normal plasma membranes prepared in the same laboratory as the CGD plasma membranes were used as indicated by asterisks.

be deficient in the 22-kDa (CGD-A) or 91-kDa (CGD-X) subunits of cytochrome b_{558} in the translocation assay in place of native membranes. In addition, red blood cell membranes were used to test whether cosedimentation was affected by the source of the plasma membranes. The results are shown in Table IV. In the absence of the agonists, the percent translocation was less than 20%; this was observed for CGD neutrophil plasma membranes, normal neutrophil plasma membranes, and red blood cell membranes. Addition of all three agonists, however, resulted in a marked increase in the percent translocation (60–75%) in all cases. When control neutrophil membranes prepared in the same lab were used to correct for any laboratory-specific preparation differences, little or no difference between normals and CGD membranes was observed. Thus, the cosedimentation in the cell-free system does not appear to be influenced appreciably by the presence of either subunit of the cytochrome, and is significant even using red cell membranes.

Effect of Salt Concentration on Activity and Apparent Translocation of Recombinant p47-phox. To investigate whether the associations of p47-phox in the cell-free system involve ionic or hydrophobic interactions, the activity and sedimentation experiments were carried out in the presence of varying concentrations of KCl. The results of these experiments are shown in Table V. With the combination of all three agonists, the maximum degree of both translocation of p47-phox and superoxide generation was observed at KCl

Table V: Effect of KCl Concentration on Translocation of Radiolabeled p47-phox and on Superoxide Generation in the Cell-Free System[a]

group [KCl] (mM)	translocation (% of total counts in pellet)		superoxide generation [nmol of cyt c reduced min^{-1} (mg of protein)$^{-1}$]	
	no agonist	SDS/diC8/GTPγS	no agonist	SDS/diC8/GTPγS
25		67.7 ± 0.6		1408 ± 64
100	18.5 ± 2.6	68.1 ± 2.2	81 ± 9	1758 ± 12
250		36.0 ± 1.3		518 ± 4
500	16.1 ± 0.5	26.5 ± 3.2	77 ± 6	114 ± 14

[a] The indicated concentrations of KCl (25–500 mM) were included in the incubation. Other treatment conditions are as described under Experimental Procedures. Translocation and superoxide generation results represent the mean ± standard error of three and four determination from a single preparation, respectively. The experiment shown is representative of two, using plasma membrane and cytosol from different donors.

concentrations of 100 mM and lower. At higher KCl concentrations, both the activity and the degree of translocation were inhibited significantly, so that at 500 mM little translocation or activation was observed. These results indicate that higher ionic concentrations can dissociate the components of respiratory burst oxidase, thereby affecting both the translocation and the activity, and suggest that interactions resulting in translocation are ionic rather than hydrophobic in nature.

Discussion

The present studies have described the development and use of a novel method for investigating the apparent translocation of soluble NADPH oxidase related proteins to sedimentable fractions. By using an in vitro translation system, biologically active radioactive protein is generated in nearly homogeneous form which can be used without purification; it can be readily quantified in pellet and soluble fractions following centrifugation. The method should be generally applicable to other translocated proteins such as protein kinase C, and requires the availability of the full-length cDNA in an appropriate vector. It has advantages in situations where the purified protein is not yet available in quantity (as in the present case), or where a specific antibody is unavailable. The method is rapid, thus allowing the processing of larger numbers of samples (including replicates) than is practical using immunoblotting or other methods, and the interpretation of the data is straightforward. In contrast, interpretation of immunoblots is frequently subjective, and may be influenced by post-translational modifications (e.g., phosphorylation) in the antibody binding region. The use of protein kinase C and [γ-^{32}P]ATP to phosphorylate and thereby quantify p47-phox levels is more quantitative, but is subject to certain assumptions (e.g. potential phosphorylation sites are not already occupied or their accessibility influenced by other changes in the protein). In addition, this method is rather specific in its applicability, since many translocated proteins are not substrates for protein kinase C. The method reported herein is also subject to certain limitations and assumptions. For example, the percent translocation rather than a measure of mass is reported. In addition, it is assumed that the translated product is posttranslationally modified in a near-normal manner so that the added radioactive protein will behave in the same way as the bulk of unlabeled cytosolic protein. This might not occur, for example, if the native protein is present in a slowly dissociating complex with other components.

In vitro translated radiolabeled p47-phox has been used in the present studies to investigate the mechanism by which

activators and inhibitors of the respiratory burst oxidase may affect function. The cell-free activation system, which utilizes plasma membrane plus cytosol along with an anionic amphiphile such as SDS or arachidonate, has become a fundamental tool to investigate the components and activation of the superoxide-generating respiratory burst oxidase. It is therefore of central importance to understand the mechanism of activation in this system and to compare activation features with those in the intact cell system.

The present studies are consistent with the hypothesis that in the cell-free system, changes in protein–protein and/or protein–membrane associations of cytosolic components such as p47-phox are a prerequisite for activation. Translocation of p47-phox in response to an anionic amphiphile was reported previously, using immunochemical and phosphorylation methods (Clark et al., 1990; Ohtsuka et al., 1990; Nauseef et al., 1990). In the present studies, various factors which perturb the activation state [inhibitors such as high salt and RGVHFIF (Kleinberg et al., 1990) and synergistic activators such as GTPγS and diacylglycerol (Burnham et al., 1990)] have been used to test the extent to which activation parallels membrane association/aggregation of p47-phox. Although activation did not strictly parallel translocation in all cases, significant activation was never seen in the absence of quantitatively significant translocation. For example, high salt, which prevents associations, also inhibits superoxide generation. Conversely, diacylglycerol and GTPγS, both of which enhance translocation, also enhance activity. Nevertheless, activators sometimes exerted their stimulatory effects in excess of their effect on translocation. For example (see Table I), addition of GTPγS to the system with SDS plus diacylglycerol did not enhance translocation significantly beyond that seen with SDS plus diacylglycerol alone. Yet, GTPγS stimulated activity in this setting by 60%. Thus, GTPγS may have additional activator functions subsequent to membrane association or aggregation of components. In contrast, the effects of both SDS and diC8 on activity paralleled closely their effects on translocation. The effects of the three activators on translocation do not seem to be unique to p47-phox. In preliminary studies using immunochemical blotting methods (Uhlinger, unpublished studies), we find that the activating factors have qualitatively similar effects on the translocation/aggregation of p67-phox as is seen in the present studies with recombinant p47-phox. Thus, these activating factors exert their effects on at least two components of the respiratory burst oxidase, and possibly other, non-oxidase-related cytosolic components.

Experiments to ascertain specificity have revealed additional features of the activation mechanism. First, at least a portion of the apparent translocation is actually due to aggregation or association with precipitable components of p47-phox and probably other proteins in the neutrophil cytosol. In the absence of any membrane, there is an appreciable precipitation of p47-phox induced by SDS. This is similar to results reported previously (Chiba et al., 1990), who noted substantial precipitation of immunochemically detectable p47-phox and p67-phox in response to anionic amphiphiles in the absence of membranes. Diacylglycerol and GTPγS both augmented the membrane-independent precipitation, in parallel with their enhancement of SDS-activated superoxide generation. These results are consistent with a major effect of anionic amphiphile as well as the synergistic activating factors diC8 and GTPγS at the level of the cytosol rather than the plasma membrane. Using an activation protocol in which the free small molecular weight activators SDS and GTPγS were separated from protein, Gabig and co-workers (Gabig et al., 1990) also concluded that the site of action of both SDS and GTPγS was initially the cytosol. Also consistent with this interpretation, we find that the translocation/aggregation of p47-phox requires other (heat-stable) cytosolic component(s), since significant translocation of recombinant p47-phox fails to occur when BSA replaces added cytosol. When the membrane is present, a somewhat larger percent translocation is seen, suggesting that activators cause p47-phox to associate either additionally or preferentially with the membrane fraction.

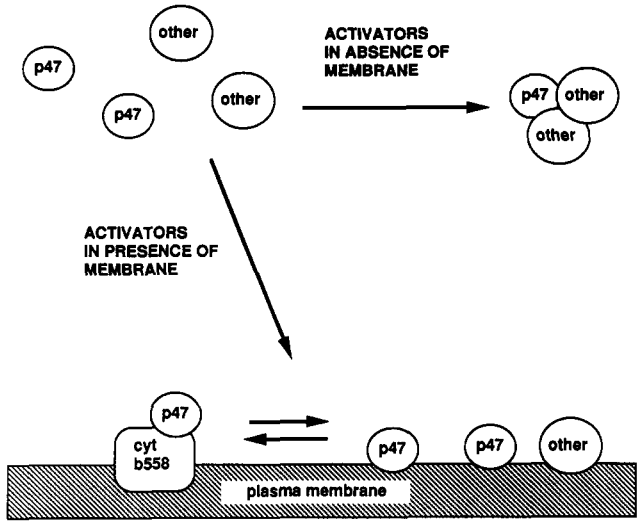

FIGURE 4: Scheme for cell-free activation and translocation of p47-phox. See text for details. It should be noted that the pellet in the presence of membranes may contain both membrane-associated material and precipitated proteins.

A second finding which derives from the specificity studies is that the enhanced sedimentation which occurs in the presence of membrane does not depend on the source of the membrane or on whether cytochrome b_{558} is present (see Table IV). This differs from the case of translocation of p47-phox and p67-phox in intact cells. Using CGD mutants lacking either the large or the small subunit of cytochrome b_{558}, Curnutte and colleagues (Heyworth et al., 1991) demonstrated that translocation of these components failed to occur except when the intact cytochrome was present, and inferred that cytochrome b_{558}, or a closely related component, forms a complex with p47-phox, which then allows p67-phox as well as other components to interact to form the active complex. Differences between the specificity of translocation in the cell-free and the intact cell systems imply that a qualitatively different mechanism of activation is occurring in the intact cell. For example, it is well-known that phosphorylation of p47-phox occurs in the intact cell (Hayakawa et al., 1986; White & Estensen, 1974; Caldwell et al., 1988; Heyworth et al., 1989; Babior, 1988) and this has been proposed to regulate activation, although in at least one case, activation is seen without phosphorylation (Badwey et al., 1989). In addition, significant phosphorylation of p47-phox can be seen in the cell-free system without detectable activation (Caldwell et al., 1988; Miyahara et al., 1988; Uhlinger et al., 1991). Thus, the physiological mechanism of activation remains unclear, but differs in some features from that in the cell-free system. In this context, it seems unlikely that arachidonate generated intracellularly in response to agonists is functioning as an activator in the same manner as it functions in the cell-free system.

A model for cell-free activation which is consistent with the data from the present studies is shown in Figure 4. We suggest that SDS binding initiates a conformational or other

change in p47-phox as well as other cytosolic neutrophil proteins, or opens up binding sites on other proteins with which p47-phox might interact. (In this context, it is interesting that the sequences of both p47-phox and p67-phox have putative cytoskeletal interaction domains.) A variant of this model is that p47 is initially complexed with other cytosolic components and the SDS alters the protein–protein interactions in such a manner as to promote association either with self, other protein components, or with the membrane when the latter is present. In any case, SDS initiates this process, and GTPγS and diacylglycerol both synergize with the SDS to enhance conformational or other changes. Although the specific targets of the three activating factors are not clear, the fact that the solubility changes occur in the absence of membrane suggests that their targets are cytosolic proteins or complexes. We suggest that when membrane is present, the components interact in a nonspecific manner with phospholipid and/or protein components in the membrane. In effect, activating factors are increasing the local concentrations of oxidase components at the plasma membrane. Specific interactions with cytochrome b_{558} are likely to represent only a small percentage of the membrane interactions so that the presence of the cytochrome does not significantly influence the overall membrane association. The model predicts that while translocation is necessary for activation, it need not be sufficient. For example, GTPγS may enhance and RGVHFIF may inhibit the specific interactions with the cytochrome without influencing the extent of translocation in a quantitatively significant manner. Thus, additional methods will be required to ascertain formation of specific complexes among oxidase components.

ACKNOWLEDGMENTS

We are grateful to Drs. Harry Malech and Thomas Leto for providing us with the pBluescript plasmid containing the p47 cDNA insert, to Dr. John Curnutte for insightful suggestions and for providing CGD and control plasma membranes, and to Linda Jones and Dr. Jan Pohl (Microchemical facility at Emory University) for providing synthetic oligonucleotides and peptides.

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Transcriptional Stimulation by Thyroid Hormone of a Cytosolic Thyroid Hormone Binding Protein Which Is Homologous to a Subunit of Pyruvate Kinase M_1

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Received August 29, 1991; Revised Manuscript Received December 19, 1991

ABSTRACT: We have recently shown that the monomer of rat pituitary pyruvate kinase subtype M_1 (p58-M_1) is a cytosolic binding protein for 3,3',5-triiodo-L-thyronine (T_3). To understand the role p58-M_1 plays in thyroid hormone action, we examined the regulation of p58-M_1 by T_3 in GH_3 cells. Expression of p58-M_1 was evaluated by metabolically labeling GH_3 cells cultured in regular medium, thyroid hormone depleted medium (T_d medium), or T_d medium supplemented with T_3 (T_d + T_3 medium) followed by immunoprecipitation. T_3 stimulates the expression of p58-M_1 by 2-fold. Analysis by pulse–chase experiments indicates that the increased expression is not due to the increase of stability of p58-M_1. Northern analysis of mRNA prepared from cells cultured in regular, T_d, or T_d + T_3 medium demonstrates that T_3 increases the accumulation of cytoplasmic mRNA by 2-fold. Nuclei from cells cultured in the three conditions were prepared, and the rates of synthesis of nascent nuclear RNA were compared by an in vitro transcription assay. Addition of T_3 stimulates the rate of transcription by 2-fold. The parallel and identical magnitude in the increase of transcription rate and the accumulation of mRNA indicates that T_3 stimulates the synthesis of p58-M_1 by increasing the transcriptional activity of its gene.

A cytosolic binding protein for 3,3',5-triiodo-L-thyronine (T_3)[1] was described as early as 1958 by Tata (Tata, 1958). In the following 3 decades, cytosolic T_3 binding protein was reported to be present in various tissues of many species and in many cultured cell lines including rat pituitary GH_1 cells and human epidermoid carcinoma A431 cells (Cheng, 1991). Using A431 cells, this cytosolic T_3 binding protein (p58-M_2) was isolated and purified (Kitagawa et al., 1987a,b). Antibodies against p58-M_2 were prepared and used to isolate the cDNA encoding p58-M_2. Analysis of nucleotide sequence indicated that p58-M_2 is a subunit of pyruvate kinase subtype

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[1] Abbreviations: T_3, 3,3',5-triiodo-L-thyronine; p58-M_1, cytosolic thyroid hormone binding protein; PKM_1, pyruvate kinase M_1; GH, growth hormone; PBS, phosphate-buffered saline; CHAPS, 3-[3-(cholamidopropyl)dimethylammonio]-1-propanesulfonate; SDS, sodium dodecyl sulfate; SDS–PAGE, SDS–polyacrylamide gel electrophoresis.