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The Human S100 Protein MRP-14 Is a Novel Activator of the β2 Integrin Mac-1 on Neutrophils

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The 14-kDa myeloid-related protein (MRP-14) and its heterodimeric partner, MRP-8, are members of the S100 family of calcium-binding proteins (S100A9 and S100A8, respectively). Their importance in neutrophil function is implied by their unusual abundance in neutrophil cytosol (~40% of cytosolic protein). Previous work from our laboratory has demonstrated the extracellular association of these proteins with vascular endothelium adjacent to transmigrating leukocytes. We report here a function for MRP-14 as a stimulator of neutrophil adhesion mediated by the β2 integrin, Mac-1. MRP-14 is an affinity regulator of Mac-1 because it promotes binding of soluble ligand and expression of an “activation reporter” epitope of high affinity β2 integrins recognized by mAb24. The activity of MRP-14 is confined to regulating integrin function because, unlike other inflammatory agonists, there was no release of L-selectin, up-regulation of cytosolic Mac-1, or induction of neutrophil respiratory burst or calcium flux. Furthermore, MRP-14 does not act as a chemoattractant or cause alterations in cell shape or cytoskeleton. MRP-8 has a regulatory role in MRP-14 activity, inhibiting the adhesion induced by MRP-14 through the formation of the heterodimer. In terms of mechanism of action, MRP-14 does not increase Mac-1 function by direct binding to this integrin but recognizes a distinct receptor on neutrophils. This receptor interaction is pertussis toxin sensitive, indicating that MRP-14-generated signals leading to a Mac-1 affinity increase are heterotrimeric G protein dependent. We postulate that MRP-14 and MRP-8 are important in vivo candidates for the regulated adhesion of neutrophils through control of Mac-1 activity. The Journal of Immunology, 1998, 160: 1427–1435.

Neutrophils are at the forefront of an inflammatory response. Their rapid recruitment from the blood to sites of tissue injury requires a cascade of well-characterized adhesion events mediated by the selectins and the leukocyte β2 integrins (CD11/CD18) (1). The function of the leukocyte integrins is regulated, however, and they become active only following triggering through other receptors. One candidate family of receptors for integrin activation are the selectins, whose interactions with ligand precede integrin binding (2). For example, Ab cross-linking of L-selectin on neutrophils has been reported to induce Mac-1 activation and ligand binding (3). It has also been reported that the interaction of ligand GlyCAM-1 with L-selectin causes β2 integrin activation (4). Other candidate activators of β2 integrins are the classical chemoattractants, such as FMLP, platelet activating factor, and the chemokines, particularly IL-8. Interaction of these chemoattractants with their heterotrimeric G protein-linked receptors on the neutrophil membrane is able to stimulate adhesion in in vitro assays (reviewed in 5). Triggering by both L-selectin and chemoattractants has also been shown to stimulate neutrophil effector functions such as calcium flux, respiratory burst, and granule exocytosis (5, 6). It remains unclear, however, which signaling molecules operate in vivo and whether stimulation of integrin adhesion, chemotaxis, and other neutrophil effector functions are all initiated by the same mediators.

In human myeloid cells, myeloid-related protein (MRP)3–8 (S100A8) and MRP-14 (S100A9) are coexpressed as a heterodimer, and, in neutrophils, this heterodimer represents 40% of the cytosolic protein (7). Specific mAbs detect these MRPs deposited on endothelia at positions where leukocytes migrate into tissues, suggesting a role in leukocyte trafficking (8). Recently several members of the S100 protein family have been defined as chemoattractants. S100L (S100A2), isolated from bovine lung, stimulated guinea pig eosinophil chemotaxis but exhibited no activity toward neutrophils or monocytes (9). Similarly, psoriasin (S100A7) is highly up-regulated in psoriatic skin and has chemotactic activity for T cells and neutrophils but not for monocytes (10). Most information is available for CP-10, a murine homologue of the human MRP-8 protein (S100A8), which is chemotactic for neutrophils and macrophages and the most potent chemotaxin to date, with optimal activity at 10−13 M (11). In this study we have examined the function of human MRP-14 and found it to be a selective stimulator of Mac-1-mediated adhesion through affinity regulation. This is the first demonstration on neutrophils of the direct activation of Mac-1 by a physiologic protein. MRP-14 activity is distinct from the other S100 family members and classic chemoattractants in promoting Mac-1 adhesion without stimulating chemotaxis or further neutrophil activation. In addition, MRP-8 is identified as a natural and specific inhibitor of MRP-14 function.

Materials and Methods

Recombinant proteins

Recombinant human MRP-14 and MRP-8 proteins (12) were prepared from pET3a expression vectors containing the cDNAs for these proteins constructed by Dr Paul Hessian in the Leukocyte Adhesion Laboratory

1 This work was supported by the Imperial Cancer Research Fund.

2 Address correspondence and reprint requests to Dr. Nancy, Hogg, Leukocyte Adhesion Laboratory, Imperial Cancer Research Fund, P.O. Box 123, Lincoln’s Inn Fields, London, WC2A 3PX, United Kingdom. Email address: hogg@europa.lif.icnet.uk

3 Abbreviations used in this paper: MRP, myeloid-related protein; F-actin, filamentous actin; H-HBSS, HBSS containing 10 mM HEPES; [Ca2+]i, intracellular calcium.
The proteins were purified to homogeneity by a combination of chromatofocusing on a Mono-P column for MRP-14 (Pharmacia Biotech, St Albans, U.K.) and using the Rotofor system for MRP-8 (Bio-Rad, Hemel Hempstead, U.K.), followed by hydroxyapatite chromatography for both proteins (Bio-Rad) (A. Coffer, unpublished observations). All purification steps were performed in the Imperial Cancer Research Fund Protein Isolation and Cloning Laboratory (London, U.K.). The purified proteins were stored at 2 mg/ml in HBSS containing 10 mM HEPES (HBSS), pH 7.5, at -70°C or over the short term at 4°C. MRP-8 was expressed in dimer form and was reduced and alkylated before use as a monomer (13). Recombinant S100A and S100B (Sigma, Poole, U.K.) were reconstituted with water to 2 mg/ml.

**Purification of MRP-8/14 heterodimer**

MRP-8/14 heterodimer was purified from neutrophil cytosol by fast performance liquid chromatography using a Mono Q anion exchange column as previously described (7).

**K562 transfected cell culture**

Mac-1-transfected (KC/16) and untransfected K562 erythroleukemic cells were maintained at 37°C and 5% CO2 in DMEM supplemented with 10% FCS (14). Geneticin G418 (0.5 mg/ml; Life Technologies, Paisley, U.K.) was added to Mac-1-transfected cell medium to maintain selection.

**Monoclonal Abs**

The following IgG1 mAbs were used in this study: LAM 1.3 (5 μg/ml), specific for L-selectin (CD62L), was a gift from Dr. Tom Tedder (Boston, MA); FAB8H4 (10 μg/ml) is specific for the Mac-1 α subunit (CD11b); LPM19c (20 μg/ml of purified mAb or 1/50 ascitic fluid), also specific for the Mac-1 α subunit (CD11b), was a gift from Dr. Karen Pulford (Oxford, U.K.); mAb24 (10 μg/ml) is a reporter of β2 integrin activation; 3.9 (10 μg/ml) is specific for the p50,95 α subunit (CD11c); 27E10 (1/50), purchased from BMA Biomedicals AG, Switzerland, is specific for the MRP-8/14 heterodimer; and 52U (10 μg/ml) is an IgG1 isotype control.

**FITC labeling of proteins**

FITC labeling of MRP-14 and fibrinogen was conducted following the method of Goding (15). Unbound FITC was removed from the FITC-conjugated protein by gel filtration in either HBSS (MRP-14) or PBS (fibrinogen) using a pre-equilibrated PD-10 column (Pharmacia Biotech).

**Neutrophil adhesion assay**

Human neutrophils were isolated from EDTA-anticoagulated whole blood by healthy volunteers by dextran sedimentation and density gradient centrifugation (16). Purified neutrophils (≥95%) were washed and resuspended at 5 × 10⁶ cells/ml in HBSS. Cells were labeled with 1 μM of the intracellular fluorescent dye 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein pentacetoxymethyl ester (BCECF/AM; Calbiochem, Nottingham, U.K.), for 30 min at room temperature. After washing, labeled cells were resuspended in HBSS at 2 × 10⁶/ml and 50 μl were added to Nunc-Immuno Maxisorp plate wells (Life Technologies) that had been precoated with 2 mg/ml fibrinogen overnight at 4°C. MRP-8 was then determined using a FACScan flow cytometer (Becton Dickinson). Cells were fixed, permeabilized, and stained for 30 min on ice by the method of Cornish et al. (17), with the exception that cells were incubated in HBSS containing 1 mM Ca²⁺, 1 mM Mg²⁺, and 10 μM Zn²⁺.

**Confocal microscopy**

For immunofluorescence analysis of F-actin staining, 13 mm glass coverslips were precoated with 2 mg/ml fibrinogen overnight at 4°C and then washed in HBSS. Buffer conditions were as for the 96-well plate adhesion assay. Unlabeled neutrophils (4 × 10⁶) were added to each coverslip in the presence or absence of stimulants in a total volume of 400 μl. Coverslips were spun at 40g and then incubated for 30 min at room temperature. Nonadherent cells were removed by gentle washing in HBSS. Cells were fixed, permeabilized, and stained for 30 min on ice by the method of Goding (15). The liquid was aspirated and the plate was blocked with 150 μl/well of PBS/0.1% Tween-20 for 1 h at room temperature. The plate was washed and incubated sequentially (including washes) with 50 μl/well of the MRP-8/14 complex-specific mAb 27E10 (18) (1/50 in PBS/0.1% Tween-20) for 1 h at room temperature, followed by 30 min at room temperature with peroxidase-conjugated goat anti-mouse Ig (1/2000; DAKO, High Wycombe, U.K.) in PBS/Tween. Bound Ig was detected using o-phenylenediamine dihydrochloride (OPD; Sigma) according to the manufacturer’s instructions.

**ELISA**

Nunc-Immuo Maxisorp 96-well plates (Life Technologies) were incubated overnight at 4°C with 2 μg of MRP-14 or MRP-8 alone, or together in HBSS/2 mM Ca²⁺/2 mM Mg²⁺/20 μM Zn²⁺ (50 μl/well). Following incubation, the liquid was aspirated and the plate was blocked with 150 μl/well of PBS/0.1% Tween-20 for 1 h at room temperature. The plate was washed and incubated sequentially (including washes) with 50 μl/well of the MRP-8/14 complex-specific mAb 27E10 (18) (1/50 in PBS/0.1% Tween-20) for 1 h at room temperature, followed by 30 min at room temperature with peroxidase-conjugated goat anti-mouse Ig (1/2000; DAKO, High Wycombe, U.K.) in PBS/Tween. Bound Ig was detected using o-phenylenediamine dihydrochloride (OPD; Sigma) according to the manufacturer’s instructions.

**Superoxide production**

The production of superoxide was measured according to the method of Smith and Weidemann (19).

**Measurement of intracellular calcium ([Ca²⁺]i)**

[Ca²⁺]i was measured using fura 2-AM according to the method of Tsien et al. (20). Stimulants were added at time points indicated on Figure 5. Fluorescence was monitored using a Perkin-Elmer LS-5 luminescence spectrophotometer (Perkin-Elmer, Beaconsfield, U.K.).

**Chemotaxis assay**

Dilutions of MRP-14 from 0 to 0.5 μM in HBSS containing 1 mM Ca²⁺, 1 mM Mg²⁺, 10 μM Zn²⁺, and 0.1% BSA (assay medium) were added to Costar 24-well tissue culture plates (Life Technologies) in a final volume of 600 μl in quadruplicate. FMLP, at 0.1 μM in the lower wells, was used as a positive control. Costar Transwells (6.5 mm diameter; 3 μm pore size; Life Technologies) were precoated on both sides with 50 μg/ml fibrinogen (Sigma) overnight at 4°C. Either 100 μl of BACECF/AM-labeled neutrophils (5 × 10⁶/ml in assay medium) alone or containing concentrations of MRP-14 from 0 to 0.5 μM were added to the top chamber. Cells were allowed to transmigrate for 1 h at 37°C. After this time, the migrated cells were detached using 5 mM EDTA and counted using a flow cytometer (Becton Dickinson).

**Results**

**The effect of MRP-14 and MRP-8 on β2 integrin Mac-1 function**

In this study we have identified the S100 protein MRP-14 as a new candidate for activation of β2 integrin-mediated adhesion. Our results show that MRP-14 is directly able to stimulate neutrophil adhesion to fibrinogen (Fig. 1). Adhesion was maximal between 0.5 to 1 μM and could be completely inhibited by CD11b mAb LPM19c, confirming that adhesion was mediated by the β2 integrin Mac-1 (CD11b/CD18) (Fig. 1, inset). This stimulation of adhesion is unique to MRP-14 because the other S100 proteins

MRP-14 in HBSS without Ca²⁺, Mg²⁺, and Zn²⁺. In experiments measuring FITC-MRP-14 binding, neutrophils or K562 cells (1 × 10⁶/sample) were incubated on ice in HBSS containing 1 mM Ca²⁺, 1 mM Mg²⁺, 10 μM Zn²⁺, and 0.1% BSA (incubation buffer) with varying amounts of FITC-MRP-14. After 30 min, the cells were washed and resuspended in ice cold incubation buffer. To test for specific binding, increasing amounts of unlabeled MRP-14 and a control S100 protein (S100A) were incubated, as above, with 2 μM FITC-MRP-14. FITC-phallolidin was used to measure filamentous actin (F-actin) according to the method of Cornish et al. (17), with the exception that cells were incubated in HBSS containing 1 mM Ca²⁺, 1 mM Mg²⁺, and 10 μM Zn²⁺.
tested, including MRP-8 (the heterodimeric partner of MRP-14), S100A (Fig. 1), and S100B (data not shown) failed to induce any adhesion. Furthermore, the native MRP-8/14 complex was unable to induce any adhesion (Fig. 1). The response to MRP-14 was equivalent to that of FMLP at saturating levels of each mediator (2 μM MRP-14 and 0.1 μM FMLP) (Fig. 1, inset, and Fig. 2). Several S100 proteins, including MRP-14, have been shown to bind Zn$^{2+}$ and, in so doing, increase their affinity for Ca$^{2+}$, presumably by a conformational change (21, 22). Our results can be similarly interpreted because the addition of 10 μM Zn$^{2+}$ increases the potency of MRP-14 as an inducer of neutrophil adhesion by approximately 10-fold (data not shown).

Experiments to assess the effect of MRP-8 on MRP-14-induced adhesion were prompted by the inability of the native MRP-8/14 heterodimer to induce neutrophil adhesion (Fig. 1). Titration of MRP-8 from 0.125 to 10 μM caused a dose-dependent inhibition of 2 μM MRP-14-induced adhesion (Fig. 2), with the 50% point occurring at an average ratio of 1:1 MRP-14 to MRP-8 (n = 6). MRP-8, in the same concentration range, had no effect on 0.1 μM FMLP-induced adhesion (Fig. 2). To eliminate the possibility that

FIGURE 1. The S100 protein MRP-14 can induce adhesion of human neutrophils. MRP-14 induces adhesion, maximal between 0.5 to 1 μM, to ligand fibrinogen via β₂ integrin Mac-1 (CD11b/CD18; αMβ₂) (●). MRP-8, the heterodimeric partner of MRP-14, shows no adhesive activity when titrated over the same dose range (□). Control S100 protein S100A (△) and native MRP-8/14 complex (▲) were similarly negative. The inset figure demonstrates the ability of CD11b mAb LPM19c at 20 μg/ml to inhibit either FMLP (0.1 μM)- or MRP-14 (1 μM)-stimulated adhesion to fibrinogen. The data shown are from one experiment representative of six.

FIGURE 2. MRP-14-induced adhesion is inhibited by the heterodimeric partner MRP-8. MRP-8 inhibits the adhesion to fibrinogen induced by 1 μM MRP-14 (□) but not that induced by 0.1 μM FMLP (○). As a control, S100A does not block MRP-14 (●) nor FMLP-induced (◆) adhesion. The data shown are from one experiment representative of six. The inset figure shows the reactivity of the heterodimer-specific mAb 27E10 with coincubated MRP-14 (2 μM) and MRP-8 (4 μM), which caused inhibition of the adhesion assay in Figure 2. By comparison, mAb 27E10 failed to react with either monomeric protein.
MRP-8 inhibition occurred by Ca\(^{2+}\) depletion, another S100 protein, S100A, was similarly titrated and had no inhibitory effect on MRP-14-induced adhesion (Fig. 2). The specificity of MRP-8 inhibition was confirmed by the expression of the MRP-8/14 complex-specific epitope, recognized by mAb 27E10 (18) upon coinubcation of MRP-8 with MRP-14 (Fig. 2, inset). The ability of MRP-8 to inhibit MRP-14-induced adhesion and the fact that heterodimer formation occurs suggests that the mechanism of MRP-8 inhibition is by direct binding to MRP-14 with a 1:1 stoichiometry between the two proteins. This identifies MRP-8 as a physiologic antagonist of MRP-14 and confirms the specificity of MRP-14 function.

**MRP-14 is a modulator of Mac-1 affinity**

Increased integrin affinity is measured by the ability to bind ligand in solution. MRP-14 induces neutrophil binding of soluble FITC-fibrinogen with saturation at 10 nM FITC-fibrinogen \(\text{(n)} (n = 6)\). The binding is cation dependent \(\text{(n)} (n = 3)\). Unstimulated cell binding of FITC-fibrinogen is indicated \(\text{a} (n = 6)\). a, MRP-14-induced binding of 20 nM FITC-fibrinogen is inhibited by unlabeled fibrinogen \(\text{c} (n = 3)\). c, FITC-fibrinogen (20 nM) binding to MRP-14-stimulated neutrophils is inhibited by the CD11b blocking mAb LPM19c, but not by the CD11c blocking mAb 3.9, nor an IgG1 control mAb (52U) \(\text{d} (n = 3)\). MAb were used at concentrations indicated in Materials and Methods.

Another characteristic of high affinity integrins is the expression of “activator reporter” epitopes. To further assess the ability of MRP-14 to positively regulate \(\beta_2\) integrin affinity, we tested for expression of an activation reporter epitope for \(\beta_2\) integrin using CD62L mAb LAM1.3. e and f, FMLP, but not MRP-14, increases expression of the \(\beta_2\) integrin Mac-1 using CD11b mAb ICRF44. MAb were used at concentrations indicated in Materials and Methods. Control Ab staining is indicated as a dashed line. Pretreatment levels of expression are indicated as a dotted line and post-treatment levels as a solid line. The data shown are from one representative experiment of six.

Inability of MRP-14 to trigger other neutrophil functions

The stimulated neutrophil undergoes a number of cell surface and intracellular changes. For example, engagement of certain cell surface receptors can invoke quantitative changes in expression of at least two adhesion molecules (26). Characteristically, for the classical chemoattractants, there is shedding of L-selectin and an up-regulation of Mac-1 on the neutrophil surface. As expected, the classical chemoattractants, there is shedding of L-selectin and an up-regulation of Mac-1 on the neutrophil surface. As expected, for maximal adhesion (0.1 \(\mu\)M), was able to induce increased mAb24 epitope expression (Fig. 4a). Similarly, MRP-14 was able to induce mAb24 epitope expression on the surface of neutrophils (Fig. 4b) in a concentration-dependent manner that mirrored the induction of adhesion (data not shown). The expression of this epitope further confirms that \(\beta_2\) integrins are in a high affinity state as a result of exposure to MRP-14 (and FMLP).

**FIGURE 3.** MRP-14 promotes binding of soluble ligand fibrinogen to neutrophil Mac-1. a, MRP-14 (1 \(\mu\)M) induces binding of FITC-fibrinogen to neutrophil Mac-1, which is saturated at 10 nM FITC-fibrinogen \(\text{(n)} (n = 6)\). The binding is cation dependent \(\text{(n)} (n = 3)\). Unstimulated cell binding of FITC-fibrinogen is indicated \(\text{a} (n = 6)\). b, MRP-14-induced binding of 20 nM FITC-fibrinogen is inhibited by unlabeled fibrinogen \(\text{c} (n = 3)\). c, FITC-fibrinogen (20 nM) binding to MRP-14-stimulated neutrophils is inhibited by the CD11b blocking mAb LPM19c, but not by the CD11c blocking mAb 3.9, nor an IgG1 control mAb (52U) \(\text{d} (n = 3)\). MAb were used at concentrations indicated in Materials and Methods.

**FIGURE 4.** MRP-14 activates \(\beta_2\) integrins without up-regulation of Mac-1 or loss of L-selectin. Alteration of neutrophil membrane phenotype was measured in response to treatment with 0.1 \(\mu\)M FMLP (a, c, e) and 1 \(\mu\)M MRP-14 (b, d, f). a and b, MRP-14 and FMLP induce expression of \(\beta_2\) integrin activation reporter epitope recognized by mAb24. c and d, FMLP, but not MRP-14, reduces expression of L-selectin using CD62L mAb LAM1.3. e and f, FMLP, but not MRP-14, increases expression of the \(\beta_2\) integrin Mac-1 using CD11b mAb ICRF44. MAb were used at concentrations indicated in Materials and Methods. Control Ab staining is indicated as a dashed line. Pretreatment levels of expression are indicated as a dotted line and post-treatment levels as a solid line. The data shown are from one representative experiment of six.
The possibility still existed that MRP-14, despite the absence of morphology, does not act as a chemoattractant or affect neutrophil neutrophil chemoattractant. For example, the murine MRP-8 homologue, CP-10, is reported to be chemotactic for neutrophils without inducing a Ca$^{2+}$ flux (17). The capacity of MRP-14 (up to 2 μM) to act as a potential chemoattractant was investigated using the Transwell system. When MRP-14 was titrated from 30 nM to 0.5 μM in either the upper or lower chambers or in both, no migration was observed above background (Table I). Concentrations up to 2 μM also failed to induce any migration (data not shown).

In contrast, approximately 50% of the total cells migrated in response to FMLP (Table I). Therefore, MRP-14 did not act as a neutrophil chemotaxin or stimulate random migration. Similarly, experiments with MRP-8, the human equivalent of CP-10, proved negative for chemotaxis (data not shown).

From the information gathered, MRP-14 function appeared to be limited specifically to affinity regulation of Mac-1 without general stimulation of other neutrophil functions or induction of chemotaxis. Integrin activation can be associated with remodeling of the cytoskeleton, with cell spreading considered to increase the efficiency of adhesion (28). From this perspective, we evaluated the effect of MRP-14 on neutrophil morphology by visualizing F-actin. A comparison of untreated (Fig. 6a) with MRP-14-treated neutrophils (Fig. 6b) shows no difference in general cell shape, whereas FMLP causes substantial alteration of cell shape and localization of F-actin (Fig. 6c). We next investigated changes in cell shape more quantitatively by assessing flow cytometric cell scatter profiles (Fig. 7a) and the kinetics of induction of F-actin (Fig. 7b). MRP-14 failed to cause distinguishable cell shape change or formation of F-actin, whereas FMLP, as expected, was able to promote an increase in cell scatter (Fig. 7a) and a 1.5-fold increase in neutrophil F-actin after 3 min of stimulation (Fig. 7b). These results confirmed that MRP-14 treatment of neutrophils had no effect on reorganization of the cytoskeleton and underlined the conclusion that MRP-14, in promoting Mac-1-mediated adhesion, is more selective in its activity than chemoattractants such as FMLP.

FIGURE 5. MRP-14 does not induce human neutrophil calcium flux (a) or respiratory burst (b). a, FMLP (0.1 μM) but not MRP-14 (1 μM) induces an intracellular calcium flux (n = 6). b,FMLP (0.1 μM) but not MRP-14 (1 μM) induces superoxide production as measured by an increase in dihydrorhodamine fluorescence (n = 3). In (b), pretreatment levels of expression are indicated as a dotted line and post-treatment levels as a solid line.

Neutrophil stimulants also cause exocytosis of granules additional to those containing Mac-1 with β-glucuronidase release from azurophilic granules frequently taken as a measure of this activity. MRP-14 failed to induce release of β-glucuronidase, further supporting the inability of MRP-14 to induce neutrophil granule exocytosis (data not shown).

In addition to changes in adhesion molecule expression, activated neutrophils can mobilize intracellular Ca$^{2+}$. FMLP gave the characteristic response of intracellular Ca$^{2+}$ flux, whereas 1 μM MRP-14 failed to induce such a Ca$^{2+}$ flux even after 30 min (Fig. 5, a and b, and data not shown). Indeed, all concentrations of MRP-14 tested, from 0.05 to 4 μM, failed to induce a Ca$^{2+}$ flux (data not shown). We then investigated the possibility that MRP-14 might activate the neutrophil respiratory burst (see (27)), FMLP at 0.1 μM (Fig. 5c), PMA, and the chemokine IL-8 (data not shown) caused neutrophil activation of the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase as detected by production of superoxide, but MRP-14 at 1 μM, or concentrations up to 8 μM (data not shown), failed to stimulate any production of superoxide (Fig. 5d).

MRP-14 does not act as a chemoattractant or affect neutrophil morphology

The possibility still existed that MRP-14, despite the absence of exocytosis, Ca$^{2+}$ flux, or superoxide production, could act as a neutrophil chemotaxin. For example, the murine MRP-8 homologue, CP-10, is reported to be chemotactic for neutrophils without inducing a Ca$^{2+}$ flux (17). The capacity of MRP-14 (up to 2 μM) to act as a potential chemotaxin was investigated using the Transwell system. When MRP-14 was titrated from 30 nM to 0.5 μM in either the upper or lower chambers or in both, no migration was observed above background (Table I). Concentrations up to 2 μM also failed to induce any migration (data not shown). In contrast, approximately 50% of the total cells migrated in response to FMLP (Table I). Therefore, MRP-14 did not act as a neutrophil chemotaxin or stimulate random migration. Similarly, experiments with MRP-8, the human equivalent of CP-10, proved negative for chemotaxis (data not shown).

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MRP-14 binds to a distinct neutrophil receptor

To determine the means by which MRP-14 altered the function of Mac-1, we investigated the interaction of MRP-14 with the neutrophil membrane. MRP-14 was found to bind to a specific receptor in a dose-dependent manner that could be blocked by unlabeled MRP-14 but not by control protein S100A (Fig. 8a, and inset). One possibility, given the limited activity of MRP-14 to modulate Mac-1 affinity, was that the MRP-14 receptor was Mac-1 itself. To investigate this possibility, we tested the binding profiles of a Mac-1 K562 transfectant (KC/16 cells) by comparison with the parent K562 cells (14, 29). Although the KC/16 cells abundantly express Mac-1 (Fig. 8b, inset), there was no difference in their ability to bind MRP-14 compared with K562 cells (Fig. 8b). The binding to both K562 and Mac-1 K562 was prevented by cold MRP-14 but not by S100A protein (data not shown). In addition, no evidence was obtained for coprecipitation of MRP-14 and Mac-1 from neutrophil lysates by an anti-MRP-14-specific mAb (data not shown). The conclusion from these experiments is that MRP-14 binds to a receptor distinct from Mac-1. Two other S100

Table 1. The migration of human neutrophils in response to various concentrations of MRP-14 compared with 0.1 μM FMLP (n = 3)

<table>
<thead>
<tr>
<th>Concentration (μM)</th>
<th>MRP-14 in upper wells</th>
<th>MRP-14 in lower wells</th>
<th>FMLP</th>
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<tr>
<td>MRP-14 0</td>
<td>8.5 ± 1.9</td>
<td>8.2 ± 0.15</td>
<td>7.7</td>
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<tr>
<td>0.03</td>
<td>7.2 ± 2.4</td>
<td>8.4 ± 1.1</td>
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<td>7.6</td>
</tr>
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<td>8.5 ± 0.70</td>
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<td>7.3 ± 1.0</td>
<td>8.0 ± 0.88</td>
<td>8.8 ± 0.79</td>
</tr>
<tr>
<td>0.5</td>
<td>7.3 ± 0.1</td>
<td>7.7 ± 0.64</td>
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<tr>
<td>FMLP 0.1</td>
<td>44.7 ± 2.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a Results are the average of quadruplicate samples and expressed as the mean % of total cells ± SD. The experiment shown is one representative of three.

- a, FMLP (0.1 μM) but not MRP-14 (1 μM) induces an intracellular calcium flux (n = 6).
- b, FMLP (0.1 μM) but not MRP-14 (1 μM) induces superoxide production as measured by an increase in dihydrorhodamine fluorescence (n = 3).
- c, FMLP failed to induce a Ca$^{2+}$ flux even after 30 min (Fig. 5a).
- d, FMLP at 0.1 μM (Fig. 5c), PMA, and the chemokine IL-8 (data not shown) caused neutrophil activation of the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase as detected by production of superoxide, but MRP-14 at 1 μM, or concentrations up to 8 μM (data not shown), failed to stimulate any production of superoxide (Fig. 5d).
- e, Results are the average of quadruplicate samples and expressed as the mean % of total cells ± SD. The experiment shown is one representative of three.
proteins have been demonstrated to bind to pertussis toxin-sensitive receptors (9, 17). To aid understanding of the function of MRP-14, we investigated the possibility that MRP-14 also operates through such a receptor. Neutrophils preincubated with pertussis toxin showed a concentration-dependent decrease in the ability of MRP-14 to stimulate adhesion (Fig. 9). The findings suggest that MRP-14 interacts with neutrophils via a G protein-coupled receptor and that the subsequent signaling leads to Mac-1 activation.

Discussion

We describe for the first time a function for the human S100 protein MRP-14 showing it to be an activator of neutrophil β2 integrin-mediated adhesion. Furthermore, MRP-14 affects the neutrophil adhesive state by directly inducing an increase in the affinity of Mac-1 on neutrophils. This is of interest because there has been a lack of identification of naturally occurring signaling molecules that directly regulate integrin function. The action of MRP-14 is very selective in that other markers of the activated neutrophil are not evident after treatment with this protein. We also show that the MRP-8/14 complex is not able to activate Mac-1 and that the function of MRP-14 is negatively regulated by its heterodimeric partner MRP-8. MRP-8/14 is expressed by myeloid cells (reviewed in 3) and has recently been shown to be released by monocytes in a novel, tubulin-dependent manner (31). Extracellular localization of these MRPs on vascular endothelium has also been observed by immunohistochemical staining (8, 30). There is some evidence from inflammatory disorders that MRP-14 is expressed alone (32) and that it can be detected in serum as a monomer (33). The conditions leading to the dissociated state, however, need further investigation.

It is becoming apparent that integrin-mediated adhesion can occur by two distinct mechanisms. By one means, a combination of integrin redistribution into clusters on the cell membrane, accompanied by accessory events such as cell spreading, acts to increase the strength of adherence to ligand (reviewed in 25, 34). This form of adhesion is dependent on rearrangement of the actin cytoskeleton and is sensitive to cytochalasin D. There are fewer examples of a second mechanism that involves conformational alteration of integrin leading to enhanced affinity for ligand. This increase in ligand binding affinity can be brought about by artificial means, such as treatment with agents such as divalent cations Mn2+ or Mg2+ and activating mAbs (25). We provide here an example of affinity regulation of Mac-1 integrin by the naturally occurring S100 protein MRP-14, which triggers Mac-1 on neutrophils to bind soluble fibrinogen with saturation at 10 nM FITC-fibrinogen. High affinity status of Mac-1 is further confirmed by the ability of MRP-14 to cause expression of the mAb24 activation reporter epitope, which also detects the Mg2+-treated Mac-1 on neutrophils (24). Therefore, MRP-14 represents one of few examples of a physiologic protein acting selectively as a modulator of integrin affinity. Another example of affinity regulation of Mac-1 follows from the treatment of monocytes with ADP, which induces Mac-1-mediated binding of soluble ligand fibrinogen (35) and Factor X (36). Also well described is the activation of platelets with thrombin or ADP, which causes αIIbβ3 to bind soluble ligand fibrinogen (reviewed in 37).

The affinity change to Mac-1 in response to MRP-14 occurs in the absence of accessory adhesion events such as neutrophil shape change, actin reorganization, or cell spreading, underlining the restricted scope of the signaling activity of MRP-14. Because intracellular Ca2+ facilitates adhesion through cell spreading for integrins such as LFA-1 (28), the fact that Mac-1 adhesion is evident...
in the absence of an increase in [Ca^{2+}], is a further indication that these accessory events are not part of the mechanism of MRP-14 action. Finally, the lack of sensitivity to cytochalasin D of MRP-14-stimulated neutrophil adhesion is additional proof that it is integrin itself that is altered and that the cytoskeleton has no role in MRP-14 function (data not shown).

These very restricted effects of MRP-14 on Mac-1 function raises the question as to its mechanism of action. One possibility is that by direct physical interaction, MRP-14 alters the conformation of Mac-1 leading to an increase in ligand-binding affinity. A precedent for this type of modification is the example of the subversion of Mac-1 function by membrane-associated urokinase-type plasminogen activator receptor (38). In this study however, we could find no evidence for such a mechanism of direct interaction with integrin. In contrast, MRP-14 binds to a distinct pertussis toxin-sensitive receptor on the neutrophil, indicating that it interacts with neutrophils via a G protein-coupled receptor and that subsequent signaling leads to Mac-1 activation. FMLP, PAF (platelet-activating factor), and the chemokines mediate their effects through pertussis toxin-sensitive 7-membrane-spanning receptors that link to heterotrimeric G proteins (reviewed in 39).

Two of the chemotactic S100 proteins, CP-10 and S100L, have been shown to signal through pertussis toxin-sensitive receptors (9, 17). The limited scope of MRP-14 signaling suggests two possibilities: first, that the G proteins coupled to their receptors differ from those associated with receptors that signal a larger array of functions, or, second, that the kinetics of receptor interaction or strength of signal might dictate the number of intracellular pathways activated. A final point is that this general class of receptor has reaction kinetics that would theoretically be sufficiently rapid to account for the rate of leukocyte response observed in vivo (39, 40).

The failure of MRP-14 to cause loss of L-selectin, Mac-1 up-regulation, and Ca^{2+} flux on neutrophils resembles the functional profile of the murine chemotactic S100 protein CP-10 (17). In contrast, the failure of MRP-14 to induce shape change, actin reorganization, or neutrophil migration indicates that MRP-14 does not act as a chemotactrant. Furthermore, human MRP-8 is not chemotactic, unlike the murine homologue (41, and data not shown). The lack of chemotactic activity of MRP-8 and MRP-14 contrasts with CP-10 (11) and the two other chemotactic S100 proteins, S100L (9) and psoriasin (10). None of these proteins have yet been tested for their ability to directly activate integrins, but it can be concluded that functional differences exist among this subset of S100 family members, which operate within the context of an immune response.

The positive effect of MRP-14 on Mac-1 affinity regulation, considered together with its localization on vascular endothelium (8, 30), implicates a function for MRP-14 in stimulating neutrophil adhesion to endothelium either by directly capturing neutrophils from the blood stream or by securing their firm adhesion. The fact that MRP-14 does not recruit new Mac-1 to the membrane is not detrimental to function because it is the constitutively expressed Mac-1 that is responsive to adhesion-activating stimuli (42). When neutrophils are stimulated with classical chemoattractants, such as FMLP, the total expression of Mac-1 on the membrane can increase by up to 10-fold (43), but this newly arrived Mac-1 is inactive until a second round of stimulation (44). If MRP-14 does supply the initial signal for adhesion, then it may be able to influence other events of the adhesion cascade. For example, the failure to activate L-selectin shedding could have a positive effect on neutrophil accumulation at sites of leukocyte trafficking. L-selectin shedding has been considered necessary for effective neutrophil

**FIGURE 8.** MRP-14 binds directly to neutrophils and K562 cells. a, FITC-MRP-14 binds to neutrophils in a dose-dependent manner (n = 6). Inset, Binding of 2-μM FITC-MRP-14 is specifically blocked by the addition of unlabeled MRP-14 (■) and not by control protein S100A (□) (n = 3). b, FITC-MRP-14 binds equivalently to both untransfected (dotted line) and Mac-1-transfected (solid line) K562 cells (see inset for Mac-1 expression).

**FIGURE 9.** Pertussis toxin sensitivity of MRP-14 and FMLP adhesion. The adhesion induced by both MRP-14 and FMLP is sensitive to pertussis toxin. Bordetella pertussis toxin at 0.5 and 1.0 μg/ml are selected as representative of a full titration range up to 1 μg/ml. These concentrations of pertussis toxin had no effect on neutrophil viability. Unstimulated neutrophils (open bar); FMLP (stippled bar); MRP-14 (solid bar). The data illustrated show one experiment of five.
rolling and tethering on the endothelium, but prevention of L-selectin cleavage causes neutrophils to roll more slowly, bringing them into close contact with the adhesive surface (45). Furthermore, under flow conditions, adhering neutrophils will recruit further neutrophils through L-selectin-mediated mediation between neutrophils (46). This route of enhanced binding is eliminated if L-selectin is shed through neutrophil activation. As well as directly influencing the above adhesion events, other classical chemotactic receptors, such as FMLP and the chemokines, serve as general neutrophil activators, inducing functions such as Ca\(^{2+}\) flux, the respiratory burst, and degranulation (see 47). Adhering neutrophils with an activated respiratory burst release harmful products, such as hydrogen peroxide and oxygen radicals (48), that damage endothelium and surrounding tissue (49). The restricted action of MRP-14 may be beneficial in minimizing the potential damage that neutrophils could inflict on vascular endothelium.

In summary, the human MRP-14 protein serves as a unique modulator of the affinity of the neutrophil β2 integrin Mac-1 acting through a pertussis toxin-sensitive receptor. Further investigation is needed to identify whether MRP-14 is able to signal the activation of other integrins on neutrophils and other leukocytes. The restricted activity of MRP-14 also suggests that it might act in conjunction with chemotactic factors such that the recruited neutrophil adherent to the vascular endothelium would then be primed and ready to respond to further signals directing it toward the injured tissue. These findings emphasize the newly recognized importance of this subset of S100 proteins in leukocyte adhesion reactions and suggest that they may function in a manner distinct from those of the classical chemotactic receptors.

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