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Myeloid-Related Protein-14 Is a p38 MAPK Substrate in Human Neutrophils

George Lominadze,* Madhavi J. Rane,† Michael Merchant,† Jian Cai,‡ Richard A. Ward,‡ and Kenneth R. McLeish2*†§

The targets of the p38 MAPK pathway that mediate neutrophil functional responses are largely unknown. To identify p38 MAPK targets, a proteomic approach was applied in which recombinant active p38 MAPK and [32P]ATP were added to lysates from unstimulated human neutrophils. Proteins were separated by two-dimensional gel electrophoresis, and phosphoproteins were visualized by autoradiography and identified by MALDI-TOF. Myeloid-related protein-14 (MRP-14) was identified as a candidate p38 MAPK substrate. MRP-14 phosphorylation by p38 MAPK was confirmed by an in vitro kinase reaction using purified MRP-14/MRP-8 complexes. The site of MRP-14 phosphorylation by p38 MAPK was identified by tandem mass spectrometry and site-directed mutagenesis to be Thr113. MRP-14 phosphorylation by p38 MAPK in intact neutrophils was confirmed by [32P]orthophosphate loading, followed by fMLP stimulation in the presence and absence of a p38 MAPK inhibitor, SB203580. Confocal microscopy of Triton X-100 permeabilized neutrophils showed that a small amount of MRP-14 was associated with cortical F-actin in unstimulated cells. fMLP stimulation resulted in a p38 MAPK-dependent increase in MRP-14 staining at the base of lamellipodia. By immunoblot analysis, MRP-14 was present in plasma membrane/secretory vesicle fractions and gelatinase and specific granules, but not in azurophil granules. The amount of MRP-14 associated with plasma membrane/secretory vesicle and gelatinase granule fractions increased after fMLP stimulation in a p38 MAPK-dependent manner. Direct phosphorylation of the MRP-14/MRP-8 complex by p38 MAPK increased actin binding in vitro by 2-fold. These results indicate that MRP-14 is a potential mediator of p38 MAPK-dependent functional responses in human neutrophils. The Journal of Immunology, 2005, 174: 7257–7267.

Circulating neutrophils are relatively benign cells that undergo a series of phenotypic changes in response to inflammatory stimuli, converting them into cells capable of adherence to vascular endothelium, migration to sites of infection, phagocytosis of bacteria, and rapid bacterial killing through exposure to proteolytic enzymes, antimicrobial peptides, and reactive oxygen species. A number of neutrophil responses, including chemotaxis, granule exocytosis, respiratory burst activity, and production of chemokines, are dependent on the activation of the p38 MAPK pathway (1–12). MAPK pathways consist of three-kinase modules formed by the terminal MAPKs, which in turn are activated by serine-threonine kinases such as p38-regulated and -activated kinase and MAPK-activated protein kinase 2 (MAPKAPK-2),3 and cell cycle regulators such as activating transcription factor 2, kinases such as p38-regulated and -activated kinase and MAPK-activated protein kinase 2 (MAPKAPK-2),3 and cell cycle regulators such as cyclin D (17–20). The only p38 MAPK substrates that have been identified in human neutrophils, however, are MAPKAPK-2 and p47(phox) (1, 9, 10, 21). The goal of the present study was to identify p38 MAPK substrates in human neutrophils using a functional proteomic approach developed in our laboratory that combines in vitro phosphorylation of neutrophil lysate by recombinant kinase, separation of proteins by two-dimensional gel electrophoresis, and phosphoprotein identification by MALDI-TOF mass spectrometry (MALDI-TOF MS) (22, 23). One of the proteins identified was myeloid-related protein-14 (MRP-14), a member of the S100 protein family of calcium binding proteins (24, 25). MRP-14 and its binding partner, MRP-8, are highly expressed in neutrophils and monocytes, comprising up to 30% of the total cytosolic protein mass in neutrophils (26, 27). In addition to the unmodified protein of 114 amino acids, an isoform with deletion of the N-terminal four amino acids (MRP-14*) also exists (24–27).

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3 Abbreviations used in this paper: MAPKAPK-2, MAPK-activated protein kinase 2; MS, mass spectrometry; MRP, myeloid-related protein; KRPB, Krebs-Ringer phosphate buffer; IPG, immobilized pH gradient; HCCA, α-cyano-4-hydroxycinnamic acid; TOF, time of flight; CID, collision-induced dissociation; Ly-GDI, lymphocyte-guanine nucleotide dissociation inhibitor; nM, mass-to-charge ratio.
Edgworth et al. (28) reported that MRP-14 and MRP-14* were phosphorylated on Thr\textsuperscript{113} in a protein kinase C-independent manner in neutrophils and monocytes after treatment with ionomycin. Bengis-Garber and Gruener (29) also reported that MRP-14 was phosphorylated in neutrophils upon cell exposure to fMLP. Phosphorylation has been implicated in translocation of the MRP-14/MRP-8 heterodimer to membranes and cytoskeleton in monocytes (30). MRP-14 was also reported to participate in the initiation of NADPH oxidase activity in human neutrophils (31, 32). The present study showed that p38 MAPK phosphorylates MRP-14 on Thr\textsuperscript{113}. fMLP stimulation of intact neutrophils induced p38 MAPK-dependent phosphorylation and translocation of MRP-14 to Triton X-100 insoluble cytoskeleton. Thus, MRP-14 is a potential mediator of p38 MAPK-dependent functional responses in human neutrophils.

Materials and Methods

**Neutrophil isolation**

Neutrophils were isolated from healthy volunteers using plasma-Percoll gradients as described by Haslett et al. (33). Trypan blue staining showed that >97% of cells were neutrophils with >95% viability. After isolation, neutrophils were suspended in Krebs-Ringer phosphate buffer (KRPB) (pH 7.2) at the desired concentration. The Human Studies Committee of the University of Louisville approved the use of human donors.

**Neutrophil lysate preparation**

Neutrophil lysate was prepared as previously described (22, 23). In brief, 10\textsuperscript{6} cells were lysed in 500 \mu l of lysis buffer containing 2 M thiourea, 7 M urea, 65 mM CHAPS, 58 mM DTT, and 4.5% amphotolys (pH 3–10). Lysates were cleared by centrifugation at 12,000 \times g for 20 min at 15°C. Before addition of exogenous p38 MAPK, lysate urea concentration was reduced to 1 M by size exclusion chromatography as previously described (22, 23).

**p38 MAPK substrate identification**

Neutrophil lysates (400 \mu g of total protein) were incubated with 10 \mu Ci of [\gamma\textsuperscript{32}P]ATP (ICN Biomedical) in the presence and absence of 400 ng of active recombinant p38 MAPK (Upstate Biotechnology) at 30°C for 1 h. kinase reactions were stopped by adding rehydration buffer (8 M urea, 2% CHAPS, 0.01 M DTT, 2% ampholytes pH 3–10, and 0.01% bromophen blue). Gel was cut into 450 \mu l slices. Proteins were separated by isoelectric focusing with 18-cm, pH 3–10 imobiline gradient (IPG) strips, followed by size separation with 10% Duracryl (Genomic Solutions), as previously described (23). Proteins were stained with SYPRO Ruby fluorescent stain (Molecular Probes), and phosphorylation was visualized by autoradiography.

**Identification of proteins by mass spectrometry**

Protein spots were excised and digested with trypsin by modification of the method of Jensen et al. (34). The excised gel pieces were incubated for 15 min in 500 \mu l of 100 mM NH\textsubscript{4}HCO\textsubscript{3} and 50% acetonitrile and were dried by vacuum centrifugation. Proteins were then reduced by incubation with 20 mM DTT for 1 h at 56°C for 45 min, followed by alkylation with 65 mM iodoacetamide in 20% acetonitrile with ion source pressure 0.5 \textmu mbar. Phosphorylation was identified by pTyr antibodies (Upstate Biotechnology) at 30°C for 1 h.

**Isolation of MRP-14/MPR-8 complex for in vitro phosphorylation**

High solubility of MRP-14/MPR-8 complex in concentrated ammonium sulfate solution was used to isolate these proteins (35). Neutrophils (3 \times 10\textsuperscript{6} cells) were resuspended in 3 ml of 10 mM Tris buffer containing 20 mM Tris (pH 7.2), 0.5% Triton X-100, 0.5% Nonidet P-40, 20 mM NaF, 0.2 mM orthovanadate, 1 mM EDTA, 1 mM EGTA, 1 mM 4-(2-aminoethyl)benzene sulfonyl fluoride, 0.005 mg/ml leupeptin, and 0.02 mg/ml aprotinin, and then they were disrupted by rotating the tube at room temperature for 15 min. Lysates were centrifuged at 14,000 \times g for 15 min to pellet the insoluble material, and supernatants were removed. Proteins in the supernatant were subjected to precipitation at room temperature by adding 100% saturated ammonium sulfate solution to reach the final concentration of 80% ammonium sulfate. Precipitated proteins were pelleted by centrifugation at 5000 \times g for 15 min, and the supernatant was dialyzed overnight at 4°C against a buffer containing 0.1 M Tris-HCl (pH 7.4), 50 mM KCl, and 20% glycerol. Protein concentration was adjusted to 1 mg/ml and aliquots were stored at −20°C.

**In vitro kinase reactions**

Phosphorylation of purified MRP-14 by p38 MAPK was performed by incubation of active recombinant p38 MAPK (1 \mu g) with 10 \mu Ci of [\gamma\textsuperscript{32}P]ATP and 12 \mu g of purified MRP-14/MPR-8 complex in 30 \mu l of kinase buffer containing 25 mM HEPES, 25 mM \beta-glycerophosphate, 25 mM MgCl\textsubscript{2}, 2 mM DTT, and 0.1 mM Na\textsubscript{2}VO\textsubscript{3} (pH 7.2). Reactions were incubated at 30°C for 30 min. Reactions were terminated with SDS sample dilution buffer, proteins were separated by SDS-PAGE on 4–12% Novex Bis-Tris gels (Invitrogen Life Technologies), gels were stained with Coomassie blue dye, and phosphorylation was visualized by autoradiography.

**Identification of the phosphorylation site in MRP-14 by mass spectrometry**

MRP-14/MPR-8 complex (40 \mu g of protein) was incubated for 2 h in 60 \mu l of kinase buffer (50 mM Tris-HCl (pH 7.5), 30 mM MgCl\textsubscript{2} containing 5 mM Na\textsubscript{2}ATP in the presence or absence of active recombinant p38 MAPKα (1 \mu g). Proteins were transferred to 100 mM ammonium bicarbonate buffer by ultrafiltration using Nanosep 3K Omega ultrafiltration devices (Pall) to the final volume of 100 \mu l. Five nanograms of sequencing grade modified porcine trypsin (Promega) was added to the protein solution and proteins were digested overnight at 37°C. Generated peptides were subjected to phosphopeptide enrichment by immobilized metal ion affinity chromatography using metal chelate ZipTipMC columns (Millipore) and Fe\textsubscript{3}O\textsubscript{4} as a source of metal ions, according to manufacturer’s instructions. Resultant peptide solutions were dried by vacuum centrifugation and re-suspended in 5 \mu l of 50 mM ammonium bicarbonate. The matrix, HCCA, was dissolved by dissolving a stock of HCCA in 1 ml of acetonitrile/water (50:50, v/v) containing 0.1% trifluoroacetic acid (TFA). The matrix solution was vortexed until all solids were dissolved. Samples were spotted as 1:1 (v/v) of protein digest/HCCA directly onto MALDI sample targets. Samples were desalted by spotting 0.7 \mu l of 0.1% trifluoroacetic acid on each dried sample spot, followed immediately by pipetting off the surface liquid. Samples were air-dried in the dark and were cleared of particulate matter with compressed gas before sample plate loading into the mass spectrometer. Positive ion MALDI-TOF mass spectra were acquired using an Applied Biosystems AB4700 protein analyzer operating in reflectron mode and with ion source pressure ~0.5 mTorr. After a 400-ns time-delayed ion extraction period, the ions were accelerated to 20 kV for time of flight (TOF) mass spectrometric analysis. A total of 600-1000 laser shots (355-nm Nd:YAG solid state laser operating at 200 Hz) were acquired and signal averaged. Data were processed using Mascot (Matrix Science), assuming 1) monoisotopic peptide masses, 2) cysteine carbamidomethylation, 3) variable oxidation of methionine, 4) a maximum of one missed trypsin cleavage, and 5) a mass accuracy of 100 ppm or better. Limitation of the original protein mass was not used within the Mascot search.

The protein candidates with Mascot significance scores of p < 0.05 were 1) analyzed for theoretical tryptic fragmentation masses using Peptide Cutter (Promega) and 2) reanalyzed by Mascot using an additional Mascot scoring parameter of serine/threonine phosphorylation. Peaks identified within the acquired MALDI-TOF spectrum matching to the predicted phosphopeptide masses were subjected MALDI-TOF-TOF analysis by collision-induced dissociation (CID) fragmentation using 1 keV collision energy and atmospheric gases. Mascot search of the MALDI-TOF-TOF data proceeded with search parameters listed above with the inclusion of a mass accuracy of 0.6 Da for peptide fragment masses. MALDI-TOF-TOF spectra were used for combined MS/MS(MS) analysis using Global Protein Server software (Applied Biosystems) and Mascot. The resultant data were
used to determine the presence or absence of phosphorylation or the sequence of the peptide.

**Expression of recombinant wild-type and mutant MRP-14**

Total RNA from human neutrophils was isolated using the RNeasy Mini kit (Qiagen). RT-PCR primers were designed according to the cDNA sequence of human MRP-14. The forward primer (5'-CTCCTGTTGATCTCTCAGGCCTTTGG-3') incorporated a BamHI restriction enzyme site, and the reverse primer (5'-CAGCTGTCAGCTTGAGGGGTCC-3') incorporated a HindIII restriction enzyme site. RT-PCR was performed using the SuperScript One-Step RT-PCR kit (Invitrogen Life Technologies) under the following conditions: 30 min at 50°C for cDNA synthesis, 2 min at 94°C for strand denaturation, followed by 40 cycles of 30-s steps at 94°C (denaturation), 55°C (annealing), and 68°C (extension), and the final extension step of 72°C for 10 min. The PCR product was digested with BamHI and HindIII (Promega) and ligated into pRSET-A plasmid (Invitrogen Life Technologies) using T4 DNA ligase (Promega). Plasmids were propagated in DH5α chemically competent *Escherichia coli* cells (Invitrogen Life Technologies). Thr114 mutation to Ala was used according to the Transformer Site-Directed Mutagenesis kit from BD Biosciences according to the manufacturer’s instructions. The mutation primer for Thr114 to Ala114 was 5'-GGGAGGGGCCCCCCCCTAAG-3' and the selection primer for pRSET-TATAM (mutating the XbaI site) was 5'-CAAGGTTTCCTTCCGAAATA-3'. Cloning and mutation were confirmed by DNA sequencing. Expression of pRSET-MRP-14 and pRSET-MRP-14AAA plasmids was conducted in BL21(DE3)pLyS chemically competent *E. coli* cells, and protein was purified using the ProBond Purification System (Invitrogen Life Technologies).

**Assessment of MRP-14 phosphorylation ex vivo**

Neutrophils were loaded with [32P]orthophosphate, as previously described by Grinstein et al. (36). Briefly, 8 × 10⁶ cells were suspended in 4 ml of loading buffer (6 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 0.25 mM CaCl₂, 10 mM glucose) for 5 min and divided into four 1-ml tubes. Two milliliters of [32P]orthophosphate was added to each tube, and the cells were rotated at 37°C for 60 min. SB203580 was added to appropriate tubes to a final concentration of 3 μM simultaneously with orthophosphate. After 1 h, IMLP (final concentration 300 nM) was added to appropriate tubes. After 5 min, cells were pelleted at 500 × g and washed once in loading buffer, and the pellet was solubilized in 600 μl of rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 0.01 M DTT, 2% Triton X-100) in a Percoll gradient formed from three 9-ml layers of Percoll, of density 1.090 g/ml, 1.050 g/ml, and 1.120 g/ml. Percoll solutions were prepared in KRPB supplemented with 1.2 mM MgCl₂ and 0.5 mM CaCl₂ at a concentration of 5 × 10⁶ cells/ml. Cells (200 μl) were added to each well of the Lab-Tek Chambered Coverglass (Nalge Nunc International) and chambers were incubated for 15 min at room temperature. SB203580 (3 μM) was added to appropriate wells, and the cells were incubated at 37°C for 1 h before addition of 300 μl IMLP for 5 min at 37°C. Cells were washed with ice-cold KRPB, and 200 μl of 0.5% Triton X-100 in KRPB was added to each well for 30 min at room temperature to extract cytosolic MRP-14 from cells. Neutrophils were fixed with 3.7% paraformaldehyde for 30 min at 4°C, blocked with 2% goat serum and 2% BSA in PBS, and incubated with mouse monoclonal anti-MRP-14 (Kamiya Biomedicals) overnight at 4°C (1/200 dilution in blocking buffers). Cells were washed with PBS and incubated at 4°C for 1 h with rhodamine-conjugated goat anti-mouse IgG (Molecular Probes; 1/1000). Cells were also stained with BODIPY-conjugated phallodin (Molecular Probes) for 1 h at 4°C (to visualize F-actin). Imaging was conducted on a Zeiss Axiovert 100 M microscope using LSM510 software.

**Two-dimensional electrophoretic analysis of granule and plasma membrane fractions**

Neutrophil subcellular fractionation was conducted by the method of Kjeldsen et al. (37). Briefly, isolated neutrophils (8 × 10⁷/ml) were treated with 10 μM diisopropyl fluorophosphates and resuspended in disruption buffer containing 100 mM KCl, 1 mM NaCl, 1 mM ATPNa₂, 3.5 mM MgCl₂, 10 mM PIPES, and 0.5 mM PMSF for 90 min. Cells were centrifuged by nitrogen cavitation for 10 min at 380 psi and 4°C. Cavitate was collected into 1.5 mM EGTA, and nuclei and intact cells were removed by centrifugation at 500 × g for 5 min. The postnuclear supernatant was layered onto a Percoll gradient formed from three 9-ml layers of Percoll, of density 1.090 g/ml, and 1.120 g/ml. Percoll solutions were prepared in buffer containing 100 mM KCl, 3 mM NaCl, 1 mM Na₂ATP, 3.5 mM MgCl₂, 1.25 mM EGTA, 10 mM PIPES, and 0.5 mM PMSF. The gradient was centrifuged at 37,000 × g for 30 min in an SS-34 fixed angle rotor in a Sorvall RC-5B centrifuge. The separated granules were recovered from the gradient interfaces by aspiration, and the Percoll was removed by ultracentrifugation at 100,000 × g for 90 min using the Ti-50.3 rotor (Beckman). Granule and plasma membrane/secretory vesicle devoid of Percoll were washed in disruption buffer by centrifugation. Washed material was dissolved in 300 μl of rehydration buffer, and lysates were cleared by centrifugation at 14,000 × g. Supernatant was loaded onto pH 3–10 IGE Zoom strip by overnight rehydration at 27°C. Proteins were separated by two-dimensional gel electrophoresis using the Zoom IGE Runner isoelectric focusing apparatus and X-Cell Sure Lock electrophoresis apparatus, as described above. Gels were stained with Coomassie blue dye.

Purity of granule fractions was determined by immunoblot analysis for CD66b (a marker of specific granules) and an ELISA for gelatinase and myeloperoxidase (a marker of azurophil granules). CD66b was found only in specific granule fractions. Distribution of gelatinase was 75% in gelatinase granules, 18% in specific granules, 5% in plasma membrane/secretory vesicles, and 2% in azurophil granules. Myeloperoxidase distribution was 76% in azurophil granules, 20% in specific granules, 3% in gelatinase granules, and 1% in plasma membrane/secretory vesicles. This degree of granule enrichment is similar to that previously reported (37).

**Association of MRP-14 with neutrophil granules and plasma membranes**

Neutrophils (9 × 10⁶ cells) were resuspended in KRPB supplemented with Ca²⁺ and Mg²⁺ and incubated with or without 300 mM IMLP for 5 min with or without pretreatment with 3 μM SB203580 for 40 min. Cells were subjected to subcellular fractionation, as described above. Granule and plasma membrane/secretory vesicle proteins were separated by SDS-PAGE on 15% gels, the gels were cut at the level of the 25-kDa marker, and the lower portions of gels were subjected to immunoblotting using standard methods. Blotted membranes were probed with mouse monoclonal anti-MRP-14 (Kamiya Biomedicals) at 1/500 dilution and with HRP-conjugated goat anti-mouse IgG (Upstate Biotechnology) at 1/2000 dilution. To ensure equal protein loading, upper portions of the cut gels were stained with Coomassie blue dye, and the staining intensities of protein bands were compared.

**F-actin co sedimentation assay**

F-actin filaments (500 μg of 5- to 10-μm length) were generated using the Actin Filament Biochem kit (Cytoskeleton) according to the manufacturer’s instructions. MRP-14/MRP-8 complexes (150 μg of protein) prepared from human neutrophils were incubated overnight at 30°C in 100 μl of KRPB containing 25 mM HEPES, 100 mM KCl, 40 mM MgCl₂ (pH 7.2), and 5 mM Na₂ATP (pH adjusted to 7.4) in the presence or absence of 2 μg of active recombinant p38 MAPK. After overnight incubation, unphosphorylated (no kinase) and phosphorylated (kinase added) MRP-14/MRP-8 complexes (100 μl) were mixed with 350-μl F-actin filaments (250 μg of actin) in the Actin Polymerization Buffer supplied with the kit. To determine the amount of nonspecific MRP-14/MRP-8 sedimentation, 100 μl of unphosphorylated and phosphorylated complexes were added to 350 μl of Actin Polymerization Buffer without actin. All solutions were incubated at 37°C for 30 min and centrifuged at 100,000 × g for 1 h in a Ti55 rotor (Beckman) at 37°C. Supernatants (450 μl) were removed completely, and pellets were dissolved in 100 μl of SDS-PAGE gel loading buffer. Twenty microfilters of the dissolved pellets and 20 μl of supernatant were loaded onto 4–12% Bis-Tris SDS-PAGE gels (Invitrogen Life Technologies), proteins were separated, and the gels were stained with colloidal Coomassie blue dye (Genomic Solutions). Gels were scanned and the intensities of MRP-14 bands were measured by densitometry.
Results
Identification of p38 MAPK substrates
To screen for p38 MAPK substrates, human neutrophil lysates were incubated with \([\gamma^{32P}]ATP\) in the presence and absence of active recombinant p38 MAPK. Proteins were separated by two-dimensional gel electrophoresis, gels were stained with Coomassie blue dye, and phosphorylated proteins were visualized by autoradiography. Autoradiographs were compared with Coomassie-stained gels to identify proteins that were p38 MAPK substrates. Protein phosphorylation was not observed on autoradiographs of neutrophil lysates incubated with \([\gamma^{32P}]ATP\) in the absence of active recombinant kinase, indicating that the method of neutrophil lysate preparation eliminated endogenous kinase activity (data not shown). In the presence of active recombinant p38 MAPK, autoradiography demonstrated at least 15 phosphorylated proteins. Three of these could be matched to Coomassie blue-stained protein spots, whereas no stained protein spots could be visualized for the remainder. Fig. 1 shows the location on two-dimensional gels of the three proteins identified by MALDI-TOF MS, MRP-14, coronin, and lymphocyte-guanine nucleotide dissociation inhibitor (Ly-GDI). MRP-14 is a known phosphoprotein that binds \(Ca^{2+}\) (24–30) and arachidonic acid (38, 39). MRP-14 also acts as a binding partner for the cytosolic factors of the NADPH oxidase (31, 32). As these findings suggest that MRP-14 may mediate some p38 MAPK-dependent neutrophil responses, additional experiments were performed to confirm that MRP-14 is a true substrate of p38 MAPK.

In vitro phosphorylation of MRP-14 by p38 MAPK
The possibility that MRP-14 phosphorylation represented a false positive was addressed by performing in vitro kinase reactions under nondenaturing conditions. In cells, MRP-14 is predominantly found in a heterodimeric complex with MRP-8 (26, 27). To assess the ability of p38 MAPK to phosphorylate MRP-14 while complexed with MRP-8, MRP-14/MRP-8 complexes were purified from human neutrophils under nondenaturing conditions. Purified complexes were incubated with active recombinant p38 MAPK and \([\gamma^{32P}]ATP\), after which proteins were separated by SDS-PAGE. Fig. 2 shows a Coomassie blue-stained gel and the corresponding autoradiograph demonstrating that MRP-14, but not MRP-8, was phosphorylated by p38 MAPK. These data show that MRP-14 is a p38 MAPK substrate while complexed with MRP-8.

MRP-14 phosphorylation by p38 MAPK in intact neutrophils
To assess whether MRP-14 is phosphorylated by p38 MAPK in intact cells, isolated neutrophils were loaded with \([32P]\)orthophosphate in the absence or presence of the p38 MAPK inhibitor SB203580.
SB203580 before stimulation of cells with fMLP. Cells were lysed and proteins separated by two-dimensional electrophoresis. Coo-massie blue-stained gels were compared with autoradigraphs to identify phosphorylation events. Fig. 3 shows that some phosphorylation of MRP-14 was detected in unstimulated cells, and this basal phosphorylation was reduced by pretreatment with 3 μM SB203580. Stimulation with 300 nM fMLP for 5 min markedly enhanced phosphorylation of MRP-14, whereas pretreatment with SB203580 abolished fMLP-induced phosphorylation. These data indicate that MRP-14 is a substrate of p38 MAPK in human neutrophils.

p38 MAPK phosphorylates Thr$^{113}$ on MRP-14

MRP-14 was reported previously to be phosphorylated on Thr$^{113}$, although the kinase was not identified (28). To determine whether Thr$^{113}$ is phosphorylated by p38 MAPK, mass spectrometry was used to identify the phosphorylated residue on MRP-14. MRP-14/MRP-8 complex alone, which included a peptide with m/z of 2177, corresponding with the C-terminal 21-aa tryptic peptide of MRP-14 (MHEGDEPGHHHKPGLGEGR) (arrow). A, Mass spectra for digests of the MRP-14/MRP-8 complex incubated with p38 MAPK. A new major peak at 2257 m/z was found, corresponding with the hypothetical mass (2256.4) of the phosphorylated C-terminal 21-aa peptide containing Thr$^{113}$. The peak with the m/z of 2257 was sequenced using the timed ion selection and CID fragmentation. C, Spectra of the ions from this fragmentation. Included in this spectra was an ion with a mass of ~2159 m/z (shown with an asterisk on Thr), which corresponds with the neutral loss of phosphoric acid (H$_3$PO$_4$) from the parent phosphorylated ion (theoretical mass of 2158). The increase of 80 Da with phospho-MRP-14 and the loss of 98 Da with CID confirm that the posttranslational modification on the peptide was phosphorylation. Observation of multiple peaks with 1-Da differences in m/z (for example, observation of peaks with m/z of 2257, 2258, and 2259) arises from the natural presence of isotopes of carbon, nitrogen, oxygen, and hydrogen in the peptides.

p38 MAPK phosphorylates wild-type human recombinant MRP-14 (hrMRP-14$^{WT}$), but not the T113A mutant (hrMRP-14$^{TA}$). Twenty micrograms of purified recombinant human wild-type and T113A mutant MRP-14 were incubated with $[^{32}$P]ATP in the presence or absence of the 50-ng active recombinant p38 MAPK for 30 min at 30°C. The upper panel shows a Coo-massie-stained gel, and the lower panel shows the corresponding autoradiograph. Wild-type MRP-14 was phosphorylated, whereas no phosphorylation of the T113A mutant was seen. The mass of the recombinant MRP-14 is ~4.5 kDa higher than that of human neutrophil MRP-14 due to the additional histidine tag.
to phosphopeptide enrichment using metal ion affinity chromatography. MALDI-TOF MS analysis of the tryptic digests confirmed the presence of MRP-14 (Mascot, \( p < 0.05 \)) in all digests. The unphosphorylated C-terminal 21-aa peptide containing Thr^{113} was predicted to have a mass-to-charge ratio (m/z) of 2176.2, whereas the phosphorylated peptide was predicted to be 2256.0 m/z due to

**FIGURE 6.** Phosphorylation-induced redistribution of MRP-14 in intact neutrophils. Control and fMLP-stimulated neutrophils were plated on confocal microscope slides and permeabilized with Triton X-100 before fixation and staining. **A**, Cells stained for F-actin using BODIPY 650/665-conjugated phalloidin (shown in green) and with monoclonal anti-MRP-14 and rhodamine-conjugated anti-mouse IgG (shown in red) and the merged pictures of the two. Unstimulated cells show minimal staining for MRP-14, confined to the periphery of the cell and colocalized with cortical F-actin (Control). Neutrophil stimulation with fMLP resulted in increased F-actin staining primarily localized to lamellipodia. An increase in MRP-14 staining was seen at the base of lamellipodia. Increased MRP-14 staining and association with lamellipodia were completely reversed by incubation of cells with SB203580 before fMLP treatment (fMLP/SB), whereas F-actin staining and lamellipodia formation were not affected. **B**, Analysis of single cell staining profiles using LSM510 software (green lines correspond with the intensity of actin staining and red with MRP-14 staining). This analysis demonstrates colocalization of F-actin and MRP-14 under basal condition (control). After fMLP stimulation, F-actin staining was most prominent in lamellipodia. MRP-14 did not colocalize with the leading edge of F-actin, but was present at the base of lamellipodia. Preincubation with 3 \( \mu M \) SB203580 (fMLP/SB) blocked translocation of MRP-14. Distance in the graphs (the x-axis) corresponds with the distance measured from the base to the tip of the line traversing cells. These results indicate that MRP-14 translocates to Triton X-100 insoluble structures at the base of lamellipodia in human neutrophils upon fMLP treatment and that the translocation is p38 MAPK dependent.
the 80-Da mass addition of a phosphoryl group (HPO₄) (40). Analysis of peptide mass spectra showed a peak at 2177.3 m/z in samples from p38 MAPK treated and untreated MRP-14 (Fig. 4, A and B). An ion of 2257.2 m/z was observed only in the digest from the sample incubated with p38 MAPK (Fig. 4B). These results suggest that the C-terminal 21-aa tryptic peptide of MRP-14 was modified by phosphorylation.

To confirm the phosphorylation of this peptide, timed ion selection of 2257 ± 3 m/z precursor ions, followed by CID fragmentation, yielded a fragmentation pattern with a Mascot ion score of 31 (significant homology (p < 0.05) at a score >18 and identity at a score of >33) for a sequence of MKMEGDEGGPGHMK PGLGEFT (114). The TOF-TOF spectrum analysis included an ion of 2158.6 m/z, which corresponds with the neutral loss of 98 m/z (the mass of phosphoric acid, H₃PO₄) from the parent phosphorylated ion (Fig. 4C). Appearance of a peptide with this m/z in addition to the mass plus hydrogen + 80 precursor ion confirms that the posttranslational modification on the peptide was phosphorylation (41–44). As Thr₁¹³ is the only residue in the peptide that could serve as a phosphate acceptor from p38 MAPK, these data suggest that p38 MAPK phosphorylates MRP-14 on Thr₁¹³.

To confirm that Thr₁¹³ is the residue phosphorylated by p38 MAPK, a mutant recombinant protein was created in which Ala was substituted for Thr₁¹³. Wild-type MRP-14 and mutant MRP-14 were expressed and incorporated in an in vitro kinase reaction with recombinant active p38 MAPK. Fig. 5 shows that wild-type MRP-14 was strongly phosphorylated under the conditions used, whereas no phosphorylation was observed when Ala replaced Thr₁¹³. Taken together, these results firmly establish that p38 MAPK phosphorylates MRP-14 on Thr₁¹³.

**p38 MAPK-dependent translocation of MRP-14 in intact neutrophils**

Phosphorylation of MRP-14 in monocytes results in increased calcium binding and translocation from cytosol to membranes and cytoskeleton (30). To determine whether a similar translocation occurred in human neutrophils, confocal microscopy was used to determine colocalization of MRP-14 and F-actin in fMLP-stimulated and unstimulated cells. Initial studies showed that the large amount of cytosolic MRP-14 resulted in intense staining of the whole cell, which obscured visualization of any changes in intracellular distribution (data not shown). Therefore, control and fMLP-stimulated neutrophils were stained with confocal microscopy slides were permeabilized with Triton X-100 to remove cytosolic MRP-14. Cells were then fixed and stained for F-actin using BODIPY 650/665-conjugated phalloidin and were stained for MRP-14 with monoclonal anti-MRP-14 and rhodamine-conjugated secondary Ab. Fig. 6A shows that minimal staining for MRP-14 associated with cortical F-actin was observed in unstimulated cells. fMLP stimulation resulted in a marked increase in MRP-14 staining and in F-actin formation, both of which were associated with lamellipodia. MRP-14 staining was localized at the base of F-actin within lamellipodia and excluded from the leading edge (Fig. 6B). The increased intensity of MRP-14 staining and localization at lamellipodia were completely prevented by pretreatment with SB203580, whereas formation of lamellipodia and increased F-actin formation were not affected. These results suggest that MRP-14 translocates to the stable actin cytoskeleton at the base of lamellipodia after fMLP stimulation of human neutrophils, and this translocation is dependent on p38 MAPK activity.

We and others reported previously that neutrophil exocytosis depends on p38 MAPK activation (6, 10–12). Therefore, localization of MRP-14 to neutrophil granule subsets and the effect of fMLP stimulation on this localization were examined. Two-dimensional electrophoretic separation of neutrophil granule and plasma membrane/secretory vesicle fraction proteins revealed that MRP-14 was present in plasma membrane/secretory vesicle fractions and on gelatinase granules and specific granules, but that it was absent from azurophilic granules (Fig. 7). The amount of MRP-14 associated with plasma membrane/secretory vesicle and granule fractions in control and fMLP-activated neutrophils was examined using Western blotting (Fig. 8). The amount of MRP-14 associated with specific granules was greater than that associated with plasma membrane/secretory vesicles or gelatinase granules. fMLP stimulation resulted in a significant increase in the amount of MRP-14 associated with plasma membrane/secretory vesicle fractions and gelatinase granules, whereas no change was observed in MRP-14 association with specific granules. This translocation was abolished by pretreatment of cells with SB203580. MRP-14 did not associate with azurophilic granules under either basal or stimulated conditions (data not shown). In separate experiments, the ability of anti-MRP-14 Ab to recognize both the nonphosphorylated and the phosphorylated forms of MRP-14 was examined. Both purified MRP-14/MRP-8 complexes and recombinant MRP-14 were incubated with ATP in the presence and absence of p38 MAPK. Immunoblot analysis showed that phosphorylated and nonphosphorylated MRP-14 were detected equally (data not shown). These results suggest that p38-MAPK-dependent phosphorylation induces translocation of MRP-14 to plasma membrane and gelatinase granules, possibly due to increased association with the actin cytoskeleton.

To determine whether MRP-14 phosphorylation regulates its binding to actin, an in vitro F-actin cosedimentation assay was performed. MRP-14/MRP-8 complexes were incubated with ATP in the presence and absence of recombinant active p38 MAPK.
Equal amounts of phosphorylated and nonphosphorylated MRP-14/MRP-8 were added to F-actin filaments, then F-actin was pelleted by ultracentrifugation. Protein in the pelleted F-actin and supernatant were separated by 4–12% gradient electrophoresis. Fig. 9A shows a Coomassie blue-stained gel demonstrating increased association of phosphorylated MRP-14/MRP-8 with actin. Fig. 9B presents the densitometric analysis of three separate experiments. The data indicate that p38 MAPK phosphorylation does the amount of MRP-14/MRP-8 complex associated with F-actin. In the absence of F-actin, MRP-14/MRP-8 complexes failed to pellet after ultracentrifugation (data not shown). These data suggest that phosphorylation by p38 MAPK regulates the translocation of MRP-14/MRP-8 to lamellipodia and granules by mediating binding to F-actin.

Discussion
The p38 MAPK pathway participates in a number of neutrophil functions critical to generation and regulation of the inflammatory response, including chemotaxis, adherence, respiratory burst activity, degranulation, and cytoskeletal reorganization (2–12). Understanding the molecular mechanisms by which p38 MAPK participates in these responses is hindered by the limited number of targets of p38 MAPK identified to date in neutrophils. MAPKAPK2 and p47(\textsuperscript{phos}) are the only clearly identified p38 MAPK targets in human neutrophils (1, 9, 10, 21). A previous study by Lewis et al. (45) determined that 20 of 25 ERK targets identified in a global screen were not previously known. Thus, it is likely that a number of important targets of p38 MAPK that participate in regulation of neutrophil responses remain to be identified. The goal of the present study was to apply a recently developed proteomic approach that allows simultaneous identification of multiple substrates of a single kinase to defining p38 MAPK substrates in human neutrophils. This approach involves in vitro phosphorylation of cellular lysate by active recombiant kinase in the presence of [\textgamma\textsuperscript{32}P]ATP, followed by protein separation by two-dimensional gel electrophoresis, alignment of autoradiogram with stained gel, and subsequent identification of phosphoproteins by MALDI-TOF MS. Using this approach, three potential p38 MAPK substrates were identified: MRP-14, Ly-GDI, and coronin.

The identification of only three of >15 phosphorylated proteins by the current approach indicates that sensitivity remains a limitation to this method. For a number of phosphorylation events identified by autoradiography, no corresponding protein spot was found or an insufficient mass spectrum was obtained for successful protein identification. The limited sensitivity may result from low abundance of these proteins, inadequate transfer of proteins from IPG strips to the second dimension gels, incomplete in-gel digestion of the protein by trypsin, or inefficient processing and ionization of phosphopeptides during mass spectrometric analysis.

All three of the proteins identified as targets of p38 MAPK have the potential of participating in neutrophil functional responses. Coronins are a family of actin binding proteins that directly regulates actin nucleation by actin-related protein 2/3 complexes (46),...
participates in chemotaxis of Dictyostelium (47), associates with the phagocytic cup (48), and binds the cytosolic components of the NADPH oxidase (49). Ly-GDI inhibits dissociation of GDP from Rho GTPases, including Rac and Cdc42, maintaining them in the inactive state and controlling distribution between cytosol and membrane (50, 51). MRP-14 and its heterodimeric partner, MRP-8, are members of the S100 family of calcium binding proteins. The heterodimer constitutes up to 30% of neutrophil and 1% of monocyte cytosolic proteins (26, 27). The MRP-14/MPR-8 complex was reported to undergo phosphorylation-dependent translocation to the plasma membrane and cytoskeleton in monocytes after cell stimulation (30). The MRP-14/MPR-8 complexes were also found to bind arachidonic acid (38, 39) and to participate in the activation of NADPH oxidase (31, 32). MRP-14/MPR-8 increased the affinity of p67phox for cytochrome b558, and oxidase activity was enhanced (32). Targeted disruption of the MRP-14 gene in mice does not produce consistent phenotypic changes (52, 53), although Manitz et al. (53) reported impaired in vitro chemotaxis and up-regulation of CD11b. Because of its abundance in human neutrophils, further studies were performed examining MRP-14.

The artificial conditions used for in vitro kinase reactions with neutrophil lysates may permit p38 MAPK phosphorylation of proteins that would not be targets in intact neutrophils. The use of cell lysate may permit access of exogenously added kinase to proteins with restricted localization in intact cells. Urea denaturation may expose phosphorylation sites that are inaccessible in properly folded proteins. Disruption of protein-protein interactions may result in dissociation of signaling modules that restrict kinase-substrate interaction. Finally, protein spots may contain more than one protein, which could lead to identification of false substrates. For these reasons, experiments were performed in which MRP-14 was confirmed to be a p38 MAPK substrate both in vitro and ex vivo. The MRP-14/MPR-8 complex was isolated under nondenaturing conditions and subjected to an in vitro kinase reaction with active recombinant p38 MAPK. Under these conditions, MRP-14, but not MRP-8, was phosphorylated. In intact neutrophils loaded with [32P]orthophosphate, MRP-14 demonstrated basal phosphorylation that was dramatically increased by FMLP stimulation. Basal and fMLP-stimulated phosphorylation of MRP-14 was dependent on p38 MAPK activity. These findings indicate that MRP-14, although in a heterodimeric complex with MRP-8, is a direct target of p38 MAPK in human neutrophils. This conclusion is supported by a recent report by Vogl et al. (54) showing that MRP-14 is phosphorylated by p38 MAPK in arsenite-stimulated human neutrophils. Based on previous reports indicating that MRP-14 phosphorylation functions from the plasma membrane/secretory vesicle fraction inArsenal-stimulated human neutrophils, we hypothesized that MRP-14 phosphorylation may be responsible for the increased staining and up-regulation of CD11b. Because of its abundance in human neutrophils, further studies were performed examining MRP-14.

To identify the p38 MAPK phosphorylation site in MRP-14, a combination of phosphopeptide enrichment using immobilized metal affinity chromatography and tandem mass spectrometry was used (41–44). Edgeworth et al. (28) showed that the penultimate residue, Thr113, was the single phosphorylated residue on MRP-14 in ionomycin-activated neutrophils and monocytes and that this phosphorylation was independent of protein kinase C activity. Our data found that in the presence of p38 MAPK, the 21-aa C-terminal MRP-14 tryptic peptide that contains Thr113 showed an 80-Da increase in mass, as compared with MRP-14 tryptic peptides in the absence of p38 MAPK. This is consistent with phosphorylation of this peptide, as the mass of a phosphoryl group (HPO3) is 80 Da (40). Use of tandem mass spectrometry allowed us to observe the β-elimination, neutral loss of 98 m/z from the precursor peptide ion, which is the mass of phosphoric acid (H3PO4) (40, 41). Peptide tag sequencing of the phosphopeptide using collision-induced dissociation showed that the sequence corresponded with that of the 21-aa C-terminal peptide of MRP-14. Confirmation that Thr113 was the amino acid residue phosphorylated by p38 MAPK was obtained by showing that mutation of Thr113 to Ala prevented phosphorylation. Similarly, Vogl et al. (54) identified Thr113 as the site of p38 MAPK-mediated phosphorylation.

Despite being the most abundant protein in human neutrophils, the functional role of MRP-14 has not been determined. The MRP-14/MPR-8 complex translocates to the plasma membrane and cytoskeleton in monocytes in a phosphorylation-dependent manner after cell stimulation (30). The present study used confocal microscopy to determine the location of MRP-14 after neutrophil stimulation with fMLP and to determine whether changes in location were dependent on p38 MAPK activity. The abundance of MRP-14 in human neutrophils resulted in marked staining of the entire cell, preventing detection of differences in MRP-14 localization between control and stimulated cells (data not shown). We presumed that the large amount of cytosolic MRP-14 was obscuring any changes in localization that could have occurred as the result of phosphorylation after cell activation. Permeabilization of cells with Triton X-100 before fixation and staining resulted in loss of cytosolic MRP-14. Under basal conditions, only a small amount of MRP-14 was present in the subplasma membrane area occupied by cortical actin. fMLP stimulation before permeabilization resulted in a significant increase in MRP-14 staining that localized to the base of the actin cytoskeleton forming lamellipodia. The increased staining for MRP-14 and its localization at the base of lamellipodia were dependent on p38 MAPK-mediated phosphorylation, as both were inhibited by SB203580 at concentrations specific for p38 MAPK inhibition (55). These data suggest that phosphorylation by p38 MAPK results in MRP-14 association with long, unbranched actin filaments that form at the base of lamellipodia. This conclusion is supported by our data demonstrating enhanced F-actin binding of phosphorylated MRP-14. Thus, the MRP-14/MPR-8 complex may play a role in stabilization of the actin network at the base of lamellipodia (56). Manitz et al. (53) reported that neutrophils from mice with targeted deletion of MRP-14 demonstrated impaired chemotaxis to IL-8 and leukotriene B4. MRP-14−/− neutrophils demonstrated increased basal levels of F-actin that did not increase with IL-8 stimulation. Impaired CD11b up-regulation in IL-8-stimulated MRP-14−/− neutrophils suggested that MRP-14 plays a role in exocytosis. Vogl et al. (54) reported that deletion of the MRP-14 gene in mice inhibited granulocyte migration, suggesting a functional role for the MRP-14/MPR-8 complex. However, Thr113 is absent from the mouse isoform of MRP-14, preventing any conclusions regarding the role of p38 MAPK-mediated phosphorylation in MRP-14 functions from studies in mice.

Permeabilization with Triton X-100 before confocal microscopic visualization of MRP-14 and F-actin solubilizes many cell structures, thereby preventing detection of translocation of MRP-14 to these structures. Using two-dimensional gel electrophoresis and MALDI-TOF MS, MRP-14 was shown to be associated with plasma membrane/secretory vesicles, gelatinase granules, and specific granules, but not with azurophilic granules, in resting human neutrophils. Based on previous reports indicating that p38 MAPK regulates neutrophil granule exocytosis (6, 10–12), the effect of MRP-14 phosphorylation on its association with these structures was determined. The data indicate that MRP-14 translocated to the plasma membrane/secretory vesicle fraction and gelatinase granules after fMLP stimulation and that this translocation was dependent on p38 MAPK activity. The failure of MRP-14 to translocate to specific and azurophilic granules upon cell activation may be related to the small amount of actin associated with these granules (G. Lominadze, R. Ward, and K. McLeish, unpublished observations). The quantitative difference in
MRP-14 association with granules, compared with gelatinase granules and plasma membrane/secretory vesicle fractions, under basal conditions remains to be explained. Taken together, the results of the present study suggest the hypothesis that phosphorylation of MRP-14 by p38 MAPK contributes to p38 MAPK regulation of cytoskeletal reorganization necessary for exocytosis. Association of MRP-14 with granule subsets may also provide a mechanism by which MRP-14 is released extracellularly at sites of inflammation (57–59).

Disclosures

The authors have no financial conflict of interest.

References


