Changing the Conformation State of Cytochrome $b_{558}$ Initiates NADPH Oxidase Activation

MRP8/MRP14 REGULATION*

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Phagocyte NADPH oxidase generates $O_2^*$ for defense mechanisms and cellular signaling. Myeloid-related proteins MRP8 and MRP14 of the S100 family are EF-hand calcium-binding proteins. MRP8 and MRP14 were co-isolated from neutrophils on an anti-p47phox matrix with oxidase cytosolic factors and identified by mass spectrometry. MRP8 and MRP14 are absent from Epstein-Barr virus-immortalized B lymphocytes and, coincidentally, these cells display weak oxidase activity compared with neutrophils. MRP8/MRP14 that was purified from neutrophils enhanced oxidase turnover of B cells in vitro, suggesting that MRP8/MRP14 is involved in the activation process. This was confirmed ex vivo by co-transfection of Epstein-Barr virus-transformed B lymphocytes with genes encoding MRP8 and MRP14. In a semi-recombinant cell-free assay, recombinant MRP8/MRP14 increased the affinity of p67phox for cytochrome $b_{558}$ synergistically with p47phox. Moreover, MRP8/MRP14 initiated oxidase activation on its own, through a calcium-dependent specific interaction with cytochrome $b_{558}$, as shown by atomic force microscopy and a structure-function relationship investigation. The data suggest that the change of conformation in cytochrome $b_{558}$, which initiates the electron transfer, can be mediated by effectors other than oxidase cytosolic factors p67phox and p47phox. Moreover, MRP8/MRP14 dimer behaves as a positive mediator of phagocyte NADPH oxidase regulation.

The phagocyte respiratory burst depends on the NADPH reduction of molecular oxygen and generation of superoxide anion $O_2^*$ upon activation of the cell with soluble (chemotactic factors) or particulate (bacteria or fungi) stimuli (1). The $O_2^*$-generating NADPH oxidase (EC 1.6.99.6) is a heterogeneous complex compartmentalized in resting cells, between plasma membrane, cytosol, and the cytoskeleton, which assembles at the plasma membrane level once activated. Cytochrome $b_{558}$ is the membrane redox core of the oxidase complex, which, after activation, transfers electrons from NADPH to molecular oxygen. It is constituted of p91phox (the β-catalytic subunit) and p22phox (α-subunit), which is necessary in phagocytes to stabilize the 1/1 (αβ) heterodimer (2). Three soluble factors, p67phox, p47phox, and p40phox, participate in the activation process, as does the monomeric G protein Rac1/2. Another monomeric G protein, Rap1A, has been identified in the membrane and proposed as a possible regulation factor of the oxidase complex (3, 4). In phagocytes, $O_2^*$ and derivatives are synthesized in large amounts and used as bactericidal tools, but uncontrolled production is also injurious to tissues and triggers inflammatory reactions (5, 6).

Phagocyte NADPH oxidase activity is transitory; it is submitted to a strict up- and down-regulation process. Up to now, oxidase activation has been investigated, but the molecular support of down-regulation has not. In fact, the role of a membrane-associated GTPase-activating protein has been described and related to the deactivation of NADPH oxidase (7, 8). Recent observations would support an allosteric regulation of NADPH oxidase activity where cytochrome $b_{558}$ is the catalytic subunit. In the oxidase assembly, p67phox is the limiting factor (9–11). The binding of p67phox to cytochrome $b_{558}$ mediates the transition from an inactive to an active conformation of cytochrome $b_{558}$ (11). In the process, p47phox and Rac1/2 are introduced as positive effectors, whereas the p40phox function remains undetermined. In chronic granulomatous disease (CGD)†, there is no oxidase activity (12).

All the components of the phagocyte NADPH oxidase complex are expressed in Epstein-Barr virus-immortalized B lymphocytes. However, in these cells, the activity of stimulated oxidase is 100 times inferior to that of human neutrophils (13). Both neutrophils and EBV-B cells are qualitatively similar in

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† The abbreviations used are: CGD, chronic granulomatous disease; AFM, atomic force microscopy; DTT, dithiothreitol; ECL, enhanced chemiluminescence; EBV, Epstein-Barr Virus; FACS, fluorescence-activated cell sorting; FAD, flavin adenine dinucleotide; GTP-γ-S, guanosine 5’-3-O-thiothriphosphate; IPQ, immobilized pH gradient; MALDI, matrix-assisted laser desorption/ionization; MRp, myeloid related protein; MRP8/MRP14, mixture (1/1) of MRP8 and MRP14; rMRP8/rMRP14, mixture (1/1) of recombinant MRP8 and recombinant MRP14; MS, mass spectrometry; PBS, phosphate-buffered saline; Phox, phagocyte oxidase; PLA₂, phospholipase A₂; PMA, phorbol myristate acetate; PMN, polymorphonuclear neutrophil; TOF, time-of-flight.

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terms of the nature of their oxidase constituents, but they differ in the concentration and stoichiometry of the cytosolic factors compared with cytochrome b$_{556}$. We have recently demonstrated that the weak oxidase activity measured in intact EBV-B lymphocytes was not only a result of the low expression of cytochrome b$_{556}$, but also of a possible defect or constraint in the assembly process because of an unfavorable membrane environment (15). In fact, once cytochrome b$_{556}$ is extracted from the membrane of both cell types, the turnover of reconstituted NADPH oxidase is similar. It is also possible that such a low activity may result from down-regulation because regulatory factors normally present in phagocytes are absent in the B cells.

Myeloid-related proteins, MRP8 and MRP14, are two proteins of the S100 family (S100A8 and S100A9, respectively) expressed by myeloid cells and some secretory epithelium (16). In myeloid cells, they represent ~45% of the cytosolic proteins in neutrophils (17) and 1% in monocytes. MRP8 and MRP14 are not expressed by resident macrophages, although they may be present in macrophages of inflammatory lesions (18). MRPs are known to be markers of inflammation. Their expression by infiltrating neutrophils was assumed to reflect activation stages (19). In inflammation, high levels of MRP8 and MRP14 were found in the extracellular medium of stimulated neutrophils (20) and these proteins could be markers of inflammatory diseases such as arthritis and ulcerative colitis (17, 21). Whether the complex is released by dying cells or is a result of active secretion has not been clarified. The two proteins are deposited onto endothelium venules through a specific MRP14-heparan sulfate proteoglycan interaction (22). It has been proposed that MRP8 and MRP14 participate in neutrophil migration, phagocytosis, and activation (21). However, their specific function remains unclear. MRP8 and MRP14 are believed to be present as a 1:1 non-covalent heterodimer, the process of dimer formation being calcium-dependent. Four MRPs isoforms have been identified, although this is not the specific function remains unclear. MRP8 and MRP14 are believed to be present as a 1:1 non-covalent heterodimer, the process of dimer formation being calcium-dependent. Four MRPs isoforms have been identified, although this is not the case for MRP8 (23). Several studies have reported that both proteins also associate with membrane and cytoskeleton in a calcium-dependent manner (24).

Recent studies have suggested that MRP8 and MRP14 play a role in potentiating activation of O$_2^-$-generating oxidase in bovine neutrophils (25). Doussiere et al. (26) also reported that MRP8/MRP14 interacts preferentially with the cytosolic factor p67$^{phox}$, which translocates to plasma membrane upon stimulation. Moreover, various phosphorylated states of MRPs may discriminate subcellular compartmentation (27).

Using a proteomics approach, we identified MRP8 and MRP14 associated with oxidase cytosolic factors in a complex isolated from neutrophil cytosol. In contrast, there was neither MRP8 nor MRP14 in the complex isolated from EBV-B cell cytosol. MRP8/MRP14 purified from human neutrophils and recombinant MRP8/MRP14 were shown to complement the cytosol of EBV-B cells and to restore a full oxidase activity, as measured in human neutrophils. These observations were confirmed ex vivo after MRP-encoding gene transfection. We demonstrated that this effect was mediated through a specific interaction with cytochrome b$_{556}$ and a change of conformation that initiated oxidase activation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Chemical reagents used in this study were obtained from the following sources: Carbolink™ coupling gel (Pierce); l-cystophosphatidylcholine type II-S, PMA, carbeneicillin, and Luminol (5-amino-2,3-dihydro-1,4-phthalalazineone) (Sigma); ECL Western blotting detection reagents, and FPML monQ anion exchange column (Amersham Biosciences, Uppsala, Sweden); immobilized linear pH gradient strips, IPG buffer, pH 3–10 (Bio-Rad); synthetic peptide corresponding to the C-terminal portion of p47$^{phox}$ (ADLILNRRSEETSKRLASV) (NeoSystem, Strasbourg, France). MRP8 (Calgranulin A C19), MRP14 (Calgranulin B C29), goat polyclonal antibody, and donkey anti-goat IgG antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); PIA (Difco); Genetin-10131–019 (G418) (Invitrogen Corp., Paisley, United Kingdom); trypsin, sequencing grade (Promega, Charbonnières, France). Rabbit antibodies (anti-MRP8/MP14) were from M. Markert (Central Laboratory of Clinical Chemistry, Lausanne, Switzerland). Mouse monoclonal antibodies 54.1 and 44.1 directed against p67$^{phox}$ and p22$^{phox}$, respectively, were prepared by J. B. Burritt and A. Jesaitis (Department of Microbiology, Montana State University, Bozeman, MT). The plasmids pRC-CMV-MRP8, pRC-CMV-MRP14, and pRC-CMV-GFP were kindly provided by H. Perron (BioMerieux, Marcy l’Etoile, France). Human recombinant MRP8 and MRP14 (28, 29) were a generous gift from Dr. C. Kerklau (Humboldt University of Berlin, Berlin, Germany). The CDNA encoding p47$^{phox}$ cloned into the plasmid pGEX-2T was kindly provided by A.W. Segal and F. Wientjes (Department of Medicine, University College London, London, United Kingdom) (30).

**Lymphoid Cell Lines and Neutrophils—**Citrate-stereile venous blood was drawn from healthy patients after obtaining their informed consent. Neutrophils fromuffy coats and B lymphocytes were isolated according to previously used methods (31). Lymphocytes were immortalized with the B9-8 strain of Epstein-Barr virus. The EBV-B lymphocyte cell lines were kept in culture using RPMI 1640 supplemented with 10% (v/v) fetal calf serum, 2 mM l-glutamine at 37 °C in 5% CO$_2$ atmosphere. The cytosolic factors from the 3 M diisopropylfluorophosphate-treated and purified neutrophils and EBV-B cells were prepared as described (32, 33). EBV-B lymphocytes from a p47$^{phox}$−deficient CDG patient were kindly provided by M.-A. Pocidalo (INSERM U479, Hôpital Bichat, Paris, France).

**Recombinant Proteins—**Full-length cDNAs encoding p67$^{phox}$, p47$^{phox}$, and Rac1 were expressed in Esherichia coli as a glutathione-S-transferase fusion protein using pGEX-3X (p67$^{phox}$) or pGEX-2T (p47$^{phox}$ and Rac1). Protein expression was induced with isopropyl-1-thio-β-D-galactopyranoside (0.2 mM at 20 °C for p67$^{phox}$ and p47$^{phox}$, 0.1 mM at 37 °C for Rac1) for 3 h. Glutathione S-transferase fusion proteins were affinity-purified from isopropyl-1-thio-β-D-galactopyranoside-induced bacteria (11). After washing in PBS, recombinant proteins were cleaved directly on the matrix using Xa factor (p67$^{phox}$) or thrombin (p47$^{phox}$ and Rac1) in PBS. Recombinant proteins (p67$^{phox}$, p47$^{phox}$, and Rac1) were stored at −20 °C or used in cell-free assay.

**Isolation of the p47$^{phox}$, p67$^{phox}$, p40$^{phox}$ Cytosolic Activation Complex—**Anti-p47$^{phox}$ immunoglobulins were immobilized onto a Carbolink™ coupling matrix. Cytosol from either EBV-B lymphocytes or neutrophils was applied to the matrix previously equilibrated in PBS with overnight recycling at 4 °C. After washing in PBS, bound proteins were eluted either with 0.1 M glycine, pH 3, or with 1 mM NaCl and then 0.1 M glycine, pH 3, or with 2 mM competitor peptide (used to generate the anti-p47$^{phox}$ antibodies). Glycine eluates were immediately buffered with 1 M Tris-HCl, pH 9.5, dialyzed against PBS containing a mixture of protease inhibitors (10 μg N-tosyl-1-L-lysine chloromethyl ketone, 1.5 μg leupeptin, 1.5 μg pepstatin) and stored at −80 °C until further use. To verify the specificity of the recovered proteins, a control experiment was conducted with cytosol from p47$^{phox}$−deficient EBV-B lymphocytes.

**MRPs and MRP14 Purification—**MRPs and MRP14 were purified from the cytosol of unstimulated neutrophils as described (35). Briefly, neutrophil cytosol was subjected to 70% (w/v) (NH$_4$)$_2$SO$_4$ precipitation. The 10,000 × g centrifugation supernatant was dialyzed against 50 mM Tris-HCl, pH 8.5, containing 1 mM DTT, 1 mM EDTA, and 1 mM EGTA, and fractionated through FPLC onto a monoQ anion exchange column. The bound MRPs and MRP14 were eluted with 0.13 M NaCl, as shown on the elution chromatogram (Fig. 1B, lane 1). This peak contained two peptide bands with an apparent molecular mass of 16 and 11 kDa, identified by Western blotting as MRP14 and MRP8, respectively (Fig. 1A, right panel, lane 2). This fraction was called MRP8/MRP14. The absence of contaminating proteins was confirmed ex vivo after MRP-encoding gene transfection. We demonstrated that this effect was mediated through a specific interaction with cytochrome b$_{556}$ and a change of conformation that initiated oxidase activation.

**Oxidase Activity Reconstitution in a Cell-free Assay with Purified Cytochrome b$_{556}$**—Cytochrome b$_{556}$ was purified from the plasma mem-
The fraction containing MRP8 and MRP14 (MRP8/MRP14) purified as Phox proteins in the purified fraction containing MRP8 and MRP14. nitro blue tetrazolium. secondary antibody coupled to alkaline phosphatase, then stained with both MRP8 and MRP14. The immune complexes were detected with a pmol) with cytosol isolated from neutrophils or from EBV-B lymphocytes and relipidated with L-cytosol by FPLC monoQ anion chromatography. A substituted with purified cytochrome b$_2$.b$_2$–1.3 M FAD, 40 g of plasmid DNA and electroporated (as described for oxidase activity reconstitution) or played. After incubation with 100 nmol of arachidonic acid at room temperature, the proteins were divided into two selected points. Protein bands of interest were excised from a high resolution two-dimensional gel electrophoresis as reported (36). Proteins (75 $\mu$g) were precipitated with 7% (v/v) isopropanol and 50% (v/v) acetone. Samples had been washed with 5 $\mu$l of 0.1% (v/v) trifluoroacetic acid before drying, as previously described by Garin (36). The MALDI spectra were analyzed by comparing a mass-to-charge ratio (peptide-mass fingerprinting) of each digested protein to available data bases at prospector.ucsf.edu/ucsfhtml4.0u/msfit.htm. Proteins were identified by MS/MS analysis when no consistent hit was found.

**Peptide Mass Fingerprinting by Matrix-assisted Laser Desorption/Ionization Time-of-flight (MALDI-TOF) Mass Spectrometry—**Mass spectra of the digest were acquired on a Biflex (Bruker Daltonik, Bremen, Germany) MALDI-TOF mass spectrometer equipped with gridless delayed extraction. The digest samples (0.5 $\mu$l) were deposited onto the target disk on a thin dry layer of matrix (mixture 4:3 (v/v) of a saturated solution of a-cyano-4-hydroxy-trans-cinnamic acid in acetone, and a solution of nitrocellulose (10 mg of nitrocellulose in 1 ml of 50% (v/v) isopropanol and 50% (v/v) acetone). Samples had been washed with 5 $\mu$l of 0.1% (v/v) trifluoroacetic acid before drying, as previously described by Garin (36). The MALDI spectra were analyzed by comparing a mass-to-charge ratio (peptide-mass fingerprinting) of each digested protein to available data bases at prospector.ucsf.edu/ucsfhtml4.0u/msfit.htm. Proteins were identified by MS/MS analysis when no consistent hit was found.

**Peptide Sequencing by Tandem Mass Spectrometry (MSMS)—**The digest was extracted with 5% formic acid and then with pure acetonitrile. The digest solution and extracts were vacuum dried, dissolved in 10 $\mu$l of 10% formic acid, and desalted with ZipTip(TM) (Millipore). After elution with 5–10 $\mu$l of 50% acetonitrile combined with 0.1% formic acid, the peptide solution was introduced into a glass capillary (MDS Protana) for nanoelectrospray ionization. Tandem mass spectrometry experiments were carried out on a Q-TOF hybrid mass spectrometer (Micromass) to obtain amino acid sequence information. MS/MS sequence information was used for data base searching using the MS-Pattern programs (prospector.ucsf.edu/ucsfhtml4.0u/mspattern.htm) or Peptide Search (www.narbiem-ebi.de).
once in a 4-mm gap electroporation cuvette at 220 V for 10 ms (BTX ECM 830™, Electro Square Porator). Then they were immediately diluted in 6 ml of complete medium containing 10% (v/v) fetal calf serum. FACS analysis of transfected cells (FACScalibur, Becton Dickinson) showed a 14% transfection efficiency 48 h after transfection. Selection of transfected cells began at 48 h after transfection by culturing cells in the presence of Geneticin (G418) (500 µg/ml). Superoxide production of transfected cells was measured by chemiluminescence.

Measurement of NADPH Oxidase Activity in EBV-B Lymphocytes

Using Chemiluminescence—Lymphocytes (5 × 10⁵ to 2 × 10⁶) suspended in 50 µl of PBS were added to 200 µl of PBS containing 0.9 mM CaCl₂, 0.5 mM MgCl₂, 20 mM glucose, 20 µM Luminol, and 10 units/ml horseradish peroxidase. Superoxide production was measured by chemiluminescence after adding 10 µl of a 2 µg/ml PMA solution (39). Photon emission was recorded at 37 °C for 1 h with a Luminoscan (Labsystem, Pontoise, France).

Polyclonal Antibodies against Cytosolic Factors—Polypeptides corresponding to the C-terminal region of p40phox (residues 325–339) and the C-terminal regions of p47phox (residues 371–390) and p67phox (residues 511–526) were synthesized by Neosystem (Strasbourg, France). Anti-gp91phox antibodies directed against gp91phox or p47phox were obtained by immunizing rabbits with monomeric recombinant proteins of gp91phox (monoclonal antibody 54.1) and p47phox (monoclonal antibody 44.1).

RESULTS

MRP8 and MRP14 Co-purify with the Cytosolic Activating Factors p67phox, p47phox, and p40phox—The NADPH oxidase-soluble components, present in the cytosol of neutrophils or EBV-B lymphocytes, were isolated on an anti-p40phox affinity matrix (Fig. 2A). SDS-PAGE analysis of the eluate protein content showed that several polypeptides other than Phox proteins were present (Fig. 2B). Similar protein profiles were observed with other elution conditions (data not shown). The specificity of bound proteins was confirmed by isolating the complex from p47phox-deficient B lymphocytes. In this case, no significant associated protein could be detected in the eluted fraction on Coomassie Blue-stained gel (Fig. 2B, lane 3). The three factors (p67phox, p47phox, and p40phox) were eluted from the matrix with glycine, pH 3, in neutrophil and B lymphocyte complexes (Fig. 2C). The immunoreactive band migrating between p67phox and p47phox corresponded to the immunoglobulin heavy chain stripped from the affinity matrix. Moreover, two low molecular weight polypeptide bands, with an apparent molecular mass of 11 and 16 kDa, were only found in the complex isolated from neutrophil cytosol (Fig. 2B, lane 1) and were completely absent from EBV-B cell cytosol (Fig. 2B, lane 2). These proteins were then identified by immunoblot as MRP8 and MRP14 (Fig. 2C). These belong to the S100 family and are also named S100A8 and S100A9, respectively. The identification of these two proteins was confirmed by MALDI-TOF mass spectrometry. The 11-kDa protein (Fig. 2B, lane 1) was clearly recognized by mass fingerprint as MRP8, with 48% of sequence recovery (Table I). The 16-kDa protein (Fig. 2B, lane 1) was identified by sequencing a peptide with tandem mass spectrometry. The peptide sequence obtained was LGH-PDTLNQGEFK and corresponded to residues 26–38 of MRP14.

Because there are several isoforms of MRP8 and MRP14, the neutrophil-isolated complex was submitted to two-dimensional gel electrophoresis to determine the specific isoforms co-purifying with Phox proteins (Fig. 3A). Interestingly, two spots with a different isoelectric point were identified as MRP8 by immunoblot (Fig. 3B, left panel), whereas only one spot corresponded to MRP14 (Fig. 3B, right panel).

![Figure 2](https://example.com/fig2.png)

**Figure 2.** Affinity purification of p47phox, p67phox, and p40phox cytosolic activating factors as a complex, from the cytosol of either neutrophils or EBV-B lymphocytes. A, elution profile. The isolation of cytosolic factors of oxidase as a complex was carried out on a specific anti-p40phox affinity matrix (Carbolink™), as described under “Experimental Procedures” and as previously reported (33, 34). Briefly, the cytosol recovered from neutrophils, corresponding to 50–100 mg of proteins, was incubated successively with the uncoupled matrix, then with the non-immune matrix, and finally with the specific anti-p47phox matrix. It was eluted after an overnight incubation at 4 °C by: 1 µl of 0.1 M NaCl followed by 0.1 M, pH 3 glycine (□), 1 µl of 0.1 M NaCl followed by 0.1 µl of 0.1 M, pH 3 glycine (●), or 2 µl competitor peptide (ADLILNRSEEETKRLASV), which was used to generate the anti-C-terminal peptide antibodies against p47phox (●). Glycine eluates were immediately buffered with 1 µl Tris-HCl, pH 9.5. For each elution condition, the eluted proteins were fractionated by SDS-PAGE. The p67phox, p47phox, and p40phox were then identified by Western blotting. On the x-axis, the fraction size is 700 µl for glycine and NaCl/glycine elutions, and 2 ml for peptide elution. B, SDS-PAGE (Coomassie Blue-stained gel) of the purified cytosolic complex (10–15 µg) isolated from neutrophil cytosol (lane 1), from B lymphocyte cytosol (lane 2) on the anti-p47phox matrix and eluted by 0.1 µl, pH 3 glycine. The specificity of the binding was verified by loading the cytosol isolated from p47phox-deficient EBV-B lymphocytes on the anti-p47phox matrix; the glycine-eluted complex was analyzed by SDS-PAGE (lane 3). C, Western blot of the purified cytosolic complex (10–15 µg) isolated either from neutrophil cytosol (lanes 1) or B lymphocyte cytosol (lanes 2) on the anti-p47phox matrix and eluted by 0.1 µl, pH 3 glycine. Immunodetection of p67phox, p47phox, p40phox, and MRP8/MRP14 was carried out using specific polyclonal antibodies. The immune complexes were stained with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate. He corresponds to the heavy chain of IgG.
The band corresponding to the 11-kDa protein (Fig. 2B, lane 1) was excised from the Coomassie Blue gel, digested by trypsin, and analyzed by MALDI-TOF mass spectrometry, as described under “Experimental Procedures.” Experimental mass fingerprint was compared with data bases of predicted peptide profiles: 6 of 8 peptides matched with the mass fingerprint of MRP8, with 48% sequence recovery.

### TABLE I

| Peptide mass | Experimental | Calculated | Position | Peptide |
|--------------|--------------|------------|----------|---------|
| 879.574      | 879.450      | 1-7        | MTLTELEK |
| 1272.727     | 1272.695     | 8-18       | ALNSIIDVVK |
| 963.500      | 963.480      | 4-31       | GNHFAYVR |
| 1434.762     | 1434.713     | 24-35      | GNHFAYRDDLK |
| 822.620      | 822.415      | 50-56      | GADVWFK |
| 982.442      | 982.423      | 86-93      | SHEESHKE |

**Fig. 3.** Two-dimensional gel electrophoresis analysis of the cytosolic activating complex isolated from PMNs. Two-dimensional gel electrophoresis was carried out, as described under “Experimental Procedures.” The cytosolic activating factor complex (75 μg), isolated from PMNs and eluted with 0.1 M glycine pH 3 (Fig. 2A), was used for high-resolution two-dimensional gel electrophoresis. A, proteins were separated first according to their isoelectric point on immobilized linear pH gradient, 3–10, and then by standard SDS-PAGE, after which the gels were silver-stained. B, Western blot of the two-dimensional gel. MRP8 and MRPI4 were immunodetected using specific polyclonal antibodies directed against MRP8 (left panel) or MRPI4 (right panel). The immune complexes were stained with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate.

**MRP8/MRP14 Increases NADPH Oxidase Activity, in Vitro and ex Vivo—**MRP8 and MRPI4 were purified from the cytosol of human neutrophils in one step on a monQ anion exchange matrix (FPLC) (Fig. 1), as described under “Experimental Procedures.” The fraction eluted at 0.13 M NaCl was called purified MRP8/MRP14. The MRP effect on reconstituted oxidase activity was investigated because they were present in the complex purified from cytosol of neutrophils, but absent from that of B cells. The purified MRP8/MRP14 preparation was first reactivated in the presence of calcium; reconstitution was performed by adding MRP8/MRP14, optimal amount of cytochrome b$_{558}$ purified from neutrophils, and the cytosol of EBV-B lymphocytes (33). Results are shown in Fig. 4A. Optimal oxidase activity was measured in the presence of cytochrome b$_{558}$ and neutrophil cytosol. It corresponded to a turnover of the reconstituted enzyme ranging close to 189 mol of O$_2$ • mol of heme $^{-1}$ (Fig. 4A, lane 5). The turnover decreased to 30–40% of the control when B-cell cytosol was used instead of neutrophil cytosol (Fig. 4A, lane 2 versus 5) (33). The reconstituted oxidase turnover became similar to the oxidase turnover measured with neutrophil fractions when Ca$^{2+}$-loaded MRP8/MRP14 was...
present (Fig. 4A, lane 4 versus 5). Purified MRP8/MRP14 was preincubated with increasing concentrations of calcium (0–1 mM), before being incubated with cytochrome b_{558} and cytosol (data not shown), to verify the calcium dependence of this effect. The results demonstrated that, in the absence of calcium, MRP8/MRP14 had no impact on the oxidase activity. The optimal amount of calcium-reactivated MRP8/MRP14 was determined with each B cell-cytosol preparation used for the cell-free assay (Fig. 4A, inset). Furthermore, the results demonstrated that Ca^{2+}-loaded MRP8/MRP14 was able, on its own, to considerably increase O_2\(^{-}\) production of repurified and purified cytochrome b_{558} in the absence of cytosol (Fig. 4A, lane 3 versus 1), suggesting a direct MRP8/cytochrome b_{558} interaction. The absence of p70^{phox}, p67^{phox}, and Rae from the fraction containing the purified cytochrome b_{558} (Fig. 4B, left panel) was assessed by Western blot and compared with positive control (neutrophil cytosol) (Fig. 4B, right panel). Because of the sensitivity of the immunoblotting assay, if potential contaminant cytosolic Phox proteins were present, their concentration would be more than 10^8 times inferior to the one necessary for cytochrome b_{558} activation in a cell-free assay.

The specificity of the interaction between MRP8/MRP14 and cytochrome b_{558} was investigated by adding purified neutrophil cytochrome b_{558} to the reconstitution medium containing Ca^{2+}-loaded MRP8/MRP14, either after (Fig. 5A, black circles) or before (Fig. 5B, black circles) adding EBV-B lymphocyte cytosol. In the absence of MRP8/MRP14, oxidase activity ranged between 50 and 70 mol of O_2\(^{-}\)·mol of heme b^– \(^{-}\) (Fig. 5, A and B, open squares). 40% of control value, as previously shown. Adding cytochrome b_{558} to the reconstitution medium containing Ca^{2+}-loaded MRP8/MRP14 induced, by itself and in the absence of EBV-B lymphocyte cytosol, a slight but significant increase in oxidase activity (Fig. 5C, black circles). This activity remained stable after 70 min of incubation on ice. These data showed that, in the presence of MRP8/MRP14, a different effect was observed depending on how cytochrome b_{558} was added to the medium. A 2-fold increase in oxidase turnover was observed when the cytochrome b_{558} fraction was added first to the MRP8/MRP14 fraction, before adding cytosol (Fig. 5B, black circles). On the other hand, the activity was similar to that reconstituted in the absence of MRP8/MRP14 when cytosol was incorporated before cytochrome b_{558} (Fig. 5A, black circle versus open squares). These results suggest a specific and necessary interaction involving MRP8/MRP14 and repurified cytochrome b_{558} to restore a total oxidase activity in EBV-B cells. Moreover, these data show that Ca^{2+}-loaded-MRP8/MRP14, on its own, is sufficient to obtain an active form of cytochrome b_{558}.

**Effect of Recombinant MRP8 and MRP14 on NADPH Oxidase Activity Reconstituted in Vitro**—A mixture (1/1) of recombinant MRP8 and MRP14 (rMRP8/rMRP14), prepared in E. coli (28, 29) and preloaded with calcium, was used instead of MRP8/MRP14 purified from neutrophil cytosol, in the reconstitution experiment that was performed with an optimal amount of purified cytochrome b_{558} and cytosol from EBV-B lymphocytes. Having rMRP8/rMRP14 in the system induced a 50% increase in reconstituted oxidase activity (88 ± 7 versus 60 ± 5 mol of O_2\(^{-}\)·mol of heme b^– \(^{-}\)) (Fig. 6A, lane 6 versus 3). However, adding either rMRP8 or rMRP14 alone had no effect (Fig. 6A, lanes 4 and 5). A significant rise in cytosol-independent activity was again measured in a medium containing exclusively cytochrome b_{558} and rMRP8/rMRP14 (Fig. 6A, lane 2 versus 1).

A similar MRP effect was observed ex vivo in EBV-B lymphocytes co-transfected with plasmids pRc-CMV-MRP8 and pRc-CMV-MRP14 encoding MRP8 and MRP14, respectively
**FIG. 6. Enhancement of NADPH oxidase activity by human recombinant MRP8 and MRP14.** A, *in vitro*, effect of recombinant MRP8 and MRP14 on NADPH oxidase activity. Oxidase activity was reconstituted, as described under “Experimental Procedures” by incubating cytochrome b$_{558}$ purified from PMNs (0.215 pmol) in the absence (lanes 1 and 2) or presence of EBV-B lymphocyte cytosol (300 μg) (lanes 3–6). Some experiments were carried out with recombinant MRP8 (rMRP8) (0.78 μg/assay) (lane 4), recombinant MRP14 (rMRP14) (0.76 μg/assay) (lane 5), or a mixture (1:1) of rMRP8 and rMRP14 (rMRP8/rMRP14) (0.76 μg/assay in total) (lanes 2 and 6). In this case and as previously described, rMRPs were pre-incubated in the presence of 500 mM CaCl$_2$ at room temperature for 20 min. Oxidase activity was expressed as oxidase turnover (mol of O$_2$)/(mol heme). As a reference value, the activity of the system containing neutrophil cytochrome b$_{558}$ and neutrophil cytochrome b$_{558}$ was 189 ± 6 mol of O$_2$/(mol heme). Results are expressed as the average of at least four experiments ± S.D. * indicates results statistically different from the control value measured in the absence of MRP (p < 0.05). B, *ex vivo*, NADPH oxidase activity of EBV-B lymphocytes transfected with cDNA encoding MRP8 and MRP14. EBV-B cells (5 x 10$^5$ living cells) transfected either with pRC-CMV-GFP (GFP) (+), pRC-CMV-MRP8 (MRP8) (△), pRC-CMV-MRP14 (MRP14) (□), or both pRC-CMV-MRP8 and pRC-CMV-MRP14 (MRP8 + MRP14) (●) were harvested between days 18 and 40 after transfection, washed twice in PBS, and stimulated with FMA as described under “Experimental Procedures”. NADPH oxidase activity was measured by chemiluminescence and expressed as relative luminescence units (RLU) measured for 1 h. The black bar corresponds to the activity of electroporated control EBV-B cells (5 x 10$^5$ living cells).

**Fig. 6.**

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**Fig. 7.** Atomic force microscopy study to investigate interaction of MRP8/MRP14 with cytochrome b$_{558}$ liposomes. A, measurement of cytochrome b$_{558}$ liposome height in the presence or absence of MRP8/MP14. For AFM experiments, the medium contained cytochrome b$_{558}$ purified from neutrophils or from EBV-B lymphocytes and incorporated into liposomes (0.2 pmol), 40 μM GTP•S, 5 mM MgCl$_2$, and 10 μM FAD. Aliquot fractions (10 μl) were collected from the mixture before and after adding 100 nmol of arachidonic acid (AA), deposited on a mica surface, and dried before observation with AFM. In some experiments, the medium contained 0.75 μg of the mixture of purified MRP8/MP14, preincubated or not with 500 mM CaCl$_2$ for 20 min at room temperature (Ca$^{2+}$). In another experiment (*), purified MRP8/MP14 was preincubated (+ AA) or not (– AA) with 100 nmol arachidonic acid before cytochrome b$_{558}$ was added. The AFM measurements were carried out using a ThermoMicroscopes Explorer AFM in non-contact mode (11). Liposome height was determined on x images (where x represents the number of liposome images acquired for the same preparation; x > 3) of three different experiments using the ThermoMicroscopes SPM-Lab software. Results are expressed as the mean value (35 < n < 80) ± S.D. B, graph representing the distribution of the measured liposome heights before (□) and after (△) adding arachidonic acid to the system containing cytochrome b$_{558}$ purified from EBV-B lymphocytes, with 40 μM of GTP•S, 5 mM MgCl$_2$, and 10 μM FAD (left panel), or to the system containing the same medium and purified MRP8/MP14 (0.75 μg) preincubated with 500 mM CaCl$_2$, as described for A (right panel). The x axis corresponds to the number of liposomes measured, and each square represents a single measurement of liposome height. ** stands for results significantly different (p < 0.05) from values obtained before stimulation.

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The cellular origin of cytochrome b$_{558}$ liposome height reached ~6 nm. We found a similar distribution of cytochrome b$_{558}$ liposome height before and after the stimulus was added (Fig. 7B, left panel). The incubation of Ca$^{2+}$-loaded MRP8/MP14 with cytochrome b$_{558}$ purified from neutrophils or from EBV-B cells, followed by the addition of arachidonic acid, led to an increase in liposome height. Fig. 7B (right panel) clearly shows the difference in the distribution of liposome height values after activation in the system containing EBV-B cell cytochrome b$_{558}$ and Ca$^{2+}$-loaded MRP8/MP14. In the absence of calcium, there was no significant conformation change upon activation (data not shown). A statistically significant increase in liposome height was observed (8 versus 10 nm) (Fig. 7A) when MRP8/MP14 was pre-incubated with arachidonic acid without calcium, and then incubated with cytochrome b$_{558}$ liposomes. These results confirm that MRP8/MP14 interact directly with cytochrome b$_{558}$ purified from neutrophils and
from EBV-B cells. This interaction depends on the presence of calcium and arachidonic acid. The effect of MRPs on NADPH oxidase assembly and activation of oxidase activity was observed with cytochrome b556, purified from neutrophil (0.215 pmol equivalent to 2.15 nM), increasing concentration of p67phox (0–300 nM), rRac1 (100 nM), GTPγS, and MgCl2 (squares). In some experiments, p47phox (185 nM) was added to the medium (circles). NADPH oxidase activity was measured after addition of arachidonic acid by measuring the cytochrome c reduction sensitive to superoxide dismutase. A mixture (1/1) of human recombinant MRPs and MRPs (rMRP8/rMRP14) (0.76 μg/assay equivalent to 300 nM) was added to the medium (black squares and black circles) to evaluate the effect of rMRP8/rMRP14 on the semi-recombinant system. In this case, rMRP8/rMRP14 was incubated in the presence of 500 nM CaCl2 at room temperature for 20 min, and then cytochrome b556 and the recombinant cytosolic factors were added.

**Table II**

| Systems | Liposome height | Oxidase turnover |
|---------|----------------|------------------|
| Cyt. b556 (PMN) + neutrophil cytosol | 5 ± 1 | 13 ± 1 | 100 ± 12 |
| Cyt. b556 (PMN) + EBV-BL Cytosol | 5 ± 2 | 12 ± 2 | 56 ± 8 |
| Cyt. b556 (EBV-BL) + neutrophil cytosol | 6 ± 2 | 13 ± 2 | 100 ± 7 |
| Cyt. b556 (PMN) | 5 ± 2 | 12 ± 2 | 17 ± 3 |
| Cyt. b556 (EBV-BL) | 6 ± 2 | 7 ± 1 | 18 ± 4 |
| Cyt. b556 (PMN) + MRP8/MRP14 (Ca2+) | 3 ± 2 | 5 ± 2 | 46 ± 12 |
| Cyt. b556 (EBV-BL) + MRP8/MRP14 (Ca2+) | 5 ± 1 | 15 ± 5 | 50 ± 4 |
| Cyt. b556 (PMN) + MRP8/MRP14 (AA) | 8 ± 3 | 10 ± 2 | 20 ± 3 |

**Fig. 8.** Effect of rMRP8 and rMRP14 in a semi-recombinant cell-free system. Reconstituted oxidase activity medium was prepared as described under “Experimental Procedures,” with cytochrome b556, purified from neutrophil (0.215 pmol equivalent to 2.15 nM), increasing concentration of p67phox (0–300 nM), rRac1 (100 nM), GTPγS, and MgCl2 (squares). In some experiments, p47phox (185 nM) was added to the medium (circles). NADPH oxidase activity was measured after addition of arachidonic acid by measuring the cytochrome c reduction sensitive to superoxide dismutase. A mixture (1/1) of human recombinant MRPs and MRPs (rMRP8/rMRP14) (0.76 μg/assay equivalent to 300 nM) was added to the medium (black squares and black circles) to evaluate the effect of rMRP8/rMRP14 on the semi-recombinant system. In this case, rMRP8/rMRP14 was incubated in the presence of 500 nM CaCl2 at room temperature for 20 min, and then cytochrome b556 and the recombinant cytosolic factors were added.

**DISCUSSION**

The main goal of this study was relative to the change of cytochrome b556 conformation, which initiates NADPH oxidase activity and can be mediated by other effectors than Phox cytosolic factors. In fact we demonstrated that MRPs can interfere with the S100 family, could initiate oxidase activity on their own through a calcium-dependent specific interaction with cytochrome b556. Moreover, MRPs are reported as allosteric effectors of phagocyte oxidase regulation.

**MRPs Control Phagocyte Oxidase Activation**—The properties of MRPs on NADPH oxidase activation, via a preferential association with the cytosolic activating factors of oxidase, mainly p67phox (26). In the present
study, MRP8 and MRP14 were co-isolated on an affinity matrix with the soluble factors of neutrophil NADPH oxidase; both components were absent from the cytosol of EBV-B lymphocytes, and coincidentally, these cells displayed a very low oxidase activity compared with neutrophils (13). We demonstrated in vitro and ex vivo, after gene transfection, that complementation of B lymphocytes with MRP8 and MRP14 was sufficient to increase oxidase activity up to control values (Figs. 4 and 6). Moreover, we showed that both MRP8 and MRP14 were necessary in this process, suggesting that at least the dimer MRP8/MP14 was involved.

There was no oxidase activity even though a normal amount of MRP8 and MRP14 was present (data not shown) in the neutrophils of CGD AR patients (12, 42). A preferential interaction between the MRP8/MP14 complex and p67phox has been suggested in resting neutrophils (26). This may suggest a co-translocation mechanism. In CGD 47p, the absence of p47phox prevents translocation of p67phox (43), whereas in CGD 67p, p47phox is present and translocates on its own to the plasma membrane, without any MRP8/MP14.

From these observations, we may conclude that, once translocated to the plasma membrane, the MRP8/MP14 complex participates in the activation of oxidase as a positive effector of regulation.

**MRP8/MP14 Interaction with Cytochrome b558 Is Mediated by Arachidonic Acid**—MRP8 needs calcium to be active. MRPs are EF-hand molecules, which bind calcium selectively and with high affinity (17). The transitory elevation of cytosolic calcium, in response to inflammatory stimuli, initiates reaction cascades beginning in cytosol with phosphorylation of oxidase cytosolic factors, mainly p47phox, but also MRP14. After calcium binding, MRPs associate in a non-covalent oligomeric MRP8/MP14 structure (heterodimer or heterotetramer) and translocate to the plasma membrane (44). In myelomonocytic cells, phosphorylation of MRP8 and MRP14 has been suggested as the main parameter of subcellular compartmentation (27, 45). The results tend to show that MRP8 and MRP14 must be assembled in an oligomeric structure to have a biological activity. A preferential translocation of phosphorylated MRP14 isoforms was reported (45) in response to a calcium signal.

Arachidonic acid has been suggested to mediate MRP association with membrane structures (46). In fact, a conformational change in the tertiary structure of the MRP8/MP14 dimer, caused by an interaction with arachidonic acid, may improve its binding on membrane or extracellular surfaces. Arachidonic acid has been shown to enhance oxidase activity in vitro (47) and to induce a significant structural change in cytochrome b558 (48). In vivo, among its multiple functions in neutrophils, arachidonic acid has been reported to have a role in NADPH oxidase activation through phospholipase A2 (PLA2). Cytosolic PLA2 was recently introduced as the major PLA2 activated in stimulated phagocytic-like cell (49, 50). Moreover, transfection of antiarachidonic PLA2 agonists completely abolished arachidonic acid release and NADPH oxidase activation in stimulated PLB985 cells, but the translocation of p47phox and p67phox was unaffected (49). These data suggest that arachidonic acid participates in the oxidase complex assembly in vivo. Biological concentrations of arachidonic acid require micromolar levels to elicit most biological activities (51). The binding of MRPs to arachidonic acid may raise its local concentration in the membrane and facilitate MRP interaction with cytochrome b558.

We have seen that both MRP8 and MRP14 were necessary to induce NADPH oxidase activation in our experiments, and that this effect was dependent on calcium and arachidonic acid (Figs. 4 and 6). Most of the S100 proteins formed homodimers, but when both MRP8 and MRP14 were expressed, the heterodimer MRP8/MP14 was preferred (17, 52). More sensitive immunochemical labeling will be necessary to determine which MRP oligomeric structure is involved, but we can reasonably suggest that the heterodimer MRP8/MP14 is indeed implicated.

**Conformation Change of Cytochrome b558 Initiates Oxidase Activation**—Other teams, as well as ours, have demonstrated in vitro that cytochrome b558 is the sole redox component and that p67phox is the limiting cytosolic factor (9, 10) that initiates both assembly of the complex and activation (11). An allosteric regulation mechanism was suggested in which p47phox behaves as a positive effector that increases the affinity of p67phox for cytochrome b558 (53, 54, 11). In vivo, phosphorylated p47phox serves as an adapter protein bringing p67phox into proximity with flavocytochrome b558 (55).

Successfully applying AFM to the structural analysis of oxidase complex assembly opened the way to exploring the structure-function relationships of cytochrome b558 incorporated into liposomes in the presence of MRP8/MP14. The results of AFM cytochrome b558 topography assessment were correlated to oxidase turnover measured at the molecular level. Purified and relipidated cytochrome b558 displays a low but measurable level of NADPH oxidase activity in vitro in the absence of cytosolic factors: this activity depends on the nature of the phospholipid environment (56, 11). The data presented here show that, in vitro, the sole Ca2+-loaded MRP8/MP14 modulates the transition from an inactive to an active conformation state of cytochrome b558 in the presence of arachidonic acid. Moreover, we found that adding MRP8/MP14 to the cytosol of p67phox-deficient EBV-B lymphocytes partly restores oxidase activity, highlighting MRP8/MP14 capacity to induce the transition between the resting and activated states of oxidase (data not shown).

When the reconstitution assay was performed with physiological effectors such as p67phox and p47phox, MRP8/MP14 increased the affinity of p67phox for cytochrome b558 and potentiated the effect of p47phox. These findings suggest that MRPs directly interact at the molecular level with cytochrome b558, this association, which depends on calcium and arachidonic acid, induces reorganization in its structural conformation (Figs. 4 and 6, lanes 2) resulting in oxidase activation. These data suggest that MRP8/MP14 will positively regulate NADPH oxidase activity in cells when cytosolic subunits of oxidase are in limited quantity.

In conclusion, conformation change of cytochrome b558 has been shown to initiate the electron transfer from NADPH to oxygen, and to generate superoxide anions. MRP8/MP14 is suggested to mediate the transition from an inactive to an active conformation state of cytochrome b558 and to behave physiologically as positive mediators of oxidase regulation. The respective function and modality of binding of both MRPs in the allosteric process should be further investigated.

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Changing the Conformation State of Cytochrome b_{558} Initiates NADPH Oxidase Activation: MRP8/MRP14 REGULATION
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