

## Biosynthesis of the Phagocyte NADPH Oxidase Cytochrome $b_{558}$

ROLE OF HEME INCORPORATION AND HETERODIMER FORMATION IN MATURATION AND STABILITY OF gp91<sup>phox</sup> and p22<sup>phox</sup> SUBUNITS\*

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The NADPH oxidase cytochrome  $b_{558}$  is a membrane heterodimer comprised of a glycosylated 91-kDa subunit, gp91<sup>phox</sup>, and a nonglycosylated 22-kDa subunit, p22<sup>phox</sup>. The role of heme in cytochrome  $b_{558}$  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<sup>phox</sup> and the mature 91-kDa form of gp91<sup>phox</sup> 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  $b_{558}$  expression and NADPH oxidase activity was prevented by adding exogenous heme and was reversible upon removal of succinyl acetone. Transgenic expression of gp91<sup>phox</sup> in monkey COS-7 and murine 3T3 cells, both of which lacked endogenous p22<sup>phox</sup> mRNA, demonstrated that p22<sup>phox</sup> was not required for maturation of gp91<sup>phox</sup> carbohydrate to complex oligosaccharides. However, coexpression of transgenic p22<sup>phox</sup> increased the abundance of the mature gp91<sup>phox</sup> glycoprotein. These results suggest that heme incorporation plays an important role in cytochrome  $b_{558}$  assembly and provide further support for the concept that stability of p22<sup>phox</sup> and the mature gp91<sup>phox</sup> subunit is increased by heterodimer formation.

The phagocyte NADPH oxidase catalyzes the formation of superoxide ( $O_2^-$ ), 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 transfer of electrons from cytosolic NADPH to molecular oxygen (1, 2). It is now established that at least three cytosolic proteins (p47<sup>phox</sup>, p67<sup>phox</sup>, and Rac) and a membrane-associated heterodimer, cytochrome  $b_{558}$ , are involved in this electron transport system, although the detailed biochemical functions of each subunit and the mechanisms of assembly remain incompletely defined (1–4).

The NADPH oxidase cytochrome  $b_{558}$ , expressed almost exclusively in phagocytic cells, is comprised of two integral membrane proteins, a glycosylated 91-kDa subunit (gp91<sup>phox</sup>) and a

22-kDa subunit (p22<sup>phox</sup>) and contains both flavin and heme groups (5–11). This heterodimer is hence believed to be the redox center of the oxidase, although p67<sup>phox</sup> has recently been shown to contain an NADPH binding site that may be important for oxidase function (12). Multiple hemes, probably two, have been reported to be incorporated within cytochrome  $b_{558}$  purified from human neutrophils, with one residing in gp91<sup>phox</sup> and a second that may be shared by the two subunits (6, 13, 14). The heme prosthetic groups have been proposed to reside within the membrane in the relatively hydrophobic  $NH_2$ -terminal portion of the gp91<sup>phox</sup> 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<sup>phox</sup> contains regions with homology to the ferredoxin-NADPH<sup>+</sup> 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),<sup>1</sup> 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  $b_{558}$ . Defects in the X-linked gene of gp91<sup>phox</sup> account for about two-thirds of cases of CGD, whereas the gene encoding p22<sup>phox</sup> is the site of mutations in a rare autosomal recessive form of CGD. Genetic defects in either the p47<sup>phox</sup> or p67<sup>phox</sup> subunit account for the remainder of autosomal recessive CGD.

Relatively little is known about the biogenesis of the cytochrome  $b_{558}$  and the role of heme incorporation in this process. In virtually all patients with cytochrome  $b_{558}$  mutations, neutrophils lack both gp91<sup>phox</sup> and p22<sup>phox</sup> polypeptides, regardless of which subunit is affected by the mutation (20, 21). This observation has suggested that formation of the gp91<sup>phox</sup>-p22<sup>phox</sup> 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<sup>phox</sup> in B cell lines from p22<sup>phox</sup>-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<sup>phox</sup> intermediate has high mannose carbohydrate side chains, indicating that the initial steps of oligosaccharide addition and processing within the endoplasmic reticulum occur in the absence of p22<sup>phox</sup>. Expression of the mature 91-kDa form of gp91<sup>phox</sup> with fully processed oligosaccharide side chains can be restored in p22<sup>phox</sup>-deficient CGD B cell lines by expression of recombinant p22<sup>phox</sup> (22, 23). The high mannose

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

gp91<sup>phox</sup> precursor has also been detected in B cell lines from four unrelated X-CGD patients who otherwise lacked mature gp91<sup>phox</sup> (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 gp91<sup>phox</sup> either by interfering with incorporation of redox cofactors or by disrupting the association with p22<sup>phox</sup>.

The heme prosthetic groups have themselves been postulated to play a role in heterodimer formation and stability of cytochrome *b*<sub>558</sub>. The hemes are tightly bound to the cytochrome *b*<sub>558</sub> 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 *b*<sub>558</sub> spectrum was absent, and indirect immunofluorescence microscopy showed markedly decreased expression of p22<sup>phox</sup>, although gp91<sup>phox</sup> expression appeared normal. The identification of CGD patients with undetectable cytochrome *b*<sub>558</sub> who have point mutations in candidate heme-binding histidine residues within gp91<sup>phox</sup> or p22<sup>phox</sup> has also been taken as indirect evidence for a role of heme incorporation in cytochrome *b*<sub>558</sub> 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 *b*<sub>558</sub>, examining the role of both heme incorporation and heterodimer formation in the maturation and stability of gp91<sup>phox</sup> and p22<sup>phox</sup> subunits. We found that succinyl acetone (SA), an inhibitor of heme biosynthesis, reversibly reduced the expression of both p22<sup>phox</sup> and the mature 91-kDa form of gp91<sup>phox</sup> but not the 65-kDa precursor of gp91<sup>phox</sup>, 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 p47<sup>phox</sup>, p67<sup>phox</sup>, and Rac2 was unaffected. Transgenic expression of gp91<sup>phox</sup> in monkey COS-7 and murine 3T3 cells, both of which lack endogenous p22<sup>phox</sup> mRNA, demonstrated that the presence of the p22<sup>phox</sup> polypeptide was not required for maturation of gp91<sup>phox</sup> oligosaccharide side chains, although coexpression of transgenic p22<sup>phox</sup> increased the abundance of the mature gp91<sup>phox</sup> glycoprotein. Taken together, these data confirm that the incorporation of heme plays an important role in the assembly of the cytochrome *b*<sub>558</sub> and suggest that the stability of phagocyte p22<sup>phox</sup> and the mature gp91<sup>phox</sup> 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- $\beta$ -*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  $\mu$ g/ml streptomycin (Life Technologies, Inc.). For granulocytic differentiation, cells at a starting density of  $1 \times 10^5$  cells/ml were exposed to 0.5% DMF in CO<sub>2</sub>-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 deter-

mined 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-aminolevulinic acid in heme biosynthesis (30). In experiments using SA, PLB-985 or HL-60 cells were differentiated with DMF in the presence of 10  $\mu$ g/ml SA as described previously (26). Cell growth and viability were similar in cells differentiated in either the presence and absence of SA. After a 6-day incubation, control DMF-induced PLB-985 cells increased to  $235 \pm 17\%$  ( $n = 3$ ) of the original cell number and were 93% viable based on trypan blue exclusion. SA treated, DMF-induced PLB-985 cells multiplied to  $246 \pm 13\%$  ( $n = 3$ ) of the initial inoculum and were 92% viable. Both SA-treated and untreated DMF-induced cells had similar morphology by cytospin and light microscopy. For SA reversal studies, PLB-985 cells cultured for 3 days in the presence of DMF with SA were collected by centrifugation at  $500 \times g$  for 5 min at room temperature and washed once with PBS, after which the cells were resuspended in fresh medium containing DMF, but no SA, and cultured further for 1, 2, or 3 days before harvest. In a separate experiment, DMF-induced differentiation of PLB-985 cells was carried out in the presence of 5  $\mu$ M hemin.

NIH 3T3 mouse fibroblasts and monkey kidney COS-7 cells were obtained from the ATCC (CRL-1658 and CRL-1651, respectively). COS-7 cells were grown in Dulbecco's modified Eagle's low glucose medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum (Sigma), 50 units/ml penicillin, and 50  $\mu$ g/ml streptomycin (Life Technologies, Inc.). 3T3 cells were cultured under the same conditions except newborn calf serum (Summit Biotechnology) was supplemented instead of fetal calf serum. Both of these cell lines are adherent and were scraped gently at about 90% of confluence for passage or harvesting.

**Transfection of 3T3 and COS-7 Cells with gp91<sup>phox</sup> and p22<sup>phox</sup> cDNAs**—For expression of recombinant gp91<sup>phox</sup> in non-phagocytic cell lines, a full-length human gp91<sup>phox</sup> cDNA was cloned into the *NotI* site of pEF-PGKpac, which is similar to the gp91<sup>phox</sup>-pEF-PGKneo mammalian expression vector (29) but contains a linked expression cassette for puromycin *N*-acetyltransferase instead of neomycin phosphotransferase. For expression of the p22<sup>phox</sup>, a full-length cDNA for human p22<sup>phox</sup> (23, 31) was cloned into the *NotI* site of pEF-PGKneo. DOTAP (Boehringer Mannheim) was used to mediate transfection of plasmid expression vectors essentially as recommended by the manufacturer. Briefly, 10  $\mu$ g of DNA in 100  $\mu$ l of sterile PBS was gently mixed with 100  $\mu$ l of DOTAP mixture consisting of 30  $\mu$ l of DOTAP reagent and 70  $\mu$ l of PBS. After a 10-min incubation at room temperature, the transfection mixture was mixed with 5 ml of fresh medium and added to the cells growing at  $\approx 60\%$  confluence in a  $100 \times 20$ -mm culture dish. The cells were transfected overnight, and the clones were selected by limiting dilution in the presence of the corresponding antibiotic (1.8 mg/ml G418 and 1  $\mu$ g/ml puromycin). To generate cell lines expressing both recombinant gp91<sup>phox</sup> and p22<sup>phox</sup>, p22<sup>phox</sup> pEF-PGKneo was transfected into clones that had been transfected previously with gp91<sup>phox</sup>-pEF-PGKpac.

**Measurement of NADPH Oxidase Activity**—Superoxide production by SA-treated and untreated differentiated PLB-985 cells was measured in a quantitative kinetic assay based on the reduction of cytochrome *c* after stimulation of cells with 0.1  $\mu$ g/ml phorbol myristate acetate. The assay was performed at 37 °C using a Thermomax microplate reader and associated SOFTMAX Version 2.02 software (Molecular Devices) as reported previously (29, 32). The nitro blue tetrazolium test on PLB-985 and HL-60 cells was performed as described previously to assay O<sub>2</sub><sup>-</sup> formation in individual cells by monitoring the intensity of formazan granules deposited from the reduction of nitro blue tetrazolium by O<sub>2</sub><sup>-</sup> (29).

**Cell Extraction and Subcellular Fractionation**—SA-treated and untreated differentiated PLB-985 and HL-60 cells were harvested by centrifugation at  $500 \times g$  for 5 min at 4 °C and washed once with cold PBS. Cells were adjusted to  $5 \times 10^7$ /ml and extracted in a Triton buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100) containing protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 20  $\mu$ g/ml chymostatin, 10  $\mu$ M leupeptin) with gentle shaking for 30 min at 4 °C. Samples were then spun at  $10,000 \times g$  for 3 min at 4 °C, and the supernatant was collected and used as cell extract. Membrane fractions from PLB-985 cells differentiated in the presence or absence of SA were prepared as described previously (33), except that cells at  $2.5 \times 10^7$ /ml in a volume of 0.5–1 ml were disrupted in 1.5 ml microtubes by sonication (Sonic 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 (Pierce). 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 gp91<sup>phox</sup> and p22<sup>phox</sup> (35) (kindly provided by D. Roos and A. Verhoeven) and rabbit polyclonal antibodies for p47<sup>phox</sup> (36) (kindly provided by D. Uhlinger), p67<sup>phox</sup> (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- $\beta$ -*N*-acetylglucosaminidase H or peptide *N*-glycosidase F was performed on membrane fractions under conditions suggested by the manufacturer. Briefly, a total of 10  $\mu$ g of membrane protein was treated with 500 units of enzyme for 2 h at 37 °C, then separated on SDS-polyacrylamide gel (12%), and electroblotted onto nitrocellulose membrane and probed with monoclonal antibodies to gp91<sup>phox</sup> and p22<sup>phox</sup>. Control membrane samples were treated similarly except that PBS was added instead of enzyme.

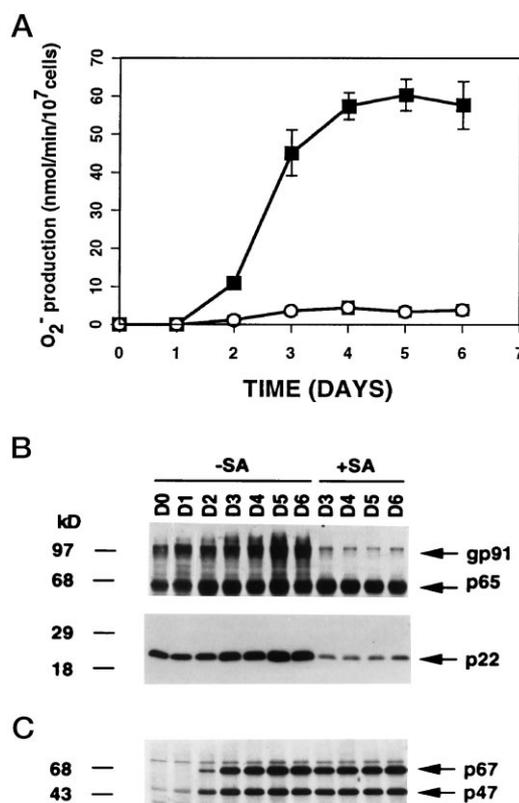
**Northern Blot Analysis**—Total cellular RNA from 3T3 cells, COS-7 cells, and SA-treated and untreated PLB-985 cells differentiated for indicated times was isolated as reported previously (39). After separation on a formaldehyde agarose gel (1%), RNA samples were transferred to Magnacharge nylon membrane (Micon Separations Inc., Westboro, MA) and hybridized with random primer-labeled human gp91<sup>phox</sup>, p22<sup>phox</sup>, and  $\beta$ -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  $\mu$ g/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 FACSscan (Becton Dickinson, San Jose, CA). Mouse IgG2b was used as an isotype control. A total of  $2 \times 10^5$  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  $\approx$ 5% activity detected at day 5 relative to non-SA-treated controls. The nitro blue tetrazolium test was also used to monitor O<sub>2</sub><sup>-</sup> 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).

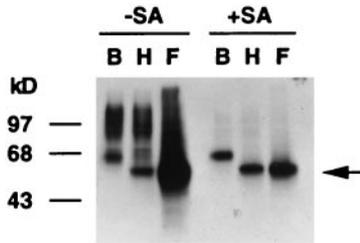
**Effect of Succinyl Acetone on Expression of NADPH Oxidase Subunits**—We hypothesized that the reduced NADPH oxidase activity in SA-treated PLB-985 and HL-60 cells was associated with the deficient expression of the oxidase cytochrome *b*<sub>558</sub> due to the inhibition of heme synthesis. The expression of NADPH oxidase subunits during DMF-induced granulocytic differentiation was examined in cell extracts by immunoblot analysis. The abundance of both the gp91<sup>phox</sup> and p22<sup>phox</sup> subunits of cytochrome *b*<sub>558</sub> 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*, O<sub>2</sub><sup>-</sup> 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  $\mu$ g/ml). The data represent the mean  $\pm$  S.D. of three experiments. *Panel B*, immunoblot analysis of whole cell extracts probed with monoclonal antibody for gp91<sup>phox</sup> (upper panel) or p22<sup>phox</sup> (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  $\mu$ g 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 p47<sup>phox</sup> and p67<sup>phox</sup>. 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  $\approx$ 65-kDa protein immunoreactive with the gp91<sup>phox</sup> monoclonal antibody was detected at a similar levels 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,<sup>2</sup> appeared to be equivalent with the 65-kDa high mannose precursor of gp91<sup>phox</sup> 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- $\beta$ -*N*-acetylglucosaminidase H, which only removes the high mannose form of *N*-linked carbohydrate, and peptide *N*-glycosidase F, which removes all *N*-linked carbohydrates. As seen in Fig. 2, endo- $\beta$ -*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  $\approx$ 58-kDa species. This species comigrates with the core

<sup>2</sup> L. Yu, L. Zhen, and M. C. Dinauer, unpublished observations.



**FIG. 2. Deglycosylation of PLB-985 granulocyte membranes.** Cellular membranes were isolated from PLB-985 cells differentiated into granulocytic forms by 5-day exposure to DMF in the absence ( $-SA$ ) or presence ( $+SA$ ) of SA. A sample of  $10 \mu\text{g}$  of membrane protein was incubated with buffer alone ( $B$ ) as a control or digested with endo- $\beta$ -N-acetylglucosaminidase H ( $H$ ) or peptide  $N$ -glycosidase F ( $F$ ), separated on SDS-polyacrylamide gel electrophoresis, and analyzed by immunoblotting with a gp91<sup>phox</sup> monoclonal antibody. The arrow indicates the  $\approx 58$ -kDa core gp91<sup>phox</sup> protein.

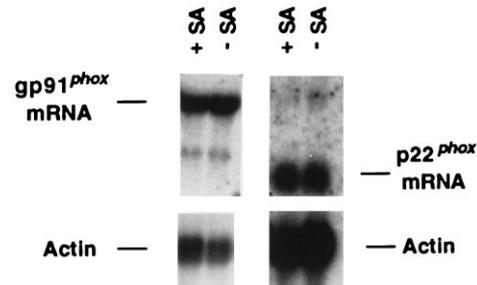
gp91<sup>phox</sup> protein seen after peptide  $N$ -glycosidase F digestion of membranes prepared from control PLB-985 cells (Fig. 2). Therefore, the 65-kDa form of gp91<sup>phox</sup> seen in both control and SA-treated PLB-985 granulocytes appears to be identical to the high mannose precursor of gp91<sup>phox</sup> reported previously (22).

In contrast to the expression of gp91<sup>phox</sup> and p22<sup>phox</sup> subunits, the expression of soluble NADPH oxidase components during DMF-induced granulocytic differentiation was unaffected by SA treatment. The p47<sup>phox</sup> 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 p67<sup>phox</sup> 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 p47<sup>phox</sup> and p67<sup>phox</sup> 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  $\beta 2$  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 gp91<sup>phox</sup> 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 gp91<sup>phox</sup> mRNA were not altered by SA treatment. Northern blot analysis for the expression of gp91<sup>phox</sup> as well as p22<sup>phox</sup> 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 p22<sup>phox</sup> and the mature form of gp91<sup>phox</sup> 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  $b_{558}$  Expression and NADPH Oxidase Activity by SA Is Reversible**—To confirm that the marked decrease in NADPH oxidase activity and expression of the p22<sup>phox</sup> and mature gp91<sup>phox</sup> polypeptides in SA-treated PLB-985 granulocytes specifically result from an impairment in heme synthesis, we supplied exogenous hemin at the onset of DMF-induced differentiation of PLB-985 cells in the presence and absence of SA. NADPH oxidase activity and cytochrome  $b_{558}$  expression were examined at day 5 (not shown). The in-



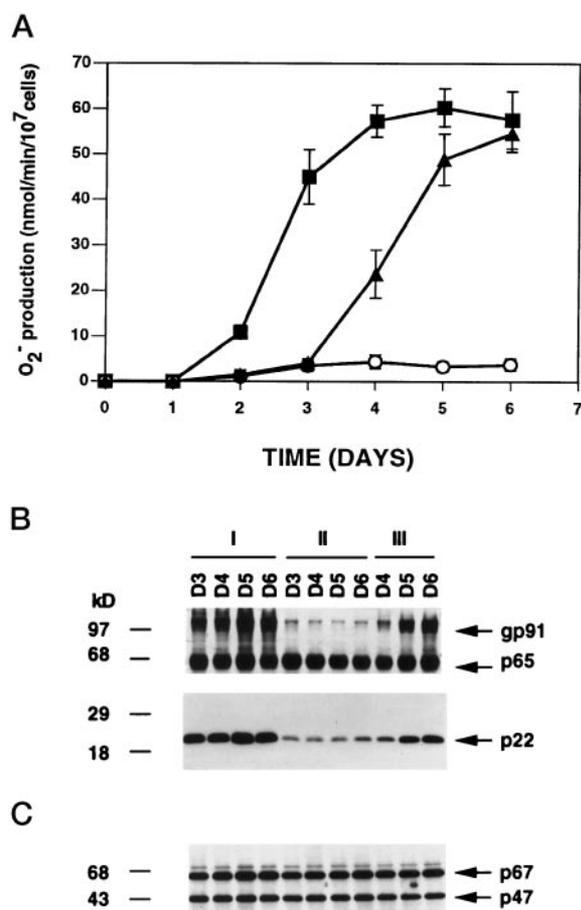
**FIG. 3. Expression of gp91<sup>phox</sup> and p22<sup>phox</sup> mRNAs in PLB-985 granulocytes.** Total cellular RNAs were isolated from PLB-985 cells differentiated by DMF for 5 days in the absence ( $-SA$ ) or presence ( $+SA$ ) of SA and analyzed by Northern blot analysis using human gp91<sup>phox</sup> (left) and p22<sup>phox</sup> cDNAs (right) as hybridization probes. The blots were also probed with  $\beta$ -actin cDNA (bottom) as a control for sample loading. A total of  $10 \mu\text{g}$  of RNA was loaded in each lane.

tensity of formazan staining in the nitro blue tetrazolium test for PLB-985 granulocytes differentiated in the presence of SA and exogenous heme was similar to control PLB-985 granulocytes. In addition, supplying exogenous heme also prevented the decreased expression of mature gp91<sup>phox</sup> and p22<sup>phox</sup> subunits of cytochrome  $b_{558}$  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  $b_{558}$  expression.

Whether the decreased expression in p22<sup>phox</sup> and the mature 91-kDa form of gp91<sup>phox</sup> 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 gp91<sup>phox</sup> and p22<sup>phox</sup> 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 gp91<sup>phox</sup>-p22<sup>phox</sup> heterodimer, resulting in the increased expression of mature gp91<sup>phox</sup> as well as its partner, p22<sup>phox</sup>. As expected, the differentiation-dependent increase in the levels of the cytosolic components p47<sup>phox</sup> and p67<sup>phox</sup> was unaffected by the presence or absence of SA (Fig. 4C).

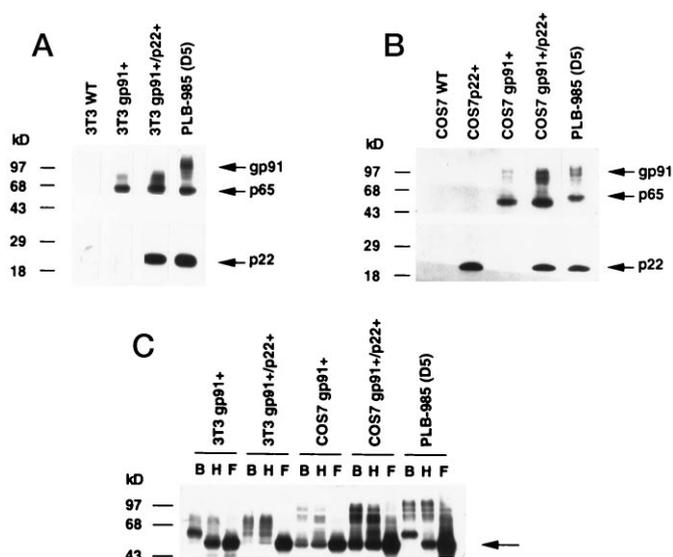
**Maturation of  $N$ -Linked Oligosaccharides in gp91<sup>phox</sup> Does Not Require Heterodimer Formation with p22<sup>phox</sup>**—To investigate further the role of gp91<sup>phox</sup>-p22<sup>phox</sup> heterodimer formation in maturation of the  $N$ -linked oligosaccharide side chains of gp91<sup>phox</sup> and stability of the two cytochrome  $b_{558}$  subunits, transgenic expression of p22<sup>phox</sup> and gp91<sup>phox</sup> 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 gp91<sup>phox</sup> carbohydrate side chains from high mannose to complex oligosaccharides is not dependent on an association of gp91<sup>phox</sup> with p22<sup>phox</sup>. However, coexpression of both p22<sup>phox</sup> and gp91<sup>phox</sup> appeared to increase the stability of the mature 91-kDa form of gp91<sup>phox</sup>.

Neither gp91<sup>phox</sup> nor p22<sup>phox</sup> 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 gp91<sup>phox</sup> cDNA, immunoblots of cell extracts were probed with a gp91<sup>phox</sup> monoclonal antibody. A prominent  $\approx 65$ -kDa band was seen (Fig. 5A), which was sensitive to endo- $\beta$ -N-acetylglucosaminidase H (Fig. 5C) and thus appears to correspond to the high mannose gp91<sup>phox</sup> intermediate. Smaller amounts of more slowly migrating



**FIG. 4. The impairment in oxidase activity and cytochrome  $b_{558}$  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 cytochrome *c* reduction assay at the indicated times. The data represent the mean  $\pm$  S.D. of three experiments. ■, -SA; ○, +SA; ▲, +SA days 1–3, -SA days 4–6. *Panel B*, immunoblot analysis of whole cell extracts collected at the indicated times were probed with monoclonal antibody for gp91<sup>phox</sup> (upper panel) or p22<sup>phox</sup> (lower panel). A total of 10  $\mu$ g of protein was loaded in each lane. I, -SA; II, +SA; III, +SA days 1–3, -SA days 4–6. *Panel C*, immunoblot analysis of the same cell extracts described in *panel B*, except that the blot was probed simultaneously with antibodies for p47<sup>phox</sup> and p67<sup>phox</sup>. The samples are loaded in the same order as in *panel B*.

gp91<sup>phox</sup> species were also detected (Fig. 5A) which were resistant to endo- $\beta$ -*N*-acetylglucosaminidase H (Fig. 5C), indicating that these contain mature, fully processed *N*-linked oligosaccharides. Subsequent transfection of 3T3-gp91<sup>phox</sup> clones with a second transgene harboring the p22<sup>phox</sup> cDNA resulted in a marked increase in the abundance of the larger gp91<sup>phox</sup> species with mature oligosaccharide side chains (Fig. 5, A and C). A similar result was seen for transgenic COS-7 cell lines expressing the gp91<sup>phox</sup> cDNA in the absence or presence of p22<sup>phox</sup>, except that in COS-7 cells, a  $\approx$ 58-kDa form of gp91<sup>phox</sup> which corresponds in size to the core gp91<sup>phox</sup> polypeptide was the prominent species detected in absence of p22<sup>phox</sup> expression (Fig. 5, B and C). Membranes prepared from either 3T3 or COS-7 cells that coexpressed both gp91<sup>phox</sup> and p22<sup>phox</sup> supported superoxide production when mixed with neutrophil cytosol in the cell free NADPH oxidase assay, indicating that the two subunits formed a functional cytochrome  $b_{558}$ ; membranes



**FIG. 5. Transgenic expression of gp91<sup>phox</sup> and p22<sup>phox</sup> 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 gp91<sup>phox</sup> and p22<sup>phox</sup> monoclonal antibodies. WT, untransfected parental cell line; gp91<sup>+</sup>, cells transfected with gp91<sup>phox</sup>-pEF-PGKpac; p22<sup>+</sup>, cells transfected with p22<sup>phox</sup>-pEF-PGKneo; gp91<sup>+</sup>/p22<sup>+</sup>, cells transfected with both gp91<sup>phox</sup>-pEF-PGKpac and p22<sup>phox</sup>-pEF-PGKneo; PLB-985 (D5), PLB-985 cells differentiated with DMF for 5 days. Each lane was loaded with 10  $\mu$ 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*-acetylglucosaminidase H (H) or peptide *N*-glycosidase F (F), then separated on SDS-polyacrylamide gel electrophoresis (12%) and analyzed by immunoblotting, probing simultaneously with gp91<sup>phox</sup> and p22<sup>phox</sup> monoclonal antibodies. The arrow indicates the  $\approx$ 58-kDa core gp91<sup>phox</sup> protein. 10  $\mu$ g of membrane protein from clones transfected with gp91<sup>phox</sup> alone and 5  $\mu$ g of protein from clones transfected with both gp91<sup>phox</sup> and p22<sup>phox</sup> were loaded in each lane. 5  $\mu$ g of membrane protein was also loaded for PLB-985 samples.

expressing either subunit alone were not active in this assay.<sup>2</sup>

For both 3T3 (not shown) and COS-7 cells transfected with a p22<sup>phox</sup> transgene (Fig. 5B), the p22<sup>phox</sup> polypeptide was expressed even in the absence of gp91<sup>phox</sup> and did not increase markedly in abundance with coexpression of recombinant gp91<sup>phox</sup>. Hence, the relative stability of the "free" p22<sup>phox</sup> polypeptide appears to differ from PLB-985 and primary neutrophils, where the genetic absence of gp91<sup>phox</sup> is associated with a marked reduction in p22<sup>phox</sup> protein expression (21, 29).

## DISCUSSION

It has now been well established that a membrane-bound cytochrome  $b_{558}$  and three cytosolic proteins, p47<sup>phox</sup>, p67<sup>phox</sup>, 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  $b_{558}$ , p47<sup>phox</sup>, and p67<sup>phox</sup>, similar to what 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 p22<sup>phox</sup> and mature gp91<sup>phox</sup>, the two subunits of cytochrome  $b_{558}$ , 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  $b_{558}$  expression.

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<sup>phox</sup> but markedly decreased p22<sup>phox</sup> expression was observed along with an increase in the relative level of p47<sup>phox</sup> (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<sup>phox</sup> and p22<sup>phox</sup> without affecting expression of the non-heme-containing oxidase subunits p47<sup>phox</sup> and p67<sup>phox</sup> 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<sup>phox</sup> was virtually undetectable, expression of the 65-kDa high mannose precursor of gp91<sup>phox</sup> was unaffected by SA treatment. Hence, it is possible that the immunoreactive gp91<sup>phox</sup> species detected by immunofluorescence represents the high mannose gp91<sup>phox</sup> intermediate.

The NADPH oxidase cytochrome  $b_{558}$  has been proposed to contain two heme groups, which mediate the final step in the transfer of electrons from NADPH to molecular oxygen to generate O<sub>2</sub><sup>-</sup> (6, 13, 14). Our results suggest that incorporation of heme plays an additional important role in the formation of the gp91<sup>phox</sup>-p22<sup>phox</sup> heterodimer. In this regard, it is noteworthy that Rotrosen and co-workers (41), in producing recombinant cytochrome  $b_{558}$  in Sf9 insect cells coinfecting with baculovirus vectors for p22<sup>phox</sup> and gp91<sup>phox</sup> expression, reported that heme supplementation was required for optimal expression of cytochrome  $b_{558}$ . In SA-treated PLB-985 cells, we observed that the reduced expression of both the mature 91-kDa form of gp91<sup>phox</sup> with *N*-linked complex carbohydrates and the p22<sup>phox</sup> polypeptide was caused by post-transcriptional mechanisms in that the expression of gp91<sup>phox</sup> and p22<sup>phox</sup> mRNAs was not altered. In addition, SA-treated PLB-985 granulocytes expressed the ≈65-kDa high mannose gp91<sup>phox</sup> intermediate at levels similar to those seen in non-SA-treated cells, indicating that translation of at least the gp91<sup>phox</sup> mRNA was not disrupted by inhibition of heme synthesis.

Overall, these data are consistent with a model of cytochrome  $b_{558}$  biosynthesis in which the heme prosthetic groups play a requisite role in the interaction of the gp91<sup>phox</sup> and p22<sup>phox</sup> 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 p22<sup>phox</sup> and gp91<sup>phox</sup> polypeptides or by acting as a direct dimerization agent. We further propose that in the absence of heme incorporation and heterodimer formation, p22<sup>phox</sup> and the mature gp91<sup>phox</sup> polypeptide are unstable in PLB-985 granulocytes, although the high mannose 65-kDa gp91<sup>phox</sup> precursor can still be detected. As discussed below, we have also shown that heterodimer formation is not required for maturation of gp91<sup>phox</sup> carbohydrate to fully processed, endo- $\beta$ -*N*-acetylglucosaminidase H-resistant forms. Thus, the reduced expression of the mature 91-kDa form of gp91<sup>phox</sup> 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<sup>phox</sup> 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<sup>phox</sup>-gp91<sup>phox</sup> complexes normally occurs as the newly synthesized cytochrome  $b_{558}$  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 translocation 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<sup>phox</sup> and/or p22<sup>phox</sup> cDNAs indicate that the maturation of *N*-linked high mannose carbohydrate residues of gp91<sup>phox</sup> to complex oligosaccharides, a marker for transport through the Golgi compartment, does not require the formation of gp91<sup>phox</sup>-p22<sup>phox</sup> heterodimers, in contrast to what has been suggested previously based on studies in B cell lines (22, 24). The unassembled p22<sup>phox</sup> and mature gp91<sup>phox</sup> 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<sup>phox</sup> and gp91<sup>phox</sup> appeared to enhance stability of the mature 91-kDa form of gp91<sup>phox</sup>, consistent with what has been described previously in p22<sup>phox</sup>-deficient CGD B cell lines (22, 23). These data are also consistent with the original observations on neutrophil cytochrome  $b_{558}$  expression in CGD, where genetic absence of either the gp91<sup>phox</sup> or p22<sup>phox</sup> cytochrome subunit results in virtually absent expression of the other cytochrome  $b_{558}$  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  $b_{558}$  heterodimer and provide further support for the concept that the stability of p22<sup>phox</sup> and the mature gp91<sup>phox</sup> subunit in granulocytic cells is increased by heterodimer formation.

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**Biosynthesis of the Phagocyte NADPH Oxidase Cytochrome *b*<sub>558</sub> : ROLE OF  
HEME INCORPORATION AND HETERODIMER FORMATION IN  
MATURATION AND STABILITY OF gp91 phox and p22 phox SUBUNITS**  
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