Isoprenoid Metabolism Is Required for Stimulation of the Respiratory Burst Oxidase of HL-60 Cells

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Abstract

The formation of oxygen radicals by phagocytic cells occurs through the activation of a multiple-component NADPH oxidase system. An unidentified low molecular weight GTP-binding protein has been proposed to modulate the activity of the NADPH oxidase. The low molecular weight GTP-binding proteins undergo posttranslational processing, including an initial covalent incorporation of an isoprenyl group. To test whether such an isoprenylation reaction might be required for the activity of the oxidase, we utilized compactin and lovastatin as inhibitors of the isoprenylation pathway. Treatment of DMSO-differentiated HL-60 cells with compactin produced a concentration-dependent inhibition of O_2^- formation in response to FMLP or phorbol myristate acetate. Cell viability was not affected nor was normal differentiation of the HL-60 cells into a neutrophil-like cell. The inhibitory effect of compactin was specifically prevented by addition of exogenous mevalonic acid to the HL-60 cells, indicating that the inhibitory effects of the drug were due to blockade of the pathway leading to isoprenoid synthesis. Addition of cholesterol, ubiquinone, or dolichol, which are also downstream products of the isoprenoid pathway, did not override the inhibitory effects of the drug. Subcellular fractions were prepared from compactin-treated cells, and the location of the compactin-sensitive factor was determined by complementation analysis in a cell-free NADPH oxidase system. The inhibited factor was localized to the HL-60 cytosol. These data suggest that an isoprenoid pathway intermediate is necessary for activation of the phagocyte NADPH oxidase. This is likely to represent the requirement for an isoprenoid moiety in the posttranslational modification of a low molecular weight GTP-binding protein. Our studies provide support for the involvement of such a low molecular weight GTP-binding protein in NADPH oxidase activation. (*J. Clin. Invest.* 1992. 89:402–408.) Key words: NADPH oxidase • prenylation • Ras-related GTP-binding proteins • neutrophils • inflammation

Introduction

Neutrophils serve as the body's primary cellular defense against bacterial infection. One of the mechanisms by which neutrophils destroy invading microorganisms is through the genera-

tion of various toxic oxygen metabolites via the so-called "respiratory burst" (1). The initial product of the respiratory burst is superoxide anion, generated by an NADPH oxidase found in neutrophils and other phagocytes (1, 2). This "enzyme" is actually a miniature electron transport chain consisting of multiple plasma membrane- and cytosol-localized protein components. The importance of the NADPH oxidase for the neutrophil's antibacterial capacity is evidenced by patients with the inherited disorder, chronic granulomatous disease. The neutrophils of patients with this disorder are unable to generate superoxide anion and are subject to persistent, severe bacterial infections, which often result in life-threatening episodes or even death (2, 3). It has been shown that several forms of this disease result from genetic defects in one of the various protein components of the NADPH oxidase system (3).

The mechanism by which the NADPH oxidase is activated by inflammatory stimuli is not well understood but appears to involve the assembly of the various components of the NADPH oxidase at the plasma membrane level to form an "active" complex (2). The processes involved in the translocation of cytosolic oxidase components to the membrane also remain to be defined. There is evidence (for review see reference 4) that a GTP-binding protein of unknown character may be involved in regulating the activation process. Indeed, a GTP-binding protein known as Rap1A has been shown to bind to the cytochrome *b* component of the NADPH oxidase (5, 6). However, the functional significance of this association is not yet clear.

The low molecular weight GTP-binding proteins (LMWG) represent a rapidly growing superfamily of GTPases that regulate a wide variety of cellular processes (7). These proteins consist of a GTP-binding monomer with a molecular weight of 19,000–28,000 and have properties that distinguish them from the various receptor-coupled G protein α subunits, including their lack of associated β/γ subunits. Although the LMWG can vary greatly in their overall amino acid sequences, they exhibit a number of features that are common to each. These include (*a*) common structural motifs; (*b*) regulation by extrinsic factors that modulate whether the protein is in a GTP- or GDP-state; and (*c*) posttranslational processing by isoprenylation, proteolytic truncation, and carboxymethylation. The latter is directed by a CAAX consensus motif found at the carboxyl terminal of all known isoprenylated proteins, where C is a cysteine residue, A is any aliphatic amino acid, and X is variable (8). Posttranslational processing involves an initial isoprenylation at the cysteine residue via a thioether bond between the protein and a C15 (farnesyl) or C20 (geranylgeranyl) isoprenyl moiety. This is followed by proteolytic truncation of the pro-

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1. *Abbreviations used in this paper:* DFP, diisopropylfluorophosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; KRHG, Krebs-Ringer Hepes with 5.5 mM glucose; LMWG, low molecular weight GTP-binding proteins.

tein, removing the three amino acids distal to the isoprenylated cysteine. The newly exposed COOH-terminal cysteine is then carboxymethylated. For the Ras proteins, each of these processing steps has been shown to be an important determinant of Ras binding to the plasma membrane (9, 10) and isoprenylation is critical for proper expression of the transforming activity of oncogenic Ras (11–13).

Pharmacologic agents able to block protein isoprenylation have been identified. These inhibitors, which include compactin and lovastatin (8, 14, 15), inhibit the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase. This enzyme synthesizes mevalonic acid, an immediate precursor of the isoprenoids. In the studies described here, we examined the effect of inhibitors of protein isoprenylation on the ability of DMSO-differentiated HL-60 cells to undergo a respiratory burst in response to receptor and nonreceptor stimuli. Compactin (as well as lovastatin) caused an inhibition of NADPH oxidase activation at micromolar concentrations that could be attributed to the ability of this compound to inhibit the pathway leading to protein isoprenylation. Using this drug, we were able to localize the compactin-sensitive component to the cytosolic fraction of differentiated HL-60 cells.

Methods

Culture of HL-60 cells. HL-60 cells were maintained in stationary culture in RPMI 1640 medium containing 10% fetal bovine serum at 37°C in an atmosphere of 95% air and 5% CO_2. The cells ($\sim 8 \times 10^5/$ml) were differentiated into neutrophil-like cells by treatment with 1.3% DMSO for 6 d (16). The cells were treated with the indicated levels of compactin or lovastatin by addition of a 1,000-fold concentrated stock solution to the appropriate concentration on day 4 of differentiation. Similar results were obtained if inhibitors were added at day 1 of differentiation, but in this case, cell viability was somewhat decreased at higher concentrations of each drug. In studies of the protective effect of mevalonic acid, the compound was added as the mevalonolactone at a concentration of 1 mM to the cell culture on days 4–6. Similarly, cholesterol was added in the form of LDL prepared according to the method of Curtiss and Edgington (17) at a final concentration of 50 µg/ml, ubiquinone was added as Coenzyme Q-10 at a final concentration of 0.5 mM, and dolichol was added as a dispersion in phosphatidylcholine (18) at a final concentration of 0.5 mM.

Preparation of HL-60 cell membranes. Cells (~ 1–2×10^8) that had been DMSO-differentiated and treated with±10 µM compactin were pelleted, washed once with isotonic NaCl, and treated with diisopropylfluorophosphate (DFP) for 15 min on ice. The cells were then pelleted and resuspended in 10 mM Pipes pH 7.3, 100 mM KCl, 3 mM NaCl, 1 mM ATP, 3.5 mM $MgCl_2$ (Relax buffer) plus 100 U aprotonin/ml, 1 mM PMSF, and 0.34 M sucrose to a final concentration of $\sim 1 \times 10^8/$ml. The cells were then disrupted on ice by 3×10 s bursts at medium setting with a Heat Systems sonicator-cell disrupter (model W-375; Heat Systems Inc., Farmingdale, NY). The homogenate was spun at 1,000 g to pellet unbroken cells and nuclei, and the remaining material was centrifuged at 165,000 g for 60 min at 2°C. The clear supernatant was collected as HL-60 cytosol. The membrane pellets were resuspended in Relax buffer with aprotonin, PMSF, and sucrose (as above) to a protein concentration of 3–5 mg/ml. Cytosol protein concentrations were typically between 1.5 and 3 mg/ml. Protein values were determined using the BCA assay (Pierce Chemical Co., Rockford, IL) with bovine serum albumin as a standard.

O_2^- assays with cells or subcellular fractions. O_2^- formation by HL-60 cells was assessed by the SOD-sensitive reduction of cytochrome c. Cells were suspended at $1 \times 10^7/$ml in Krebs-Ringer Hepes buffer with 5.5 mM glucose (KRHG), and 50–100 µl of cells was added to a cuvette containing 100 µM cytochrome c (type III, Sigma Chemical Co., St.

Louis, MO)±300 µg/ml SOD in 700–750 µl KRHG. Cytochalasin b was added to a final concentration of 5 µg/ml, and the cells were incubated at 37°C for 5 min while a stable baseline at $\lambda = 550$ nm was obtained. O_2^- formation was initiated by the addition of 1 µM N-formylmethionylleucylphenylalanine (FMLP) or 1 µg/ml phorbol myristate acetate (PMA). Cytochrome c reduction at 550 nm was continuously monitored and maximal rate and extent of the reaction calculated.

To assess O_2^- formation using HL-60 membrane and cytosol, the cell-free system of Curnutte et al. (19, 20) was utilized. Briefly, HL-60 membrane pellet (50–60 µg) or human neutrophil membrane (6.35 $\times 10^6$ cell equivalents) was added to a cuvette containing 100 µM cytochrome c, 6.25 mM $MgCl_2$, 93 mM KCl, 2.8 mM NaCl, 9.3 mM Pipes pH 7.3, 0.8 mM ATP, 0.16 mM NADPH, 10 µM GTPγS, 225 µg HL-60 cytosol, or 250 µg (1×10^7 cell equivalents) of human neutrophil cytosol and±300 µg/ml SOD. After a 3-min equilibration at 25°C, the formation of O_2^- was initiated by the addition of 100 µM SDS. O_2^- generation was monitored continuously as the SOD-sensitive reduction of cytochrome c at 550 nm. Human neutrophil plasma membranes (γ-GSP) and cytosol (GSS) were prepared as described in reference 19 and were the kind gift of Dr. John Curnutte (this institution).

Flow cytometric analysis of cell viability and differentiation. Cell viability was determined by uptake of propidium iodide, detected from the emission fluorescence at 625/35 nm using a DM560 dichronic mirror (21). Expression of N-formyl peptide receptor was determined using fluorescein-labeled N-CHO-Nle-Leu-Phe-Nle-Tyr-Lys essentially by the method of Sklar and Finney (22). Specificity of ligand binding was determined in the presence of 4 mM unlabeled T-boc peptide. CD14 expression was determined using monoclonal antibody 3C10 at a dilution of 1:1,000 (23), with detection using a fluorescein-labeled secondary antibody. Flow cytometric analyses were performed on a FACSIV® (Becton-Dickinson, San Jose, CA) equipped with a 2W argon laser (Coherent Inc., Palo Alto, CA) using a flow rate of < 1,000 cells/s. Data were collected in list mode and analyzed using the Consort 30 program (Becton-Dickinson) after collecting 10,000 events.

Immunological procedures. Western blots were performed as described in reference 24. Anti–Rap1 antibody R61, anti–G protein β subunit antibody, R3,4 and anticytochrome 22-kD subunit antibody are all specific and are described in references 24–26, respectively.

Analysis of Rap1 processing in HL-60 cells was performed by labeling cells ($8 \times 10^5/$ml) with 200 µCi/ml ^{35}S-*Trans*-label (ICN Biomedicals, Inc., Costa Mesa, CA) for 5 d during cell differentiation with 1.3% DMSO in Cys-Met-free medium containing 12% dialyzed fetal bovine serum. Various concentrations of compactin were included as indicated. Metabolically labeled cells were collected, washed once with isotonic NaCl, treated with 2.5 mM DFP for 15 min at 0°C, and then washed and suspended in 1 ml of 50 mM Hepes pH 7.5, 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 µM PMSF, and 100 U aprotonin. After a 15-min incubation on ice, the cell lysates were pelleted in a microfuge for 2 min, and then the supernatant was transferred to clean tubes containing 100 µl 4 M NaCl, 5 µl 10% SDS, and 50 µl 10% deoxycholate. The samples were boiled for 2 min, pelleted, and transferred to clean tubes containing 2 µl of the primary antibody, 142-24E05 (27, 28). Immune precipitates were then worked up as previously described (25), except that the pellets were washed six times with 1 ml 50 mM Hepes pH 7.5, 500 mM NaCl, 0.1% Triton X-100, and 0.059% SDS before preparation for SDS-PAGE.

Materials. SOD, cytochrome c type III, cytochalasin b, mevalanolactone, PMA, FMLP, ubiquinone 50 (Coenzyme Q-10), and dolichol were from Sigma Chemical Co. (St. Louis, MO). Propidium iodide (537-059) was from Calbiochem-Behring Corp. (La Jolla, CA). Fluorescein-labeled N-CHO-NLeu-Leu-Phe-NLeu-Tyr-Lys and fluorescein-labeled goat anti–mouse were from Molecular Probes, Inc. (Eugene, OR) and BRL (Gaithersburg, MD), respectively. LDL was a generous gift of Linda Curtiss (this institution). Rap1A was prepared as in reference 29. Compactin was provided by Channing J. Der (La Jolla Cancer Research Foundation, La Jolla, CA) and lovastatin was provided by William Maltese (Geisinger Clinic, Danville, PA). Anti–cytochrome b

antibody was the gift of Mark T. Quinn (Montana State University, Bozeman, MT).

Results

Compactin inhibits O_2^- generation in DMSO-differentiated HL-60 cells. The HL-60 cell line is a promyelocytic line that, when induced to differentiate into a neutrophil-like cell by DMSO, develops a fully activatable NADPH oxidase (16, 30–32). We treated DMSO-differentiated HL-60 cells with various concentrations of compactin and observed a dose-dependent inhibition of O_2^- formation in response to either the receptor stimulus, FMLP, or the protein kinase C activator, PMA (Table I). Both the rate and extent of O_2^- formation were diminished by the drug. Inhibition was half-maximal between 0.4 and 2 μM compactin and reached nearly complete inhibition (> 85%) at 10 μM compactin. HL-60 cell viability was not affected by the compactin treatment as assessed by staining with propridium iodide (Table I).

The inhibitory effect of compactin on FMLP-stimulated O_2^- formation could conceivably be due to an effect of the drug to prevent normal HL-60 differentiation into a neutrophil-like cell. It has been previously shown that HL-60 differentiation causes a marked increase in the expression of N-formyl peptide chemoattractant receptor (33). Also, a number of known components of the NADPH oxidase system have been shown to increase upon HL-60 differentiation (31, 32). We assessed HL-60 differentiation using two distinct markers: appearance of N-formyl peptide receptors and appearance of the CD14 antigen. Both induction of N-formyl peptide receptor expression and CD14 expression were normal in the compactin-treated cells (Table I). The inhibitory effect of compactin on FMLP-stimulated O_2^- formation was therefore not due to a loss of high affinity cell surface receptors for this chemoattractant. The loss in the ability of PMA to stimulate O_2^- formation also argued that a process downstream of receptors and their associated heterotrimeric G protein was being affected. Since cell differentiation appeared normal, it is also unlikely that other oxidase components were not expressed at normal levels. This was confirmed by analysis of cytochrome b_{558} levels in treated vs. control cells using a specific anti–cytochrome antibody (26) on Western blots (data not shown).

We also determined that compactin did not cause inhibition via direct short-term toxic effects upon the NADPH oxidase system. Treatment of peripheral blood neutrophils with 10 μM compactin for up to 2 h produced no effect upon the ability of these cells to support a respiratory burst in response to FMLP. Additionally, using a cell-free oxidase system, the inclusion of 10 μM compactin in the assay cuvette did not inhibit O_2^- formation. Finally, we observed similar inhibition of the NADPH oxidase in HL-60 cells treated with 25 or 50 μM lovastatin, another inhibitor of HMG-CoA reductase (data not shown). It seems unlikely that both compounds would produce similar nonspecific toxic effects on the NADPH oxidase.

Compactin inhibition is specifically reversed by mevalonic acid. Inhibition of protein isoprenylation can interfere with LMWG function without inhibiting overall cellular function (8). Since compactin inhibits at the enzymatic step proceeding mevalonic acid synthesis, we should be able to reverse effects of compactin due specifically to inhibition of this pathway by addition of exogenous mevalonic acid. Addition of 1 mM mevalonic acid (added as the mevalonolactone) to DMSO-differentiated HL-60 cells that had been treated with 4 or 10 μM compactin nearly completely reversed the inhibitory effect of this agent (Table II). Mevalonate itself had no consistent effect on control rate or extent of O_2^- formation, although in several experiments the responses were slightly enhanced. In contrast, the supplementation of the HL-60 cells with 50 μg/ml LDL cholesterol, with ubiquinone 50 (0.5 mM), or with dolichol (0.5 mM) had no effect on compactin blockade of oxidase activity.

Compactin inhibits LMWG isoprenylation in HL-60 cells. The ability of the concentrations of compactin we were using to inhibit endogenous HL-60 cell LMWG isoprenylation was assessed. Inhibition of Rap1 isoprenylation was determined in two ways. We used Western blotting to evaluate the presence of Rap1 in cytosol or membrane fractions of compactin-treated vs. untreated cells (Fig. 1). In untreated cells, Rap1 was present largely in the membrane fraction, with very little Rap1 in the cytosol. This is consistent with our previous observations on the subcellular distribution of Rap1 in mature human neutrophils (25). In contrast, cells treated with 10 μM compactin showed the appearance of large amounts of Rap1 in the cytosolic fraction. We estimated by densitometric analysis that $\sim$ 60–70% ($n = 3$) of the total Rap1 previously associated with

Table I. Effects of Compactin on HL-60 Cell Viability, Differentiation, and Respiratory Burst Activity

| Compactin | FMLP-stimulated O_2^- formation | | Percent viable cells | Percent cell differentiation | |
	Rate	Extent		NFPR	CD14
μM	% of control		%	%	
0	100	100	100	100	100
0.4	77±10	71±10	100±1.6	102 ($n = 2$)	98 ($n = 2$)
2	47±11	36±11	95±1.2	100 ($n = 2$)	93 ($n = 2$)
4	39±4.1	22±1.0	97±0.9	103±2.6	ND
10	15±2.6	12±2.1	96±1.2	97±0.6	94 ($n = 2$)

Values are means±SEM of three or more experiments, except as indicated.
NFPR, N-formyl peptide receptor; ND, not done.

Table II. Prevention of Compactin Inhibition by Mevalonic Acid

| Condition | FMLP-stimulated O_2^- formation | |
	Rate	Extent
	% of control	
No addition	100	100
+1 mM mevalonate	120±10	111±8.0
+4 μM compactin	43±5.0	28±3.5
4 μM compactin + 1 mM mevalonate	97±4.0	96±8.0
+10 μM compactin	19±3.9	13±0.4
10 uM compactin + 1 mM mevalonate	115±7.6	114±1.6
+50 μg/μl cholesterol	90 ($n = 2$)	99 ($n = 2$)
10 μM compactin + 50 μg/ml cholesterol	28 ($n = 2$)	22 ($n = 2$)

Values are means±SEM of three experiments, except as indicated.
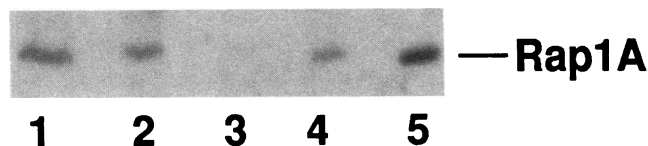
Figure 1. Western blot analysis of Rap1 in HL-60 subcellular fractions. HL-60 cells were treated with±10 μM compactin, and membrane and cytosol fractions were prepared as described in Methods. Equal amounts of membrane protein (100 μg) and cytosol protein (70 μg), respectively, were loaded in each lane. Total membrane protein obtained in this experiment from ~ 3 × 10⁸ cells was ~ 3 mg and cytosolic protein was ~ 2 mg. The samples were analyzed on 15% SDS-polyacrylamide gels, transferred to nitrocellulose, and blotted with specific anti-rap1 antibody, R61 at 1:500 dilution. Detection was with ¹²⁵I-goat anti–rabbit IgG and autoradiograms were exposed for 24–36 h with intensifying screen at −70°C. (Lane *1*) Untreated HL-60 membrane. (Lane *2*) 10 μM compactin-treated HL-60 membrane. (Lane *3*) untreated HL-60 cytosol. (Lane *4*) 10 μM compactin-treated HL-60 cytosol. (Lane *5*) Approximately 300 ng Rap1A standard.

the membrane pellet was now soluble. This observation is consistent with the ability of isoprenylation to promote membrane association of LMWG. Isoprenylation of Rap1 was also assessed by metabolic labeling of the HL-60 cells with [³⁵S]met/cys, immune precipitation of Rap1, and analysis of the 22-kD processed and 23-kD unprocessed forms of Rap1 (34). These experiments (data not shown) confirmed a dose-dependent decrease in the 22-kD processed form, with a proportional increase in the 23-kD unprocessed (re-nonisoprenylated) form.

Subcellular localization of the compactin-sensitive factor. To localize the compactin-sensitive factor required for NADPH oxidase activity to either the cytosol, membrane, or both, we prepared subcellular fractions from control or compactin-treated differentiated HL-60 cells. The membrane fraction and cytosol from the cells were then analyzed using the cell-free NADPH oxidase system. When membrane and cytosol from the untreated HL-60 cells were combined in the cell-free assay, O_2^- formation in response to 100 μM SDS was rapid and was similar to rates obtained with more purified subcellular fractions obtained from (human peripheral blood) neutrophils (Table III). In contrast, the fractions prepared from the cells pretreated with 10 μM compactin were unable to sustain a normal respiratory burst in vitro, with both the rate and extent of the burst decreased to < 10% of control values.

We determined whether the inhibited factor(s) was present in the membrane fraction or cytosol by performing complementation experiments using cytosol or membranes from normal human neutrophils. These data are presented in Table III and Fig. 2. The combination of membrane from the compactin-treated HL-60 cells with cytosol from normal neutrophils gave a rate of O_2^- formation that was essentially identical to that obtained with an equal amount of cytosol (protein/protein) from untreated HL-60s. This rate was slightly less than the rate obtained with both membrane and cytosol from control neutrophils, but we have not adjusted for the difference in purity and protein value between the highly purified neutrophil membranes and the relatively crude HL-60 membranes. It was apparent that the compactin treatment of the HL-60 cells, somewhat surprisingly, had not affected a membrane-associated oxidase component. When we used cytosol from the

Table III. Analysis of O_2^- Formation by Subcellular Fractions from HL-60 Cells±Compactin Treatment

Condition	Rate of O_2^- formation		
	Experiment 1	Experiment 2	Experiment 3
	nmol/min		
Untreated cytosol + untreated membrane	2.2	2.1	—
Compactin cytosol + compactin membrane	0.1 (1)	0.4 (5)	—
Neutrophil cytosol + neutrophil membrane	4.5	4.7	6.8
Neutrophil cytosol + untreated membrane	2.0	2.6	4.1
Neutrophil cytosol + compactin membrane	1.9 (95)	2.6 (100)	4.0 (98)
Untreated cytosol + neutrophil membrane	3.3	4.0	5.1
Compactin cytosol + neutrophil membrane	1.1 (33)	1.5 (37)	2.4 (47)

HL-60 cells were treated ±10 μM compactin, as described in Methods. Untreated cytosol/membrane and Compactin cytosol/membrane refer to the fractions obtained from these cells. Values are the result of duplicate determinations within each experiment. Values in parentheses represent the percent of the rate obtained for the respective untreated HL-60 sample.

compactin-treated HL-60 cells with membranes from normal neutrophils, however, the ability to form O_2^- was markedly reduced over that obtained with an equivalent amount of cytosol from untreated HL-60 cells. The rate of O_2^- formation was de-
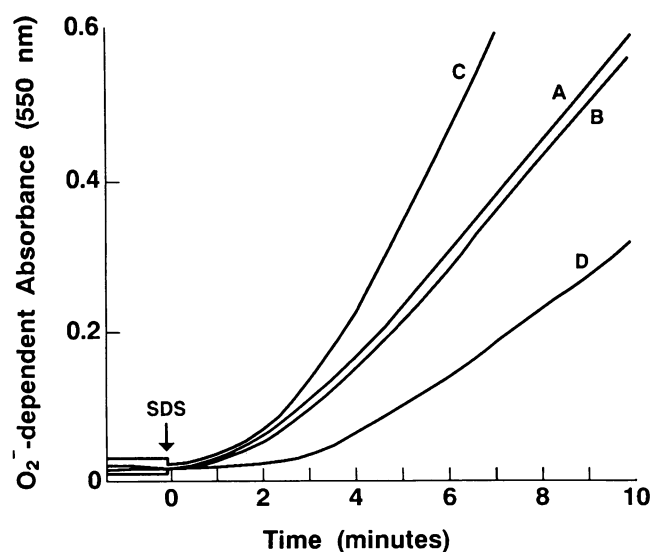
Figure 2. Complementation analysis of O_2^- formation by control vs. compactin-treated HL-60 cells. O_2^- formation was analyzed in a cell-free assay as described in Methods. (Trace *A*) Untreated HL-60 membrane + neutrophil GSS. (Trace *B*) Compactin-treated HL-60 membrane + neutrophil GSS. (Trace *C*) Untreated HL-60 cytosol + neutrophil γGSP. (Trace *D*) Compactin-treated HL-60 cytosol + neutrophil γGSP.

creased to about one-third of that seen with the untreated HL-60 cytosol. Additionally, as can be seen in Fig. 2, not only was the rate of O_2^- formation decreased, but there was also a marked increase in the lag period that occurs after the addition of the SDS and until the rate of O_2^- formation becomes maximum. It was apparent that the compactin-sensitive point in O_2^- formation was localized to a factor present in the HL-60 cell cytosol.

Discussion

Various studies have identified a multiplicity of cellular proteins that appear to be covalently modified by isoprenyl groups (8, 35). The electrophoretic pattern of these proteins is remarkably similar from one cell to another, and the proteins generally fall into two size classes. A group of 44–69-kD isoprenylated proteins are largely localized to the nucleus and the associated nuclear matrix. Within this group are the nuclear lamins (8, 12, 36). A second class of 20–24-kD isoprenylated proteins are more widely distributed within the cell and appear to represent the LMWG (37). A common feature of all known isoprenylated proteins is the presence of a CAAX motif at the carboxyl terminus. This sequence appears to represent a signal for protein isoprenylation and is present in most of the LMWG that have been identified (8, 35).

Compactin and lovastatin are known to block isoprenoid synthesis by inhibiting the enzyme HMG-CoA reductase and can thus prevent this covalent modification of the LMWG (37). The NADPH oxidase of phagocytes has been shown to be regulated by an apparent LMWG that has not yet been purified or characterized. Reasoning that if this GTP-binding protein was of the Ras-related or low molecular weight superfamily, then it would also be posttranslationally isoprenylated, we tested these drugs as a pharmacologic means to intervene in NADPH oxidase activation.

We observed that compactin (and lovastatin) markedly inhibited the ability of HL-60 cells to generate O_2^- in response to both receptor stimuli and downstream activators of the respiratory burst oxidase. The inhibitory effects of compactin occurred at concentrations between 0.4 and 10 μM. These concentrations are somewhat lower than those that have been previously reported to inhibit the overall isoprenylation of cellular proteins (8, 10–13). This may reflect the relative sensitivity of the compactin-sensitive factor in HL-60 cells to the lack of isoprenoid substrate (38). Several pieces of data indicate that the effect of compactin on the oxidase was of a specific nature. At the concentrations of drug used in these studies, HL-60 cell viability was not affected; neither was the ability of these cells to differentiate normally upon exposure to DMSO, as evidenced by analysis of two independent markers of HL-60 differentiation, the N-formyl peptide receptor (33) and the CD14 antigen (39). The effect of compactin is unlikely to be due to inhibition of cholesterol, ubiquinone, or dolichol synthesis, pathways that are also blocked by inhibitors of HMG-CoA reductase. It is known that neutrophils/HL-60 cells do not rely upon endogenous synthesis of cholesterol but largely obtain their cholesterol from exogenous sources. Indeed, since the cells are cultured in 10% fetal calf serum, they are always supplemented with cholesterol and probably dolichol as well. When we supplied the compactin-treated HL-60 cells with additional cholesterol, ubiquinone, or dolichol, we were still unable to overcome the block in NADPH oxidase activity seen. In contrast, we were able to totally restore cellular responsiveness by supplying exogenous mevalonic acid to the cells, indicating that the synthesis of isoprenoids was likely to be the limiting factor that prevented oxidase activation.

A number of components of the neutrophil NADPH oxidase system have been cloned, including p47, p67, and cytochrome b_{558} (40–43). None of these proteins contains the CAAX consensus isoprenylation motif nor have they been reported to be isoprenylated. Expression of cytochrome b_{558} was normal in HL-60 cells treated with 10 μM compactin, suggesting the treated cell contained adequate levels of dolichol for glycosylation of this membrane protein. Although we cannot rule out the possibility that an as-yet unidentified oxidase component unrelated to the LMWG is isoprenylated, we suggest that it is the regulatory LMWG known to be involved in the oxidase system that is being affected. It has been explicitly demonstrated that various forms of Ras (10–13), Rap1 (34, 44), Rap2 (45), G25K (46), Rac (47, 48), Ral (48), and Rab (49, 50) are isoprenylated posttranslationally. In the present study, we observed that the processing of endogenous HL-60 Rap1 was inhibited by the concentrations of compactin used to inhibit NADPH oxidase activity, although we do not claim any direct correlation between the inhibition of Rap1 isoprenylation and the blockade of oxidase activation observed.

We were able to make use of the essentially irreversible effect of compactin to inhibit posttranslational isoprenylation to identify the subcellular location of the putative LMWG. Somewhat surprisingly to us, the compactin-sensitive component was found to reside in the HL-60 cytosol. This was indicated by the ability of the membrane fraction from the compactin-treated cells to fully support a normal respiratory burst, whereas the cytosol from such cells was largely defective in supporting a normal oxidative response. Since analysis of Rap1 indicated that a portion of this GTP-binding protein was still membrane associated after treatment with 10 μM compactin, it is possible that we have been unable to decrease all compactin-sensitive membrane components to rate-limiting levels. We interpret these results, however, to indicate that the relevant component (LMWG?) required for oxidase activation is resident in the cytosol and that it must be isoprenylated in order to carry out its normal function in supporting a respiratory burst. We would hypothesize, as depicted in Fig. 3, that this protein might have to interact with the membrane at some point during the activation of the oxidase and that the isoprenyl group is necessary for this transient interaction to occur effectively. Alternatively, if this component is a LMWG, it may require this posttranslational modification for efficient interaction with regulatory components, such as guanine nucleotide dissociation stimulators or inhibitors (51). It is clear from previous work that isoprenylation of a protein is not sufficient in itself for membrane localization (8–10, 13, 34). Indeed, many isoprenylated proteins are cytosolic (8) and both Ras and G25K are found in cytosolic and membrane-associated isoprenylated forms (9, 10, 45). Although the γ subunits of the heterotrimeric G proteins have been shown to be isoprenylated (8), the localization of the compactin-sensitive factor to the cytosol would apparently rule out the possibility that the G protein γ subunit is the necessary factor. G protein β/γ subunits are membrane localized and we did not detect β/γ subunits in human neutrophil cytosol by Western blotting (24).

These findings demonstrate the existence of a protein that is required for activity of the NADPH oxidase and whose activity can be inhibited by inhibitors of protein isoprenylation. This
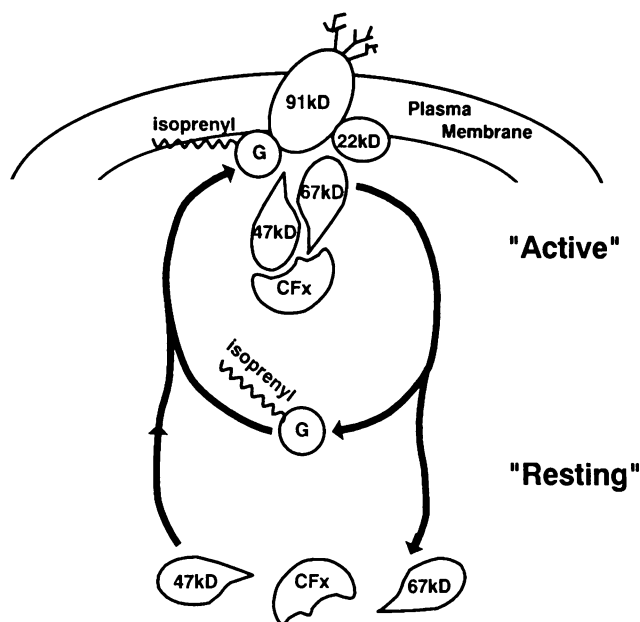
Figure 3. Hypothetical model depicting the potential cycling of an isoprenylated LMWG (G) between a cytosolic form in the "resting" oxidase and a membrane-associated form in the "active" oxidase. 91 and 22 kD represent the subunits of the oxidase-associated cytochrome *b;* 47 and 67 kD represent other known and as yet unidentified (CFx) cytosolic NADPH oxidase components.

protein, which is likely to be a regulatory LMWG, is localized to the HL-60 cytosol. An important area of future investigation will be to identify this particular component and to define its role in regulating the NADPH oxidase system. Our results suggest that it may be possible to develop new classes of antiinflammatory drugs whose targets are the enzymatic machinery that carries out the posttranslational processing of this regulatory component. Similar strategies are currently being pursued in attempts to develop inhibitors of cell transformation caused by the *ras* oncogene (52, 53).

Acknowledgments

Note Added in Proof: We have recently identified a LMWG that stimulates NADPH oxidase activity as Rac2 (U. G. Knaus, et al. 1991. *Science* [Wash. DC] 254:1512–1515).

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