Cloning of Murine gp91phox cDNA and Functional Expression in a Human X-Linked Chronic Granulomatous Disease Cell Line

By Helga Björngvinsdóttir, Ling Zhen, and Mary C. Dinauer

The phagocyte cytochrome b58a, a heterodimer comprised of gp91phox and p22phox, is a flavocytochrome that mediates the transfer of electrons from NADPH to molecular oxygen in the respiratory burst oxidase. The human gene encoding the glycosylated gp91phox subunit is the site of mutations in X-linked chronic granulomatous disease (CGD). Reverse transcriptase-polymerase chain reaction was used to obtain a full-length clone for the murine gp91phox cDNA, which was 87% identical to the human gp91phox cDNA. The encoded murine protein had 39 amino acids out of 570 that differed from the human, many of which were conservative substitutions. Nonconservative replacements occurred in hydrophilic regions outside of domains previously implicated in binding to NADPH, flavin, and the cytosolic oxidase subunit p47phox. Some substitutions altered potential N-glycosylation sites, which is likely to explain why the glycosylated murine protein migrates with an apparent molecular mass of 58 kD instead of 91 kD as seen for the human protein. Expression of murine gp91phox in a human myeloid cell line with a null gp91phox allele using a mammalian expression plasmid or a retroviral vector rescued stable expression of the p22phox subunit and fully reconstituted respiratory burst activity. This suggests that the murine gp91phox subunit forms a functional cytochrome b58a heterodimer with human oxidase subunits, consistent with the high degree of identity between the mouse and human proteins in domains implicated in cytochrome function.

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MATERIALS AND METHODS

Cell lines. PLB-985 cells24 and derivatives were maintained as described.25 X-CGD PLB-985 cells were previously generated by targeted disruption of the gp91phox gene, and lack endogenous gp91phox and respiratory burst activity.25 For granulocyte differentiation, logarithmically growing PLB-985 and derivative cell lines at a density of 1 to 3 x 10^6 cells/mL were exposed to 0.5% dimethylformamide (DMF) for 6 days.26 Retrovirus packaging lines GP + E6627 and GP+envAm1228 were provided by Dr A. Bank (Columbia University, New York, NY) and maintained in 50% Ham's F12 and 50% Dulbecco's modified Eagles medium (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (Sigma, St Louis, MO).

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infect GP using DOTAP (Boehringer Mannheim). Transfected packaging cells m91 plasmid was transfected into GP+E86 ecotropic packaging cells different vectors after changing the 5’ colonies. To estimate a quantitative titer, 3T3 cells were infected as G418-resistant m91neoAm2 cell population was used for subsequent amplifications were sequenced to ensure that the sequence obtained did not include PCR-induced mutations. Translation of the nucleotide sequence and best-fit alignments with the sequence for the murine gp91phox cDNA has been described. Synthetic oligonucleotide primers derived from the human gp91phox cDNA sequence, were prepared to amplify the murine gp91phox cDNA using PCR. 5'-ACAATCTCGAGACCATG GGG AAC TGG GCT GTG AAT GAA 3' (forward primer) and 5'-ACTTAGGATCCTTA GAA GTT TTC CTT GTT GAA AAT GAA GTG-3' (reverse primer). Sequences from three independent amplifications were sequenced to ensure that the sequence obtained did not include PCR-induced mutations. Translation of the nucleotide sequence and best-fit alignments with the human gp91phox sequence were performed using the Genetics Computer Group molecular genetics software (Program Manual for the Wisconsin Package, Version 8; Genetics Computer Group, Madison, WI). The full-length murine gp91phox cDNA was cloned into two different vectors after changing the 5’ Xho I site to EcoRI: the mammalian expression vector pEF-PGKneo to generate the plasmid, pEFneo-m91, and the plasmid shuttle vector for the human elongation-factor promoter,25 and a retroviral vector, MSCVneoEB, in which cDNA expression is driven by a modified Maloney leukemia virus LTR.2627

RESULTS

To generate a full-length murine gp91phox clone, synthetic oligonucleotide primers derived from the human gp91phox sequence were used to amplify the murine sequence from first-strand cDNA prepared from mouse macrophage mRNA. A single ~1,700-bp product was generated, whose nucleotide sequence was 87% identical to the human gp91phox cDNA* (not shown). The amino acid sequence of the encoded murine protein is shown in Fig 1, and is aligned with the human gp91phox sequence for comparison. The murine and human sequences are identical in 531 out of 570 amino acids, although note that the N-terminal 8 and C-terminal 9 amino acids in the murine sequence were specified by the oligonucleotide primers. Seventeen of the nonidentical amino acids represent conservative substitutions. Since the residues that are dissimilar between the two species involve consensus sequences for N-linked glycosylation (N-X-S/T), with only two potential sites shared between the murine and human sequences (Fig 1).

We investigated whether the murine gp91phox protein could rescue p22phox expression and participate in superoxide generation in human X-CGD PLB-985 cells. Two different expression systems were used: a mammalian plasmid expression vector, pEF-PGKneo, in which the cDNA is under control of the human elongation-factor 1-a promoter,26 and a retroviral vector, MSCVneoEB, in which cDNA expression is driven by a modified Maloney leukemia virus LTR.2627 pEFneo-m91 was introduced into X-CGD PLB cells by electroporation, and stable transfectants isolated by limiting dilution. Twelve clones were randomly chosen for analysis, of which seven were NBT-positive, which demonstrated both successful transfection of the entire transgene as well as functional reconstitution of respiratory burst activity. On Northern blot analysis, NBT-negative clones lacked mRNA for gp91phox (data not shown), which suggested that the transgene was nonfunctional in these cells. Four of the NBT-positive clones were chosen for further detailed analysis. Cell extracts were prepared from granulocyte-induced cells and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting to examine the expression of cytochrome b558 using antibodies for both gp91phox and gp91phox cDNA. Triton X-100 extracts and cellular membranes of nonadherent mouse bone marrow (BM) cells and of PLB-985 and derivative cells were prepared as previously described.2526 Protein concentration was measured by BCA protein assay (Pierce, Rockford, IL). On a per-cell basis, membrane extracts typically yield ~20% to 35% of the protein obtained from whole-cell extracts. Immunoblot analysis was performed as described28 using either affinity-purified p22phox antibody or C-terminal antibody for gp91phox as probes.32

Assay of superoxide formation. The nitroblue tetrazolium (NBT) assay was performed on granulocyte-induced PLB-985 cells or derivatives.29 For quantitative measurement of superoxide formation elicited by phorbol myristate acetate (PMA)-stimulated whole cells after granulocyte differentiation, the continuous cytochrome c assay was used.30
Fig 1. Amino acid sequence of murine and human gp91 phox sub-units of phagocyte cytochrome b$_{552}$. The amino acid sequence of the murine gp91 phox polypeptide as predicted from its cDNA sequence is shown, and is aligned with the human gp91 phox for comparison (solid lines denote identical residues, double dots indicate high degree of similarity, and single dots indicate lower degree of similarity). The alignment was performed using the Genetics Computer Group software program, Best Fit. Hydrophobic regions are enclosed in shaded rectangles. Potential N-linked glycosylation sequences (N-X-S/T) are indicated in both the murine and human sequences using dashed rectangles. Regions with homologies to the FNR family of flavoenzymes are indicated in the human sequence with solid lines, as follows: (a) flavin isoalloxazine ring; (b) flavin ribityl chain; (c) NADP pyrophosphate; (d) NADP ribose; (e) NADP 2'-phosphate, NADP ribose 2'-phosphate adenine ring; (f) nicotinamide C-4 atom.

and p22$^{phox}$. Untransfected X-CGD PLB-985 cells lack gp91$^{phox}$ and display markedly reduced levels of p22$^{phox}$ (Fig 2A, lane 6 and Fig 2B, lane 5). NBT-positive transfectants (Fig 2A, lanes 1 through 4) all expressed a protein of ~58 kD that was immunoreactive with the gp91$^{phox}$ antibody and which comigrated with the gp91$^{phox}$ species detected in non-adherent murine BM cells (of which ~50% are neutrophils and ~30% mononuclear phagocytes) (Fig 2A, lanes 7 and 8). There was some clone-to-clone variation in the level of expression, which had previously been seen for expression of recombinant human gp91$^{phox}$ using this vector. The relative level of recombinant murine gp91$^{phox}$ was estimated to be 15% to 30% of that detected in murine BM cells. Murine gp91$^{phox}$ was also expressed in X-CGD PLB-985 cells using a retroviral vector, MSCVneoEB. A G418-resistant population was collected for analysis. Membrane extract was prepared from granulocyte-induced cells for immunoblot analysis of gp91$^{phox}$ expression (Fig 2A, lane 5), which was comparable to that seen using the pEFneo-m91 vector. Finally, expression of recombinant murine gp91$^{phox}$ resulted in markedly increased levels of the p22$^{phox}$ subunit in human X-CGD cells, as assessed by immunoblotting (Fig 2B).

A continuous cytochrome c reduction assay was used to quantitate respiratory burst activity in granulocyte-induced X-CGD PLB-985 cells that expressed recombinant murine gp91$^{phox}$ (Table 1). As previously shown, X-CGD PLB cells lacked respiratory burst activity, whereas wild-type PLB-985 cells had levels similar to those seen in normal human neutrophils. Expression of recombinant murine

**Fig 2. Immunoblot analysis of expression of murine cytochrome b$_{552}$ subunits in human PLB-985 cell lines and murine BM cells. Membrane or whole-cell extracts were prepared from granulocyte-induced PLB-985 cells and derivatives and from wild-type mouse nonadherent BM cells. Molecular-weight standards and the estimated size of the recombinant human gp91$^{phox}$ species detected in non-adherent murine BM cells (of which ~50% are neutrophils and ~30% mononuclear phagocytes) are indicated in both the murine and human sequences using dashed rectangles. Potential N-linked glycosylation sequences (N-X-S/T) are indicated in both the murine and human sequences using dashed rectangles. Regions with homologies to the FNR family of flavoenzymes are indicated in the human sequence with solid lines, as follows: (a) flavin isoalloxazine ring; (b) flavin ribityl chain; (c) NADP pyrophosphate; (d) NADP ribose; (e) NADP 2'-phosphate, NADP ribose 2'-phosphate adenine ring; (f) nicotinamide C-4 atom.**

**MURINE gp91$^{phox}$-CDNA**

mouse 1 1 MDNMRNRLSFRYTPINLTVSGKDETVVTVCVNFRKQGKD30
human 1 1 MDNMRNRLSFRYTPINLTVSGKDETVVTVCVNFRKQGKD30
mouse 51 450 TYPFNYFRPLLYTGLSNFNLVYDEARNQGPDGCND30
human 51 450 TYPFNYFRPLLYTGLSNFNLVYDEARNQGPDGCND30
mouse 100 900 YNLNGVGRIVGIYGIAPSSLYIFYFIQGFC40
human 100 900 YNLNGVGRIVGIYGIAPSSLYIFYFIQGFC40
mouse 151 220 EVLNPARRK9I9VQVTVLVIQVLTV45
human 151 220 EVLNPARRK9I9VQVTVLVIQVLTV45
mouse 200 270 TVHFPRTLQEQQKDFEKQYVIVKPCVQKSL40
human 200 270 TVHFPRTLQEQQKDFEKQYVIVKPCVQKSL40
mouse 251 320 WGDIECSPFPAGQNNMDTVVEMYGPYQPVBR50
human 251 320 WGDIECSPFPAGQNNMDTVVEMYGPYQPVBR50
mouse 301 370 ISNHGRPGSRTFRKPTQKNTISN50
human 301 370 ISNHGRPGSRTFRKPTQKNTISN50
mouse 351 420 FSNHKRYVQAVLQNAQNLKQK40
human 351 420 FSNHKRYVQAVLQNAQNLKQK40
mouse 401 470 YEVQNVQIVITVYTFASVLKSVYQVCMN45
human 401 470 YEVQNVQIVITVYTFASVLKSVYQVCMN45
mouse 451 500 EIDPALDQQLEQMNQNLQVYLVQQA50
human 451 500 EIDPALDQQLEQMNQNLQVYLVQQA50
mouse 501 530 LTVQILKQYVRGPMONQFRTSKAISS30
human 501 530 LTVQILKQYVRGPMONQFRTSKAISS30
mouse 551 600 ISNHEDGGPFQSPFRKTPF570
human 551 600 ISNHEDGGPFQSPFRKTPF570
Table 1. Superoxide Generation by PLB-985 Cell Lines After Granulocytic Differentiation

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>( V_{max} ) (nmol O(_2)/min/10(^6) cells)</th>
<th>Cumulative O(_2) (nmol/30 min/10(^6) cells)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>56 ± 21</td>
<td>622 ± 56</td>
<td>6</td>
</tr>
<tr>
<td>X-CGD</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>X-CGD + pEFneo-m91</td>
<td>53 ± 13</td>
<td>649 ± 28</td>
<td>3</td>
</tr>
<tr>
<td>Clone 1</td>
<td>66 ± 28</td>
<td>645 ± 16</td>
<td>3</td>
</tr>
<tr>
<td>Clone 4</td>
<td>48 ± 19</td>
<td>653 ± 27</td>
<td>3</td>
</tr>
<tr>
<td>Clone 6</td>
<td>68 ± 21</td>
<td>715 ± 43</td>
<td>3</td>
</tr>
<tr>
<td>Clone 7</td>
<td>64 ± 22</td>
<td>654 ± 57</td>
<td>2</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The phagocyte flavocytochrome \( b_{558} \) heterodimer functions as the redox center of the respiratory burst oxidase, mediating the transfer of electrons from NADPH to oxygen upon translocation of cytosolic oxidase subunits to the plasma membrane. Although the relative functions of each subunit have not been fully defined, current data suggest that each subunit contains a heme center and that both flavin and NADPH binding sites are localized to gp91\(_{ph}^{+ \text{pos}} \). The human gp91\(_{ph}^{+ \text{pos}} \) polypeptide has 570 amino acid residues, and contains multiple hydrophobic domains in addition to five potential N-linked glycosylation sites (Fig 1). No polymorphisms have been reported for the human sequence and all identified amino acid substitutions have been associated with X-linked CGD, suggesting that there are stringent requirements in the primary sequence to preserve structure and/or function.

Here we report cloning of a cDNA encoding the murine gp91\(_{ph}^{+ \text{pos}} \) subunit by RT-PCR, using oligonucleotide primers derived from the human sequence. The predicted murine and human gp91\(_{ph}^{+ \text{pos}} \) protein sequences differ by only 39 out of 570 amino acids (93% identity) (Fig 1). Positions where amino acid substitutions have been identified in X-CGD patients are all conserved in the murine gp91\(_{ph}^{+ \text{pos}} \) protein.

Amino acids that are not conserved between the human and murine polypeptides are largely localized to hydrophilic regions of the polypeptide, including several substitutions that alter potential N-glycosylation sites (Fig 1). The murine gp91\(_{ph}^{+ \text{pos}} \) protein contains four potential N-linked glycosylation sites, of which the two most carboxyl-terminal lie in what is most likely an intracytoplasmic domain. Utilization of fewer N-linked glycosylation sites is likely to explain why the murine gp91\(_{ph}^{+ \text{pos}} \) migrates with an apparent molecular mass of \(~38\) kD, compared with \(~91\) kD for the human protein. Upon deglycosylation, the murine and human core proteins both migrate at \(~54\) kD. The C-terminal sequences of gp91\(_{ph}^{+ \text{pos}} \) suggested to form the NADPH binding site are all conserved in the murine sequence (Fig 1). Specific residues involved in flavin binding are less clear, but are believed to reside within residues 290 through 390. This region is identical in the mouse and human gp91\(_{ph}^{+ \text{pos}} \) sequences. There are only two nonconservative substitutions within hydrophobic domains of the mouse and human proteins. Some or all of these regions are likely to be transmembrane or intramembranous domains in which the heme prosthetic groups are embedded. Raman and electron paramagnetic resonance spectroscopy have suggested that the hemes are coordinated with imidazole or imidazolate ligands supplied by histidine residues. All histidines are conserved between the murine and human sequences (Fig 1). Finally, several regions within gp91\(_{ph}^{+ \text{pos}} \) that may interact with p47\(_{ph}^{+ \text{pos}} \) (residues 86 through 93 and 450 through 457) are conserved between the mouse and human gp91\(_{ph}^{+ \text{pos}} \) proteins. The degree of similarity in a carboxy-terminal terminal sequence of gp91\(_{ph}^{+ \text{pos}} \) (residues 559 through 565), also implicated as a binding site for p47\(_{ph}^{+ \text{pos}} \), cannot be assessed, because most of this region of the murine sequence was derived from the reverse PCR primer.

In the studies reported here, the murine gp91\(_{ph}^{+ \text{pos}} \) was capable of both rescuing stable expression of human p22\(_{ph}^{+ \text{pos}} \) and of complementing other human oxidase subunits to fully reconstitute respiratory burst activity in human X-CGD cells. These observations strongly suggest that a functional heterodimeric complex is formed between murine gp91\(_{ph}^{+ \text{pos}} \) and human p22\(_{ph}^{+ \text{pos}} \) subunits. Moreover, the kinetics of superoxide formation were indistinguishable from wild-type human PLB-985 cells, suggesting that the assembly of the multi-subunit active oxidase complex is unaffected. These observations are consistent with the conservation of sequences between human and murine gp91\(_{ph}^{+ \text{pos}} \) in domains likely to be involved in redox function and oxidase assembly. These results also underscore the high degree of cross-species conservation in the NADPH oxidase complex.

Other studies have also suggested that NADPH oxidase subunits from different species have a high degree of similarity in both amino acid sequence and function. Comparisons of porcine and human p22\(_{ph}^{+ \text{pos}} \) showed 83% amino acid similarity, and of murine and human p22\(_{ph}^{+ \text{pos}} \) revealed 88% protein identity. Murine and human p47\(_{ph}^{+ \text{pos}} \) have 82% amino acid identity. Anti-human antisera to the cytochrome \( b_{558} \) components cross-react with the guinea pig cytochrome subunits in immunoblots. Heterologous cell-free oxidase assays have demonstrated functional cross-species complementation between human B-cell cytosolic factors and bovine membranes, rabbit membranes, and bovine cytosolic factors, and murine p47\(_{ph}^{+ \text{pos}} \) combined with human oxidase subunits.

Allogeneic BM transplantation has successfully been used to treat some individuals with CGD and somatic gene therapy is a potential treatment option in the future. MSCV retroviral vectors have been shown to exhibit long-term ex-
pression in vivo after transduction of murine hematopoietic stem cells. 30 Although the level of expression of murine gp91phox conferred by the MSCVneo-m91 retroviral vector was modest, high levels of respiratory burst oxidase reconstitution were observed in a human X-CGD cell line. We are presently investigating the efficacy of this vector for phenotypic correction of the X-CGD mouse 32 by gene transfer into pluripotent hematopoietic stem cells.

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