p22-phox–Deficient Chronic Granulomatous Disease: Reconstitution by Retrovirus-Mediated Expression and Identification of a Biosynthetic Intermediate of gp91-phox

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Chronic granulomatous disease (CGD) results from defects in the phagocyte nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, central to which is the membrane-bound cytochrome b-245. The cytochrome is composed of two protein subunits, the larger (gp91-phox) being deficient in X-linked CGD. In this study, we have analyzed expression of the cytochrome subunits in B-cell lines from two autosomal CGD patients for whom the disease is caused by deficiency of p22-phox, the smaller subunit. We report the presence of a 65-kD precursor of gp91-phox in the membrane fraction of both p22-phox–deficient cell lines, corresponding to the core protein with N-linked carbohydrate side chains in the high mannose form. Expression of p22-phox in these cells resulted in functional correction of NADPH oxidase. In addition, gp91-phox in the reconstituted cells was processed to its terminally glycosylated form. These data suggest that the association of the 65-kD gp91-phox precursor with p22-phox is a prerequisite for processing of the carbohydrate side chains to the complex form in the Golgi. The detection of this precursor will enable characterization of mutations disrupting the subunit interaction (either naturally occurring or derived by in vitro mutagenesis) and so aid in structure-function analysis of cytochrome b-245. Reconstitution of p22-phox–deficient cells shows the potential of gene therapy for this autosomal form of CGD.

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The phagocyte nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is a multicomponent system whose function is to reduce molecular oxygen to superoxide anion (O2) at the cell surface and within the phagocytic vacuoles of neutrophils and macrophages. Four components are specific to this system and defects in any of these cause chronic granulomatous disease (CGD), a rare inherited immunodeficiency resulting in inability to cope with bacterial and fungal infections. Two of the components are cytosolic (p47-phox and p67-phox) and of unknown function. The other two components, p22-phox and gp91-phox, are the small (or α) and large (or β) subunits, respectively, of a membrane b-type cytochrome. This cytochrome is termed b-245 or b558 after its midpoint potential of −245 mV and absorption peak at 558 nm. Flavin adenine dinucleotide is involved in the electron transport process, and sequence analysis indicates that the binding sites for this cofactor as well as for the substrate NADPH are formed primarily by the β-subunit. The binding site for heme has not been identified.

The gene for gp91-phox is on the X chromosome, whereas those for p22-phox, p47-phox, and p67-phox are all autosomal. Therefore, CGD has both X-linked (X-CGD) and autosomal (A-CGD) forms, the former accounting for two-thirds of patients. The majority of A-CGD cases are caused by defects of p47-phox, many of them resulting from a common mechanism. X-CGD defects encompass deletions, splicing mutations, and a variety of point mutations, which generally result in loss of gp91-phox and absence of the heme spectrum in patient neutrophils. Similarly, rare mutations in the p22-phox gene generally result in loss of both heme spectrum and protein expression. In both cases, there is concomitant loss of the second cytochrome component. There is a single known example for each subunit in which the mutation abrogates function but not expression. In each case, as for the normal cytochrome, the heme spectrum and both subunits are present. Little is known concerning the biosynthesis of cytochrome b-245; however, these data, in which absence of the heme spectrum correlates with loss of both constituent subunits regardless of which has the primary genetic lesion, suggest a mutual dependence for stability.

NADPH oxidase is also expressed and functional in B cells, although at low levels relative to neutrophils. Epstein-Barr virus (EBV)-transformed B-cell lines have proven useful reagents in the study of the disease, because those derived from CGD patients display the disease phenotype of failure to make O2. Such studies have shown that O2 production by B cells uses at least the components p22-phox, p47-phox, and gp91-phox of neutrophil NADPH oxidase and strongly suggest the equivalence of the system in both cell types. B-cell lines have been used as targets for the expression of both p47-phox and gp91-phox using retroviral vectors, resulting in reconstitution of function in the appropriate patient cell lines. Episomal EBV-based vectors have also been used for expression of p47-phox and p22-phox in B-cell lines.

In this study, we examined expression of the cytochrome b-245 subunits in B-cell lines and neutrophils derived from two A-CGD patients with p22-phox gene defects. Both cell lines were found to express a 65-kD high-mannose glycoprotein precursor of gp91-phox in the absence of p22-phox expression. This intermediate form of glycosylation is characteristic of the stage of processing in the endoplasmic reticulum (ER) without subsequent modification of the car-
hydrate side chains to the complex form in the Golgi. Retrovirus-mediated transfer of the p22-phox cDNA was used to restore expression of the α-subunit and consequently to enable completion of the posttranslational modification of the β-subunit. NADPH oxidase function was also reconstituted. These data suggest that the 65-kD species is a biosynthetic intermediate of mature gp91-phox and that association with the α-subunit is a prerequisite for completion of β-subunit processing. This association most likely occurs in the ER or at the ER/Golgi interface.

MATERIALS AND METHODS

CGD patient-derived B-cell lines. The EBV-transformed B-cell lines used in this study were derived from two patients (R.H. [female] and A.S. [male]) with A-CGD caused by p22-phox deficiency and from one patient (D.D.) with X-CGD. All these cell lines are defective in O2·− production (see below). The A-CGD patients are unrelated. Diagnosis for patient R.H. was based on a lack of detection of p22-phox in Western blot analysis of neutrophil extracts and an autosomal mode of inheritance (C. Casimir, personal communication, March 1992). The precise gene defects are unknown for this patient, but Southern blot, Northern blot, and polymerase chain reaction analyses indicate a small reduction in transcript size without detectable gene deletion, suggestive of a splicing defect (Porter et al., in preparation). Diagnosis for patient A.S. was confirmed by functional complementation in monocyte hybrids, and the defect is known to be a homozygous point mutation resulting in substitution of Gln for Arg at amino-acid position 90 of p22-phox.

Western blot analysis of p22-phox and gp91-phox expression. Cellular membrane fractions were prepared by hypotonic lysis, and the proteins (50 μg) were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 6% acrylamide gels for the analysis of gp91-phox or 12% for the analysis of p22-phox). Western blot analysis, after transfer to Hybond-C Nelson, (Amersham International, Buckinghamshire, UK) by semidybl blotting, was as described, using either monoclonal antibody (MoAb) 48 (for p22-phox) or 449 (for p22-phox) and enhanced chemiluminescence detection. For some experiments, filters were stripped by incubation for 30 minutes at 50°C in 62.5 mmol/L Tris-HCl (pH 7.4), 2% SDS, and 100 mmol/L β-mercaptoethanol, were washed thoroughly, and were reused with an antibody specific for CD45 (Bra-11; Sigma Immunochemicals, St. Louis, MO). To examine the glycosylation status of proteins detected with MoAb 48 membrane preparations were denatured and treated with peptide:N-glycosidase F (PNGaseF) or endoglycosidase H (endoH; both obtained from New England Biolabs, Beverly, MA) before SDS-PAGE and Western blot analysis. The proteins (50 μg) were digested with 2,000 U for 2 hours at 37°C in 20 μL reaction mixture containing 50 mmol/L Tris (pH 8.0), 2 mmol/L MgCl2, 2 mmol/L CaCl2, 10 mmol/L β-mercaptoethanol, 50 μg/mL each of PNGaseF and endoglycosidase H, 10 μL of a 20 mg/mL solution of tunicamycin for 48 hours to inhibit N-linked glycosylation. In this study, we similarly analyzed B-cell lines from two patients (R.H. and A.S.) with p22-phox-deficient A-CGD using either this antibody or MoAb 449, which detects p22-
Fig 1. Western blot analysis of p22-phox expression with MoAb 449 (A) and of gp91-phox expression with MoAb 48 (B) in cellular membrane fractions. N, unaffected B-cell line; RH and AS, B-cell lines from p22-phox-deficient A-CGD patients R.H. and A.S; and DD, B-cell line from gp91-phox-deficient X-CGD patient D.D. Membrane fractions from patient B-cell lines transduced with Mβ1.SP (α) or Mβ2.SP (β) were also analyzed. The positions of protein size markers (in kilodaltons [Kd]) are indicated. A signal of heterogeneous high molecular weight detected with MoAb 48 indicates expression of mature, variably glycosylated gp91-phox.

**phox.** Both cell lines lack expression of p22-phox but express a 65-kD species that was detected with MoAb 48 (Figs 1A and B). The lack of signal heterogeneity suggests that this species is not variably glycosylated. Treatment of membrane preparations from either of the A-CGD cell lines with PNGaseF resulted in loss of the 65-kD species and in the appearance of the 55-kD core protein, showing that the reduction in molecular weight is not caused by truncation of the protein moiety (Fig 2). To examine further the nature of the 65-kD species, we used digestion with the enzyme endoH, which also removes N-linked carbohydrate but is specific for high-mannose modifications and does not remove carbohydrate that has been processed to the complex form. Consistent with this specificity, treatment with endoH did not affect the signal detected with MoAb 48 in membrane preparations from unaffected cells. However, such treatment resulted in a reduction in size of the 65-kD species detected in the A-CGD samples to a size indistinguishable from the 55-kD core protein generated by PNGaseF digestion (Fig 2).

The core protein of gp91-phox has five putative N-linked glycosylation sites, three of which are predicted to be extracytoplasmic and are, therefore, potentially glycosylated. If all three sites were modified and in the high-mannose configuration, the molecular weight would be expected to increase by approximately 5 to 6 kD. The data are not inconsistent with this, given the reduced apparent molecular weight
Normal | RH | RH/α | AS | AS/α
---|---|---|---|---
C | H | F | C | H | F | C | H | F | C | H | F

Kd
120 | 80 | 46 | 35

Fig 2. Glycosidase analysis of gp91-phox expression. Cellular membrane fractions were digested with PNGaseF (F) or endoH (H) before Western blot analysis with MoAb 48, as in Fig 1B. Controls (C) were treated equivalently but without addition of enzyme. The membrane fractions were prepared from an unaffected B-cell line (normal), from the two B-cell lines from p22-phox-deficient A-CGD patients R.H. and A.S., and from these same cell lines after transduction with MBα.SP for reconstitution of p22-phox expression (RH/α and AS/α). The positions of protein size markers (in kilodaltons [Kd]) are indicated. The faint signal detected at 120 kD, also present in the B-cell line from X-CGD patient D.D. (see Fig 1B), is nonspecific.

of the core protein (55 kD) compared with its calculated molecular weight (65 kD), bearing in mind that electrophoretic mobility is only an indication of molecular weight. To quantitate the abundance of the gp91-phox precursor in B-cell lines, membrane preparations were treated with PNGaseF to allow comparison of signal strength resulting from detection of the core protein. Varying amounts of digested preparations from an unaffected cell line were used to facilitate this estimation. Such experiments indicated an approximate relative abundance of 5% for the precursor in A-CGD cell lines compared with that of the mature glycoprotein in unaffected cell lines (Fig 2 and data not shown). A trivial explanation for the inability of these A-CGD cell lines to complete the processing of carbohydrate side chains from the high-mannose to the complex form during the synthesis of gp91-phox would be a generalized defect in the glycosylation pathway. To test this explanation, membrane preparations were analyzed for CD45 expression by Western blot analysis. This surface glycoprotein exists in several high molecular weight isoforms and was detectable at comparable levels in the A-CGD as well as the unaffected and X-CGD B-cell lines (data not shown), showing an intact glycosylation pathway.

The foregoing data suggest that both A-CGD cell lines express a high-mannose, modified gp91-phox precursor, consistent with synthesis, posttranslational modification, and transport as far as the ER but with no subsequent modification in the Golgi. Therefore, completion of the posttranslational modification process of the β-subunit of cytochrome b_245 is apparently dependent on the presence of the α-subunit.

p22-phox and gp91-phox expression in patient neutrophils.

The observation of a stable precursor of gp91-phox in the absence of p22-phox in these A-CGD B cell lines was unexpected in the light of published data that show correct expression of these two subunits is mutually dependent in neutrophils. We were also able to detect expression of p22-phox in the X-CGD B-cell line D.D., in the absence of gp91-phox expression (Fig 1A), which similarly contradicted this notion. A neutrophil extract from patient R.H. was made available to us for Western blot analysis (kindly provided by M. Chetty, University College London School of Medicine, London, UK). As with patient B-cell line membrane preparations, a PNGaseF-sensitive, endoH-sensitive 65-kD species was detected (Fig 3). However, this species was less abundant in neutrophils than in the B cells. After PNGaseF digestion, the level of gp91-phox core protein in patient neutrophils indicated a relative abundance of the precursor of less than 1% (Fig 3). These data indicate that the mutual dependence of the cytochrome subunits is greater in neutrophils, perhaps reflecting a more proteolytically active environment and/or less ER membrane in these terminally differentiated cells.

Retroviral transduction of A-CGD B-cell lines for reconstitution of p22-phox expression: Analysis of gp91-phox expression. To study further the apparent dependence of maturation of gp91-phox on p22-phox, we reconstituted p22-phox expression in both A-CGD B-cell lines (R.H. and A.S.). Because of our interest in developing a gene therapy approach to CGD, we used retrovirus-mediated gene transfer. An X-CGD B-cell line (D.D.) was also used as a target for infection to control for specificity. As a further control, the cell line R.H. was similarly transduced with a retrovirus mediating expression of gp91-phox, as used previously to reconstitute X-CGD B-cell lines. The retroviruses used endowed resistance to paromycin, allowing for selection of resistant cell populations. The structures of the respective proviruses MBα.SP and MBβ.SP are shown in Fig 4A. DNA from transduced cells was digested with Kpn I, and Southern blot analysis confirmed the presence of provirus at approxi-
RECONSTITUTION OF p22-PHOX–DEFICIENT CGD

We examined protein expression in cellular membrane fractions prepared by hypotonic lysis of the transduced B-cell lines. Western blot analysis indicated that p22-phox was synthesized and present at levels comparable with those detected for unaffected B cells in all three B-cell lines transduced with MBα.SP (Fig 1A). This protein was not detected in R.H. transduced with MBβ.SP; therefore, MBα.SP efficiently and specifically directed p22-phox expression. Fully mature gp91-phox was detected in the A-CGD, but not the X-CGD, B-cell lines transduced with MBα.SP (Fig 1B), at levels similar to those for unaffected B cells (by comparison of levels of deglycosylated protein; see Fig 2, and data not shown). Analysis of R.H. transduced with MBβ.SP did not show such high molecular weight material but showed the 65-kD gp91-phox precursor, as was the case for nontransduced R.H., which is consistent with the absence of p22-phox in these cells. The glycosylation status of gp91-phox in corrected A-CGD B-cell lines was confirmed by glycosidase digestion. In contrast to the presence of a PNGaseF- and endoH-sensitive 65-kD species in the nontransduced cells, heterogeneous material of high molecular weight that was PNGaseF-sensitive but endoH-resistant was detected (Fig 2). Therefore, restoration of p22-phox expression to the two A-CGD B-cell lines permitted completion of the posttranslational modification of gp91-phox, and the cells displayed an essentially normal phenotype with respect to expression of both subunits of cytochrome b-245. This experiment confirms that the glycosylation pathway in these cells is intact (see above).

Functional reconstitution of NADPH oxidase in transduced A-CGD B-cell lines. Consistent with the patient phenotype, the two A-CGD cell lines showed no response above baseline in chemiluminescence assays for NADPH oxidase activity. However, after reconstitution of p22-phox expression, cells transduced with MBα.SP were shown to be functionally restored using the sensitive luminol-based assay for H2O2 formed by dismutation of O2 (Fig 5). A-CGD B cells transduced with MBβ.SP were not functionally reconstituted. Similarly, functional reconstitution of the X-CGD B-cell line D.D. was observed after transduction with MBβ.SP but not with MBα.SP (Porter et al16 and data not shown). These experiments show that the function of cytochrome b-245 in active NADPH oxidase is absolutely dependent on the presence of both subunits. As was the case for unaffected B-cell lines, NADPH oxidase activity in reconstituted cells was shown to be via activation of protein kinase C, by virtue of its dose-dependence on PMA and its lack of response to the PMA-ME analogue. It was abrogated by the free-radical scavenger Tiron. Reconstituted cells also showed a response using the lucigenin-based chemiluminescence assay for O2; for which specificity was shown using superoxide dismutase and Tiron as inhibitors (Porter et al14 and data not shown).

DISCUSSION

The data presented here provide new information on the biosynthesis of cytochrome b-245. We have shown that both A-CGD B-cell lines used in this study, in the absence of expression of p22-phox, express a membrane-associated species of 65-kD molecular weight that is immunoreactive with the gp91-phox–specific MoAb 48. Glycosidase digestion sensitivity showed that this is a high-mannose precursor of gp91-phox. The simplest interpretation of these data is that, at least for B cells, this high-mannose intermediate of gp91-phox requires association with p22-phox, perhaps by virtue

![Glycosidase analysis](image-url)
Fig 4. (A) Schematic representation of the provirus in cells infected with the recombinant retrovirus MBα.SP (for expression of p22-phox) or MBβ.SP (for expression of gp91-phox). The positions of Kpn I (K) restriction sites and the BamH I (B) and Bgl II (Bg) restriction sites used during construction are indicated. (B) Southern blot analysis of Kpn I-digested DNA using the p22-phox cDNA probe. The high molecular weight fragment results from endogenous gene sequences, whereas the 2.4-kb fragment (arrow) indicates the presence of the MBα.SP provirus. HeLa and RH: DNA samples of HeLa cells or the B-cell line from patient R.H., nontransduced (−) or transduced with MBα.SP (α) or MBβ.SP (β). The provirus in the HeLa cell DNA was previously determined to be single copy per diploid genome. (C) An equivalent blot to that in (B) using the gp91-phox cDNA probe. The two high molecular weight fragments result from endogenous gene sequences, whereas the 4.5-kb fragment (arrow) indicates the presence of the MBβ.SP provirus.
of a necessity for heme incorporation, to enable translocation
to the Golgi for continuation of the process of posttransla-
tional modification. It is likely that this is also true for synthe-
sis in neutrophils, because the 65-kD intermediate was de-
tectable, albeit at low abundance, in an extract of neutrophils
obtained from one of the patients (R.H.). This reduced abun-
dance may reflect the more proteolytically hostile environ-
ment and reduced ER content of neutrophils. Expression of
p22-phox in the B-cell lines led to expression of mature
gp91-phox and functional reconstitution. During the comple-
tion of this report, a work describing reconstitution of p22-
phox-deficient A-CGD B-cell lines using an EBV-based
vector was published but did not report characterization of
a gp91-phox precursor as described here; however, a low
molecular weight immunoreactive protein was detected.

It is well established in the literature that the α- and β-
subunits of cytochrome b_{245} show a mutual dependence
for stable expression in neutrophils. Therefore, CGD patient
neutrophils for which the heme spectrum is missing lack
both subunits, regardless of whether the defect is in the gp91-
phox gene (X--CGD)\textsuperscript{3,4,9,11} or in the p22-phox gene (A--
CGD).\textsuperscript{8,10} A single case of X-CGD (with a Pro to His muta-
tion at amino-acid position 415 of gp91-phox) has been
described for which the heme spectrum is normal (X+-CGD);\textsuperscript{9}
both subunits were detected at normal levels.\textsuperscript{11} Similarly, a
single case of A-CGD caused by a p22-phox gene defect
(with a homozygous Pro to Gln mutation at amino-acid posi-
tion 156) has been described for which the heme spectrum
was normal; again, both subunits were expressed at normal
levels.\textsuperscript{12} It is likely that heme incorporation is required for
the structural integrity of the cytochrome heterodimer.

In no case have these neutrophil studies reported a stable
precursor of the β-subunit such as that described here. In
pursuance of the use of CGD patient-derived B-cell lines
as a gene therapy model, we have identified a biosynthetic
intermediate of the β-subunit in B-cell lines that requires
interaction with the α-subunit to enable further processing.
The precursor was also detected in A-CGD patient neutro-
phils at low abundance, implying the same biosynthetic path-
way but indicating a reduced stability in these cells. Simi-
larly, we have detected α-subunit apart from expression of
the β-subunit in X-CGD B-cell lines, although this has not
been observed in X-CGD patient neutrophils. Therefore, the
reduced interdependence of the cytochrome subunits in
B-cell lines has enabled the identification of a biosynthetic
intermediate of the β-subunit. Detection of this stable precu-
sor may facilitate structure-function studies of cytochrome
b_{245}. Point mutations in the gp91-phox gene that abolish
stable protein expression could be mutations for which the
β-subunit is intrinsically unstable, or for which it is unable
to associate with the α-subunit. The latter class of mutations
would be equivalent to the expression of the normal β-sub-
unit in p22-phox-deficient A-CGD cells. Therefore, screening
X-CGD B-cell lines for the presence of the 65-kD β-
subunit precursor would identify mutations disrupting the
interaction of the subunits and, thus, delineate regions of the
β-subunit involved in their association. Such regions would
be expected to include the heme-binding site and sites of
protein-protein contact. Similarly, given the recent success-
ful expression of gp91-phox,\textsuperscript{16} it would be possible to per-
form in vitro mutagenesis with expression in X-CGD B-cell
lines to identify such regions. We have expressed gp91-
phox in murine fibroblast (3T3) cells and have detected the
presence of both the precursor and mature forms, which may
be indicative of a weak association of murine p22-phox with
human gp91-phox (data not shown).
Our studies on the subunits of cytochrome b-245 in two A-CCG B-cell lines, and in these same lines after functional reconstitution, have enabled dissection of part of the pathway of the biosynthesis of this heterodimer. The data suggest the following model. The 55-kD beta-subunit core protein and the alpha-subunit are synthesized in the ER membrane. The beta-core protein is N-glycosylated at its luminal surface and processed to the high-mannose, modified form (65-kD) within the ER. It is not possible to determine whether the alpha/beta association normally occurs before or after N-glycosylation. However, this association must occur before exit from the ER and transfer to the Golgi. It is likely that the heme moiety is attached when the subunits associate, thus stabilizing this interaction. The processing of the carbohydrate side chains to the complex form occurs in the Golgi before the mature cytochrome is finally translocated and expressed at the cell surface. Another example of an oligomeric membrane-associated complex for which the completion of glycosylation of one subunit is dependent on association with another is the lymphocyte function-associated molecule-1/Mac-1/p150,95 leukocyte adhesion molecule family. It has also been proposed that the invariant chain (II) is required for major histocompatibility complex class II expression, because it facilitates movement through the ER/Golgi system.

We have previously used retrovirus-mediated transfer of the gp91-phox cDNA to functionally correct EBV-transformed B-cell lines derived from X-CCG patients, showing the feasibility of gene therapy for treatment of this disease. Retroviral vectors are currently the system of choice for such an approach. B-cell lines from A-CCG patients with defects in the gp91-phox expression have also been used as targets for correction using such vectors. In this study, we have used retrovirus-mediated expression of p22-phox to functionally reconstitute two B-cell lines derived from A-CCG patients with defects in the gene for this protein, which combines with gp91-phox to form cytochrome b-245, the membrane component of NADPH oxidase. We have shown membrane association of the expressed protein, completion of posttranslational modification of gp91-phox (see above), and appropriate regulation of the reconstituted system, as previously described for our reconstituted X-CCG B-cell lines.

This report extends the in vitro model system for gene therapy of CGD to encompass three of the four genetic forms of the disease. It is likely that reconstitution of p67-phox deficiency is also feasible and that all forms of CGD would be amenable to a gene therapy approach. Such a therapeutic option could offer significant advantages over current treatments, for which there is still a 50% mortality rate by the age of 20 years. One possibility is to target myeloid progenitors obtained from peripheral blood, as has recently been described for p47-phox--deficient A-CCG, although repeated therapeutic intervention would be required. Clearly, introduction of the appropriate cDNA into patient hematopoietic stem cells would be a desirable approach. Recent studies with retroviral vectors for expression of ADA or CD18 have shown efficient infection and functional correction in patient bone marrow grown in long-term culture in vitro. Preliminary data from our laboratory show that this is also possible for correction of X-CCG patient-derived bone marrow (in preparation).

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RECONSTITUTION OF p22-phox–DEICIENT CGD


p22-phox-deficient chronic granulomatous disease: reconstitution by retrovirus-mediated expression and identification of a biosynthetic intermediate of gp91-phox

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