The S100 Family Heterodimer, MRP-8/14, Binds with High Affinity to Heparin and Heparan Sulfate Glycosaminoglycans on Endothelial Cells*

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The S100 family proteins MRP-8 (S100A8) and MRP-14 (S100A9) form a heterodimer that is abundantly expressed in neutrophils, monocytes, and some secretory epithelia. In inflamed tissues, the MRP-8/14 complex is deposited onto the endothelium of venules associated with extravasating leukocytes. To explore the receptor interactions of MRP-8/14, we use a model system in which the purified MRP-8/14 complex binds to the cell surface of an endothelial cell line, HMEC-1. This interaction is mediated by the MRP-14 subunit and is mirrored by recombinant MRP-14 alone. The cell surface binding of MRP-14 was blocked by heparin, heparan sulfate, and chondroitin sulfate B, and the binding sites were sensitive to heparinase I and trypsin treatment but not to chondroitinase ABC. Furthermore MRP-8/14 and MRP-14 did not bind to a glycosaminoglycan-minus cell line. MRP-14 has a high affinity for heparin (Kd = 6.1 ± 3.4 nM), and this interaction mimicked that with the endothelial cells. We therefore conclude that the MRP-8/14 complex binds to endothelial cells via the MRP-14 subunit interacting chiefly with heparan sulfate proteoglycans. CD36 and RAGE, two other putative receptors for MRP-8/14, were not expressed by HMEC-1 cells. This binding activity may explain the immobilization of the MRP-8/14 complex on endothelium that is observed in vivo.

The S100 proteins are a family of small (10–14 kDa) calcium-binding proteins (1, 2). The majority of the S100 genes are tightly clustered together on chromosome 1q21 in man and chromosome 3 in the mouse, but the individual proteins are expressed in distinctive cell types. Generally, the functions of S100 proteins are poorly characterized. However, there is increasing evidence that some S100 proteins have extracellular activities, particularly in the immune response. Several S100 proteins have been reported to act as chemoattractants with potencies in the 10−10−13 M range. Thus, S100L (S100A2) from the lung acts as a chemoattractant for eosinophils (3); psoriasin (S100A7) acts as a chemoattractant for neutrophils and CD4+ T lymphocytes (4); murine MRP-8 (CP-10; S100A8) acts as a chemoattractant for myeloid cells (5); human MRP-8 acts as a chemoattractant for peridontal ligament cells (6); and S100A12 (ENRAGE) acts as a chemotactrant for human monocytes (7) and neutrophils (8).

Because the S100 proteins appear to have extracellular functions, there has been an interest in the nature of the receptors for these proteins and the downstream events that they might induce. The chemotactrant effects of two S100 proteins, S100L and CP-10, are sensitive to pertussis toxin, suggesting a receptor interaction linked to small G proteins (3, 9). The proinflammatory protein S100A12 binds to the receptor for advanced glycation end products (RAGE)1 (7). RAGE is a scavenger-type receptor belonging to the immunoglobulin superfamily that signals to the NFκB pathway following ligation. In addition to S100A12, it also binds advanced glycation end products, amyloid fibrils, and amphoterin (reviewed in Ref. 10). Recently S100B and S100A1 have also been shown to bind RAGE (11); thus, it has been speculated that RAGE may be a general receptor for the S100 family of proteins.

The MRP proteins MRP-14 (S100A9) and MRP-8 (S100A8) are expressed by myeloid cells and some secretory epithelium (12). In myeloid cells, MRP-8 and MRP-14 form a heterodimer that constitutes 45% of the cytosolic protein in neutrophils and 1% in monocytes (13). Determining the function of these proteins has been difficult, particularly because their abundance has lead to a propensity to contaminate functional assays. Recently, the MRP-8/14 heterodimer isolated from keratinocytes (14) and myeloid cells (14−16) has been demonstrated to bind to a class of unsaturated fatty acids, including arachidonic acid. MRP-8/14 has been reported to aid uptake of arachidonic acid by binding CD36 (17), which is now recognized as a fatty acid transporter protein (18).

Immunohistochemical studies have localized MRP-8/14 to venules associated with extravasating myeloid cells (19). In this study we show that, in human inflammatory disease, the source of the MRP proteins is not the endothelium but the associated myeloid cells. Therefore, we have sought to identify the molecules to which MRP-8/14 binds on endothelium. Our findings suggest that the primary binding partner is not a protein receptor but a sulfated glycosaminoglycan structure.

EXPERIMENTAL PROCEDURES

Reagents—The native complex of MRP-8/14 was isolated from fresh human neutrophils by Mono Q and Mono S chromatography, as described previously (13). Recombinant (r) human MRP-8 and -14 were expressed in Escherichia coli and purified by previously defined protocols (20). The purity and integrity of each protein was verified by SDS-PAGE.

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1 The abbreviations used are: RAGE, receptor for advanced glycation end products; GAG, glycosaminoglycan; HBSS, Hank’s balanced salt solution; H-HBSS, HEPES-buffered HBSS; mAb, monoclonal antibody; MRP, myeloid-related protein; r, recombinant; PBS, phosphate-buffered saline; BSA, bovine serum albumin; CHO, Chinese hamster ovary.

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Monoclonal antibodies (mAbs) 1H9, 1F5, and 6F5, specific for human MRP-14, and mAb 7C12, specific for human MRP-8, were generated as a result of mouse immunization with human rMRP-14 and rMRP-8, respectively. The mAbs were characterized by Western blotting and enzyme-linked immunosorbent assay with the assistance of Dr. John Gallagher (Imperial Cancer Research Fund Central Cell Services). The Fab' preparations were made by the Immunopure Fab preparation kit (Pierce) according to the manufacturer's instructions. The monospecific rabbit anti-MRP-14 and MRP-8 sera have previously been described (21). mAb 10E4, specific for heparan sulfate, was from Seikagaku Corp., and mAb CS-56, specific for chondroitin sulfate, was from Sigma. CD45 mAb, clone 2B11/PD7/26 was from Dako. The anti-CD36 mAb, CLBIVC7, was donated by Dr. Ian Dransfield (University of Edinburgh), and the rabbit anti-RAGE by was donated by Dr. Ann-Marie Schmidt (New York).

The human CD36 construct in pcDNA3.1 was a kind gift from Dr. Maria Febbraio (Cornell University). The human RAGE cDNA was generously donated by Dr. Igor Bronstein (University of York), and Dr. Paula Stanley (Imperial Cancer Research Fund) inserted it into pIRE2-EGFP and pEGFP-N2 (both from CLONTECH). All glycosaminoglycan and modified hepatic RAGE preparations were purchased from Sigma, with the exception of [3H]heparin (PerkinElmer Life Sciences). These preparations were made up at 1 mg/ml in HBSS (without Ca" or Mg") buffered with 10 mM Hepes, pH 7.4 (HB-HEPSS), just prior to use in the assay, except hyaluronan, which was solubilized in 0.3 M Na2HPO4, pH 5, at 50 °C.

Cell Culture—The human microvascular endothelial cell line, HMEC-1 (22) was generously donated by Dr. R. Bicknell (Imperial Cancer Research Fund) with the permission of Dr. T. Lawley and maintained by culturing on gelatin-coated flasks in Dulbecco's modified Eagle’s medium (Sigma) supplemented with 10% fetal calf serum (Bio- clear), 10 ng/ml epidermal growth factor (Sigma), and 1 mg/ml hydrocortisone (Sigma). Before use, the cells were cultured on tissue culture plastic for one passage, removed with 0.5 mM EDTA in PBS, and washed in HB-HEPSS containing 0.2% BSA (fatty acid free, ICN) for use in assays. The CHO-KI cell line and clone pgA-745, which is mutated in xylosyl transferase and is unable to synthesize heparan or chondroitin sulfate GAGs (23), were obtained from the Imperial Cancer Research Fund Cell Services Department and Dr. John Gallagher, respectively, and were maintained in Kigha's modified Ham’s F-12 medium supplemented with 10% fetal calf serum. These cells were harvested as above.

CHO Cell Transfections—The CHO-KI cell line and GAG-minus clone pgA-745 (see above) were transfected using LipofectAMINE (Invitrogen) with the CD36 construct, and expression was monitored by binding of rMRP-14 to CLBIVC7 after 48 h. RAGE was similarly transfected into the same CHO cell lines. Successful transfection (50–70% total cells) was detected by both green fluorescent protein expression and Western blotting for RAGE using the rabbit anti-RAGE antibody. Immunochemistry—Paraffin-embedded tissues sections were dewaxed, the endogenous peroxidase was blocked, and the slides were washed in H2O2 in PBS (0.06%) for 2 min at room temperature. Finally, the slides were incubated in 3,3'-diaminobenzidine solution (0.05% 3,3'-diaminobenzidine/H2O2) for 2 min at room temperature. The slides were washed and then incubated for 45 min with either biotinylated swine anti-rabbit IgG (1:100; DAKO) followed by phycoerythrin streptavidin (1:100; Jackson ImmunoResearch). After washing, the slides were resuspended in PBSA + 2% formaldehyde and analyzed by flow cytometry using a FACScan (Becton Dickinson).

To determine the dative cation dependence of rMRP-14 binding to cell surfaces, the cells were resuspended in binding buffer containing 1 mM rMRP-14 and either no divalent cations; 1 mM MgSO4 and 10 mM ZnSO4; 1 mM CaCl2 and 1 mM MgSO4; 10 mM ZnSO4 and 1 mM CaCl2 or all three divalent cations. After the cells were incubated on ice for 40 min, they were washed three times with binding buffer containing the same divalent cations and then another three times with binding buffer containing all three divalent cations. The amount of bound rMRP-14 was determined as above.

Heparin Binding Assay—96-well Immunol plates (Dynex Technologies) were coated with mAb I9H at 100 μg/ml in PBSA overnight at 4 °C. The plate was then blocked with 2% BSA in PBSA for 1–2 h at room temperature and washed three times with H-HEPSS. 0.1 mM rMRP-14 in H-HEPSS containing 2% BSA and divalent cations (1 mM CaCl2, 1 mM MgSO4, and 10 mM ZnSO4) was added, and the plate was incubated for 1 h at room temperature. After washing three times with H-HEPSS/cations containing 0.1% Tween 20, 100 μl/well [3H]heparin in H-HEPSS/cations/BSA with and without blocking agents was added to the plate. The plate was incubated for 1 h (unless otherwise stated) at 37 °C and then washed. The bound [3H]heparin was solubilized by 0.5 mM NaOH containing 1% SDS for 30 min at 37 °C. The contents of each well were added to 5 ml of liquid scintillation mixture (Ecolite +; ICN) and counted (Beckman scintillation counter LS6500). The specific binding was determined by subtraction of binding in the presence of 100 μg/ml cold heparin.

In assays with blocking agents, a parallel experiment was performed to test whether the blocking agent was able to compete the rMRP-14 from the anchoring mAb. The plates were coated, blocked, and incubated with 0.01 μM rMRP-14 as above. The rMRP-14 was then treated with anti-MRP-14 Fab' as in the [3H]heparin binding assay. After washing, the amount of bound rMRP-14 was determined by enzyme-linked immunosorbent assay using rabbit anti-MRP-14 followed by horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (DAKO), both diluted 1:1000 in H-HEPSS/cations/BSA. After washing, the bound antibody was detected by O-phenylenediamine (Sigma) according to the manufacturer's instructions. The absorbance at 492 nm was read by a Multiscan spectrophotometer reader (Tietzert).

The salt sensitivity of rMRP-14 binding to heparin was analyzed by the above assay with an additional wash step (washing three times with H-HEPSS/cations with and without NaCl) following the incubation with [3H]heparin. Like the blocking assays, a parallel experiment with rabbit anti-MRP-14 determined that the rMRP-14 was not released from the mAb I9H.

Enzymic Digestion of HMEC-1 Cell Membrane GAGs—HMEC-1 cells were resuspended at 1 × 106 cells/ml in binding buffer containing protease inhibitors (20 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 10 μg/ml leupeptin) and various concentrations of heparinase I (Sigma), or alternatively, H-HEPSS containing 0.2% BSA, protease inhibitors, 0.5 mM EDTA, and various concentrations of chondroitinase ABC (Sigma). The digestion was performed for 4–6 h at room temperature. Alternatively, the cells were resuspended in PBSA containing 0.5 mM EDTA with and without 0.25% trypsin for 5 min at room temperature. After enzyme treatment, the cells were washed four times in binding buffer and used in the cell surface binding assay as above. The removal of heparan sulfate or chondroitin sulfite was monitored by autoradiography using antisense heparinase I. The removal of glycosaminoglycan was confirmed by autoradiography using antisense chondroitinase ABC. Both heparinase I and chondroitinase ABC were from Boehringer Mannheim.
RESULTS
Expression of MRP-8 and MRP-14 in Vivo—Immunohistochemical studies have localized MRP-8/14 to venules featuring extravasating myeloid cells (19). A survey of noninflamed tissues revealed little positive staining for MRP-8 and -14 (data not shown). However, in inflammatory conditions, such as found in Crohn’s disease, small venules frequently stained with both anti-MRP-14 (Fig. 1A) and anti-MRP-8 (data not shown). The staining of the two subunits was always coincident. The CD45-positive total leukocyte infiltrate (Fig. 1B) contained CD15-positive neutrophils and CD68-positive monocytes (data not shown) but was negative for MRP-8 and -14 (Fig. 1A).

Together these results suggest that in an inflammatory setting, myeloid cells lose expression of MRP-8 and -14 during transmigration, and this can be associated with MRP positive endothelium.

To investigate whether the source of the endothelial-associated MRP-8/14 is the endothelium itself or the transmigrating leukocyte, we tested for MRP mRNA expression within blood vessels of small intestine tissue from Crohn’s disease. When small vessels positive for MRP-8 protein (Fig. 1C) were examined by in situ hybridization, no MRP-8 mRNA could be detected in the endothelial cells (Fig. 1D). In contrast, associated myeloid cells were positive for both MRP-8 protein and mRNA. Identical results were obtained for MRP-14 (data not shown). These results indicate that the endothelial cells do not synthesize the MRP proteins found on their surface and provide direct evidence that associated leukocytes are the source of the deposited protein.

To further test human endothelial cells for their ability to synthesize the MRP proteins, we stimulated the microvascular endothelial cell line HMEC-1 (22) for 20 h with a concentration range of the agonists tumor necrosis factor α, interleukin-1β, interferon-γ, or lipopolysaccharide. As determined by immunohistochemistry and enzyme-linked immunosorbent assay for the MRP-8/-14 complex, the HMEC-1 cells were found not to express the MRP proteins even after stimulation (data not shown).

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binding as detected by the anti-MRP-8 mAb, 7C12 (data not shown). Similar MRP-8/14 and rMRP-14 blocking data were also obtained with another anti-MRP-14 mAb, 6F5 (data not shown). Thus, the MRP-8/14 heterodimeric protein complex interacts with endothelial cells via the MRP-14 subunit, and rMRP-14, but not rMRP-8, mirrors this binding.

rMRP-14 has been demonstrated to bind both calcium (25) and zinc (26) ions. The binding of rMRP-14 to HMEC-1 cells required the presence of both Ca\(^{2+}\) and Zn\(^{2+}\) but was independent of a third divalent cation, Mg\(^{2+}\) (Fig. 3). This result suggests that only the calcium and zinc ion-bound conformation of MRP-14 was able to bind to endothelial cell surfaces.

Heparin Blocks MRP-14 Binding to Endothelial Cells—Chemokines are immobilized onto the vascular lumen by binding to GAG structures on endothelial cells (27, 28). To evaluate the contribution of GAGs to MRP-14 binding, a range of GAGs were used as blocking agents. When the GAGs were titrated from 0.1–100 \(\mu\)g/ml, heparin potently inhibited the binding of rMRP-14 to HMEC-1 cells with an \(IC_{50}\) of 0.1 \(\mu\)g/ml (approximately 7 nM; Fig. 4A and data not shown). Both heparan sulfate and chondroitin sulfate B (dermatan sulfate) reduced the binding of rMRP-14 to endothelial cells but were less potent than heparin. Chondroitin sulfate A (chondroitin-4-sulfate) and chondroitin sulfate C (chondroitin-6-sulfate) were poor inhibitors. Hyaluronic acid (data not shown) and keratan sulfate did not affect the interaction.

Dextran sulfate often interferes with protein-GAG interactions that are dependent on sulfation of the GAG. 100 \(\mu\)g/ml dextran sulfate, but not 100 \(\mu\)g/ml dextran, inhibited rMRP-14 binding to HMEC-1 cells (Fig. 4B). Together these data show that the endothelial receptors for MRP-14 are highly modified and sulfated GAG structures.

The Interaction between rMRP-14 and Heparin—rMRP-14, which was immobilized through the nonblocking mAb 1H9, bound \(^{3}H\)heparin. In the absence of MRP-14 or in the presence of 100 \(\mu\)g/ml cold heparin, the binding of \(^{3}H\)heparin was eliminated, demonstrating that the interaction was specific (Fig. 5A and data not shown). After 1 h of incubation, the binding curve demonstrated that the interaction was saturable and of high affinity, with Scatchard analysis determining the \(K_d\) to be 79 ± 44 ng/ml (n = 5; Fig. 5B). As the \(K_d\) and maximum binding determined after 30 min and 2 h did not significantly differ from those determined after 1 h, the binding was considered to be at equilibrium, and the 1-h time point was chosen for further studies. Using the mid-point value of the heparin molecular mass range (i.e. 13,000 Da) yielded a \(K_d\) = 6.1 ± 3.4 nM. Therefore, MRP-14 has very high affinity for heparin, as compared with most GAG-protein interactions (29).

mAbs 1F5 and 6F5 inhibited the interaction between \(^{3}H\)heparin and rMRP-14 (data not shown). This blocking indicates that the properties of MRP-14 binding to heparin mimic the binding of MRP-14 to endothelial cell surfaces.

The Nature of the MRP-14 to Heparin Interaction—To investigate whether the interaction between rMRP-14 and heparin was dependent on sulfation, modified preparations of heparin were used to block 100 ng/ml \(^{3}H\)heparin binding to rMRP-14. Heparin blocked the interaction with an \(IC_{50}\) of 10–100 ng/ml (~0.7–7 nM; Fig. 6A). This \(IC_{50}\) is slightly lower than the concentration of \(^{3}H\)heparin, which probably indicates that the two preparations of heparin differed somewhat. Removal of the amino-linked sulfate groups of heparin (de-N-sulfated heparin) reduced the potency of heparin as a blocking agent by about 3 orders of magnitude. N-Acetylation of the de-N-sulfated heparin (N-acetyl heparin) did not further affect the blocking of the heparin-rMRP-14 interaction. Removal of O-linked sulfate groups from this N-acetyl heparin (N-acetyl-de-O-sulfated heparin) completely removed the capacity of heparin to block the interaction. Parallel experiments demonstrated that these preparations of modified heparin at 500 \(\mu\)g/ml did not significantly reduce the amount of rMRP-14 immobilized on mAb 1H9 (data not shown). These results suggest that the binding of rMRP-14 to heparin is dependent on both N- and O-linked sulfate substitutions.

To further investigate the nature of the interaction between heparin and rMRP-14, a salt wash was used in the \(^{3}H\)heparin...
binding assay. [3H]Heparin binding to rMRP-14 was disrupted by washing with assay buffer containing 0.5 M NaCl (Fig. 6B). A parallel experiment demonstrated that a 0.5 M salt wash did not reduce the amount of rMRP-14 anchored by mAb 1H9 (data not shown). This indicates that the interaction between rMRP-14 and heparin was largely ionic in nature, further supporting the involvement of the sulfate groups of heparin.

MRP-8/14 Binding to GAGs on Other Cell Types —Next we wanted to confirm that MRP-14 and the complex were binding specifically to GAGs and to exclude the possibility that soluble heparin sequesters the MRP-14 from binding to another receptor on endothelial cells. Therefore, we took advantage of a CHO-KI-derived cell line, pgsA-745, which is defective in GAG synthesis (23). rMRP-14 bound in a saturable manner to the parental CHO-KI cells, but binding to the GAG-minus CHO cells was completely absent (Fig. 7). Similarly, the MRP-8/14 complex at 1 ng/ml was bound to rMRP-14 and washed three times with assay buffer containing a range of NaCl concentrations. The data are the means ± S.D. of triplicate experiments, and representative experiments of two are shown.

MRP-14 Binds to Heparinase I-sensitive Endothelial Proteoglycans —Because GAG moieties vary greatly between tissues, the blocking data by GAG preparations isolated from other tissues often give a false impression of the nature of the target GAG. Consequently, we evaluated the contribution that chondroitin sulfate and heparan sulfate made to MRP-14-binding sites on HMEC-1 cells by digesting these structures with specific enzymes. Heparinase I digestion of HMEC-1 cell surfaces consistently reduced the number of MRP-14-binding sites by 60–70% (Fig. 8A). However, chondroitinase ABC treatment had little effect on the number of MRP-14-binding sites (Fig. 8B). The enzyme was further titrated between 0.2 milliunit/ml and 8 units/ml without affecting rMRP-14 binding (data not shown). The removal of the GAGs was confirmed by the depletion of the heparan sulfate-specific 10E4 epitope (Fig. 8A) and the chondroitin sulfate specific epitope CS-56 (Fig. 8B). The loss of rMRP-14 binding following heparinase I treatment was specific, because removal of binding sites was not affected by proteinase inhibitors but was inhibited by 0.5 mM EDTA. In addition, the digestion by both enzymes did not reduce the expression of the other GAG species or the abundantly expressed membrane proteins CD29 and CD44 (data not shown). The binding of MRP-14 was also eliminated by trypsin treatment of the HMEC-1 cells (Fig. 8C). Again this was mirrored by the depletion of the 10E4 epitope (data not shown). These
MRP-8/14 Binds Glycosaminoglycans

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**DISCUSSION**

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**Involvement of CD36 and RAGE as MRPs**

CD36 has been reported to act as a receptor for MRP-8/14 (17), and RAGE has been proposed as a receptor for S100 proteins (7). Therefore, we evaluated the contribution of these receptors to MRP-14 binding sites on HMEC-1 cells. Resting HMEC-1 cells express little CD36 as a receptor for S100 proteins, and they express no RAGE. Therefore, we conclude that the MRP complex can bind to heparan sulfate structures of endothelial cell surface proteoglycans. We also demonstrate that rMRP-14 binds heparin directly and that this appears to be dependent on ionic interaction with the N- and O-linked sulfate substitutions of the GAG.

**GAG modifications vary greatly between tissues.** The sulfation pattern recognized by MRP-14 appears to be widespread, because the recombinant protein binds to several cell lines, including T lymphoblasts, neutrophils, myeloid cell lines, COS cells (data not shown), and CHO cells, with a similar or slightly reduced affinity compared with HMEC-1 cells. The only tested cell line to which rMRP-14 did not bind was a GAG-minus CHO cell mutant, thus confirming the nature of the MRP-14 receptor. In addition, the amount of rMRP-14-binding sites and therefore the target GAGs do not alter in expression following endothelial cell stimulation (data not shown), suggesting that these GAGs are stable membrane structures.

rMRP-14 binds to heparin with a high affinity for a GAG-protein interaction (Kd = 6.1 ± 3.4 nM) which ranges from 10^-9 to 10^-8 M. For example chemokines, such as RANTES (regulated on activation normal T cell expressed and secreted) and interleukin-8, that also bind to endothelial GAGs have Kd values in the μM range (28). Identifying heparin binding sequences in proteins has been difficult but consensus motifs, such as XBBBXXBX, XBBXBX, or TXXBXXTBXXXTBB, have been suggested (29). MRP-14, MRP-8, and other S100 proteins do not contain these motifs (data not shown). It is therefore likely that the heparin-binding motif is formed as a result of the tertiary structure of MRP-14. This conjecture is backed up by the finding that 15–20 residue peptides spanning the se-

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The binding of MRP-14 to the endothelial cells is dependent upon Ca\(^{2+}\) and Zn\(^{2+}\). The structure of several S100 proteins undergoes conformational change in the presence of Ca\(^{2+}\), which includes the exposure of a putative receptor binding cleft (32). In addition, the structure of Ca\(^{2+}\)–occupied S100A7 (psoriasis), a close homologue of MRP-14, is altered on ligation of Zn\(^{2+}\) (33). Ca\(^{2+}\) and Zn\(^{2+}\) are known to bind to the MRP-8/14 complex and induce a conformational change (16). Therefore, we propose that the divalent cation regulation of the MRPs will be critical for their function.

It has been reported that the scavenger receptors CD36 (17) or potentially the RAGE might serve as cell surface receptors for the MRP-8/14 heterodimer. Interestingly, the proinflammatory functions of S100A12, a close homologue of MRP-14, are attributed to ligation of and signaling through RAGE (7). RAGE also mediates the neuronal outgrowth stimulated by S100A1 and S100B proteins (11). The HMEC-1 cells in this study expressed little CD36 and no detectable RAGE with CD36 mAb (solid line) or control mAb (dashed line). C, the binding of 1 μM rMRP-14 to vector alone (gray bar) and CHO-K1 cells and GAG-less pgsA-745 cells. Inset, CHO-K1 cell expression of CD36 on CD36 transfected (solid line) and mock transfected cells (dashed line) as detected with mAb CLBIVC7; HMEC-1 cells labeled with CD36 mAb (solid line) or control mAb (dashed line).

A recent paper by Srikrishna et al. (34) reported that MRP-14 and MRP-8/14 complex bind to novel carboxylated N-glycans on endothelial cells and that the binding was blocked by the N-glycan-specific mAb GB3.1. This interaction may be distinct from the GAG binding we describe here, because mAb GB3.1 binding is insensitive to heparin (up to 250 μg/ml) and heparan sulfate (up to 50 μg/ml). It is interesting to speculate that the N-glycan reactivity may account for the heparinase I-insensitive binding of MRP-8/14.

The function of the MRP complex within inflammation is poorly defined. MRP-14 antiserum reportedly inhibit transmigration of monocytes expressing MRP complex (35). The results in our study could provide a mechanism for this observation because cell surface bound MRP-8/14 could act as an endothelial cell receptor. In addition, the immobilization on GAGs is also consistent with and in fact provides a localization mechanism for an anti-oxidant activity of MRPs in protecting the endothelium against oxidative damage by leukocytes, as proposed by Geczy and co-workers (37).

The binding of the MRP complex to GAGs resembles that of the chemokines. It is thought that immobilization on proteoglycans prevents chemokines from being washed away in the blood flow, localizing them to the site of inflammation (27, 38). Additionally, signaling by these inflammatory mediators is believed to be enhanced by their presentation on endothelium to rolling leukocytes expressing their receptors (28). Chemokines then signal to inflammatory cells via interacting with G protein-coupled receptors. Similarly GAGs may facilitate the binding of the MRP complex to an additional receptor still to be

\footnote{G. Srikrishna and H. Freeze, personal communication.}
identified that might then signal into the cell.

In summary, we have demonstrated that the major receptor for the MRP-8/14 complex on endothelium is a heparan sulfate moiety. The widespread expression of such GAGs suggests a certain nonselectivity in MRP complex binding. However, it is probable that a specific binding stimulus induces release of these proteins from myeloid cells on vessels near an inflammatory site where they will function. Such stimuli have still to be identified. Our study provides a mechanism for the presentation of the vessel-associated MRP-8/14 and may, as a basis for further investigation, help elucidate the extracellular functions of the MRP proteins.

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REFERENCES

1. Schafer, B. W., and Heizmann, C. W. (1996) Trends Biochem. Sci. 21, 134–140
2. Donato, R. (1999) Biochim. Biophys. Acta 1456, 191–231
3. Komada, T., Araki, R., Nakatani, K., Yada, I., Naka, M., and Tanaka, T. (1996) Biochim. Biophys. Res. Commun. 220, 871–874
4. Jinquian, T., Vorum, H., Larsen, C. G., Madsen, P., Rasmussen, H. H., Gesser, B., Etzerodt, M., Honore, B., Celis, J. E., and Thestrup-Pedersen, K. (1996) J. Invest. Dermatol. 107, 5–10
5. Lackmann, M., Cornish, C. J., Simpson, R. J., Moritz, R. L., and Gecky, C. L. (1992) J. Biol. Chem. 267, 7499–7504
6. Nishimura, F., Terranova, V. P., Sawa, T., and Murayama, Y. (1999) J. Dent. Res. 78, 1251–1255
7. Hofmann, M. A., Drury, S., Fu, C., Qu, W., Taguchi, A., Lu, Y., Avila, C., Kambham, N., Bierhaus, A., Nawroth, P., Neurath, M. F., Slattery, T., Beach, D., McClary, J., Nagashima, M., Morser, J., Stern, D., and Schmidt, A. M. (1999) Cell 97, 889–903
8. Yang, Z., Tao, T., Rafferty, M. J., Youssef, P., Di Girolamo, N., and Gecky, C. L. (2001) J. Leukocyte Biol. 69, 986–994
9. Cornish, C. J., Devery, J. M., Poronnik, P., Lackmann, M., Cook, D. I., and Gecky, C. L. (1996) J. Cell. Physiol. 166, 427–437
10. Schmidt, A. M., Yan, S. D., Yan, S. F., and Stern, D. M. (2000) Biochim. Biophys. Acta 1498, 99–111
11. Huttunen, H. J., Kuja-Panula, J., Sorci, G., Agneletti, A. L., Donato, R., and Rauvala, H. (2000) J. Biol. Chem. 275, 40096–40105
12. Hessian, P. A., Edgeworth, J., and Hogg, N. (1993) J. Leukocyte Biol. 53, 197–204
13. Edgeworth, J., Gorman, M., Bennett, R., Freemont, P., and Hogg, N. (1991) J. Biol. Chem. 266, 7706–7713
14. Siegenthaler, G., Boulin, K., Chatellard-Gruaz, D., Hotz, R., Saurat, J. H., Hellman, U., and Hagans, G. (1997) J. Biol. Chem. 272, 9371–9377
15. Klempt, M., Melkonyan, H., Nacken, W., Wiesmann, D., Holtkemper, U., and Sorg, C. (1997) FEBS Lett. 408, 81–84
16. Kerkhoff, C., Klempt, M., Kaeve, V., and Sorg, C. (1999b) J. Biol. Chem. 274, 32672–32679
17. Kerkhoff, C., Sorg, C., Tandon, N. N., and Nacken, W. (2001) Biochemistry 40, 241–248
18. Abumrad, N. A., el-Maghrabi, M. R., Amri, E. Z., Lopez, E., and Grimaldi, P. A. (1993) J. Biol. Chem. 268, 17665–17668
19. Hogg, N., Allen, C., and Edgeworth, J. (1989) Eur. J. Immunol. 19, 1053–1061
20. Newton, R. A., and Hogg, N. (1998) J. Immunol. 160, 1427–1435
21. Robinson, M. J., and Hogg, N. (2000) Biochem. Biophys. Res. Commun. 275, 865–870
22. Ades, E. W., Candal, F. J., Swerlick, R. A., George, V. G., Summers, S., Bosse, D. C., and Lawley, T. J. (1992) J. Invest. Dermatol. 99, 683–690
23. Esko, J. D., Stewart, T. E., and Taylor, W. H. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 3197–3201
24. Senior, P. V., Critchley, D. R., Beck, F., Walker, R. A., and Varley, J. M. (1988) Development 104, 431–446
25. Hunter, M. J., and Chazin, W. J. (1998) J. Biol. Chem. 273, 12427–12435
26. Rafferty, M. J., Harrison, C. A., Alewood, P., Jones, A., and Gecky, C. L. (1995) Biochem. J. 316, 285–293
27. Rot, A. (1992) Immuno. Today 13, 291–294
28. Kuschert, G. S., Coulin, F., Power, C. A., Proudfoot, A. E., Hubbard, R. E., Hoogewerf, A. J., and Wells, T. N. (1999) Biochemistry 38, 12959–12968
29. Hileman, R. E., Fromm, J. R., Weiler, J. M., and Linhardt, R. J. (1998) Bioessays 20, 156–167
30. Yen, T., Harrison, C. A., Devery, J. M., Leong, S., Ismaan, S. E., Yoshimura, T., and Gecky, C. L. (1997) Blood 90, 4812–4821
31. Casu, B., Peticou, M., Provasoli, M., and Sinay, P. (1988) Trends Biochem. Sci. 13, 212–225
32. Matsumura, H., Shiba, T., Inoue, T., Harada, S., and Kai, Y. (1998) Structure 6, 233–241
33. Brodersen, D. E., Nyborg, J., and Kjeldgaard, M. (1999) Biochemistry 38, 1695–1704
34. Srikrishna, G., Panneerselvam, K., Westphal, V., Abraham, V., Varki, A., and Freeze, H. H. (2001) J. Immunol. 166, 4678–4688
35. Eue, I., Pietz, B., Storck, J., Klempt, M., and Sorg, C. (2000) Int. Immunol. 12, 1583–1604
36. Deleted in proof
37. Passey, R. J., Xu, K., Hume, D. A., and Gecky, C. L. (1999) J. Leukocyte Biol. 66, 549–556
38. Tanaka, Y., Adams, D. H., and Shaw, S. (1993) Curr. Top. Microbiol. Immunol. 184, 99–106
