Functional Reconstitution of the Phagocyte NADPH Oxidase by Transfection of Its Multiple Components in a Heterologous System

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The phagocyte NADPH oxidase system, as previously defined by cell-free reconstitution, is comprised of five essential components, three of which are produced during late phagocytic differentiation—namely, two cytosolic proteins, p47- and p67-phox—and the large subunit of cytochrome b558. gp91-phox. To confirm that these are the only phagocyte-specific components necessary for oxidase activity in whole cells, the recombinant NADPH oxidase was reconstituted in a heterologous cell line. An undifferentiated multipotent leukemic cell line, K562, which expresses endogenous Rac and the small subunit of the flavocytochrome b558, was cotransfected with episomal expression vectors containing cDNAs for the three other oxidase components. After 4 days of selection, the complete oxidase system was functionally reconstituted in transfected cells stimulated with phorbol myristate acetate or calcium ionophore. These easily transfected cells provide an ideal model system in which several oxidase components can be genetically manipulated and readily expressed. This system can be used to test the effects of mutations associated with any of the genes affected in chronic granulomatous disease and will facilitate studies on structure-function relationships within several oxidase components. This system will also aid in delineation of upstream regulators functioning through various signaling pathways. This is a US government work. There are no restrictions on its use.

NADPH OXIDASE is an enzyme complex found in phagocytes that generates oxygen metabolites toxic to invading microorganisms. After cell stimulation, the NADPH oxidase catalyzes reduction of molecular oxygen to superoxide anion (O2−) using NADPH as an electron donor. Chronic granulomatous disease (CGD) is a genetic disorder resulting from mutations in any one of four genes that encode distinct oxidase components.1,2 Phagocytes from affected individuals cannot produce superoxide, which results in recurrent and severe infections. Activation of the respiratory burst involves assembly of three cytosolic components (p47- and p67-phox) and a small guanosine triphosphatase (GTPase), Rac1 or Rac2 with a heterodimeric transmembrane glycoprotein, flavocytochrome b558. The cytochrome is comprised of two subunits (gp91- and p22-phox) that contain heme and putative binding domains for flavin and NADPH.3,4

The K562 cell line, derived from a patient with chronic myelogenous leukemia in blast crisis,5 exhibits multilineage markers,6 indicating that it is a highly undifferentiated, multipotent, hematopoietic precursor cell line. After exposure to various stimuli, this cell line has been known to undergo either erythroid or megakaryocytic differentiation and, more recently, granulocytic differentiation. The K562 line has been shown to constitutively express normal levels of p22-phox transcript.7 The transcript for Rac is also widely expressed in a number of human tissues and cell lines.8 These observations are in contrast to the specificity of expression of other oxidase components; gp91-, p67-, and p47-phox are limited primarily to phagocytic lineages capable of generating superoxide.9,10,11

Functional reconstitution of this enzyme in a cell-free system with purified components has delineated minimum requirements for enzymatic activity,12,13 namely, the two cytosolic components, Rac and the cytochrome b558. However, the extent to which the cell-free system mimics activation processes occurring in whole cells is unclear. The cell-free reconstitution has not provided information concerning the different signaling pathways involving protein kinase C or other kinases elicited by a variety of stimuli that lead to oxidase activation in vivo.14,15 The physiologic relevance of anionic amphiphilic activators used in the cell-free system, such as arachidonic acid or sodium dodecyl sulfate (SDS), has not been established. Structural requirements within oxidase proteins also appear to differ in the whole cell and cell-free systems. For example, both SH3 domains in p67-phox are needed for oxidase activity and for p67-phox translocation to membranes in whole cells, while a truncated form of this protein that lacks both SH3 domains (residues 1-246) is active in vitro.16 Furthermore, translocation of the cytosolic components to membranes during activation was shown to be accompanied by phosphorylation of p47-phox17,18 although phosphorylation is not required in the cell-free system.19,20 Therefore, expression of the multicomponent recombiant oxidase within a multipotent precursor cell would confirm minimum phagocyte-specific components necessary for activation and function in whole cells. This system would also enable delineation of functional and regulatory domains involved in assembly of the oxidase.

Although the genes for all the oxidase components have been cloned,5,7,17,19 their specific roles in the assembled enzyme are poorly understood. Because of the restricted expression patterns of NADPH oxidase components, few transferrable cell models are available. B cells transformed with Epstein-Barr virus (EBV) use the same oxidase components identified in phagocytes to generate small amounts of superoxide.21 So far, EBV B cell lines derived from CGD patients deficient in single oxidase component have been used as transferrable cell models,22-26 although these cells are neither easily transfected nor efficient for expression of oxidase pro-
TRANSFECTION OF NADPH OXIDASE IN K562 CELLS

The purpose of this study was also to establish an easily manipulated cell model to study the role of each oxidase component within the NADPH oxidase. This study represents the first reconstitution of the whole recombinant NADPH oxidase system in an early multipotent leukemic cell line by rapid and stable cotransfection of mammalian vectors encoding three engineered components of the oxidase: p47-, p67-, and gp91-phox.

MATERIALS AND METHODS

Cell Line

K562 cells were grown in RPMI 1540-glutamine supplemented with 10% fetal calf serum (Life Technologies, Inc, Grand Island, NY), penicillin (100 U/mL), and streptomycin (100 μg/mL).

Subcloning of p47-, p67-, and gp91-phox in K562 Cell Lines

All constructs were engineered in pREP4 or pREP10 (Invitrogen, San Diego, CA), episomal expression vectors that allow selection based on hygromycin resistance. Vector pREP10 was used previously to correct the oxidase in p67-phox-deficient CGD EBV B cells.

Cotransfections

Transfections were performed by electroporation, as previously described, with the following modifications. K562 cells (10^6) were electroporated in the presence of 20 μg of each plasmid DNA at 250 V, 960 μF using a Gene pulser (Bio-Rad, Melville, NY). At 48 hours posttransfection, 10^6 cells per milliliter were selected for 5 days in complete medium containing 250 μg/mL hygromycin B and then maintained in 150 μg/mL hygromycin B.

Immunoblotting

Cytosol fractions were obtained by differential centrifugation of sonicated cells and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (8% to 16% gel) immunoblot analysis for recombinant p47- and p47-phox production, as previously described. K562 cells were detected from immunoblotting membranes that were treated with endoglycosidase F before electrophoresis, as previously described.

Superoxide Production Assays

Chemiluminescence. Assays were performed as previously described with 2 μg/mL phorbol myristate acetate (PMA) using an enhanced luminol-based substrate (DIOGENES; National Diagnostics, Atlanta, GA). Both experimental and superoxide dismutase (SOD)-inhibited control reactions were observed for 10 to 30 minutes at 37°C using a luminoskan luminometer (Labystem, Hayward, CA).

Nitroblue tetrazolium (NBT) reduction. Cells (1.25 × 10^6) were resuspended in 100 μL of Hanks’ balanced salt solution with Ca^2+, Mg^2+, 0.05% (wt/vol) NBT, and 2 μg/mL PMA in 96-well plates and incubated at 37°C for 30 minutes before assessing NBT reduction by formation of a dark blue formazan precipitate.

Double Immunofluorescence Staining

K562 cells were spun onto slides (cytospin), fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) for 10 minutes, and then permeabilized in 0.1% Triton X-100 in PBS for 5 minutes at room temperature, as described. Nonspecific binding was blocked with a 1:4 dilution of normal rabbit serum in PBS (1 hour). Cells were stained with mouse anti-p47-phox (MoAb 5017; gift from J.T. Curnutte, Genentech Inc, South San Francisco, CA) and goat polyclonal anti-p67-phox (both at a dilution of 1:500 in PBS) for 2 hours at 37°C in a humidified chamber. Secondary antibodies (dichorotriazinylamino fluorescein [fluorescein]-conjugated rabbit anti-goat IgG F(ab')2 and lissamine rhodamine sulfonyl chloride [rhodamine]-conjugated rabbit anti-mouse IgG (H+L; Jackson Immunoresearch, Westgrove, PA)) were both used at a dilution of 1:500 in PBS for 1.5 hours at 37°C. Cells were mounted in 10% polyvinyl alcohol/10 mM N-propyl gallate and observed by fluorescence microscopy.

RESULTS AND DISCUSSION

Superoxide production was assayed by SOD-inhibitable chemiluminescence using two different stimuli (PMA and A23187) after the selection of hygromycin B-resistant K562 cells. The enhanced luminol-based chemiluminescence assay was used due to its higher sensitivity compared with the cytochrome c reduction assay. After 4 days of selection (Fig 1), the amount of O_2^- produced by PMA-stimulated K562 cells cotransfected with all three constructs (pREP4p47, pREP4p67, and pREP10gp91) was beyond the range of O_2^- generated by normal EBV-transformed B cells [chemiluminescence peak: 119 ± 31.6 relative chemiluminescence units (RLU) 0.25 × 10^6 cells, n = 5; 34 ± 20.8 RLU/0.25 × 10^6 cells, n = 7, respectively]. This activity in normal EBV B cells was equivalent to 3.7 ± 2.05 nmol (n = 6) nmoles of superoxide per minute per 10^6 cells, when calculated from the SOD-inhibitable reduction of cytochrome c (data not shown). Consistent with previous work, normal EBV B cell oxidase activity represented about 1% of human neutrophil activity, while cotransfected K562 cells produced four times more superoxide than did EBV B cells. In contrast to the wide range of oxidase activity exhibited by a number of normal EBV B cell lines tested, the amount of superoxide produced by transfected K562 cells was within a relatively narrow range. In addition to the higher yield of superoxide, the reproducible baseline of oxidase activity observed in the cotransfected K562 cells offers significant advantages over EBV B cells, particularly in experiments...
were cotransfected with all three constructs. Low levels of activity previously reported in studies that used similar EBV-based episomal vectors to transfect p22- and p47-phox-deficient EBV B cells may be explained by the use of different promoters (Rous sarcoma virus LTR in pREP4SV40). Only partial restoration was achieved when gp91-phox cDNA in pREP10 was transfected into EBV B cells from X-linked CGD patients, suggesting that a similarly low level of expression of gp91-phox in cotransfected K562 cells might represent the limiting factor affecting superoxide yield.

A major disadvantage of the EBV-B cell transfection model has been low transfection and slow growth rates. After transfection, these CGD EBV B cells typically required greater than 3 weeks of hygromycin selection and a subsequent growth period of at least 2 weeks to obtain a sufficient number of cells for superoxide production assays. Resistant colonies of EBV B cells transfected with retroviral vectors containing p47- or gp91-phox also required selection for 2 to 6 weeks. In contrast, the cotransfected K562 cells required only 3 days of selection in hygromycin to obtain a maximum in superoxide production by chemiluminescence.

designed to compare activities of structurally modified forms of these oxidase components.

Both cell lines exhibited distinct kinetics of oxidase activation (Fig 1). Like neutrophils, the transfected K562 cells showed a plateau in activity within 5 to 10 minutes of activation, while the peak of activity observed in EBV B cells occurred between 20 and 30 minutes. Stimulation of control untransfected K562 cells with PMA did not result in any superoxide production, and omission of transfection of any one of the three oxidase genes resulted in a complete absence of superoxide generation. These findings confirm that all three genes, gp91-, p47-, and p67-phox, are essential to reconstitute oxidase activity and that they are the only phagocyte-specific genes needed for activation of oxidase assembly and function in whole cells.

Stimulation of cotransfected K562 cells with 8 μmol/L calcium ionophore (A23187) yielded low, albeit significant, levels of superoxide anion that were also dependent on co-transfection with all three genes. In contrast to EBV B cells, concanavalin A stimulation of cotransfected K562 cells also resulted in limited superoxide production. However, both agents elicited only a fraction of the activity observed with PMA stimulation (1% to 2% and 0.1%, respectively; data not shown).

To further characterize these oxidase gene constructs, several EBV B cell lines derived from all four genetic subtypes of CGD patients were also transfected. Full restoration of oxidase activity relative to seven normal EBV-transformed B cell lines was observed with three of the four oxidase components (p22-, p47-, or p67-phox; Fig 2). Low levels of activity previously reported in studies that used similar EBV-based episomal vectors to transfect p22- and p47-phox-deficient EBV B cells may be explained by the use of different promoters (Rous sarcoma virus LTR in pREP4SV40). Only partial restoration was achieved when gp91-phox cDNA in pREP10 was transfected into EBV B cells from X-linked CGD patients, suggesting that a similarly low level of expression of gp91-phox in cotransfected K562 cells might represent the limiting factor affecting superoxide yield.

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suggesting that a high transfection efficiency was achieved and that a significant number of cells coexpressing the three genes was already selected. After selection, the amount of superoxide produced was reproducible at any given time posttransfection. This activity persisted even a month after poration, where it represented about one half of the activity seen 5 days after transfection (data not shown). This gradual decrease can be attributed to the absence of unique selectable markers necessary to retain all three essential gene constructs within the same cell. We have explored cotransfection protocols using more than one selectable marker for independent expression of more than one oxidase gene. The neomycin resistance gene in pREP 9 (Invitrogen) was less advantageous, in that it required at least 2 weeks of selection process with G418. The short selection process required for cotransfection of all three genes linked to hygromycin resistance, together with the high transfection efficiency, rapid growth rate, and consistent yield of superoxide of K562 cells, enables rapid and reproducible reconstitution of a functional recombinant oxidase within a week of electroporation.

The number of K562 cells containing all three gene constructs within the same cell was determined by an NBT reduction assay after PMA stimulation, which permits staining of single cells capable of producing superoxide by formation of an insoluble formazan product that can be quantitatively assessed by cell counting in a hemocytometer chamber (Fig 3). After 4 days of selection, a significant and relatively constant number of cells produced superoxide in six separate transfection experiments (25.2% ± 5.6%). Both stimulated-untransfected and nonstimulated-cotransfected cells were devoid of NBT staining. Using double-labeling immunofluorescence staining of cotransfected K562 cells (Fig 4), about 35% of the cells stained strongly for both cytosolic components together (p47- and p67-phox). Untransfected K562 cells, as well as control cotransfected cells probed without primary antibodies, were totally devoid of fluorescence. A number of cells also stained strongly positive for either component alone or weakly positive for both. Together, these data indicated that a consistent number of cells were capable of rapidly coexpressing the three genes

Fig 3. Detection of superoxide production by NBT reduction in cotransfected K562 cells. Cells co-transfected with the three oxidase constructs used in Fig 1 (A) and untransfected K562 cells (B) were stained with 0.05% NBT after 2 μg/mL PMA stimulation (see Materials and Methods).
and producing superoxide at considerably higher levels than EBV B cells.

Production of both recombinant cytosolic components was also determined by immunoblotting of K562 cytosol with p67- and p47-phox antibodies (Fig 5A). Transfected K562 cells produced both components in significantly higher quantities than EBV B cells, especially p47-phox. Cells in which transfection of cytosolic factor genes were omitted did not produce any detectable amounts of the corresponding proteins.

The presence of the small GTPase necessary for NADPH oxidase activity was confirmed by immunoblotting untransfected K562 cytosol with Rac1 and Rac2 peptide antibodies. Endogenous Rac2 was detected in the cytosol of untransfected K562 cells at levels comparable with neutrophils, based on equivalent cell number loadings (Fig 5D). Like neutrophils, Rac1 was not detected in K562 cells with this antibody reagent (data not shown), although a role for Rac1 in the oxidase was noted in macrophages.

A low level of recombinant gp91-phox protein was detected in transfected K562 cells with the mouse MoAb 4838 (Fig 5B). No gp91-phox protein was detected in non-transfected cell membranes. In contrast to earlier findings, where p22-phox transcript but not the protein was observed in untransfected K562 cells, we did detect low levels of endogenous p22-phox protein with mouse MoAb 44938 (Fig 5C). The endogenous p22-phox levels did not appear to be affected by recombinant gp91-phox expression in K562 cells. Several investigators have noted the absence of both

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Fig 4. Double immunofluorescence of rp67- and rp47-phox in co-transfected K562 cells. K562 cells transfected with the three constructs used in Fig 1 were probed with both goat anti-p67-phox and mouse monoclonal anti-p47-phox antibodies and detected by rabbit anti-goat fluorescein (p67-phox; A) or anti-mouse rhodamine-conjugated (p47-phox; B) secondary antibodies. (C) Phase photomicrograph of the same field shown in panels A and B.

Fig 5. Western blotting of recombinant (A, B) and endogenous (C, D) NADPH oxidase proteins in K562 cells. (A) Immunodetection of p47- and p67-phox in cytosols (3 x 10^6 cell equivalents) from cells co-transfected with all three or only two of the constructs using goat anti-p47-phox and p67-phox antibodies. UN, untransfected K562 cells; All, K562 cells co-transfected with pREP4p47, pREP4p67, and pREP1OgpS1; (-) 67, with pREP1Ogp47 and pREP1Ogp91; (-) 47, with pREP4p67 and pREP1Ogp91; (-) 91, with pREP4p47 and pREP4p67; NB, normal EBV B cells (6 x 10^6 cell eq). (B) Immunodetection of recombinant gp91-phox in Endo-F-treated membrane fractions from K562 cells. UN, untransfected (100 μg); gp91, cotransfected with pREP1Ogp91 (100 μg); PMN, neutrophils (10 μg; see Materials and Methods). (C) Detection of endogenous p22-phox in membrane fractions from K562 cells (60 μg) and neutrophils (10 μg), defined in panel B. (D) Detection of endogenous Rac2 in cytosol fractions from untransfected K562 cells (UN; 5 x 10^6 cell eq per lane) and neutrophils (PMN; 0.5 x 10^6 cell eq per lane) on SDS-PAGE (4% to 20%) immunoblots probed with anti-Rac2 antibody. Migration of molecular weight standards is indicated in kilodaltons.
cytochrome subunits in neutrophils from CGD patients with genetic lesions that affect production of either cytochrome subunits, suggesting that the cytochrome is stabilized in the heterodimeric form. In contrast to the rescue of 91-phox expression observed after transfection of p22-phox cDNA in p22-deficient CGD EBV B cells, we saw no evidence for p22-phox stabilization by gp91-phox expression in the transfected K562 cell model. Thus, this apparent lack of an interdependence of expression of gp91- and p22-phox in this transfected cell model may not permit assessment of mutations that affect stability of cytochrome b558 in other cells.

To directly assess whether recombinant cytochrome from cotransfected K562 cells was functional, cell-free oxidase activity was assayed by combining K562 cell membranes with neutrophil cytosol. Membranes from transfected K562 cells complemented neutrophil cytosol in generating superoxide in vitro, while untransfected cell membranes did not (data not shown). Thus, transfection of gp91-phox cDNA was an absolute requirement for reconstitution of NADPH oxidase activity both in whole K562 cells and in membranes preparations tested in vitro with neutrophil cytosol. This activity was 1.5% to 2% of the activity seen with an equivalent amount of neutrophil membranes, again suggesting that membrane cytochrome content was the limiting factor in transfected K562 cells.

Results obtained by increasing the amount of p22-phox by transfection of p22-phox cDNA along with the other three oxidase genes in K562 cells suggested that endogenous levels of p22-phox did not limit oxidase activity reconstituted in these cells. In this case, the overexpression of p22-phox actually resulted in a significantly lower oxidase activity (10% of maximum activity yielded with the three other oxidase genes; data not shown). In these cells, a higher level of expression of recombinant p22-phox was confirmed by Western blotting. Cotransfection with an irrelevant cDNA in place of the p22-phox cDNA, such as the vector alone or pREPcAT, along with the other oxidase genes (gp91-, p67-, and p47-phox) also resulted in a similar decrease in oxidase activity and a corresponding decreased number of oxidase-competent cells (data not shown). These results can be explained by the decreased probability of all three essential genes being coexpressed in the same cell, likely due to the dilution of the DNA mixture by an additional construct that also endows hygromycin resistance. However, as enhanced production of p22-phox did not result in any increase in oxidase activity over that observed with transfection with a nonspecific cDNA, it appears that the endogenous p22-phox is sufficient for maximum superoxide generation, and gp91-phox is likely to be the limiting component in the recombinant oxidase system produced in these cells.

This very flexible whole-cell model possesses overwhelming advantages, including high and consistent transfection efficiencies, a rapid host cell growth rate, short selection periods, high levels of production of recombinant proteins, reproducible yields of superoxide, and the potential for genetically manipulating the structures of three oxidase proteins within the same transfected cell. This system will permit a rapid assessment of functional consequences of mutations in three of the four genes affected in CGD. We have recently shown an effect of overexpressed mutated p22-phox on p47-phox translocation in cotransfected K562 cells, demonstrating that mutations in the fourth component can also be studied with this cell assay system.

The reconstitution of an active NADPH oxidase system in this erythroleukemia line has confirmed that p47-, p67-, and gp91-phox are the only phagocyte-specific components needed for function in whole cells. This model system will also allow the identification of other phagocyte-specific upstream signaling molecules involved in various oxidase activation pathways triggered in response to a variety of stimuli.

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