Biosynthesis of the Phagocyte NADPH Oxidase Cytochrome b\textsubscript{558}

ROLE OF HEME INCORPORATION AND HETERODIMER FORMATION IN MATURATION AND STABILITY OF gp91\textsuperscript{phox} and p22\textsuperscript{phox} SUBUNITS*

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The NADPH oxidase cytochrome b\textsubscript{558} is a membrane heterodimer comprised of a glycosylated 91-kDa subunit, gp91\textsuperscript{phox}, and a nonglycosylated 22-kDa subunit, p22\textsuperscript{phox}. The role of heme in cytochrome b\textsubscript{558} biosynthesis was studied using succinyl acetone, an inhibitor of heme synthesis, in PLB-985 myeloid cells undergoing granulocytic differentiation. Succinyl acetone markedly reduced expression of p22\textsuperscript{phox} and the mature 91-kDa form of gp91\textsuperscript{phox} but not its 65-kDa high mannose precursor, in association with a profound reduction in NADPH oxidase activity. Expression of non-heme-containing cytosolic oxidase components was unaffected. The reduction in cytochrome b\textsubscript{558} expression and NADPH oxidase activity was prevented by adding exogenous heme and was reversible upon removal of succinyl acetone. Transgenic expression of gp91\textsuperscript{phox} in monkey COS-7 and murine 3T3 cells, both of which lacked endogenous p22\textsuperscript{phox} mRNA, demonstrated that p22\textsuperscript{phox} was not required for maturation of gp91\textsuperscript{phox} carbohydrate to complex oligosaccharides. However, coexpression of transgenic p22\textsuperscript{phox} increased the abundance of the mature gp91\textsuperscript{phox} glycoprotein. These results suggest that heme incorporation plays an important role in cytochrome b\textsubscript{558} assembly and provide further support for the concept that stability of p22\textsuperscript{phox} and the mature gp91\textsuperscript{phox} subunit is increased by heterodimer formation.

The phagocyte NADPH oxidase catalyzes the formation of superoxide (O\textsubscript{2}·), the precursor to a variety of potent oxidants that are important for the host defense against invading microorganisms (1). Dormant in resting phagocytes, the oxidase is assembled rapidly upon phagocyte activation to mediate the host defense against invading microorganisms. The oxidase is dormant in resting phagocytes, an oxidase in association with a profound reduction in granulocytic differentiation. Succinyl acetone markedly reduced expression of p22\textsuperscript{phox} and the mature 91-kDa form of gp91\textsuperscript{phox} but not its 65-kDa high mannose precursor, in association with a profound reduction in NADPH oxidase activity. Expression of non-heme-containing cytosolic oxidase components was unaffected. The reduction in cytochrome b\textsubscript{558} expression and NADPH oxidase activity was prevented by adding exogenous heme and was reversible upon removal of succinyl acetone. Transgenic expression of gp91\textsuperscript{phox} in monkey COS-7 and murine 3T3 cells, both of which lacked endogenous p22\textsuperscript{phox} mRNA, demonstrated that p22\textsuperscript{phox} was not required for maturation of gp91\textsuperscript{phox} carbohydrate to complex oligosaccharides. However, coexpression of transgenic p22\textsuperscript{phox} increased the abundance of the mature gp91\textsuperscript{phox} glycoprotein. These results suggest that heme incorporation plays an important role in cytochrome b\textsubscript{558} assembly and provide further support for the concept that stability of p22\textsuperscript{phox} and the mature gp91\textsuperscript{phox} subunit is increased by heterodimer formation.

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The NADPH oxidase cytochrome b\textsubscript{558} expressed almost exclusively in phagocytic cells, is comprised of two integral membrane proteins, a glycosylated 91-kDa subunit (gp91\textsuperscript{phox}) and a 22-kDa subunit (p22\textsuperscript{phox}) and contains both flavin and heme groups (5–11). This heterodimer is hence believed to be the redox center of the oxidase, although p67\textsuperscript{phox} has been shown to contain a NADPH binding site that may be important for oxidase function (12). Multiple hemes, probably two, have been reported to be incorporated within cytochrome b\textsubscript{558} purified from human neutrophils, with one residing in gp91\textsuperscript{phox} and a second that may be shared by the two subunits (6, 13, 14).

The heme synthetic pathways have been proposed to reside within the membrane in the relatively hydrophobic NH\textsubscript{2}-terminal portion of the gp91\textsuperscript{phox} polypeptide (1, 2, 13, 15) and appear to be in a six-coordinated state with axial imidazole or imidazolate ligands supplied by histidine residues (16, 17). The hydrophilic carboxyl-terminal half of gp91\textsuperscript{phox} contains regions with homology to the ferredoxin-NADPH+ reductase flavoenzyme family, including flavin and NADPH binding domains (7, 8, 18).

The physiologic importance of the NADPH oxidase is illustrated by the inherited immunodeficiency, chronic granulomatous disease (CGD), which results from genetic defects in different oxidase subunits (1, 19, 20). Affected patients develop recurrent, severe bacterial and fungal infections caused by the deficient generation of phagocyte oxidants. Two genetic subgroups involve mutations in the cytochrome b\textsubscript{558}. Defects in the X-linked gene of gp91\textsuperscript{phox} account for about two-thirds of cases of CGD, whereas the gene encoding p22\textsuperscript{phox} is the site of mutations in a rare autosomal recessive form of CGD. Genetic defects in either the p47\textsuperscript{phox} or p67\textsuperscript{phox} subunit account for the remainder of autosomal recessive CGD.

Relatively little is known about the biogenesis of the cytochrome b\textsubscript{558} and the role of heme incorporation in this process. In virtually all patients with cytochrome b\textsubscript{558} mutations, neutrophils lack both gp91\textsuperscript{phox} and p22\textsuperscript{phox} polypeptides, regardless of which subunit is affected by the mutation (20, 21). This observation has suggested that formation of the gp91\textsuperscript{phox} p22\textsuperscript{phox} heterodimer is important for stable expression of each subunit within the neutrophil. Porter and co-workers (22) have identified a partially processed 65-kDa precursor of gp91\textsuperscript{phox} in B cell lines from p22\textsuperscript{phox}-deficient CGD patients, where it may be more stable compared with neutrophils or more abundant because of an increased content of endoplasmic reticulum. This 65-kDa gp91\textsuperscript{phox} intermediate has high mannosne carbohydrate side chains, indicating that the initial steps of oligosaccharide addition and processing within the endoplasmic reticulum occur in the absence of p22\textsuperscript{phox}. Expression of the mature 91-kDa form of gp91\textsuperscript{phox} with fully processed oligosaccharide side chains can be restored in p22\textsuperscript{phox}-deficient CGD B cell lines by expression of recombinant p22\textsuperscript{phox} (22, 23).

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1 The abbreviations used are: CGD, chronic granulomatous disease; SA, 4,6-dioxoheptanoic acid or succinyl acetone; DMF, N,N-dimethylformamide; PBS, phosphate-buffered saline.

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gp91phox precursor has also been detected in B cell lines from four unrelated X-CGD patients who otherwise lacked mature gp91phox (24). The patients all had missense mutations or an in-frame deletion of the coding sequence, and it was postulated that these mutations impaired normal biosynthetic processing of gp91phox either by interfering with incorporation of redox cofactors or by disrupting the association with p22phox.

The heme prosthetic groups have themselves been postulated to play a role in heterodimer formation and stability of cytochrome b558. The hemes are tightly bound to the cytochrome b558 heterodimer, and the detectable spectrum is always accompanied by the stable expression of both subunits (2, 19, 20, 25). When heme synthesis was inhibited in myeloid leukemia HL-60 cells induced to undergo granulocytic differentiation, cells failed to show the normal increase in NADPH oxidase activity (26). The typical cytochrome b558 spectrum was absent, and indirect immunofluorescence microscopy showed markedly decreased expression of p22phox, although gp91phox expression appeared normal. The identification of CGD patients with undetectable cytochrome b558, who have point mutations in candidate heme-binding histidine residues within gp91phox or p22phox, has also been taken as indirect evidence for a role of heme incorporation in cytochrome b558 expression (20). However, three of the four of the aforementioned mutations involve His → Arg substitutions within hydrophobic domains, which could also have a nonspecific effect on protein stability.

In the current study, we have investigated further the biosynthesis of cytochrome b558, examining the role of both heme incorporation and heterodimer formation in the maturation and stability of gp91phox and p22phox subunits. We found that succinyl acetone (SA), an inhibitor of heme biosynthesis, reversibly reduced the expression of both p22phox and the mature 91-kDa form of gp91phox but not the 65-kDa precursor of gp91phox, in association with a profound reduction in NADPH oxidase activity in cultured PLB-985 myeloid cells undergoing granulocytic differentiation. Expression of the cytosolic oxidase components p47phox, p67phox, and Rac2 was unaffected. Transgenic expression of gp91phox in monkey COS-7 and murine 3T3 cells, both of which lack endogenous p22phox mRNA, demonstrated that the expression of the p22phox polypeptide was not required for maturation of gp91phox oligosaccharide side chains, although coexpression of transgenic p22phox increased the abundance of the mature gp91phox glycoprotein. Taken together, these data confirm that the incorporation of heme plays an important role in the assembly of the cytochrome b558 and suggest that the stability of phagocyte p22phox and the mature gp91phox subunit is dependent on both heme incorporation and heterodimer formation.

EXPERIMENTAL PROCEDURES

Materials—The following reagents were purchased from Sigma: SA, hemin (hemin chloride), nitro blue tetrazolium, N,N-dimethylformamide (DMF), phorbol myristate acetate, and cytochrome c. Endo-β-N-acetylglucosaminidase H and peptide N-glycosidase F were obtained from New England Biolabs, Beverly, MA. Fluorescein isothiocyanate-conjugated mouse anti-human CD11b and mouse IgG2b isotype were purchased from Immunotech, Inc, Westbrook, ME.

Cell Culture and Differentiation—Human myeloid leukemia PLB-985 (27) cells (obtained from P. Newburger, University of Massachusetts) and HL-60 (28) cells (obtained from ATCC CCL 240) were maintained in RPMI 1640-glutamine (Life Technologies, Inc.) supplemented with 10% fetal calf serum (Sigma), 50 units/ml penicillin, and 50 μg/ml streptomycin (Life Technologies, Inc.). For granulocytic differentiation, cells at a starting density of 1 × 10⁶ cells/ml were exposed to 0.5% DMF in CO₂-independent medium (Life Technologies, Inc.) in the absence of fetal calf serum but supplemented with Nutridoma-SP (Boehringer Mannheim) and 2 mM l-glutamine. The medium was changed once at day 3 during the period of differentiation. Under these conditions, at least 85% of the cells had undergone granulocytic differentiation by 4–6 days based on acquisition of respiratory burst oxidase activity as determined using the nitro blue tetrazolium test (29).

SA is a specific inhibitor of the enzyme 5-aminolevulinic acid dehydratase that catalyzes the formation of porphobilinogen from 5-aminolevulinate in heme biosynthesis (30). In experiments using SA, PLB-985 or HL-60 cells were differentiated with DMF in the presence of 10 μM SA, as described previously (25, 26). Cells grown in the presence of SA were prepared as described previously (33), except that cells at 2.5 × 10⁵/ml in a volume of 0.5–1 ml were disrupted in 1.5 ml microtubes by sonication (Sonics and Materials, Inc., Danbury, CT) three times at 20% power for 5 s each. Protein concentration of cell extracts and membrane fractions was determined by BCA assay (Fierce). Whole cell and membrane extracts were made from 3T3 and COS-7 cell lines following
essentially the same protocols described for PLB-985 cells except that cells were harvested by gentle scraping.

**Immunoblot and Deglycosylation Analysis of Whole Cell and Membrane Extracts—**Separation of Triton X-100-extracted protein samples on 12% SDS-polyacrylamide gels and immunoblotting was performed as described previously (29, 34), using monoclonal antibodies for gp91phox and p22phox (35) (kindly provided by D. Roos and A. Verhoeven) and rabbit polyclonal antibodies for p47phox (36) (kindly provided by D. Uhlinger), p67phox (37) (kindly provided by P. Heyworth), and Rac2 (38) (kindly provided by G. Bokoch). After blots were developed using the ECL chemiluminescence method (Amersham), densitometry was performed as described previously (34).

Digestion of glycoprotein N-linked oligosaccharides with endo-β-N-acetylgalactosaminidase H or peptide N-glycosidase F was performed on membrane fractions under conditions suggested by the manufacturer. Briefly, a total of 10 μg of membrane protein was treated with 500 units of enzyme for 2 h at 37 °C, then separated on SDS-polyacrylamide gel (12%), and electrophoretically onto nitrocellulose membrane and probed with monoclonal antibodies to gp91phox and p22phox. Control membrane samples were treated similarly except that PBS was added instead of enzyme.

**Northern Blot Analysis—**Total cellular RNA from 3T3 cells, COS-7 cells, and SA-treated and untreated PLB-985 cells differentiated for indicated times was isolated as reported previously (39). After separation on a formaldehyde agarose gel (1%), RNA samples were transferred to Magnacharge nylon membrane (Micron Separations Inc., Westboro, MA) and hybridized with random primer-labeled human gp91phox, p22phox, and β-actin full-length cDNAs according to the protocols recommended by the manufacturer (29).

**Flow Cytometric Analysis of CD11b (MAC-1) Expression—**PLB-985 cells differentiated for 5 days in the presence or absence of SA (10 μg/ml) were collected by centrifugation and washed once with PBS, then stained with fluorescein isothiocyanate-conjugated mouse anti-human CD11b monoclonal antibodies as recommended by the manufacturer. The expression of CD11b on the cell surface was measured by FACScan (Becton Dickinson, San Jose, CA). Mouse IgG2b was used as an isotype control. A total of 2 × 10⁶ cells were analyzed.

**RESULTS**

**Induction of Superoxide-generating Activity during Differentiation of PLB-985 Cells Was Impaired by Succinylacetone Treatment—**Human myeloid leukemia PLB-985 cells are arrested at the early promyelocytic stage and can be induced to differentiate into granulocytic forms with DMF. During differentiation, expression of NADPH oxidase subunits is induced, resulting in a marked increase in superoxide-generating activity, similar to what has been described for HL-60 myeloid leukemia cells (40). As shown in Fig. 1A, NADPH oxidase activity in DMF-induced control PLB-985 cells, as measured by the cytochrome c reduction assay, appeared at day 2 and reached a maximum level at day 5. In the presence of the heme synthesis inhibitor SA, DMF-induced cells exhibited only very low levels of superoxide production (Fig. 1A), with ∼5% activity detected at day 5 relative to non-SA-treated controls. The nitro blue tetrazolium test was also used to monitor O2
red production in SA-treated and untreated PLB-985 and HL-60 cells during DMF-induced differentiation. In contrast to DMF-induced control cells, where the majority contained numerous dark purple formazan deposits 30 min after phorbol myristate acetate stimulation, SA-treated cells contained only a few formazan deposits (data not shown).

**Effect of Succinyl Acetone on Expression of NADPH Oxidase Subunits—**We hypothesized that the reduced NADPH oxidase activity in SA-treated PLB-985 cells (Fig. 1A) was associated with the deficient expression of the oxidase cytochrome b₅₅₈ due to the inhibition of heme synthesis. The expression of NADPH oxidase subunits during DMF-induced granulocytic differentiation was examined in cell extracts by immunoblot analysis. The abundance of both the gp91phox and p22phox subunits of cytochrome b₅₅₈ increased during differentiation in non-SA-treated control PLB-985 cells, which were only detected at extremely low levels in SA-treated cells (Fig. 1B).

**Fig. 1. Effect of SA on superoxide-generating activity and expression of the NADPH oxidase subunits during granulocytic differentiation of PLB-985 cells.** Granulocytic differentiation of PLB-985 cells was induced with DMF in the absence or presence of succinyl acetone. Cells were harvested each day and analyzed for NADPH oxidase activity and expression of oxidase components. Panel A, O2 production in intact cells was determined by using a continuous cytochrome c reduction assay at the indicated times during DMF-induced differentiation in the absence (●) or presence (○) of SA (10 μg/ml). The data represent the mean ± S.D. of three experiments. Panel B, immunoblot analysis of whole cell extracts probed with monoclonal antibody for gp91phox (upper panel) or p22phox (lower panel). Samples were collected at the indicated times during DMF-induced granulocytic differentiation in the absence (−SA) or presence (+SA) of SA. A total of 10 μg of protein was loaded in each lane. Panel C, immunoblot analysis of the same cell extracts described in panel B, except that the blot was probed simultaneously with antibodies for p47phox and p67phox. The samples are loaded in the same order as in panel B.

Similar results were also obtained when HL-60 cells were differentiated with DMF in the presence of SA (data not shown). Interestingly, a ∼65-kDa protein immunoreactive with the gp91phox monoclonal antibody was detected at a similar level in both SA-treated and untreated differentiated cells, increasing modestly with differentiation (Fig. 1B). This 65-kDa species, which is more abundant in PLB-985 cells grown in serum-free medium compared with those grown with fetal calf serum, appeared to be equivalent with the 65-kDa high mannose precursor of gp91phox first described in B cell lines (22). To verify this point, cellular membranes prepared from the PLB-985 cells differentiated in the absence or presence of SA for 5 days were digested with the enzyme endo-β-N-acetylglucosaminidase H, which only removes the high mannose form of N-linked carbohydrates, and peptide N-glycosidase F, which removes all N-linked carbohydrates. As seen in Fig. 2, endo-β-N-acetylglucosaminidase H treatment of membranes prepared from both control and SA-treated PLB-985 granulocytes resulted in the disappearance of the 65-kDa band and the appearance of a ∼58-kDa species. This species comigrates with the core...
gp91phox protein seen after peptide N-glycosidase F digestion of membranes prepared from control PLB-985 cells (Fig. 2). Therefore, the 65-kDa form of gp91phox seen in both control and SA-treated PLB-985 granulocytes appears to be identical to the high mannose precursor of gp91phox reported previously (22).

In contrast to the expression of gp91phox and p22phox subunits, the expression of soluble NADPH oxidase components during DMF-induced granulocytic differentiation was unaffected by SA treatment. The p47phox subunit was detected in small amounts at day 0 and increased during differentiation to reach a plateau at day 3 in control PLB-985 cells (Fig. 1C). The p67phox subunit was readily detected by day 2, consistent with the onset of measurable oxidase activity, and reached a plateau at day 4. A very similar increase in expression of p47phox and p67phox was observed in cells induced to differentiate with DMF in the presence of SA (Fig. 1C). No differences in the pattern and the relative amounts of Rac2 expression were seen between the SA-treated and untreated control cells (data not shown).

Differentiation of PLB-985 cells in the presence of SA also did not affect the cell surface expression of the β2 integrin, CD11b/CD18 (MAC-1), a glycosylated plasma membrane heterodimer that does not contain heme. As studied by flow cytometry of unpermeabilized cells using an antibody directed against CD11b, no differences in MAC-1 expression were observed in PLB-985 cells differentiated with DMF for 5 days in the presence of SA compared with DMF-induced cells not exposed to SA (data not shown).

Although very little of the mature form of gp91phox was detected in PLB-985 cells differentiated in the presence of SA, the presence of its 65-kDa high mannose precursor suggested that transcription and translation of the gp91phox mRNA were not altered by SA treatment. Northern blot analysis for the expression of gp91phox as well as p22phox mRNAs was performed using their full-length cDNAs as probes and showed no differences between PLB-985 cells differentiated in the absence or presence of SA (Fig. 3). Taken together, these observations suggest that the decrease in the abundance of both p22phox and the mature form of gp91phox in SA-treated cells is post-translational and related to deficient heme incorporation and heterodimer formation in the absence of heme synthesis.

The Inhibition of Cytochrome b558 Expression and NADPH Oxidase Activity by SA Is Reversible—To confirm that the marked decrease in NADPH oxidase activity and expression of the p22phox and mature gp91phox polypeptides in SA-treated PLB-985 granulocytes specifically result from an impairment in heme synthesis, we supplied exogenous heme at the onset of DMF-induced differentiation of PLB-985 cells in the presence and absence of SA. NADPH oxidase activity and cytochrome b558 expression were examined at day 5 (not shown). The intensity of formazan staining in the nitro blue tetrazolium test for PLB-985 granulocytes differentiated in the presence of SA and exogenous hemin was similar to control PLB-985 granulocytes. In addition, supplying exogenous heme also prevented the decreased expression of mature gp91phox and p22phox subunits of cytochrome b558 as determined by immunoblot analysis. Addition of exogenous heme to PLB-985 cells differentiated in the absence of SA did not significantly alter NADPH oxidase activity or cytochrome b558 expression.

Whether the decreased expression in p22phox and the mature 91-kDa form of gp91phox seen with SA exposure was reversible was also examined. PLB-985 cells were treated with SA for 3 days from the beginning of DMF-induced differentiation, then SA was removed, and the cells were cultured further for an additional 3 days. As shown in Fig. 4A, NADPH oxidase activity rapidly increased beginning 1 day after removal of SA, and after an additional 2 days in culture it reached 93% of the activity seen in control cells differentiated in the absence of SA. In parallel, the expression of the mature 91-kDa form of gp91phox and p22phox increased to levels similar to those observed in non-SA-treated controls (Fig. 4B). These observations are again consistent with a role of heme in formation of the gp91phox-p22phox heterodimer, resulting in the increased expression of mature gp91phox as well as its partner, p22phox. As expected, the differentiation-dependent increase in the levels of the cytosolic components p47phox and p67phox was unaffected by the presence or absence of SA (Fig. 4C).

Maturation of N-Linked Oligosaccharides in gp91phox Does Not Require Heterodimer Formation with p22phox—To investigate further the role of gp91phox-p22phox heterodimer formation in maturation of the N-linked oligosaccharide side chains of gp91phox and stability of the two cytochrome b558 subunits, transgenic expression of p22phox and gp91phox was studied in two non-phagocytic cell lines, NIH 3T3 murine fibroblasts and monkey kidney COS-7 cells. As described below, the results show that processing of gp91phox carbohydrate side chains from high mannose to complex oligosaccharides is not dependent on an association of gp91phox with p22phox. However, coexpression of both p22phox and gp91phox appeared to increase the stability of the mature 91-kDa form of gp91phox.

Neither gp91phox nor p22phox mRNA expression was detected by Northern blot analysis of the parental 3T3 or COS-7 cell lines (not shown). After stable transfection of 3T3 cells with an expression vector containing the gp91phox cDNA, immunoblots of cell extracts were probed with a gp91phox monoclonal antibody. A prominent ~65-kDa band was seen (Fig. 5A), which was sensitive to endo-β-N-acetylglucosaminidase H (Fig. 5C) and thus appears to correspond to the high mannose gp91phox intermediate. Smaller amounts of more slowly migrating
gp91phox species were also detected (Fig. 5A) which were resistant to endo-$\beta$-N-acetylgalcosaminidase H (Fig. 5C), indicating that they contain mature, fully processed N-linked oligosaccharides. Subsequent transfection of 3T3-gp91phox clones with a second transgene harboring the p22phox cDNA resulted in a marked increase in the abundance of the larger gp91phox species with mature oligosaccharide side chains (Fig. 5, A and C). A similar result was seen for transgenic COS-7 cell lines expressing the gp91phox cDNA in the absence or presence of p22phox, except that in COS-7 cells, a $\sim$58-kDa form of gp91phox which corresponds in size to the core gp91phox polypeptide was the prominent species detected in absence of p22phox expression (Fig. 5, B and C). Membranes prepared from either 3T3 or COS-7 cells that coexpressed both gp91phox and p22phox supported superoxide production when mixed with neutrophil cytosol in the cell free NADPH oxidase assay, indicating that the two subunits formed a functional cytochrome $b_{556}$; membranes expressing either subunit alone were not active in this assay.2

For both 3T3 (not shown) and COS-7 cells transfected with a p22phox transgene (Fig. 5B), the p22phox polypeptide was expressed even in the absence of gp91phox and did not increase markedly in abundance with coexpression of recombinant gp91phox. Hence, the relative stability of the “free” p22phox polypeptide appears to differ from PLB-985 and primary neutrophils, where the genetic absence of gp91phox is associated with a marked reduction in p22phox protein expression (21, 29).

**DISCUSSION**

It has now been well established that a membrane-bound cytochrome $b_{556}$ and three cytosolic proteins, p47phox, p67phox, and Rac, are required for superoxide-generating activity of the phagocyte NADPH oxidase (1–4). In this study, acquisition of NADPH oxidase activity during granulocytic differentiation of PLB-985 cells was closely correlated with the increased expression of cytochrome $b_{556}$, p47phox, and p67phox. This fact has been described previously for differentiating HL-60 myeloid cells (40). The presence of SA, an inhibitor of heme biosynthesis, during PLB-985 differentiation produced a marked decrease in the expression of p22phox and mature gp91phox in the two subunits of cytochrome $b_{556}$ and in NADPH oxidase activity. This effect could be prevented by the addition of exogenous heme, was reversible upon removal of SA and did not affect the expression of other NADPH oxidase subunits or the $\beta_2$ integrin MAC-1 during granulocytic differentiation. Therefore, we conclude that the effect of SA on NADPH oxidase activity is a direct result of the disruption of heme synthesis and a concomitant decrease in cytochrome $b_{556}$ expression.

**FIG. 4.** The impairment in oxidase activity and cytochrome $b_{556}$ expression in PLB-985 cells differentiated in the presence of SA is reversible. PLB-985 cells were induced with DMF to differentiate for 6 days in the absence of SA (I), the presence of SA (II), and in the presence of SA from day 1 to day 3, which was then removed for continued DMF-induced differentiation from day 4 to day 6 (III). Cells were harvested at the indicated times and analyzed for NADPH oxidase activity and expression of oxidase components. Panel A, superoxide production in intact cells was determined by using a continuous cytometric assay that measures changes in flow cytometric parameters in response to an extracellular stimulus. Panel B, immunoblot analysis of whole cell extracts described in panel A, except that the blot was probed simultaneously with antibodies for p47phox and p67phox. The samples are loaded in the same order as in panel B.

**FIG. 5.** Transgenic expression of gp91phox and p22phox polypeptides in NIH 3T3 and COS-7 cells. Panels A and B, whole cell extracts from the indicated sources were examined by immunoblot analysis using gp91phox and p22phox monoclonal antibodies. WT, untransfected parental cell line; gp91phox, cells transfected with gp91phox-pEF-PGKpac; p22phox, cells transfected with p22phox-pEF-PGKneo; gp91phox/p22phox, cells transfected with both gp91phox-pEF-PGKpac and p22phox-pEF-PGKneo; PLB-985 (B9), PLB-985 cells differentiated with DMF for 5 days. Each lane was loaded with 10 g of protein. Panel C, cell membrane extracts from the indicated cell lines were either incubated with buffer alone (panel B) or digested with endo-$\beta$-N-acetylgalcosaminidase H (H) or peptide N-glycosidase F (F), then separated on SDS-polyacrylamide gel electrophoresis (12%) and analyzed by immunoblotting, probing simultaneously with gp91phox and p22phox monoclonal antibodies. The arrow indicates the $\sim$58-kDa core gp91phox protein. 10 g of membrane protein from clones transfected with gp91phox alone and 5 g of protein from clones transfected with both gp91phox and p22phox were loaded in each lane. 5 g of membrane protein was also loaded for PLB-985 samples.
In the only other report in which the effect of SA on phagocyte NADPH oxidase activity was studied, NADPH oxidase activity in differentiating HL-60 myeloid leukemia cells was also found to be reduced (26). Normal expression of gp91<sub>phox</sub> but markedly decreased p22<sub>phox</sub> expression was observed along with an increase in the relative level of p47<sub>phox</sub> (26). It is unclear why these latter observations differ from our results, which showed that inhibition of heme synthesis by SA resulted in a decrease in both mature gp91<sub>phox</sub> and p22<sub>phox</sub> without affecting expression of the non-heme-containing oxidase subunits p47<sub>phox</sub> and p67<sub>phox</sub> during granulocytic differentiation of PLB-985 and HL-60 cells. One explanation might be the different methods used to assess oxidase subunit expression. In the report by Henderson and co-workers (26), indirect immunofluorescence microscopy was used to monitor expression of the oxidase subunits, whereas we used immunoblotting of cell and membrane extracts. We found that although the mature 91-kDa form of gp91<sub>phox</sub> was virtually undetectable, expression of the 65-kDa high mannose precursor of gp91<sub>phox</sub> was unaffected by SA treatment. Hence, it is possible that the immunoreactive gp91<sub>phox</sub> species detected by immunofluorescence represents the high mannose gp91<sub>phox</sub> intermediate.

The NADPH oxidase cytochrome b<sub>558</sub> has been proposed to contain two heme groups, which mediate the final step in the formation of the 65-kDa high mannose precursor of gp91<sub>phox</sub> (41). Incorporation of heme is an essential step in the formation of gp91<sub>phox</sub>-p22<sub>phox</sub> heterodimer. We further propose that in the absence of heme incorporation into the cytochrome subunit results in virtually absent expression of the other cytochrome subunit (21).

Overall, these data are consistent with a model of cytochrome b<sub>558</sub> biosynthesis in which the heme prosthetic groups are incorporated into the cytochrome subunits, with N-linked complex carbohydrates and the 22<sub>phox</sub>-polypeptide being caused by post-transcriptional mechanisms in that the expression of gp91<sub>phox</sub> and p22<sub>phox</sub> mRNAs was not altered. In addition, SA-treated PLB-985 granulocytes expressed the ~65-kDa high mannose gp91<sub>phox</sub> intermediate at levels similar to those seen in non-SA-treated cells, indicating that translation of at least the gp91<sub>phox</sub> mRNA was not disrupted by inhibition of heme synthesis.

Overall, these data are consistent with a model of cytochrome b<sub>558</sub> biosynthesis in which the heme prosthetic groups play a requisite role in the interaction of the gp91<sub>phox</sub> and p22<sub>phox</sub> polypeptides and their subsequent stable expression and electron transport function in granulocytic cells. We propose that incorporation of heme promotes heterodimer formation either indirectly by facilitating proper folding of the 22<sub>phox</sub>- and gp91<sub>phox</sub> polypeptides or by acting as a direct dimerization agent. We further propose that in the absence of heme incorporation and heterodimer formation, p22<sub>phox</sub> and the mature gp91<sub>phox</sub> polypeptide are unstable in PLB-985 granulocytes, although the high mannose 65-kDa gp91<sub>phox</sub> precursor can still be detected. As discussed below, we have also shown that heterodimer formation is not required for maturation of gp91<sub>phox</sub> carbohydrate to fully processed, endo-β-N-acetylglucosaminidase H-resistant forms. Thus, the reduced expression of the mature 91-kDa form of gp91<sub>phox</sub> with inhibition of heme synthesis may not be caused directly by impaired intracellular processing of the 65-kDa high mannose intermediate but instead may reflect the instability of mature gp91<sub>phox</sub> in granulocytic cells in the absence of heterodimerization. Additional issues that remain to be clarified include whether heme incorporation occurs cotranslationally, as has been suggested for globin chain biosynthesis (42), or post-translationally, as has been shown for mitochondrial cytochrome c (43) and myeloperoxidase (44–46). The compartment in which the formation of p22<sub>phox</sub>-gp91<sub>phox</sub> complexes normally occurs as the newly synthesized cytochrome b<sub>558</sub> subunits are transported through the endoplasmic reticulum and Golgi to the plasma membrane is also as yet unknown.

Intracellular protein processing has been shown in other instances to be influenced by multimer assembly or by the incorporation of prosthetic groups. The formation of heterooligomers can affect protein turnover, and rapid degradation of unassembled subunits has been observed for the acetylcholine receptor and the T cell antigen receptor (47, 48). The insertion of prosthetic groups into apoprotein precursors can also play a role in peptide stability and post-translational processing. Newly synthesized chlorophyll apoproteins require chlorophyll for stable accumulation and maturation (49), and complete translation of cytochrome c across the outer mitochondrial membrane is closely coupled to attachment of heme (43). In neutrophils, the incorporation of heme has been shown to play an important role in the intracellular processing of myeloperoxidase, a hemoprotein whose mature form is located in azurophilic granules. In this case, the heme group is inserted post-translationally in an apoprotein precursor of myeloperoxidase in the endoplasmic reticulum; but if heme synthesis is inhibited by SA, the apoprotein fails to undergo transport to the lysosome and further proteolytic processing and is instead degraded (44–46, 50). Hence, it has been proposed that heme incorporation induces conformational changes in the apoprotein which is otherwise not processed correctly (44). Heme insertion and subsequent apoprotein processing have also been shown to be defective in a mutant form of myeloperoxidase with a R569W substitution due to a point mutation in the myeloperoxidase gene, which has been identified as a common cause of human myeloperoxidase deficiency (51).

Studies on COS-7 and 3T3 cells transfected with gp91<sub>phox</sub> and/or p22<sub>phox</sub> cDNAs indicate that the maturation of N-linked high mannose carbohydrate residues of gp91<sub>phox</sub> to complex oligosaccharides, a marker for transport through the Golgi compartment, does not require the formation of gp91<sub>phox</sub>-p22<sub>phox</sub> heterodimers, in contrast to what has been suggested previously on studies performed in B cell lines (22, 24). The unassembled p22<sub>phox</sub> and mature gp91<sub>phox</sub> polypeptides appear to be more stable in these non-phagocytic cells relative to PLB-985 and B cell lines, perhaps because of differences in the proteolytic environment. However, both for COS-7 and 3T3 cells, coexpression of both p22<sub>phox</sub> and gp91<sub>phox</sub> appeared to enhance stability of the mature 91-kDa form of gp91<sub>phox</sub>, consistent with what has been described previously in p22<sub>phox</sub>-deficient CGD B cell lines (22, 23). These data are also consistent with the original observations on neutrophil cytochrome b<sub>558</sub> expression in CGD, where genetic absence of either the gp91<sub>phox</sub> or p22<sub>phox</sub> cytochrome subunit results in virtually absent expression of the other cytochrome b<sub>558</sub> subunit (21).

In conclusion, the studies reported here provide indirect but compelling support for an essential role for heme incorporation in the assembly of the cytochrome b<sub>558</sub> heterodimer and provide further support for the concept that the stability of p22<sub>phox</sub> and the mature gp91<sub>phox</sub> subunit in granulocytic cells is increased by heterodimer formation.

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Biosynthesis of the Phagocyte NADPH Oxidase Cytochrome b558: ROLE OF HEME INCORPORATION AND HETERODIMER FORMATION IN MATURATION AND STABILITY OF gp91 phox and p22 phox SUBUNITS

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