Reconstitution of Defective Respiratory Burst Activity With Partially Purified Human Neutrophil Cytochrome B in Two Genetic Forms of Chronic Granulomatous Disease: Possible Role of Rap1A

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Neutrophil plasma membranes from patients with the X-linked and autosomal recessive forms of chronic granulomatous disease (CGD) that lack cytochrome b are incapable of generating superoxide anion (O$_2^-$) in vivo and in vitro. The O$_2^-$-generating activity of these defective membranes was reconstituted with the addition of partially purified human neutrophil cytochrome b in a detergent-based, cell-free activation system. Depending on the detergent system used, 50% to 100% of the activity of control membranes was recovered, and this activity was directly dependent on the cytochrome b concentration. However, when cytochrome b was purified to 99% homogeneity, the reconstitutive capability of the cytochrome was lost, possibly because of subtle denaturation of the cytochrome or the removal of an additional required cofactor. Examination of the latter possibility with respect to a protein known to coassociate with the cytochrome, ie, Rap1A, indicated that this ras-like protein was present in the partially purified cytochrome preparation used to reconstitute activity in CGD membranes, but was missing in the highly purified preparation. However, the finding that Rap1A was present in normal amounts in the neutrophil membranes from all four major types of CGD (including those missing cytochrome b) suggested that the conditions required of the reconstitution assay did not favor the reassociation of the membrane-derived Rap1A with exogenously added cytochrome b or that another unidentified membrane component was lost during the final purification step. The normal expression of Rap1A in CGD cell membranes also indicates that this protein is not responsible for the absence of O$_2^-$ production in the X-linked and autosomal recessive cytochrome b-negative forms of CGD. Finally, these results show that the expression of Rap1A in the plasma membrane is not dependent on the coordinate expression of cytochrome b, despite the close association shown for these two proteins in the normal cell membrane.

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CHRONIC GRANULOMATOUS disease (CGD) is a heterogeneous group of inherited disorders in phagocytic cells resulting in severe, recurrent infections that usually begin early in life. Different forms of CGD can be distinguished by mode of inheritance, with approximately 55% of the cases involving X-linked inheritance, and the remaining 45% following an autosomal recessive pattern of inheritance.

Phagocytes from patients with CGD can ingest microorganisms normally; however, their inability to produce superoxide anion (O$_2^-$) and related microbicidal active oxygen species renders them ineffective at killing a large number of pathogenic bacteria and fungi. The enzyme system responsible for the generation of O$_2^-$, and the site of the biochemical defects in CGD cells, is the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a multicomponent complex that assembles in the plasma membrane following phagocyte stimulation. Activation of this system appears to involve the translocation of cytosolic oxidase components to the plasma membrane where they associate with a low-potential b-type cytochrome to form an electron transport chain that transfers electrons from cytosolic NADPH to molecular oxygen. The putative terminal component of this chain is this low-potential cytochrome b$^{10,12,13}$ (referred to as cytochrome b$_{559}$, b$_{388}$, or b$_{-248}$), which is a heterodimer$^14$ of a glycosylated 91-Kd heavy chain (gp91-phox)$^{15,16}$ and a 22-Kd light chain (p22-phox)$^{15,17}$ Defects in the gene for gp91-phox result in the majority of cases of X-linked CGD (termed X-linked/cytochrome negative or X- CGD)$^{18,19,20}$, however, a rare type of autosomal recessive CGD is caused by a defect in the gene for the light chain (termed autosomal recessive/cytochrome negative or A- CGD)$^{21,22}$

Several other neutrophil proteins and cofactors have been implicated in the NADPH oxidase system, including cytosolic proteins of 47 and 67 Kd (p47-phox and p67-phox, respectively)$^{23,24}$ Both of these proteins have been shown to translocate to the plasma membrane during oxidase activation.$^2$ Recent studies have also shown that a deficiency of p47-phox is responsible for the most common type of autosomal recessive CGD$^{25,27,29,30}$ and a deficiency of p67-phox causes a rarer form of autosomal recessive CGD.$^3,25,28,31$ Other putative oxidase components include NADPH-binding proteins,$^{32,34}$ flavoproteins,$^{33,41}$ and guanosine triphosphate (GTP)-binding proteins,$^{42,47}$ including a ras-related protein, Rap1A, previously shown by us to be directly associated with cytochrome b.$^48$ In addition, very recent reports suggest a cytosolic low molecular weight
GTP-binding protein (Racl or Rac2) may also play a role in the NADPH oxidase system.49,50

One of the most powerful biochemical tools for understanding the interaction of both membrane proteins as well as cytosolic proteins in the activation of the oxidase is the use of reconstitution systems. This approach has recently been used with the soluble cytosolic proteins in which partially purified components have been identified through their ability to reconstitute cytosol factor activity in two types of autosomal recessive CGD.25-28,31,32 However, similar experiments have not been performed in those forms of CGD in which the membrane component is defective. To date, all of the defects involving the membrane in CGD have been ascribed to the absence of cytochrome b.1 To characterize the interaction of the cytochrome with the other oxidase components, we have attempted to reconstitute oxidase activity in two forms of CGD in which the membrane-bound cytochrome b is absent: X- and A- CGD. The development of techniques for activating the oxidase in a cell-free system33-34 with deoxycholate-solubilized membranes35 as well as the purification of human neutrophil cytochrome b15 have provided the necessary means to attempt such membrane reconstitutions. In this report, we describe the correction of the severe defect in the membranes of X- and A- CGD with partially purified cytochrome b from normal human neutrophil membranes. The role played by Rap1A in these reconstitution reactions and in the membrane pathology of X- and A- CGD is also explored.

MATERIALS AND METHODS

Neutrophil subcellular fractions. Blood from both normal and CGD patients was obtained by venipuncture after obtaining informed consent. In the case of normal subjects, neutrophils from 5 U of fresh blood (each from a different donor) were pooled before nitrogen cavitation. Cytosol and solubilized membranes for oxidase reactions were prepared from resting human neutrophils using nitrogen cavitation to disrupt the neutrophils and discontinuous Percoll density gradients to resolve cytosol from membranes as previously described.55,56 The latter were then solubilized in deoxycholate,57 centrifuged at 430,000g, and the supernatant used as a source of dormant membrane oxidase components (Fig 1). Protein concentrations of these fractions were previously determined57 to be as follows: cytosol, 250 ± 16 µg/10^7 cell equivalents (mean ± SD); solubilized membranes, 25.7 ± 1.4 µg/10^7 cell equivalents (mean ± SD).

Neutrophil membranes used for purification of cytochrome b were prepared as described previously.15

Purification of human neutrophil cytochrome b. Heparin-Ultragel (IBF Biotechnics Inc, Savage, MD) purified cytochrome b (90% pure) was prepared from stimulated, degranulated neutrophils as previously described,15 with the final detergent system for eluting the cytochrome from the heparin-Ultragel affinity column being either 0.1% Triton X-100 (Boehringer Mannheim Biochemicals, Indianapolis, IN) or 0.5% octyl glucoside (CalBiochem, La Jolla, CA).

Partially purified cytochrome b, prepared as above, was purified to more than 99% homogeneity by concentrating it to 4 µmol/L cytochrome b using Centricon 30 microconcentrators (Amicon, Inc, Danvers, MA) and subjecting it to velocity sedimentation on linear sucrose gradients (5% to 20% wt/vol, 5 mL) containing 0.1% Triton X-100 or 1.0% octyl glucoside, depending on the detergent system being tested.15 Fractions of 0.35 mL were collected from the top. The cytochrome b in the peak fractions was more than 99% pure as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Reconstitution of oxidase activity. As summarized in Fig 1, the method used to reconstitute oxidase activity in deoxycholate-solubilized CGD membranes involved mixing solubilized CGD membranes (2.5 µL containing 1.5 × 10^7 cell Eq of membranes) with either the heparin-Ultragel eluate or sucrose gradient fractions (7.5 µL containing 0 to 22.5 pmol cytochrome b) at 20°C for 5 minutes. The extent to which this membrane/cytochrome b mixture could be activated to produce superoxide in the cell-free activation system was then determined by adding aliquots (10 µL) of this mixture to both the sample and reference cuvettes used for the oxidase activation reaction that also contained normal cytosol (10^7 cell Eq), cytochrome c (100 µmol/L), NADPH (160 µmol/L), and buffer (100 mmol/L KCl/3 mmol/L NaCl/3.5 mmol/L MgCl2/10 mmol/L Pipes, pH 7.3) in a total volume of 750 µL as previously described.58 The reference cuvette also contained 45 µg of superoxide dismutase. After a 3-minute preincubation, SDS (40 µmol/L) was added to each cuvette and the rate of O_2^- production measured continuously at 550 nm. The maximum rate of O_2^- production was used as the measure of oxidase activity as previously described.55,56
was included in this system, NADPH oxidase activity was enhanced approximately twofold over the activity levels observed in the absence of FAD.

Because Triton X-100 itself had an inhibitory effect on the NADPH oxidase reconstitution system, studies were also performed in parallel using an octyl glucoside detergent system. The results of these two systems were essentially identical; however, the amplitude of the activities observed using the octyl glucoside system (which did not inhibit oxidase activity) was approximately 8 to 10 times greater than that using the Triton system.

Biochemical assays. Cytochrome b was quantitated by dithionite reduced minus oxidized difference spectroscopy on a PerkinElmer Lambda 4C dual beam spectrophotometer (Perkin-Elmer Corp, Newark, NJ) or a Shimadzu UV-3000 dual beam spectrophotometer (Shimadzu Corp, Kyoto, Japan) assuming an extinction coefficient at 559 nm of 29.3 mmol/L/cm. Protein was measured by the BCA method as described by Pierce (Rockford, IL) using bovine serum albumin as a protein standard.

Electrophoresis. SDS-PAGE was performed at room temperature using 7% to 18% gradient gels containing 0.1% SDS (wt/vol) as described previously. Western blotting. Electrophoretic transfer of proteins from SDS-PAGE onto nitrocellulose and Western blotting were performed as described previously. Nitrocellulose transfers were incubated for 3 hours with 1/1,000 dilutions of rabbit anti-Rap1A/B peptide (amino acids 131 through 140) antisera (characterized to be highly specific for Rap1A/B [6]), anti-gp91-phox peptide antibodies, or anti-p22-phox antibodies, washed, incubated with an alkaline phosphatase conjugated goat anti-rabbit IgG (1 μg/mL) for 1 hour, and developed using a commercial kit (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Because Rap1A and Rap1B are nearly identical (95% homologous), and the peptide used for antibody preparation is conserved between Rap1A and Rap1B, we were unable to distinguish between these two proteins. However, in light of recent data that have demonstrated that the neutrophil rap1 protein is predominantly, if not exclusively Rap1A [6], this distinction was not necessary. Western blots were quantitated by scanning the blots with a Zeineh 1D/2D scanning laser densitometer (Biomed Instruments, Fullerton, CA).

RESULTS

Neutrophils from patients with the X- and A- forms of CGD are, by definition, devoid of cytochrome b based on absorbance spectrum and Western blot analyses. Moreover, membranes from these patients fail to express not only the mutated cytochrome b polypeptide, but also the other subunit as well [5]. Consequently, such membranes in combination with the appropriate cytosolic factors should provide a medium to test the ability of purified cytochrome b from normal neutrophils to reconstitute superoxide generating activity. Using an SDS-activatable cell free system containing deoxycholate-solubilized CGD (X- or A-) membranes, we attempted to reconstitute this oxidase activity with partially purified cytochrome b fractions eluted from a heparin-Ultrogel affinity column, the penultimate step in cytochrome b purification.

The magnitude of the reconstituted activity is displayed in Fig 2 as a function of the corresponding cytochrome b content in each of the fractions used as determined by reduced minus oxidized difference spectroscopy. This result suggests that reconstitution is directly dependent on the cytochrome content and that it is equally effective using membrane extracts from neutrophils from X- and A- patients, as is shown in the upper and lower panels of Fig 2, respectively. This reconstitution also paralleled the cytochrome subunit content as determined by SDS-PAGE.
is prepared in octyl glucoside, the relationships observed remain the same, but 8 to 10 times more activity is observed. When similar reactions were conducted omitting the patient membranes and using the cytochrome b fractions alone, no oxidase activity was observed for cytochrome prepared in Triton X-100 and only 10% of the control activity for cytochrome prepared in octyl glucoside (see Table 1). The reconstitution of both X- and A- CGD oxidase activity was clearly dependent on the presence of the membranes, cytosol, and the cytochrome b fractions as is shown in Table 1. When corrected for detergent and buffer effects (Triton X-100 had an inhibitory effect in this system), the levels of reconstitution observed were approximately 50% to 80% (Triton X-100) or equivalent (octyl glucoside) to those seen with normal membranes.

To obtain an estimate of the stoichiometry of the reconstitution reaction, the dependence on cytochrome b concentration was determined as shown in Fig 3. Reconstitution appeared to saturate at ~200 pmol cytochrome b/10^7 cell equivalent membrane, a ratio that is approximately 6 to 10 times that seen in normal neutrophil membranes. In contrast, superoxide generating activity estimated on a mole O_2^-/per mole cytochrome b basis is 1.030 ± 151.2 mol O_2^-/min/mol cytochrome b (mean ± SD; n = 11), which is approximately one tenth that observed in intact neutrophils. These results suggest that either the reconstituted oxidase is less efficient than the native system in intact cells or that only a fraction of the cytochrome added has become incorporated into the oxidase and that some of the cytochrome may be inaccessible because of sequestering in micelles.

The reconstitution experiments described thus far were conducted using approximately 90% pure cytochrome b (heparin-Ultrogel column eluate), However, these preparations contained minor amounts of other contaminating proteins. Therefore, to investigate the potential effect of these contaminants, we further purified cytochrome b utilizing velocity sedimentation in detergent-containing sucrose density gradients as described previously. Cytochrome b prepared using this method is essentially homogeneous, comprising more than 99% of the total protein as determined by SDS-PAGE analysis followed by silver staining. Using highly purified cytochrome, prepared using both types of detergent systems, we were unable to reconstitute oxidase activity in X- or A- CGD samples (data not shown), suggesting that either the structure of the cytochrome was perturbed by this purification step (even though the visible spectrum remained intact) or that additional necessary cofactors were lost. Since CGD cells should contain any cofactor not requiring association with cytochrome b, the results suggest the putative cofactor would also have to be cytochrome associated.

A candidate cofactor is the GTP-binding ras-related

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Table 1. Reconstitution of NADPH Oxidase Activity in Deoxycholate-solubilized X- and A- CGD Membranes

<table>
<thead>
<tr>
<th>Reaction Conditions</th>
<th>Superoxide Production (pmol/min)</th>
<th>Super oxide Generation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Triton X-100</td>
<td>Octyl Glucoside</td>
</tr>
<tr>
<td>Complete reconstitution mixture</td>
<td>(X- CGD)</td>
<td>(A- CGD)</td>
</tr>
<tr>
<td>with CGD membranes</td>
<td>6.2</td>
<td>3.4</td>
</tr>
<tr>
<td>Omit cytochrome b fraction</td>
<td>0.3</td>
<td>0.4</td>
</tr>
<tr>
<td>Omit membranes</td>
<td>0.4</td>
<td>0.1</td>
</tr>
<tr>
<td>Omit cytosol</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Control membranes</td>
<td>53.1</td>
<td>61.4</td>
</tr>
<tr>
<td>Control membranes plus Triton X-100 buffer</td>
<td>7.6</td>
<td>6.1</td>
</tr>
<tr>
<td>Percent reconstitution</td>
<td>82.0</td>
<td>56.0</td>
</tr>
</tbody>
</table>

Reconstitution experiments were performed as described in Materials and Methods. For experiments using Triton-solubilized cytochrome b, 22.5 pmol of cytochrome b was added to the reaction containing 1.5 x 10^9 cell Eq of membrane, while for experiments using octyl glucoside-solubilized cytochrome b, 17.8 pmol of cytochrome b was added to the reaction. Percent reconstitution was calculated by comparing the activities measured using the complete reconstitution mixture with CGD membranes to the control values corrected for buffer effects (Triton X-100 system) or the control values alone where no buffer effects were observed (octyl glucoside system). The results are representative of 10 similar experiments, 6 with X- CGD membranes (3 with each detergent system) and 4 with A- CGD (2 with each detergent system) membranes.
protein, Rap1A, which we previously showed to be associated with human neutrophil cytochrome b. Therefore, we considered the role of Rap1A in these experiments. As shown in Fig 4, immunoblot analysis of partially purified cytochrome b for the presence of Rap1A using Rap1-specific antibodies demonstrated that Rap1A codistributes and is associated with cytochrome b in the heparin-Ultrogel column fractions and would, therefore, always be present in the reconstitution assay when this type of cytochrome preparation is used. On the other hand, the highly purified cytochrome prepared by subsequent velocity gradient sedimentation contained no Rap1A as determined by Western blot analysis (Fig 5). These results indicated that Rap1A was indeed removed during final purification, providing a possible explanation for the loss of reconstitutive activity. However, when neutrophil membranes prepared from patients with the four known types of CGD were immunoblotted for Rap1A using Rap1-specific antibodies, they were found to contain normal amounts of Rap1A compared with control cell membranes (Fig 6; X- [lane 2], A- [lane 3], A+ p67-phox negative [lane 4], A+ p47-phox negative [lane 5], and X- gp91-phox point mutation [lane 6]). Therefore, Rap1A was always present in the reconstitution assay in the detergent extracts of the CGD membranes, although at an Rap1A to cytochrome b ratio far lower than that found in normal membranes.

**DISCUSSION**

We have used purified human neutrophil cytochrome b to reconstitute superoxide generating activity in solubilized membrane extracts of neutrophils from patients with X- and A- CGD. These cells are incapable of producing O2•− and in each case lack both subunits of cytochrome b (i.e., gp91-phox and p22-phox). Because 50% to 100% of normal oxidase activity (depending on the detergent system used) can be restored by the addition of exogenous 90%-purified cytochrome b from normal cells to the CGD membranes, our results confirm that the absence of cytochrome b is responsible for the severe membrane defect in X- and A- CGD cells. However, because the reconstitution is ineffective with nearly homogeneously purified cytochrome b (> 99%), our results also raise the possibility that the absence of other membrane-associated cofactors, such as a putative flavoprotein, may contribute to the oxidase defect. These cofactors may then be lost during the final purification of the cytochrome. Alternatively, the cytochrome structure or its associations may be subtly altered during the velocity sedimentation step, resulting in an inactive cytochrome. Analysis of the highly purified membrane extracts of neutrophils from patients with X- and A- CGD. These cells are incapable of producing O2•− and in each case lack both subunits of cytochrome b (i.e., gp91-phox and p22-phox). Because 50% to 100% of normal oxidase activity (depending on the detergent system used) can be restored by the addition of exogenous 90%-purified cytochrome b from normal cells to the CGD membranes, our results confirm that the absence of cytochrome b is responsible for the severe membrane defect in X- and A- CGD cells. However, because the reconstitution is ineffective with nearly homogeneously purified cytochrome b (> 99%), our results also raise the possibility that the absence of other membrane-associated cofactors, such as a putative flavoprotein, may contribute to the oxidase defect. These cofactors may then be lost during the final purification of the cytochrome. Alternatively, the cytochrome structure or its associations may be subtly altered during the velocity sedimentation step, resulting in an inactive cytochrome. Analysis of the highly purified

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**Fig 4.** Codistribution of Rap1A with cytochrome b in heparin-Ultrogel column fractions. Human neutrophil cytochrome b was purified as described in Materials and Methods and the heparin-Ultrogel column fractions analyzed for cytochrome b spectrum (O). Percent of maximal activity is plotted where 100% activity level represents 3.5 μmol/L cytochrome b. Cytochrome b distribution was confirmed by immunoblotting column fractions with anti-gp91-phox and anti-p22-phox peptide antibodies (data not shown). Rap1A distribution was determined by immunoblot analysis of the column fractions with anti-Rap1A peptide antibodies as described in Materials and Methods. Blots were quantitated using a scanning soft laser densitometer and the band densities plotted as percent of maximal activity where the densest band represents 100% activity. Data are representative of three separate experiments (three separate cytochrome preparations).

**Fig 5.** Analysis of partially purified and highly purified cytochrome b preparations for Rap1A. Partially purified cytochrome b from heparin-Ultrogel column eluate (15 pmol) (lane 1) and highly purified cytochrome b from sucrose density gradients (15 pmol) (lane 2) were subjected to SDS-PAGE and immunoblotted with anti-Rap1A peptide antibodies as described in Materials and Methods. As a control, both samples positively blotted with anti-gp91-phox and anti-p22-phox antibodies, confirming the presence of cytochrome b in these samples (data not shown). Data are representative of four separate experiments (two using cytochrome b purified in Triton X-100-containing gradients and two using cytochrome b purified in octyl glucoside-containing gradients).
Fig 6. Identification of the RaplA protein in CGD neutrophil membranes. Neutrophil membranes prepared from normal (lane 1), X– CGD (lane 2), A– CGD (lane 3), A+/p67-phox– CGD (lane 4), A+/p47-phox– CGD (lane 5), and X–/point mutation CGD (lane 6) cells were subjected to SDS-PAGE (10 μg sample/lane) and immunoblot analysis using anti-RaplA peptide antibodies as described in Materials and Methods. The minor bands stained on these blots are caused by nonspecific antibody binding, based on competition experiments with excess synthetic peptide that did not inhibit their labeling (labeling of the RaplA band was completely blocked under the same conditions). No RaplA was observed in the corresponding cytosols (data not shown). At least two samples (from different donors) were analyzed for each type of CGD, except for X–/point mutation CGD, where only one sample was available. Identical results were obtained when deoxycholate-solubilized membranes were analyzed (data not shown). Blots were repeated three separate times to confirm the results.

cytchrome suggests that, in first approximation, this is not the case, ie, SDS-PAGE shows normal 91- and 22-Kd subunits, reduced minus oxidized spectroscopy indicates a normal spectrum, and Western blotting with anti-cytochrome b antibodies indicates no apparent proteolysis or breakdown of the subunits (M.T. Quinn, C.A. Parkos, and A.J. Jesaitis, unpublished observations, March 1988). However, subtle changes in conformation may nonetheless be present that were not detected in these analyses.

Based on the extensive genetic data linking mutations in the gp91-phox and p22-phox genes to absent (or nonfunctioning) cytchrome b and clinical CGD, it is clear that cytchrome b is an essential membrane-bound component of the NADPH oxidase. The reconstitution experiments presented in this report add further support to this concept. However, what is less certain is whether there may be other membrane oxidase components or cofactors (possibly cytochrome-associated) required for oxidase activation or catalytic activity. A recent report by Knoller et al provides strong evidence that cytchrome b was the only oxidase component in the membranes of guinea pig-elicited peritoneal macrophages required for oxidase activation in a cell-free system containing macrophage cytosol and an anionic amphiphile (SDS). However, the presence or absence of membrane-bound oxidase cofactors in human neutrophils was not examined in the studies by these investigators.

One possible candidate for such a cofactor would be the cytochrome-associated raplA protein. Indeed, a recent report from Eklund et al suggested that recombinant soluble (truncated C-terminus) Krev-1 (aka. RaplA) could reconstitute cytosome depleted of ras-related proteins in a cell-free NADPH oxidase reconstitution assay. Because many investigators, including Eklund et al, use cytosome prepared from cells that are sonicated (a treatment that releases RaplA from the membrane), these results are consistent with RaplA being a necessary cofactor. Comparison of the two cytosome b preparations used in the experiments reported here showed the dissociation of RaplA from the cytosome during the velocity sedimentation step, suggesting that the loss of this cofactor could explain the inability of highly purified cytosome to reconstitute activity. However, immunoblot analysis of the CGD membranes for the presence of RaplA showed normal amounts of this protein in the membranes from X– and A– neutrophils, as well as in those from the other known forms of CGD. The inability of the CGD membrane RaplA to replace the loss of normal RaplA can be explained by several possibilities. One is that the conditions of the soluble reconstitution assay do not favor or allow the reconstitution of the cytosome b and RaplA. Recently, we found that the in vitro reconstitution of cytosome b-RaplA complexes is highly dependent on salt and detergent conditions as well as phosphorylation state. Another possibility is that the CGD raplA protein, although physically identical to the normal protein, could be defective (eg, defective GTPase activity, etc). Currently, there is no evidence to support this possibility, although further studies are necessary to rule it out. A third possibility is that another, as yet unidentified, cytochrome-associated cofactor was removed. Again, further studies are required to address this possibility.

The presence of normal amounts of RaplA in neutrophil membranes from CGD cells, regardless of the defect, suggests that the expression of this protein is not controlled by the presence or absence of membrane-bound or cytosolic oxidase components. It also shows that, although RaplA is known to associate with the cytosome, cytosome b is not necessary for RaplA to be present in the membrane. Finally, these results show that the previously observed absence of p47-phox translocation to the plasma membrane in X– and A– CGD neutrophils is not attributable to the absence of RaplA, although it has been suggested that p47-phox might associate with RaplA. Our findings support previous conclusions that the absence of p47-phox translocation is attributable to the absence of cytosome b, but do not rule out the possibility that RaplA could also be interacting with this component.

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REFERENCES

33. Umei T, Takeshige K, Minakami S: NADPH binding component of the superoxide-generating oxidase in unstimulated neutrophils and neutrophils from the patients with chronic granulomatous disease. Biochem J 243:467, 1987
RECONSTITUTION OF OXIDASE ACTIVITY IN CGD

44. Ligeti E, Doussiere J, Vignais PV: Activation of the O$_2^-$-generating oxidase in plasma membrane from bovine polymorphonuclear neutrophils by arachidonic acid, a cytosolic factor of protein nature, and nonhydrolyzable analogues of GTP. Biochemistry 27:193, 1988
63. Segal AW: Absence of both cytochrome b$_{559}$ subunits from neutrophils in X-linked chronic granulomatous disease. Nature 326:88, 1987
67. Bokoch GM, Quilliam LA, Bohl BP, Jesaitis AJ, Quinn MT: Inhibition of Rap1A binding to cytochrome b$_{559}$ of NADPH oxidase by phosphorylation of Rap1A. Science 254:1794, 1991
Reconstitution of defective respiratory burst activity with partially purified human neutrophil cytochrome B in two genetic forms of chronic granulomatous disease: possible role of Rap1A

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