The S100 family protein MRP-14 (S100A9) has homology with the contact domain of high molecular weight kininogen

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Abstract The heterodimeric molecule MRP-8/MRP-14 (S100A8/S100A9) is abundantly expressed in circulating monocytes and neutrophils. We report here an homology between the C-terminal 'tail' region of MRP-14 (S100A9) and sequences within the plasma protein, high molecular weight kininogen (HMWK) which are involved in binding to negatively charged surfaces such as kaolin. MRP-14 also binds to kaolin and is competitively inhibited by HMWK and by peptides corresponding to MRP-14 tail and the HMWK 'contact' regions. Furthermore both MRP-14 and the tail peptide inhibit the coagulation cascade in vitro giving functional relevance to the homology between MRP-14 and HMWK. At inflammatory sites, MRP-8/14 is localised to areas of close contact between myeloid cells and endothelium. The results of this study identify a potential binding region in MRP-14 and suggest that it could function by interfering with fibrin formation at sites of leukocyte transendothelial migration.

Key words: S100 protein; MRP-8; S100A8; MRP-14; S100A9; Calcium-binding protein; Cell adhesion; Coagulation

1. Introduction

The proteins MRP-8 (S100A8) and MRP-14 (S100A9) are members of the S100 family abundantly expressed as a heterodimeric complex in the cytosol of circulating monocytes and neutrophils [1–5]. The presence of two of the helix-loop-helix, calcium-binding motifs (known as EF-hands) in both MRP-8 and MRP-14 is a general characteristic of S100 family members which divides the proteins into an amino (N)-terminal region, a 'hinge' region between the two EF-hands and a carboxyl (C)-terminal region [5–10]. Throughout the S100 family there is a significant divergence in sequence in these three regions which is thought to confer specificity to functions of individual proteins. For the limited number of S100 proteins where function has been assigned, this hypothesis has been confirmed. The C-terminal region of human p11, the annexin II light chain, mediates complex formation with the annexin II heavy chain, p36 [11], while the hinge region of the murine MRP-8 homologue, CP-10, has potent chemotactic activity [12].

In MRP-14, the C-terminal sequence (residues 89–114) is unusually extended leaving the protein with an obvious 'tail', a feature which has highlighted this region as an area of potential functional significance. The sequence of this region has previously been shown to be identical to the neutrophil immobilising factor (NIF) peptides [13], suggesting that this protein may have a role in the localisation of neutrophils and monocytes to inflammatory sites [14]. Phosphorylation of the penultimate threonine residue (Thr^{113}) of the C-terminal region occurs with elevated $(Ca^{2+})_i$ implicating MRP-14 in the sequence of events associated with neutrophil or monocyte activation [15]. A search of protein sequences revealed homology between the C-terminal tail region of MRP-14 and functional domains within high molecular weight kininogen (HMWK) [16,17]. This plasma protein, which has been considered to be involved in the initiation of intrinsic coagulation, is also likely to have additional activities. In this study, we explore the implications of the MRP-14 and HMWK homology and present results which suggest a function for MRP-14.

2. Materials and methods

2.1 Protein isolation and iodination

The MRP-8/14 complex was purified from neutrophil cytosol using Fast Protein Liquid Chromatography ion exchange and gel filtration as previously described [2]. MRP-8 and MRP-14 subunits were further separated by C_{18}-reverse phase HPLC. Purified MRP-14 was iodinated to a specific activity of 5×10^4–10^6 cpm $\cdot \mu g^{-1}$ using Iodobeads as recommended by the manufacturer (Pierce, UK). Kallikrein-digested, two chain HMWK (Enzyme Research Laboratories, UK), bovine S100a (S100A1/B) and S100b (S100B/B) (Sigma, USA) were obtained commercially and their purity confirmed by SDS-PAGE.

2.2 Oligopeptide synthesis

Peptides spanning the amino terminus (amino peptide; MRP-14/5–25), the hinge region between the two EF-hand Ca^{2+}-binding domains (hinge peptide, MRP-14/45–62), the C-terminal EF-hand Ca^{2+}-binding region (EF-hand peptide; MRP-14/59–79) and the C-terminal tail region (tail peptide; MRP-14/89–114) of MRP-14 and the HG-rich (HG-rich peptide; HMWK/443–475) and HGK-rich (HGK-rich peptide; HMWK/493–520) regions of HMWK [17] were prepared by solid phase synthesis using *f*-methoxycarbonyl chemistry [18] and characterised by amino acid analysis and plasma desorption mass spectrometry. The relative positions of these peptides are detailed in Fig 1A.

2.3 Competitive inhibition of MRP-14 binding to kaolin

Binding to kaolin was used to measure the ability of MRP-14 to interact with an anionic surface Ten μl of inhibitory protein or peptide solutions at the concentrations indicated, were mixed in 0.65 ml microcentrifuge tubes (Low protein binding, Sorensen Bioscience Inc., USA) with an equal volume of kaolin (1.25 mg $\cdot$ ml^{-1} in 20 mM Tris-HCl, pH 7.4, 0.15 M NaCl; ICN Biochemicals, USA) for 30 min at room temperature prior to the addition of 2 μM ^{125}I-labelled MRP-14 for a further 15 min. Supernatant was removed after centrifugation at 13,000 $\times$ *g* for 2 min and the pellet counted.

2.4. Inhibition of intrinsic plasma coagulation assay

An activated partial thromboplastin time (APTT) assay with kaolin was used to assess the effect of the proteins or peptides on intrinsic coagulation. The assay was performed as described elsewhere [19] with minor modification In brief, 100 μl volumes of prewarmed kaolin (5 mg $\cdot$ ml^{-1} in 0.15 M NaCl) and Bell and Alton platelet substitute (Diagen; Diagnostic Reagents Ltd , UK) solutions were mixed in a glass tube for 30 seconds prior to the addition of 50 μl of

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appropriate protein, peptide solution or Tris-buffered saline control and 100 μl of normal plasma (diluted 1:4 in PBS; Organon Teknika, UK). After 2 min incubation at 37°C, the mixture was recalcified (100 μl of 25 mM $CaCl_2$), and the time necessary for clot formation was measured on duplicate samples.

3. Results

3.1. Sequence homology searches

A search of protein sequences in the Owl database (release 22) [20] revealed significant homology between the MRP-14 tail and three regions within domain 5 (D5) of the light chain of plasma HMWK. The relative positions of these homologous regions are detailed in Fig. 1A. The most extensive alignment spans 23 amino acids of the MRP-14 tail and includes 11 identical residues and 5 conserved substitutions for amino acids in the histidineglycine (HG)-rich region of HMWK (residues 442–464) (Fig. 1B). In addition, the second half of the MRP-14 tail sequence can be aligned with a continuation of the HG-rich region (residues 465–474) and with sequence in the histidine-glycine-lysine- (HGK)-rich region of HMWK at residues 494–504 (Fig. 1B). When MRP-14 is compared with these three sequence alignments, only two residues do not represent either an identity or a conservative change.

3.2. Purified neutrophil MRP-14 binds to kaolin

The HG- and HGK-rich regions of HMWK both contain a 'contact site' which independently enable HMWK to bind to anionic surfaces in model systems [17]. The similarity in sequence suggested that MRP-14 might also function like HMWK in binding to a negatively charged surface such as kaolin. The MRP-8 and MRP-14 complex and individual subunits were all purified to homogeneity from neutrophil lysates (Fig. 2) and the MRP-14 subunit iodinated. Fig. 3A shows that ^{125}I-labelled MRP-14 bound to kaolin and that this binding was inhibited in a dose dependent manner by MRP-14, the MRP-8/14 complex and by HMWK with fifty percent inhibition achieved between 1 and 5 μM. In contrast, MRP-8 and two other related S100 family members, bovine S100a (S100A1/B or αβ heterodimer) and S100b (S100B/B or ββ homodimer) did not interfere with MRP-14 binding. Thus, like HMWK, monomer MRP-14 was able to bind to kaolin and this activity was not affected by complexing with MRP-8 and was not shared with at least two other S100 protein family members. Removal of Ca^{2+} (1 mM EDTA) or addition of Ca^{2+} (1 mM) to these assays did not influence MRP-14 binding to kaolin (data not shown) suggesting that this activity functioned independently of the two EF-hand Ca^{2+} binding regions of the native MRP-14 protein.

3.3. C-terminal sequence of MRP-14 mediates anionic surface binding

In order to test whether it was specifically the tail region of MRP-14 which was homologous in function as well as sequence to HMWK, we assessed the ability of MRP-14 to bind to kaolin in the presence of a series if MRP-14 and HMWK peptides (see

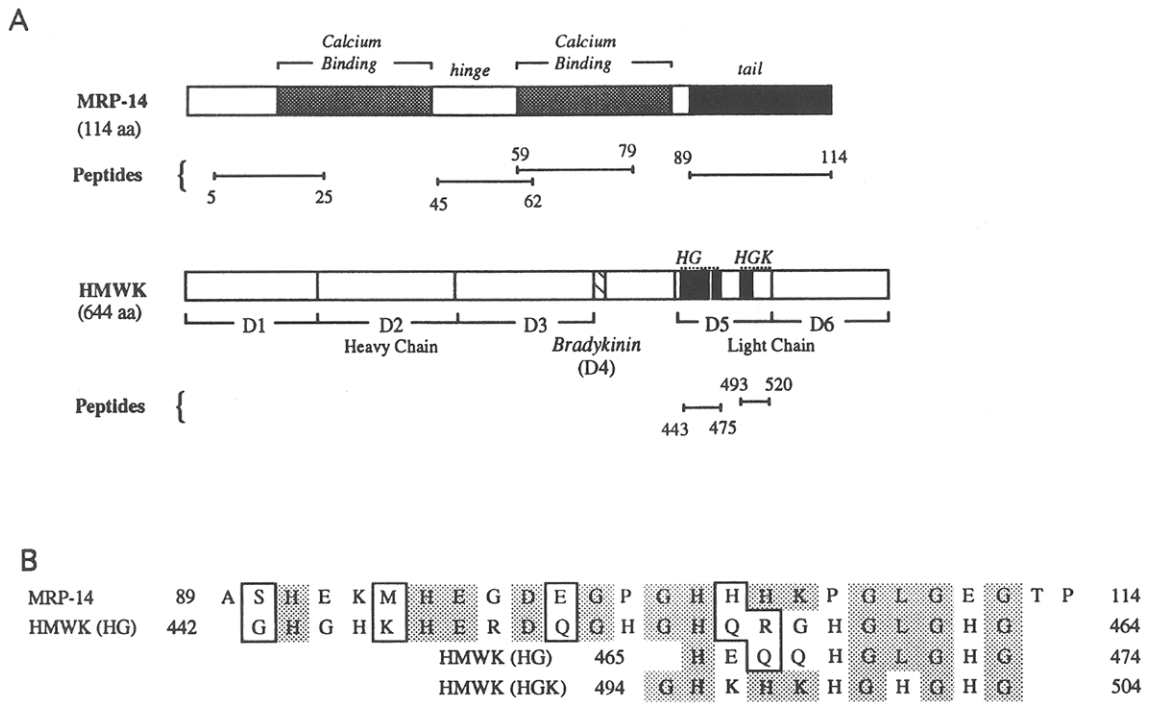

Fig. 1. Comparative homology between MRP-14 and HMWK. (a) A schematic representation of the MRP-14 and HMWK proteins illustrating the domain structure of each protein. The relative positions of the MRP-14 'tail' and the three regions in HMWK with which it is homologous are shown as dark shading. MRP-14 consists of two EF-hand type Ca^{2+} binding domains which divide the protein into N-terminal, 'hinge' and C-terminal 'tail' regions. HMWK is divided into the six domains (D1–D6) which constitute the mature single chain protein prior to release of the vasoactive kinin, bradykinin (striped shading), and full processing of the light chain by kallikrein cleavage. Domain 5 of HMWK contains the HG- and HGK-rich 'contact' regions. The positions of peptides used in this study are indicated. (b) Detailed amino acid comparisons of the C-terminal 'tail' sequence of MRP-14 (aa 89–114) and the three regions of homology in HMWK: residues 442–464 (HG-rich region); residues 465–474 (a continuation of sequence in the HG-rich region); and 494–504 (HGK-rich region). The shaded residues are identical with those in the MRP-14 sequence; unshaded boxed residues indicate conservative substitutions for those in the MRP-14 sequence.

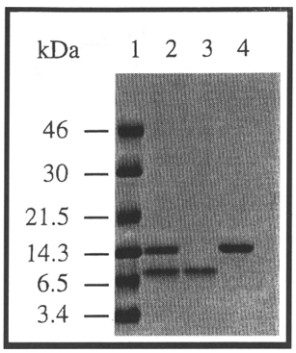

Fig. 2. Analysis of MRP-8/MRP-14 complex and separated subunits. Proteins were purified as described in section 2. Shown under reducing conditions are molecular weight standards (Amersham, Rainbow markers; lane 1); MRP-8/MRP-14 complex isolated from neutrophil lysates (lane 2), and separated MRP-8 (lane 3); and MRP-14 subunits (lane 4). Analysis was using SDS-15% PAGE followed by Coomassie blue staining.

section 2 and Fig. 1 for details). Of four MRP-14 peptides, only the C-terminal tail peptide significantly inhibited MRP-14 kaolin binding in a dose dependent manner (Fig. 3B). This result indicates that the binding of MRP-14 to kaolin is specifically mediated by the extended C-terminal tail region. As this inhibiting activity occurs at concentrations two orders of magnitude higher than the intact protein, the tail peptide must depend on other parts of MRP-14 for optimal conformation or presentation. It is also important to note that the interaction is not charge dependent as the tail peptide is slightly negatively charged (like kaolin) under these conditions (data not shown).

The effects of peptides spanning the HG- and HGK-rich regions of HMWK were also investigated. Both these peptides inhibited MRP-14 binding in a dose dependent manner although there was a difference in the concentration range over which they were effective (Fig. 3B). Notably, the HG-sequence peptide, displaying high homology with MRP-14 tail sequence, inhibited with greater efficiency than the tail peptide itself. This difference could reflect the fact that this peptide contains a duplicated HG-homologous region (see Fig. 1). Collectively, the results show the binding of MRP-14 to negatively charged surfaces is specifically mediated by the C-terminal tail region of the protein which simulates the action of the HG-rich and HGK-rich regions in mediating the binding of HMWK to similar surfaces.

3.4. Effect of MRP-14 on intrinsic coagulation

As well as the HG-rich and HGK-rich contact regions in domain 5, the light chain of HMWK contains, in domain 6, binding sites for coagulation factor XI and prekallikrein. All these areas are requisite for HMWK function in the initial stages of intrinsic plasma coagulation [21]. As MRP-14 appears to share only the contact sequence, we predicted that this protein might be able to interfere with the coagulation cascade. Therefore, in order to obtain further parallels between MRP-14 and HMWK, we tested the effect of MRP-14, MRP-8 and the MRP-8/14 complex in an in vitro assay of the intrinsic plasma coagulation. Both MRP-14 and MRP-8/14 complex, but not MRP-8 were able to significantly delay the onset of coagulation (Table 1). We next tested peptides from several regions of MRP-14. This approach was advantageous because amounts of MRP-8 and MRP-14 were limited (~ 50 nM) compared with average concentration of plasma HMWK at ~1 μM [22] and because the peptides allowed the identification of critical regions in MRP-14 which were active in the coagulation assay. It also allowed a comparison with peptides covering the HG- and HGK-rich regions of HMWK, previously shown to inhibit such assays [17,19]. Figure 4 shows that coagulation was inhibited by the MRP-14 tail peptide. At concentrations of this peptide which more than doubled the time required for clot

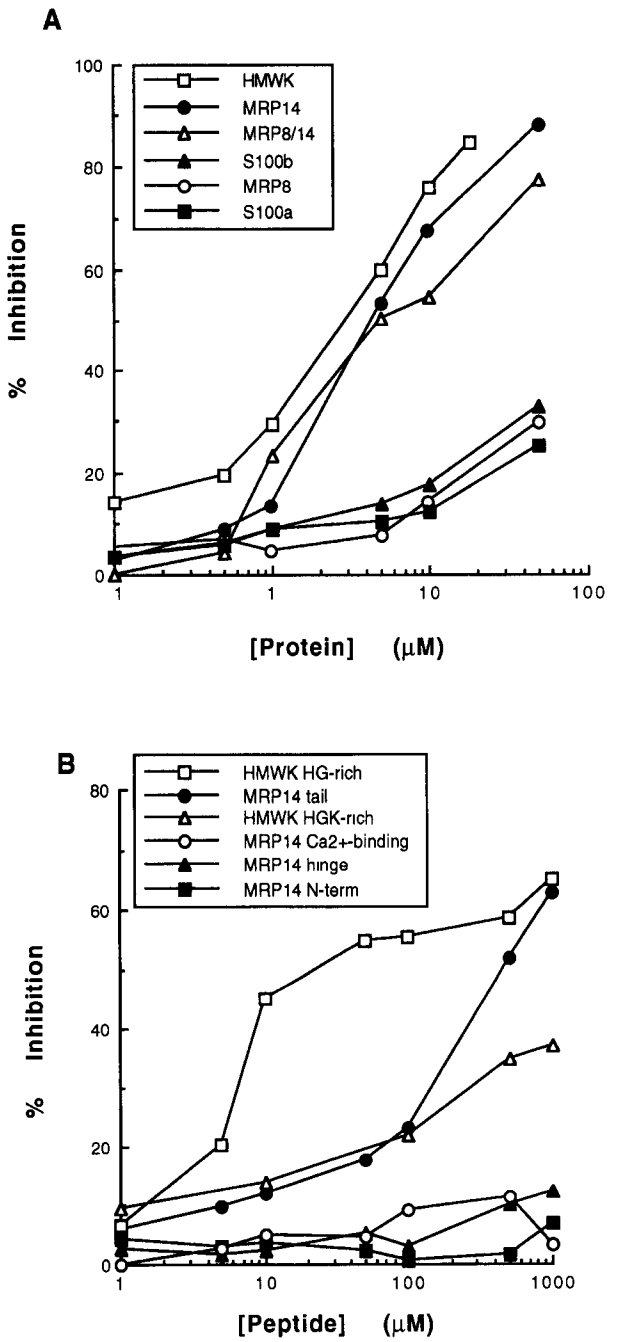

Fig. 3. The C-terminal domain of MRP-14 mediates binding to anionic surfaces. The binding of ^{125}I-labelled MRP-14 to kaolin in (a) the presence of varying concentrations of purified and unlabelled MRP-14, kallikrein-digested two chain HMWK, MRP-8, the MRP-8/MRP-14 complex and other S100 proteins, bovine S100a and bovine S100b or (b) the presence of peptides spanning areas of MRP-14 and HMWK. All points are shown as the mean of duplicate values and each curve is representative of 2–4 analyses

Table 1
MRP-14 inhibits plasma coagulation

	Clotting time (s)	*P* value
Control	179 ± 3*	–
MRP-14	212 ± 7 (18.4%)	<0.001
MRP-8/14	198 ± 13 (10.6%)	0.005
MRP-8	174 ± 4 (−2.4%)	n.s.

The effect of MRP-14, MRP-8, and the MRP-8/14 complex on assays of intrinsic plasma coagulation. Protein inhibitory activity is measured as the increased time necessary for clot formation and is displayed as mean ± standard deviation (seconds) of 4 separate experiments. Mean values were compared with the control using an unpaired *t*-test. Values given are for final assay protein concentrations of 5×10^{-8} M, which approximates the detectable plasma levels of the proteins in inflammatory diseases [25].
*Values are mean ± standard deviation (seconds); bracketed values represent the inhibitory activity (%).

formation, the MRP-14 hinge peptide (Fig. 4) and those from other regions of MRP-14 had no significant effect (data not shown). The inhibitory activity could be titrated but was always proportionately less than the inhibition produced by the HG-region peptide from HMWK. The peptide from the HGK region of HMWK was also inhibitory (data not shown). Thus the presence of the contact sequence in MRP-14 is sufficient to inhibit coagulation.

4. Discussion

Previous studies have shown that abundant amounts of MRP-8/14 are localised on inflammatory endothelium when adherence and transmigration of cells is in progress [1,7]. Of interest is the fact that adhesion of monocytes to matrix proteins fibronectin and collagen cause expression of a membrane form of MRP-8/9 although it is not known how it is secreted [23]. Such observations suggest that the MRP-8/14 complex might have a function in the sequence of adhesion receptor-ligand interactions leading to leukocyte entry into inflamed tissues. Here we show homology between the MRP-14 C-terminal tail and the two regions of HMWK which direct binding of the protein to anionic surfaces. Using kaolin as a model anionic surface, we show that the C-terminal tail sequence of MRP-14 performs a similar functional role. From available evidence, the more efficient site for HMWK binding appears to be contained within the HG-rich region [17,19], although it has previously been suggested that this area of the HMWK light chain has arisen by gene duplication [24]. Thus it is significant that it is with the HG-rich region that the MRP-14 tail is most homologous and that a peptide covering the region is the most effective inhibitor of MRP-14 binding to kaolin. As activated endothelium becomes more anionic and serves as a procoagulant surface [25], it may be speculated that the kaolin binding feature of the MRP-14 tail reflects a means by which the MRP-8/MRP-14 complex or the MRP-14 monomer might be retained at regions of endothelium involved in inflammation. This remains to be directly tested.

Assays of contact activation using kaolin have been used to mimic the role of HMWK as a trigger for the intrinsic coagulation pathway. The presence of either the HG- or HGK-rich region together with binding sites for kallekrein and factor XI in HMWK are essential for this function [17]. The fact that MRP-14 bears only the contact sequence and lacks the other procoagulant features of HMWK has implications for a possible role which bound protein could adopt in vivo. Fibrin deposited as a result of coagulation is anti-adhesive, apparently creating a barrier to leukocyte access to endothelium [26]. If the C-terminal tail-mediated binding of MRP-14 were able to counter fibrin deposition, more efficient interaction of leukocytes with endothelium or extracellular matrix would result. Of interest is the fact that in our studies, monomer MRP-14 or MRP-8/MRP-14 complex inhibit the coagulation assay at concentrations approximating those detected in plasma in inflammatory disease [27]. A second pathway of coagulation, known as the extrinsic pathway, initiated by tissue factor [28] was not affected by MRP-14 or the MRP-8/14 complex (data not shown). It is thought that in vivo the extrinsic pathway is the initiator of fibrin formation and the intrinsic pathway is the primary route for continued growth of the fibrin clot [29]. The possibility that both pathways could be activated at inflammatory sites raises questions about their importance during leukocyte adhesion and transendothelial migration and the contribution of HMWK and MRP-14. In addition, activated enzymes occurring downstream in the coagulation pathways are important in a variety of processes, many of which have a bearing on the efficiency of inflammation.

Accumulating evidence suggests domain 5 which contains the contact domain forms an important receptor recognition/binding region for HMWK. It is now speculated that the contact sites of HMWK mediate binding to heparin [30] and by promoting interactions between proteinases (e.g. kallikrein or Factor XI) and inhibitors such as antithrombin, HMWK might facilitate the inactivation of these proteases [31]. Such speculation suggests that, dependent on the local environment, HMWK is capable of both pro- and anti-coagulant activity. In addition domain 5 reported to interact with the leukocyte β_2

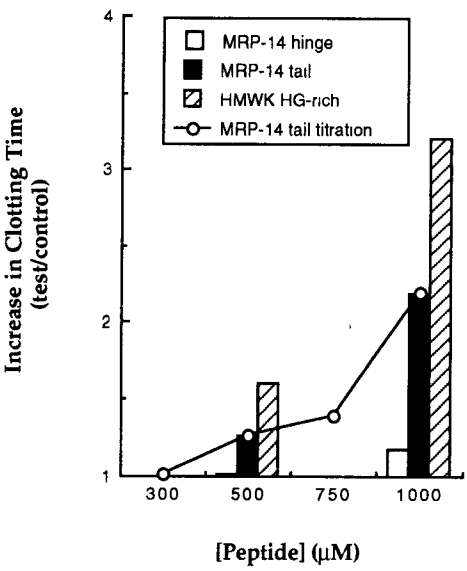

Fig. 4. The C-terminal tail of MRP-14 mediates the inhibition of coagulation. A comparison of the effect on coagulation of the MRP-14 tail peptide and the HG-region peptide from HMWK at concentrations of 500 μM and 1000 μM. Inhibitory activity of the MRP-14/89–114 tail peptide can be titrated (line graph) and is abolished at concentrations between 300–500 μM. Equivalent concentrations of the hinge peptide from MRP-14 were compared. Coagulation times in the absence of peptide (control) were within the range shown in Table 1.

integrin Mac-1 (CD11b/CD18) [32] and provides part of a composite site [33] recognised by additional receptors on endothelium [34], platelets [35] and neutrophils [36]. The utilisation of any one of the functional domains of HMWK upon interaction with these receptors is likely to have a unique effect, given the different roles of these cell types in the inflammatory process. Thus domain 5 may also direct the involvement of HMWK in additional activities which would have implications for the role of MRP-14 in inflammation. This study shows that the C-terminal tail sequence of the S100 family protein, MRP-14, is highly homologous to the contact sites of HMWK. The homology extends to functional similarities, in that the tail sequence specifically mediates the binding of MRP-14 to surfaces similar to those recognised by HMWK and MRP-14 can inhibit one activity attributable to HMWK, that of intrinsic coagulation. Obviously more information about the function of these proteins will aid our understanding of how the contact domain operates in vivo.

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