Biosynthesis of Flavocytochrome b$_{558}$

gp91$^{phox}$ IS SYNTHESIZED AS A 65-kDa PRECURSOR (p65) IN THE ENDOPLASMIC RETICULUM*

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The redox center of the phagocyte NADPH oxidase is flavocytochrome b$_{558}$, a transmembrane protein with two subunits, gp91$^{phox}$ and p22$^{phox}$. In this study we investigated the identity, subcellular localization, and maturation of a putative 65-kDa gp91$^{phox}$ precursor (p65). Expressing the gp91$^{phox}$ cDNA in an \textit{in vitro} transcription and translation system, we found that synthesis of p65 required endoplasmic reticulum (ER) microsomes. Sucrose density gradient centrifugation of postnuclear supernatants obtained from a PLB-985 derived cell line with a constitutively expressed gp91$^{phox}$ transgene demonstrated that p65 co-sedimented with the ER marker protein calreticulin and myeloperoxidase precursors. Unexpectedly, the majority of p22$^{phox}$ was found in subcellular compartments containing the mature 91-kDa form of gp91$^{phox}$ and not with p65, suggesting that heterodimer formation may occur in a post-ER compartment. The heme synthesis inhibitor, succinyl acetone, reduced the abundance of mature gp91$^{phox}$ and p22$^{phox}$ but had little or no impact on p65. These studies demonstrate (a) gp91$^{phox}$ is synthesized as a glycosylated 65-kDa precursor in the ER, (b) heterodimer formation is not a co-translational process, and (c) heme insertion is a determinant in the formation of a stable heterodimer but does not appear to affect the stability of p65.

Activated phagocytes release granule contents and generate reactive oxygen species to kill ingested microorganisms. Potent reactive oxygen species such as hydrogen peroxide and hypochlorous acid generated by phagocytes originate from a superoxide anion (O$_2^-$) produced by the NADPH-dependent oxidase (1). The NADPH oxidase is a multicomponent enzyme complex that includes an integral membrane protein, flavocytochrome b$_{558}$ (2–4), and four cytosolic protein components, p47$^{phox}$ (5–7), p67$^{phox}$ (5, 8), p40$^{phox}$ (9), and a small GTP-binding protein (Rac) (10, 11). In unstimulated phagocytes the oxidase is un-protected in SA-treated PLB-985 cells in which both p22$^{phox}$ and p65$^{phox}$ subunits were able to replace the intact gp91/p22 heterodimer in supporting O$_2^-$ production in a cell-free NADPH oxidase reconstitution assay, indicating that assembly of the fully functional enzyme complex requires specific interactions between subunits (21). In addition, stable expression of gp91$^{phox}$ in phagocytes closely correlates with expression of p22$^{phox}$ as has been observed in CGD patients with mutations in either flavocytochrome b$_{558}$ subunit (22, 23) and has also been shown by \textit{in vitro} studies using the heme synthesis inhibitor, succinyl acetone (SA) (24).

Clarification of the biosynthetic pathway of flavocytochrome b$_{558}$ is a logical step toward understanding the determinants for the association of gp91$^{phox}$ and p22$^{phox}$, which are important for both stability and function of the cytochrome. The mature form of gp91$^{phox}$ migrates as a broad band in SDS-PAGE with an average size of $\sim$91 kDa because of variable carbohydrate processing (25). In previous studies, we and others have described a 65-kDa immunologically related intermediate of gp91$^{phox}$ with high mannose carbohydrate side chains (p65) (24, 26, 27). This species was detected in SA-treated PLB-985 cells in which both p22$^{phox}$ and mature gp91$^{phox}$ are absent (24) and also in B-cell lines from p22$^{phox}$-deficient CGD patients (26). The mature form of gp91$^{phox}$ has post-translationally modified N-linked carbohydrate, which resists digestion with endoglycosidase H, indicating that side chains undergo additional processing by mannosidases in the Golgi complex (16, 24, 26). However, the actual sequence of events in the post-translational processing of gp91$^{phox}$ and the subcellular localization

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§ These abbreviations used are: CGD, chronic granulomatous disease; phox, phagocyte oxidase; SA, succinyl acetone; PAGE, polycrylamide gel electrophoresis; PNGase F, peptide N-glycosidase F; Endo H, endoglycosidase H; CRT, calreticulin; MPO, myeloperoxidase; ER, endoplasmic reticulum; \( \beta \)-COP, a 110-kDa subunit of the coat proteins.

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of the putative 65-kDa precursor have not yet been characterized.

We have previously established a cultured cell model of X-linked CGD by targeted disruption of the gp91<sub>phox</sub> gene in PLB-985 cells (28). After the stable transfection of wild-type gp91<sub>phox</sub> cDNA into X-CGD PLB-985 cells, expression of the putative 65-kDa precursor and mature gp91<sub>phox</sub> was constitutive, making it possible to monitor the relationship of their co-expression in biosynthetically active cells. These studies show that gp91<sub>phox</sub> is generated as a glycosylated 65-kDa precursor in the ER and further suggest that its association with p22<sub>phox</sub> is not co-translational but is augmented by heme insertion.

**EXPERIMENTAL PROCEDURES**

**Materials—**[35S]Methionine/cysteine (7.18 mCi/0.5 ml) was obtained from Amersham Pharmacia Biotech. Peptide N-glycosidase F (PNGase F), endoglycosidase H (Endo H), and fluorescein isothiocyanate-conjugated goat anti-mouse IgG were obtained from Boehringer Mannheim. A rabbit reticulocyte lysate kit for transcription and translation reactions was purchased from Promega, Inc. (Madison, WI). Mouse anti-CD11b-fluorescein isothiocyanate and mouse IgG2b-fluorescein isothiocyanate were from Immunotech, Inc. (Westbrook, ME). All other reagents were purchased from Sigma. Antibodies specific for gp91<sub>phox</sub> and p22<sub>phox</sub>, monoclonal antibodies 48 and 449, respectively, were kindly provided by D. Roos and A. Verhoeven, and monoclonal antibodies 54.1 and p22<sub>phox</sub> (control) for 5 days were stained with 7D5 (a generous gift of Dr. Michio Nakamura, Nagasaki University, Japan), a gp91<sub>phox</sub>-monoclonal antibody that reacts with an extracellular epitope of the cytochrome (32, 33). Surface expression of CD11b (Immunotech) was assessed in a similar fashion, and IgG1 and IgG2b were used as antibody isotype controls for 7D5 and anti-CD11b, respectively. Following staining, samples were analyzed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA). SDS-PAGE and Immunoblotting—Intact cells (5 × 10<sup>5</sup>/ml) were solubilized in a buffer containing 1% Triton X-100, 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 1 mM EDTA (24), and proteins were resolved by 12% SDS-PAGE (34). Alternatively, 20 μl of each sucrose gradient fraction was resolved by 10% SDS-PAGE. Proteins were transferred to nitrocellulose (35), and blots were probed with antibodies specific for gp91<sub>phox</sub> (p22<sub>phox</sub>, p47<sub>phox</sub>, p67<sub>phox</sub>, myeloperoxidase (MPO), CRT, and β-COP (see above). Immunoblots were developed using an enhanced chemiluminescence detection system (SuperSignal Substrate, Pierce) according to the manufacturer’s instructions.

**RESULTS**

The gp91<sub>phox</sub> Precursor, p65, Is Synthesized and Co-translationally Modified in Endoplasmic Reticulum—We and others have postulated that gp91<sub>phox</sub> is synthesized as a 65-kDa precursor (p65) in the ER and that subsequent carbohydrate processing in the Golgi complex yields mature gp91<sub>phox</sub> (24, 26). To identify the initial precursor of gp91<sub>phox</sub>, we used cDNA encoding gp91<sub>phox</sub> to prime biosynthesis in an in vitro transcription and translation system. We have previously demonstrated that in this system the nonglycosylated core polypeptide precursor of MPO is generated using MPO cDNA (30). When purified ER microsomes were then added to the system, both unglycosylated and high-mannose type N-linked glycosylated forms of precursor MPO were produced (30). Using gp91<sub>phox</sub> cDNA in the absence of ER microsomes, gp91<sub>phox</sub> was translated as a 58-kDa polypeptide (Fig. 1), a finding consistent with the size of the gp91<sub>phox</sub> core protein previously described by Parkos et al. (16) and others following removal of carbohydrate with PNGase F (24, 25, 26). In the presence of added ER microsomes, both the 58-kDa core polypeptide and p65 were generated (Fig. 1). Because generation of p65 was dependent on the presence of ER microsomes containing the necessary machinery for N-linked glycosylation, the data clearly indicate that p65 is a precursor of the 91-kDa forms of gp91<sub>phox</sub> and is synthesized in the ER. Using a similar assay system to delineate sites of N-linked glycosylation, Wallach and Segal (37) observed that gp91<sub>phox</sub> cDNA was translated into polypeptides of ~50 kDa in the absence of ER microsomes and variably glycosylated species of 53–60 kDa when ER microsomes were present. Thus, the identity of the primary translation product was not estab-
lished. By contrast, our data demonstrate that these species are 58 kDa when translated without microsomes and 65 kDa with added microsomes. The reasons for these differences are uncertain, but may be related to technical variations in the in vitro translation and/or gel electrophoresis system.

We have previously established a cultured cell model of X-linked CGD by targeted disruption of the gp91phox gene in PLB-985 myelomonoblastic cells (28). These cells are unable to produce O2 after granulocytic differentiation because of the absence of endogenous gp91phox (29). Stable transfection of X-CGD PLB-985 cells with the wild-type gp91phox cDNA under control of the constitutively active EF1α promoter resulted in continuous expression of gp91phox even in undifferentiated cells and restored O2 generating capacity upon granulocytic differentiation (28, 29). To further address the subcellular location of p65 in gp91phox-transfected cells (gp91 PLB), we separated subcellular organelles by sucrose density gradient centrifugation as described previously (30). Following centrifugation, 0.5-ml fractions were collected, and organelles were identified by the presence of specific marker proteins as described previously (30). ER (CRT and 90-kDa precursor MPO), lysosomes (59-kDa heavy subunit of mature MPO), Golgi (β-COP), plasma membrane (cell surface biotinylation), and cytosol (p47phox) were identified and compared with the sedimentation of gp91phox, p22phox, and p65 (Fig. 2A). Fractions containing peak levels of gp91phox (fractions 10–13) corresponded to those containing peak levels of p22phox (fractions 10–13) (Fig. 2A). gp91phox in fraction 12 was resistant to digestion with Endo H, indicating that its subcellular localization is likely that of a post-ER and/or post-Golgi vesicle, because it has already undergone carbohydrate modification (Fig. 2B). Fractions 7–9 contained peak levels of p65 that co-sedimented with ER markers CRT and precursor MPO (90 kDa) (Fig. 2A). In contrast to gp91phox, p65 was found to be susceptible to digestion with both Endo H and PNGase F consistent with previous reports (24, 26) and with its localization in the ER (Fig. 2B). Though peak levels of p65 were found in fractions 7–9, its decreasing distribution toward lesser density in the gradient was countered by the increasing distribution of gp91phox in those same fractions (fractions 7–13) (Fig. 2A). The overall distributions of p65 and gp91phox revealed an apparent ER-to-Golgi continuum separated in the sucrose density gradient as fraction 7 contained mainly p65, whereas fraction 13 contained only gp91phox (Fig. 2A, top panel). Only the mature form of gp91phox was found in fractions corresponding to the β-COP-associated organelles, implying that p65 had been completely processed to mature gp91phox prior to reaching the region of the Golgi associated with β-COP (Fig. 2A). It is notable that the distribution of β-COP may reflect only a subset of Golgi vesicles and/or part of the Golgi complex (Fig. 2A). We additionally observed that the majority of gp91phox and p22phox was intracellular with only a small fraction associated with biotinylated plasma membrane proteins (fractions 16–18) (Fig. 2A). This observation has been confirmed by immunofluorescence microscopy as well (29). Finally, p22phox distribution appeared unassociated with ER-associated p65, suggesting that heterodimer formation was not a co-translational event and likely occurred in a post-ER compartment (Fig. 2A).

Although both gp91phox and p22phox were associated with organelles that co-sedimented with those containing a 59-kDa heavy subunit of mature MPO (lysosomes and/or azurophilic granules), we found that the intracellular pool of gp91phox and p22phox was not lysosomal, because plasma membrane expression of the cytochrome was not up-regulated when these cells were differentiated and then treated with dihydrocytochalasin B and 1 mM formylmethionylleucylphenylalanine (data not shown). However, β-glucuronidase, a lysosomal marker, was released following treatment with dihydrocytochalasin B and formylmethionylleucylphenylalanine, indicating that degranulation had occurred (data not shown). Therefore, the intracellular pool of gp91phox and p22phox was not contained within lysosomal or azurophilic granule membranes.

Heme Insertion Augments gp91/p22 Heterodimer Formation

FIG. 2. Subcellular distribution of gp91phox, p22phox, and p65. Postnuclear supernatants of undifferentiated gp91 PLB cells expressing gp91phox were separated by centrifugation on 10–60% sucrose gradients as described under “Experimental Procedures.” A, gradient fractions were resolved by 10% SDS-PAGE and then probed with antibodies to gp91phox, p22phox, CRT, MPO, p47phox, and β-COP, as indicated. A gradient from PLB-985 X-CGD cells was included (gp91phox KO) to show nonspecific immunoreactivity just below 65 kDa. The bottom panel indicates distribution of surface-biotinylated proteins derived from gp91 PLB cells. Results are representative of at least three to four separate experiments.

B, 20–40 μl of the indicated gp91 PLB gradient fractions were digested with Endo H or PNGase F and separated by SDS-PAGE, and the immunoblots were probed with antibody to gp91phox. Results are representative of at least three to four separate experiments.

Heme Insertion Augments gp91/p22 Heterodimer Formation at the Post-translational Level—Synthesis of heme is essential for complete processing of hemoproteins such as MPO and for enzymatic activity (38–40). To determine whether inhibition of heme synthesis affected flavocytochrome b558 biosynthesis in gp91 PLB cells, we treated these cells with SA and examined the expression of gp91phox, p22phox, and p65 after 5 days of granulocytic differentiation with N,N-dimethylformamide. As shown in Fig. 3A, differentiated gp91 PLB cells developed
FIG. 3. Effect of SA on O$_2^-$-generating capacity and expression of NADPH oxidase components in differentiated gp91 PLB cells. Undifferentiated (D0) gp91 PLB cells or those differentiated (D5) for 5 days in the absence (−SA) or presence (+SA) of SA were analyzed for O$_2^-$-generating activity (A), expression of gp91$^{phox}$ and p22$^{phox}$ (B), or expression of p47$^{phox}$ and p67$^{phox}$ (C) by immunoblotting as described under “Experimental Procedures.” The 65-kDa precursor of gp91$^{phox}$ is indicated by an arrow. Results are expressed as the mean ± S.D. of at least three experiments.

significant O$_2^-$-generating capacity in comparison with that in undifferentiated cells that do not express the cytosolic oxidase components p47$^{phox}$ and p67$^{phox}$ (Fig. 3C). The O$_2^-$-generating capacity in differentiated gp91 PLB cells was reduced significantly upon SA treatment (Fig. 3A). Immunoblot analysis of whole cell extracts showed that decreased activity was associated with reduced expression of both p22$^{phox}$ and gp91$^{phox}$ (Fig. 3B). The induced expression of other oxidase components p47$^{phox}$ and p67$^{phox}$, non-heme-containing proteins, was unaffected by SA treatment (Fig. 3C). The SA-dependent reduction of transgenic gp91$^{phox}$ expression is consistent with our previous findings on endogenously expressed gp91$^{phox}$ in differentiated PLB-955 cells (24), providing further evidence that heme incorporation is important for stable expression of the gp91/p22 heterodimer. By contrast, expression of p65 was unaffected by SA treatment, which is a finding similar to what has been described in SA-treated PLB-955 cells (Fig. 3B) (24). Furthermore, these data suggest that p22$^{phox}$ does not interact with p65 in the absence of heme incorporation, because stable expression of p22$^{phox}$ is not supported by the continued presence of p65 in SA-treated cells.

Unaltered expression of p65 in SA-treated cells suggested that transcription and/or translation of gp91$^{phox}$ mRNA was not blocked by inhibition of heme synthesis. Northern blot analysis showed similar levels of transgenic gp91$^{phox}$ mRNAs in both undifferentiated and differentiated gp91 PLB cells in the absence or presence of SA (Fig. 4). The abundance of endogenous p22$^{phox}$ mRNA increased following differentiation and was unaffected by SA treatment (Fig. 4). Thus, the decreased expression of the 91-kDa form of gp91$^{phox}$ and of p22$^{phox}$ by SA was not because of reduced levels of gp91$^{phox}$ or p22$^{phox}$ transcripts. These data along with the presence of p65 in SA-treated cells suggest that heme plays a role in the post-translational processing of flavocytochrome b$_{558}$ rather than gene expression.

We have previously shown that the transgenic expression of gp91$^{phox}$ also resulted in its plasma membrane association, indicating it is fully processed in these cells (29). Because a small portion of mature gp91$^{phox}$ was found in the plasma membrane-enriched fractions of gp91 PLB cells (Fig. 2A) (fractions 15–18), reduced expression of mature gp91$^{phox}$ in SA-treated gp91 PLB cells should correlate with its reduced cell surface expression. To confirm this hypothesis, we examined plasma membrane expression of gp91$^{phox}$ in SA-treated or untreated differentiated gp91 PLB cells by flow cytometry using the monoclonal antibody, 7D5 (32, 33). 7D5 recognizes an extracellular epitope of gp91$^{phox}$ (21). As shown in Fig. 5A, cells not treated with SA contain surface-expressed gp91$^{phox}$. In contrast, treatment with SA reduced gp91$^{phox}$ surface expression to a level comparable with that of the IgG1 control antibody, a finding consistent with data obtained by immunoblotting (compare Figs. 3B and 5A). Decreased expression of gp91$^{phox}$ by SA treatment was specific for heme-containing proteins, as the cell surface expression of the b$_2$-integrin (CD11b/CD18), a non-heme-containing heterodimer, was unaffected, demonstrating that normal protein processing was unaffected by SA treatment (Fig. 5B).

Discussion
Flavocytochrome b$_{558}$ is primarily expressed in phagocytic cells. In neutrophils, it has been well established that ~85% of the cytochrome is found in the membranes of specific granules and gelatinase-containing granules with the remainder residing in the secretory vesicles and plasma membrane (31, 41). Stability of flavocytochrome b$_{558}$ is dependent on co-expression of both gp91$^{phox}$ and p22$^{phox}$ (16, 22). Recently, a putative 65-kDa precursor (p65) of gp91$^{phox}$ was described in Epstein-Barr virus-transformed B-cell lines derived from individuals with X-CGD and p22$^{phox}$-deficient CGD (26, 27) and also in the

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digestion (24); however, mature gp91
somes. Previously, we found that p65 contained high mannose-
phox expressed. No 58-kDa gp91
gp91
phox in the transfected cells, suggesting that the addition of aspara-
phox. No 58-kDa gp91
phox was undetectable in mature neutrophils, though antibodies specific for the peptide backbone of gp91
phox was Endo H-resistant, consistent with its acquisition of complex carbohydrate side chains after further processing in the Golgi complex (16, 24).

The distribution of p22phox in sucrose gradients paralleled that of the 91-kDa form of gp91phox which suggested that p22phox did not associate with p65 in the ER but that heterodimer formation occurs later in flavocytochrome b558 biosynthesis. Our co-sedimentation data are consistent with the observations in SA-treated gp91 PLB cells in which p65 was persistently expressed in contrast to the decreased expression of p22phox and the mature 91-kDa form of gp91phox and also supported by the observations of Porter et al. (26) who were able to detect p65 in p22phox-deficient CGD individuals who lacked the 91-kDa form of gp91phox secondarily. We have previously demonstrated that both complete processing of N-linked carbohydrates and correct targeting of gp91phox to plasma membranes can be achieved in the absence of p22phox in non-myeloid COS7 cells (24, 21), indicating that an association of p65 with p22phox is not a prerequisite for these events. These earlier findings are consistent with our current results, because co-sedimentation of p22phox with gp91phox in sucrose gradients is not observed until gp91phox is modified to its 91-kDa form (Fig. 2A) (fractions 9–14). The compartment in which heterodimer formation occurs between p22phox and gp91phox or an intermediate species as it is processed from its p65 to mature 91-kDa form is likely to be post-ER or Golgi. Heterodimer formation appears to be important for increased stability of both subunits against degradation in the proteolytic environment in phagocytes and may also facilitate the subsequent targeting of the gp91/p22 heterodimer to the plasma membrane or, in neutrophils, specific granule membranes.

In SA-treated cells, p65 was more stable than either p22phox or mature gp91phox, suggesting that heme incorporation augments heterodimer formation in an as of yet undefined way. Previous studies using p22phox-deficient lymphoblasts also revealed that the abundance of a 65-kDa protein detected with antibody specific for gp91phox was unaffected in the absence of p22phox (26); however, formation of the mature form of gp91phox could be rescued by expression of transgenic p22phox (26). Because p22phox neither binds heme nor is glycosylated, its stability appears to be regulated by the abundance of heme-containing gp91phox. Heme insertion has no impact on p65 stability either because it occurs during or after carbohydrate modification to produce the 91-kDa form of gp91phox or because heme-associated gp91phox is required for subsequent p22phox binding. Because we have recently shown that both heme prosthetic groups are contained solely within gp91phox, neither heme is directly involved in dimerization but may impart a required conformation for heterodimer formation (21). We recently reported that impaired MPO biosynthesis because of a missense mutation (R569W) resulted in the inability of MPO to acquire heme and subsequent peroxidase activity in transfected K562 cells (40). In those cells, MPO containing the R569W mutation (R569W) resulted in the inability of MPO to acquire heme and subsequent peroxidase activity in transfected K562 cells (40). In those cells, MPO containing the R569W mutation was not processed to mature heavy and light subunits, suggesting that heme insertion was necessary for protein maturation (40). In a similar fashion, impaired heme insertion resulting from SA treatment blocked heterodimer formation in flavocytochrome b558. Because p65 expression is unaffected by SA treatment and heterodimers are not formed, the fate of p65 synthesized in the absence of heme synthesis is unknown.

Further studies are necessary to elucidate how the association between gp91phox and p22phox promotes their increased expression in comparison with that of the unassembled subunits. The sites of interaction between the two subunits have not yet been described, and further studies will also be needed to address the timing of heme insertion and its role in flavocytochrome b558 stability. It is likely that observations in this

human promyelocytic leukemia cell line, PLB-985 (24). Although antibodies specific for the peptide backbone of gp91phox also recognize p65, p65 is undetectable in mature neutrophils, perhaps because of their low biosynthetic activity and the highly proteolytic environment.

Our current studies investigated directly the processing of gp91phox using gp91phox-transfected PLB-985 X-CGD cells in which mature gp91phox as well as p65 were constitutively expressed. No 58-kDa gp91phox core polypeptide was detected in the transfected cells, suggesting that the addition of asparagine N-linked glycosylation was a co-translational process during biosynthesis. Our data from in vitro transcription and translation assays using gp91phox cDNA strongly support that gp91phox is synthesized and co-translationally modified as p65. These results are supported by the earlier work of Wallach and Segal (37) who found that the initial translational product of gp91phox cDNA is glycosylated in the presence of ER microsomes. Previously, we found that p65 contained high mannose-type carbohydrates as determined by its sensitivity to Endo H digestion (24); however, mature gp91phox was Endo H-resistant, consistent with its acquisition of complex carbohydrate

![Figure 5](http://www.jbc.org/)

**Fig. 5.** SA-dependent reduction of plasma membrane-associated flavocytochrome b558. Undifferentiated gp91 PLB cells not treated (−SA) or those treated (+SA) with SA for 5 days were stained with 7Di, which recognizes an extracellular epitope of gp91phox and was analyzed by flow cytometry (A). Mouse IgG1 was used as an isotype control in A. Alternatively, these cells were analyzed for surface expression of CD11b to determine the effect of SA on normal protein processing (B). IgG2b was used as an isotype control in B. Results shown are from one representative experiment performed three times.
system will have application to the biosynthesis and targeting of other heme-containing multisubunit proteins.

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