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Identification of a common hyaluronan binding motif in the hyaluronan binding proteins RHAMM, CD44 and link protein

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We have previously identified two hyaluronan (HA) binding domains in the HA receptor, RHAMM, that occur near the carboxyl-terminus of this protein. We show here that these two HA binding domains are the only HA binding regions in RHAMM, and that they contribute approximately equally to the HA binding ability of this receptor. Mutation of domain II using recombinant polypeptides of RHAMM demonstrates that K423 and R431, spaced seven amino acids apart, are critical for HA binding activity. Domain I contains two sets of two basic amino acids, each spaced seven residues apart, and mutation of these basic amino acids reduced their binding to HA–Sepharose. These results predict that two basic amino acids flanking a seven amino acid stretch [hereafter called B(X_7)B] are minimally required for HA binding activity. To assess whether this motif predicts HA binding in the intact RHAMM protein, we mutated all basic amino acids in domains I and II that form part of these motifs using site-directed mutagenesis and prepared fusion protein from the mutated cDNA. The altered RHAMM protein did not bind HA, confirming that the basic amino acids and their spacing are critical for binding. A specific requirement for arginine or lysine residues was identified since mutation of K430, R431 and K432 to histidine residues abolished binding. Clustering of basic amino acids either within or at either end of the motif enhanced HA binding activity while the occurrence of acidic residues between the basic amino acids reduced binding. The B(X_7)B motif, in which B is either R or K and X_7 contains no acidic residues and at least one basic amino acid, was found in all HA binding proteins molecularly characterized to date. Recombinant techniques were used to generate chimeric proteins containing either the B(X_7)B motifs present in CD44 or link protein, with the amino-terminus of RHAMM (amino acids 1–238) that does not bind HA. All chimeric proteins containing the motif bound HA in transblot analyses. Site-directed mutations of these motifs in CD44 sequences abolished HA binding. Collectively, these results predict that the motif of B(X_7)B as a minimal binding requirement for HA in RHAMM, CD44 and link protein, and occurs in all HA binding proteins described to date.

Key words: binding motif/CD44/cell motility/hyaluronan binding proteins/hyaluronan receptors/link protein/RHAMM

Introduction

Extracellular matrix molecules are involved in modifying cell responses to growth factors and cytokines, and in regulating cell adhesive interactions, motility and growth. These molecules, which include collagen, fibronectin, laminin, proteoglycans and glycosaminoglycans, interact with receptors on the cell surface. Emerging data indicate that these matrix–receptor associations occur via protein–protein and protein–carbohydrate interactions. Thus, integrins interact with extracellular matrix proteins via several sequences (for review see Schnaper *et al.*, 1993), the RGD motif being the best studied (Ruoslahti and Yamaguchi, 1991). The motifs that mediate protein–carbohydrate interaction have not been as well described. Heparin binding domains for vascular proteins (Sobel *et al.*, 1992), basic fibroblast growth factor (Zhu *et al.*, 1991; Guimond *et al.*, 1993) and fibronectin (Wilke *et al.*, 1991) have been shown to involve specific basic amino acids. Some vascular proteins interact with heparin via a specific motif, BBXXBBBXXBB (Sobel *et al.*, 1992). We have shown that the binding of hyaluronan (HA) to one of its receptors, RHAMM, is dependent, in part, upon ionic interactions and thus also probably involves basic amino acids (Yang *et al.*, 1993). We describe here a motif with which HA interacts with receptors/binding proteins, that is an important regulator of cell motility.

Hyaluronan is a large glycosaminoglycan containing repeating disaccharide units of *N*-acetyl glucosamine and glucuronic acid; it occurs in the extracellular matrix and on cell surfaces. It has been shown to be critical for maintaining the structure of the extracellular matrix and to affect such cell behavior as adhesion, motility and growth when it occurs on cell surfaces. Its production has been linked to a variety of disease, developmental and physiological processes (Laurent and Fraser, 1992; Turley, 1992).

A number of cell-associated and extracellular HA binding proteins have been described that mediate the biological effects of HA (Toole, 1990; Turley, 1991). These include link protein (Deak *et al.*, 1986; Doege *et al.*, 1987; Goetinck *et al.*, 1987), CD44 (Underhill *et al.*, 1985, 1987; Stamenkovic *et al.*, 1989, 1991; Culty *et al.*, 1990; Gunthert *et al.*, 1991; Jackson *et al.*, 1992; Lesley *et al.*, 1992), RHAMM (Turley *et al.*, 1987), aggrecan (Doege *et al.*, 1991), versican (Zimmermann and Ruoslahti, 1989), bacterial HA synthase (Lansing *et al.*, 1993), a hyaluronidase (Hope *et al.*, 1993), collagen VI (McDevitt *et al.*, 1991) and TSG-6 (Lee *et al.*, 1992). Although considerable information on the structure of some of these proteins is available, the specific requirements for their binding to HA are not known.

The tandem repeats found in the B loops of link protein contain peptides that bind to HA (Goetinck *et al.*, 1987), and homology to these repeats as a whole has been used to predict the HA binding nature of other proteins. However, the primary sequence of these peptides within the B loops

of link protein are not homologous to other HA binding proteins. Further, the recently cloned HA receptor RHAMM (Hardwick *et al.*, 1992), the bacterial HA synthase (Lansing *et al.*, 1993) and hyaluronidase (Hope *et al.*, 1993) all bear no sequence homology to other HA binding proteins, and yet bind HA with high affinity (K_d = 2 nM) and specificity (Hoare *et al.*, 1993). These anomalies and recent information that the binding site of the related glycosaminoglycan, heparin, to some proteins is based upon positive charge clustering (Ferran *et al.*, 1992; Sobel *et al.*, 1992) caused us to consider that an HA binding domain of the above proteins may likewise be based upon a charge motif rather than a specific primary amino acid sequence.

In a recent report (Yang *et al.*, 1993), we localized the HA binding domain of RHAMM to two, 10 amino acid sequences located near the carboxyl-terminus of this protein. Here we show that these sequences contain a motif of seven amino acids flanked by either an arginine or a lysine residue that is critical for HA binding. We also show that sequences of link protein and CD44 that contain this motif also bind HA. Further, we show that mutation of the motif's critical basic amino acids in these proteins abolishes their binding to HA. These results indicate that there exists at least one discrete binding motif for the glycosaminoglycan HA. This motif differs from heparin binding motifs that have been described and it occurs in all HA binding proteins characterized to date.

Results

Domains I and II are the only HA binding domains of RHAMM

We have previously reported that amino acids (aa) 401–411 (domain I) and 423–432 (domain II) of RHAMM are HA binding domains (Yang *et al.*, 1993). We sought to determine if these two domains were the only HA binding domains of this receptor. To accomplish this, we deleted domain II and mutated K405 and K409 in domain I to E, as shown in the strategy depicted in Figure 1A. Preliminary experiments had suggested that alterations of these amino acids abolished binding of HA to domain I (and see below). Fusion protein expressed from this deleted/mutated cDNA reacted with a rabbit polyclonal antibody to RHAMM but did not bind HA (Figure 1C). These results indicate that all of the HA binding activity of RHAMM resides in aa 401–432, as previously predicted (Yang *et al.*, 1993).

Effect of mutating basic amino acids on the binding of HA to RHAMM

We have previously shown that the binding of HA to RHAMM requires ionic interactions and that domains I and II contain a relatively high concentration of basic amino acids (Yang *et al.*, 1993). Furthermore, random placing of basic charges within these domains severely reduces their HA binding ability (Yang *et al.*, 1993). We first sought to determine which of the basic amino acids of these domains were critical to HA binding.

We utilized a technique (Yang *et al.*, 1993) wherein we constructed a recombinant RHAMM protein containing the amino-terminal fragment of RHAMM (aa 1–238) that does not bind HA fused to domain II. The ability of the resulting fusion protein product to bind biotinylated HA was then determined in a transblot assay (Hoare *et al.*, 1993; Yang *et al.*, 1993). Using this method, we constructed a series of mutations that were designed to identify the minimum critical basic amino acids that contribute to the HA binding ability of domain II in RHAMM (Figure 2A). In this assay, intact domain II (aa 423–432) bound biotinylated HA as previously described (Yang *et al.*, 1993). Mutation of the three carboxyl-terminal amino acids, K430, R431 and K432, to H residues (mutation I, Figure 2D, lane 2) abolished HA binding identifying these three basic residues as critical for interactions with HA. Mutation of K432 to I (mutation II) only slightly reduced binding (Figure 2D, lane 3), while mutation of R431 to S (mutation III, Figure 2A) resulted in a 62% reduction in HA binding (Figure 2D, lane 4). Mutation of K423 to N (mutation IV, Figure 2A) reduced HA binding by >80% (Figure 2D, lane 5). Mutation of both K423 and R431 [to N and S respectively (mutation V)] abolished binding (Figure 2D, lane 6). These results collectively suggest that K423 and R431 are critical for binding. These results further suggest that the HA binding site can be approximated by $B(X_7)B$ where B is critical. Since some binding did occur when only one of these amino acids (R431) was mutated, other basic amino acids may also contribute to binding in a minor fashion.

In domain I, we observed that four basic amino acids were each spaced seven amino acids apart a spacing identical to that occurring between the two critical basic amino acids observed in domain II. Changing K409 and K411 to L, in peptides otherwise mimicking domain I, abolished this spacing arrangement and significantly reduced binding of these peptides to HA-Sepharose (Figure 3). These results are consistent with those obtained for domain II, indicating that basic amino acids spaced precisely apart represent minimal requirements for binding.

Creation of artificial peptides that bind HA

Using the above recombinant techniques, we prepared peptides that contained basic amino acids oriented in the motifs depicted in Figure 4. The simplest peptide, $R(G_7)R$, bound to HA very weakly. Although the mutation analyses described above predict that the flanking basic amino acids are necessary for HA binding, these studies indicate that other factors enhance binding. Increasing the charge density on either end of the motif or adding a basic amino acid in the centre of the peptide enhanced binding so that an artificial peptide, $R(G_3)R(G)_2R_2$, exhibited binding to the level of either domain I or II as determined by densitometric analysis.

Effect of acidic amino acids on the binding of RHAMM peptides to HA

Examination of the RHAMM sequence (Hardwick *et al.*, 1992) indicates that there are three regions other than aa 401–411 and 423–432 where there are two basic amino acids separated by seven intervening amino acids. However, our mutational analysis shows that they do not bind HA. Interestingly, these apparently non-functional sites contain varying amounts of acidic residues between the flanking basic amino acids. To assess whether such residues affect binding, we mutated V429 of domain II to D (mutation VI, Figure 2A) and assayed the fusion protein product for its ability to bind HA in a transblot assay. Inclusion of D in domain II significantly reduced HA binding, indicating that the presence of an acidic amino acid is detrimental to HA

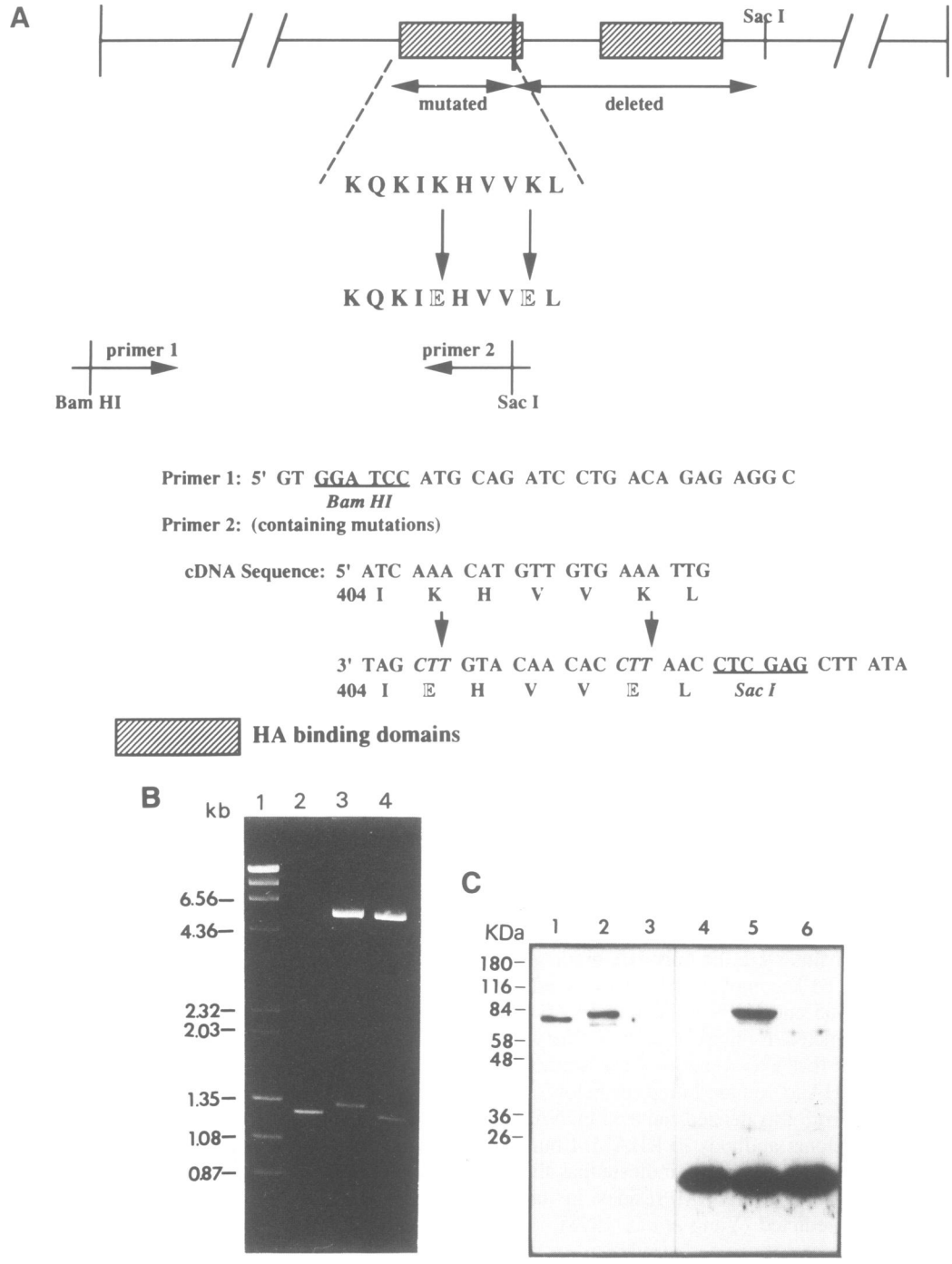

Fig. 1. Deletion and mutation of HA binding domains in RHAMM. **(A)** The HA binding domain II (aa 423−432) was completely deleted and the HA binding domain I (aa 401−411) was partially deleted. The remaining domain (aa 401−411) was altered by mutating K405 and K409 to E. **(B)** The PCR product (Figure 2A, lane 2) was ligated into the plasmid-containing fragment (5.3 kb in lane 3) and transformed into *E.coli* HB101. The clone containing the correct insert (lane 4) was used to prepare RHAMM fusion protein. **(C)** Cell lysates containing the complete fusion proteins (lanes 2 and 5), deleted fusion protein (lanes 1 and 4) and HB101 lysate (lanes 3 and 6) were prepared by sonication, then separated by SDS−PAGE and immunoblotted. RHAMM protein was visualized using anti-RHAMM antibody (lanes 1−3) or biotin-labelled HA (lanes 4−6). The results show that after deletion of domains I and II, RHAMM lost its ability to bind to biotin-labelled HA (lane 4). The bacterial lysate contains an HA binding protein of <26 kDa that is not related to RHAMM (lanes 4−6).

binding (Figure 2D, lane 7). The alteration of V208 and V209 in domain I to D also reduced HA binding (Figure 3).

The two HA binding domains of RHAMM contribute equally to its HA binding ability

Having established that aa 401−411 and aa 423−432 are the only two HA binding domains of RHAMM, we next sought to determine the relative contribution of the two binding sites to HA binding activity by examining the ability of RHAMM fusion protein that was deficient in either domain I or domain II to bind biotinylated HA. Since we had shown above that K405 and K409 were critical for the HA binding ability of domain I, we mutated each of these residues to E. The mutated cDNA was used to generate a fusion protein which was analyzed for its ability to bind HA. Results shown in Figure 5 indicate that binding of HA to

A Site-directed mutations of peptide 423-432:

1 ⊢//— 720 T AAA CTG CGA TCT CAG CTT GTT AAA AGG AAA GAA TTC ATT CAT 3'
K L R S Q L V K R K *EcoRI*

I: GAC GCT AGA GTC GAA CAA GTA GTG GTA CTT AAG TAA GTA 5'
L R S Q L V H H H *EcoRI*

II: CAA TTT TCC TAT CTT AAG TAA GTA 5'
V K R I *EcoRI*

III: CAA TTT TCG TTT CTT AAG TAA GTA 5'
V K S K *EcoRI*

IV: A TTA GAC GCT AGA GTC GAA CAA TTT TCC TTT CTT AAG TAA GTA 5'
N L R S Q L V K R K *EcoRI*

V: A TTA GAC GCT AGA GTC GAA CAA TTT TCG TTT CTT AAG TAA GTA 5'
N L R S Q L V K S K *EcoRI*

VI: GAA CTA TTT TCC TTT CTT AAG TAA GTA 5'
L D K R K *EcoRI*

B Resultant amino acid sequences:

Peptide 423-432:	K L R S Q L V K R K
Mutation I:	K L R S Q L V H H H
Mutation II:	K L R S Q L V K R I
Mutation III:	K L R S Q L V K S K
Mutation IV:	N L R S Q L V K R K
Mutation V:	N L R S Q L V K S K
Mutation VI:	K L R S Q L D K R K

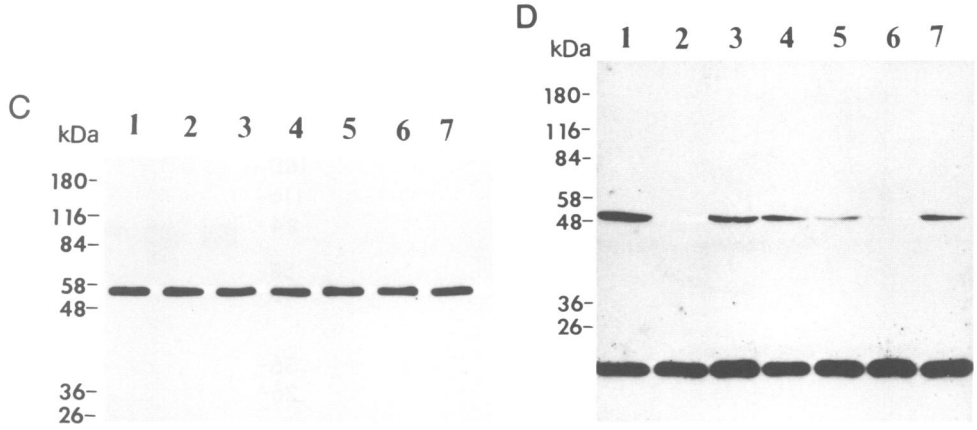

Fig. 2. Strategy for defining the critical basic amino acids that determine the HA binding properties of domain II. To investigate the importance of basic amino acids in domain II for HA binding, six independent mutations were carried out and a RHAMM fusion peptide was generated by fusing domain II with the amino-terminus (aa 1−238) of RHAMM using a recombinant technique. (**A**) The primers used to generate the altered cDNAs. (**B**) The resultant amino acid sequences. Highlighted amino acids indicate mutations. Six cDNAs, each containing site-directed mutation(s) were generated in the PCRs diagrammed in panels A and B, using RHAMM $cDNA^{1-720}$ as the template DNA and containing the oligonucleotide encoding aa 423−432 of RHAMM with different mutations. PCR products from the six primers containing the mutated nucleotides were doubly digested with *Bam*HI+*Eco*RI, ligated into pGEX-2T and transformed into HB101. Selected clones were confirmed to contain correct inserts by double digestion with *Bam*HI+*Eco*RI and electrophoresis on agarose gels. Fusion proteins were prepared from clones and analyzed in Western blots with either anti-RHAMM antibody to visualize RHAMM protein (**C**) or biotin-labelled HA (**D**) to assay HA binding activity. The results show that the HA binding ability of mutations I−VI (panel D) was reduced to 0, 67, 38, 21, 2 and 40%, of the control (lane 1), respectively. Lane 1, control; lane 2, mutation I; lane 3, mutation II; lane 4, mutation III; lane 5, mutation IV; lane 6, mutation V; lane 7, mutation VI.

RHAMM containing the mutated domain I dropped by 62% relative to the intact RHAMM fusion protein (compare lane 5 with lane 4). In domain II, K430, R431 and K432 were targeted for mutation. The mutated cDNA was expressed as a fusion protein and then analyzed for its HA binding ability with the transblot assay (Figure 6). The results indicate that the site-directed mutations of K430, R431 and K432 to N, W and E respectively, decreased the HA binding capability of RHAMM to 64% (Figure 6, lane 4) relative to the original RHAMM fusion protein (Figure 6, lane 3). These results indicate that the two domains contribute approximately equally to the HA binding activity of RHAMM.

Mutation of basic amino acids in intact RHAMM fusion protein abolishes HA binding

To disrupt the spacing (BX_7B) of the basic amino acids noted above to be required for the binding of domains I and

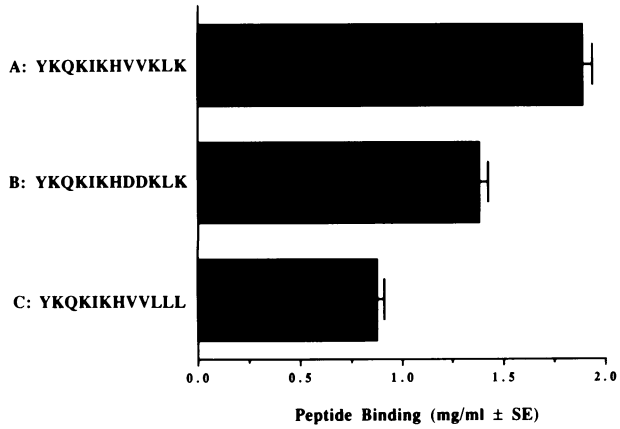

Fig. 3. Affinity of mutated peptides in domain I to HA−Sepharose. HA−Sepharose affinity gel was prepared according to the manufacturer's instructions. Peptides in PBS containing 0.15 M NaCl were applied to HA−Sepharose. Unbound peptides were removed by washing the gel with PBS containing 0.15 M NaCl. The binding peptides were eluted with PBS containing 1.5 M NaCl. The amounts of peptides applied, unbound peptides and binding peptides were determined by measuring their OD_{280}. The results indicated that HA binding domain I bound to HA−Sepharose with highest affinity. Alteration of basic amino acids within a $B(X_7)B$ motif of domain I reduced binding by ~50%. Insertion of two acidic amino acids also significantly reduced HA binding affinity. Alteration of basic amino acids caused the greatest reduction in HA binding affinity.

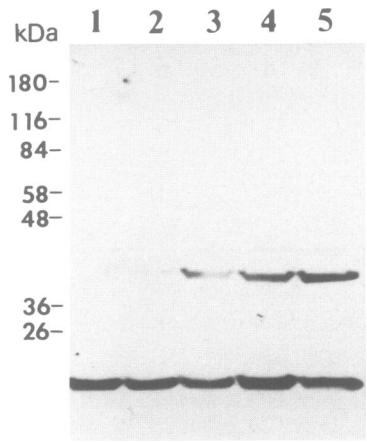

Fig. 4. Preparation of artificial peptides that bind to HA. Four peptides that mimicked aspects of the HA binding domains of RHAMM were prepared and linked to the non-HA binding fragment of RHAMM, aa 1−125, as described in Materials and methods. These included RGGGGGGGR (lane 2), RGGGRGGGR (lane 3), RGGGRGGRR (lane 4) and RGGGRGRRR (lane 5), and were generated as fusion proteins. The ability of these artificial domains to bind HA were tested in a transblot assay using biotinylated HA. Results showed a ranking of HA binding activity of RGGGRGRRR > RGGGRGGRR > RGGGRGGGR > RGGGGGGGGR. RGGGRGRRR binding activity was equivalent to domain II of RHAMM. Bacterial lysate was used as a control (lane 1).

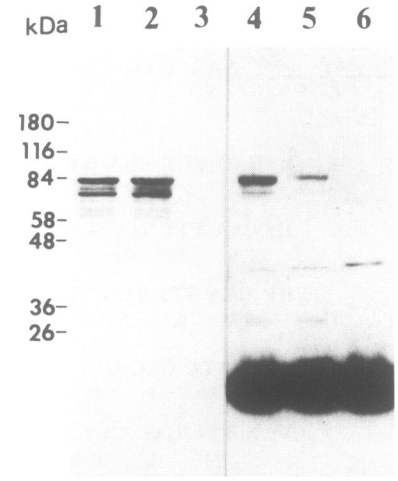

Fig. 5. Site-directed mutagenesis of HA binding domain I (aa 401−411) in RHAMM. In this experiment, K405 and K409 in binding domain I were each to be mutated to E while domain II was left intact, as described in Materials and methods. Fusion protein was prepared from both of the clones containing the mutations (lanes 2 and 5) and from the intact RHAMM cDNA (lanes 1 and 4) then electrophoresed on SDS−polyacrylamide gels. HB101 lysate without the RHAMM cDNA (lanes 3 and 6) was also prepared. The separated proteins were transblotted and electrophoresed onto nitrocellulose membranes and stained with antibody to RHAMM (lanes 1−3) or biotin-labelled HA (lane 4−6). The results showed that the HA binding ability of RHAMM mutated in domain I was reduced by ~62% (lane 4 versus lane 5).

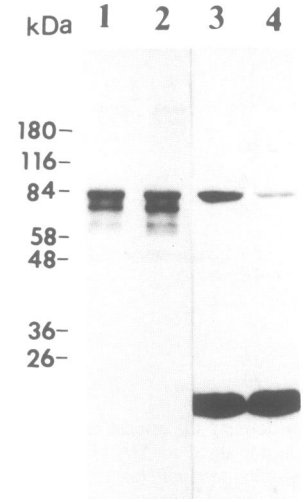

Fig. 6. Mutagenesis of domain II (aa 423−432) in RHAMM. In this experiment, K430, R431 and K432 were mutated to N, W and E, respectively, as described in Materials and methods. Cell lysates were prepared from the mutated clone (lanes 2 and 4). Intact RHAMM fusion protein (lanes 1 and 3) was used as a control. Proteins in cell lysates were separated by SDS−PAGE and Western blotting was conducted using an anti-RHAMM antibody to visualize RHAMM (lanes 1 and 2) and biotin-labelled HA to assay HA binding ability (lanes 3 and 4). The results show that a 36% decrease in the ability of the mutated RHAMM fusion protein to bind HA (lane 4).

II to HA, K405 and K409 of domain I were both altered to E, and K430, R431 and K432 of domain II were altered to N, W and E, respectively. This site-directed mutagenesis was accomplished in two steps using primers containing base pair changes that would lead to single amino acid mutations. The first step created a RHAMM cDNA that was mutated at positions 405 and 409 in domain I. In the second step, using the mutated RHAMM cDNA as a template in PCR, K430, R431 and K432 were altered to create a mutated domain II. This resulted in a cDNA that was mutated in both HA binding domains. Fusion protein was prepared and assayed for its HA binding ability using the transblot assay. As shown in Figure 7, the above mutations abolished the ability of RHAMM to bind to HA identifying these five basic

amino acids as part of the critical determinants that bestow HA binding capability to intact RHAMM.

A basic amino acid motif is found in other HA binding proteins

An examination of other molecularly characterized HA binding proteins reveals the occurrence of a $B(X_7)B$ motif within these proteins (Table I). Thus, for example, link protein contains two such motifs; CD44 contains three, hyaluronidase two, versican one and aggrecan two motifs. The amino acid sequences of these motifs are not otherwise homologous to one another. Consistent with the results presented in the section above entitled 'Creation of artificial peptides that bind HA', all of these domains contain clusters of basic amino acids at either end of the motif or in the intervening amino acid sequence.

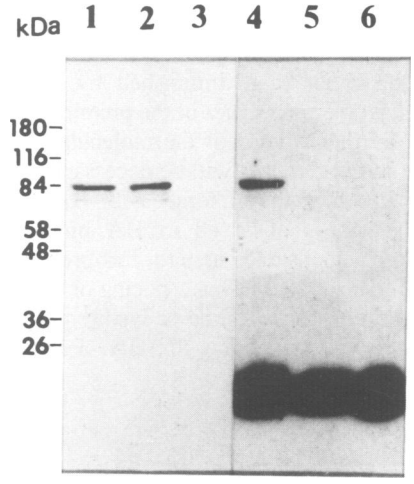

Fig. 7. Site-directed mutagenesis of K405, K409, K430, R431 and K432 (HA binding domains I and II). The K405 and K409 mutated cDNA in Figure 5 was used to generate the additional mutations shown in Figure 6, resulting in mutations occurring at K405, K409, K430, R431, K432 in RHAMM. The mutated cDNA was expressed as fusion protein for Western blot assays (lanes 2 and 5). Intact RHAMM fusion protein (lanes 1 and 4) and bacterial lysate (lanes 3 and 6) were used as controls. All proteins were stained with anti-GST antibody (lanes 1–3) or biotinylated HA (lanes 4–6). The results indicate the complete loss of HA binding ability in the mutated RHAMM (lane 5).

Sequences of CD44 and link protein that contain a $B(X_7)B$ motif bind HA

Peptides mimicking the amino acid sequence of link protein have previously been shown to bind to HA–Sepharose (Goetinck *et al.*, 1987). To assess the accuracy of our binding assay and to determine whether the $B(X_7)B$ motif contained in this previously identified sequence (Table I), also binds HA, we molecularly engineered the link protein sequence (aa 316–325, RYPISRPRKR) to the non-HA binding amino-terminal region of RHAMM (aa 1–125). As shown in Figure 8A and B, the link sequence predicted to bind HA by the presence of the $B(X_7)B$ motif bound HA, confirming the previous results of Goetinck *et al.* (1987).

As shown in Table I, we have identified three regions in CD44 that contain the motif identified in this report. The first, aa 38–46, is within the B loops previously thought to bind to HA (Murakami *et al.*, 1990); the second (aa 150–162) is outside this domain; the third (aa 292–300) is in the cytoplasmic region of CD44 (Table I). When each sequence was joined by molecular engineering to the amino-terminus of RHAMM, as above (also see Figure 9A), all resultant fusion proteins bound HA (Figure 9B and 9C). Furthermore, mutation of R46 in aa 38–46, K158 and R162 in aa 150–162, and K298, K299 and K300 in aa 292–300 of CD44, abolished binding of these sequences to HA (Figure 9B and C).

Discussion

Extracellular matrix molecules interact with their receptors via discrete and conserved motifs that can be either based upon primary sequence, as in the RGD sequence of many proteins that bind to integrins, or upon ionic interactions, as has been predicted for some interactions between heparin and vascular proteins (Sobel *et al.*, 1992). It was first suggested by Tengblad (1981) that the interactions between HA and some binding proteins were of an ionic nature, a prediction that was later confirmed by others (Kimata *et al.*, 1982; Lyon, 1986; Goetinck *et al.*, 1987; Yang *et al.*,

Table I. HA binding motifs $[B(X)_7B]$ of HA binding proteins

Proteins	Binding region	Amino acid sequence	Binding confirmed	Reference
RHAMM	401–411	^{401}KQKIKHVVKLK^{411}	+	Yang *et al.*, 1993
RHAMM	423–432	^{423}KLKSQLVKRK^{432}	+	Yang *et al.*, 1993
Link protein	316–325	^{316}RYPISRPRKR^{325}	+	This report; Goetinck *et al.*, 1986
CD44	38–46	^{38}KNGRYSISR^{46}	+	This report
CD44	150–162	^{150}RDGTRYVQKGEYR^{162}	+	This report
CD44	292–300	^{292}RRRCGQKKK^{300}	+	This report
HAse	96–104	^{96}RGTRSGSTR^{104}	+	Hope *et al.*, 1993
HAse	106–117	^{106}RRRKKIQGRSKR^{117}	+	Hope *et al.*, 1993
Link protein	103–112	^{103}RKSYGKYQGR^{112}		Goetinck *et al.*, 1986
Versican	25–33	^{25}KVGKSPPVR^{33}		Zimmermann and Ruoslahti, 1989
Versican	2319–2327	^{2319}KTFGKMKPR^{2327}		Zimmermann and Ruoslahti, 1989
Aggrecan	71–79	^{71}RIKWSRVSK^{79}		Doege *et al.*, 1987
Aggrecan	2109–2117	^{2109}KRTMRPTRR^{2117}		Doege *et al.*, 1987
Human GHAP	S4	^{34}KVGKSPPVR^{42}		Perides *et al.*, 1989
TSG-6	39–48	^{39}HREARSGKYK^{48}		Lee *et al.*, 1992

1993). We confirm their results by characterizing an HA binding motif that is common to all HA binding proteins described to date, is based upon a motif of basic amino acids, and is predicted to be a universal motif defining protein–HA interactions.

The simplest explanation for results presented in this report is that HA can optimally bind to an amino acid sequence designated by the precisely spaced motif $B(X_7)B$, where B is either R or K and X is any amino acid except acidic residues. Although the flanking basic amino acids are required for binding as demonstrated by site-directed mutagenesis, they are apparently not sufficient to bind HA with high affinity. Rather, our peptide analysis suggests that clustering of basic amino acids at the ends of motifs or the occurrence of basic amino acids within the motif result in an HA binding motif that interacts with HA to the same degree as native domain I or II. These results further indicate that the conformation of the peptides, which are helical in nature (Hardwick *et al.*, 1992), is not a major factor in binding. Several observations in this report support the suggestion that HA binds to proteins via a definable motif. It is unlikely that simple basic charge densitry is crucial for HA binding, since HA does not bind well to polylysine (Goetinck *et al.*, 1987). We have shown that (i) the motif occurs in all HA binding proteins particularly within regions previously shown to bind HA, and (ii) site-directed mutagenesis and peptide mimicry of either one or both basic amino acids spaced seven amino acids apart abolishes the ability of proteins such as RHAMM and CD44 to bind HA. Since mutation of K431 reduces binding less than is predicted by the motif, the flanking K430 or K432 may contribute to binding in the absence of K431. This is consistent with the peptide analysis that indicated clustering of flanking amino acids enhances binding to HA and the observation that an artificial sequence composed of $RG_3RG_2R_2$ binds to HA to the same extent as the HA binding domain II (aa 423–432) of RHAMM. In domain II, K423 appears to be slightly more important than R431, a property not predicted by a simple $B(X_7)B$ motif. Again, this is probably related to the ability of K430 and K432 to compensate for loss of binding to R431. The motif described here occurs in the two regions of CD44 recently shown to contain HA binding activity (Peach *et al.*, 1993) and mutation of K41 of CD44, which removes the basic amino acid within the $B(X_7)B$ motif predicted by our studies to be necessary for HA binding, impairs binding to HA. It is therefore interesting that HA–CD44 interactions are enhanced rather than diminished by ionic strength, suggesting that other properties of the protein also contribute to binding. Further studies of the molecular alignment of amino acids in these motifs will be necessary to investigate the manner in which they interact with HA.

All of the motifs observed in HA binding proteins, including those demonstrated here for link protein and CD44, are nine amino acids long. Thus, spacing of the amino acids internal to the flanking arginine or lysine residues appears to be critical for its binding to HA. Peptide mimicry

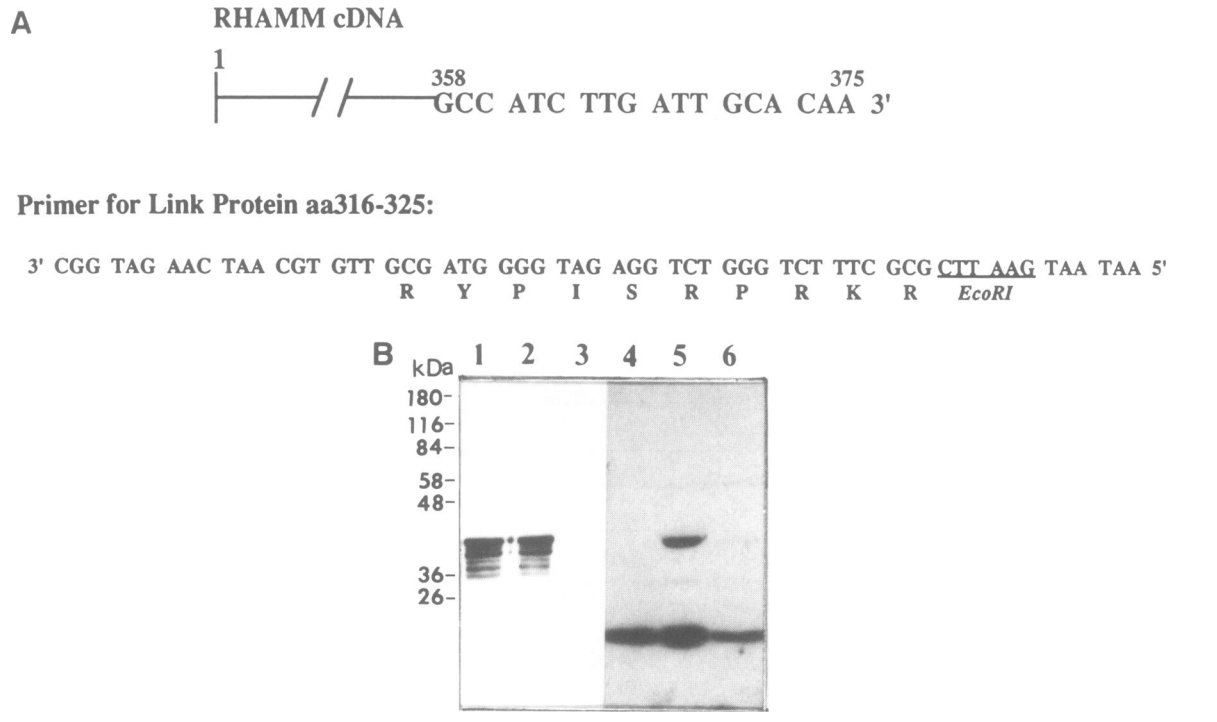

Fig. 8. Preparation of chimeric link peptide aa 316–325–RHAMM peptide. (**A**) An oligonucleotide encoding aa 316–325 of link protein was aligned by PCR to a cDNA encoding an amino-terminal polypeptide of RHAMM (aa 1–238) that does not bind HA. This was carried out using a primer that was complementary to nt 358–375 of RHAMM and nt 946–975 of link protein that created an *Eco*RI site and that was in the same reading frame. A recombinant cDNA encoding a chimeric RHAMM–link peptide was generated with this primer and another primer mimicking nt 1–22 of RHAMM creating a *Bam*HI site linked to nucleotide 1. The PCR product was then doubly digested with *Bam*HI+*Eco*RI, ligated into pGEX-2T, transformed into HB101 and confirmed to be present in the selected clones by restriction endonuclease digestion. (**B**) The fusion protein from the above chimera was prepared from the original RHAMM aa 1–238 only (lanes 1 and 4) and the clone containing RHAMM/link aa 316–325 (lanes 2 and 5). HB101 lysate without a RHAMM cDNA insert was used as a control (lanes 3 and 6). The fusion proteins were analyzed with Western blot assays using anti-RHAMM antibody to visualize RHAMM (lanes 1–3) and biotin-labelled HA to assay for HA binding activity (lanes 4–6). The result show that chimeric link peptide–RHAMM polypeptide bound to HA (lane 5).

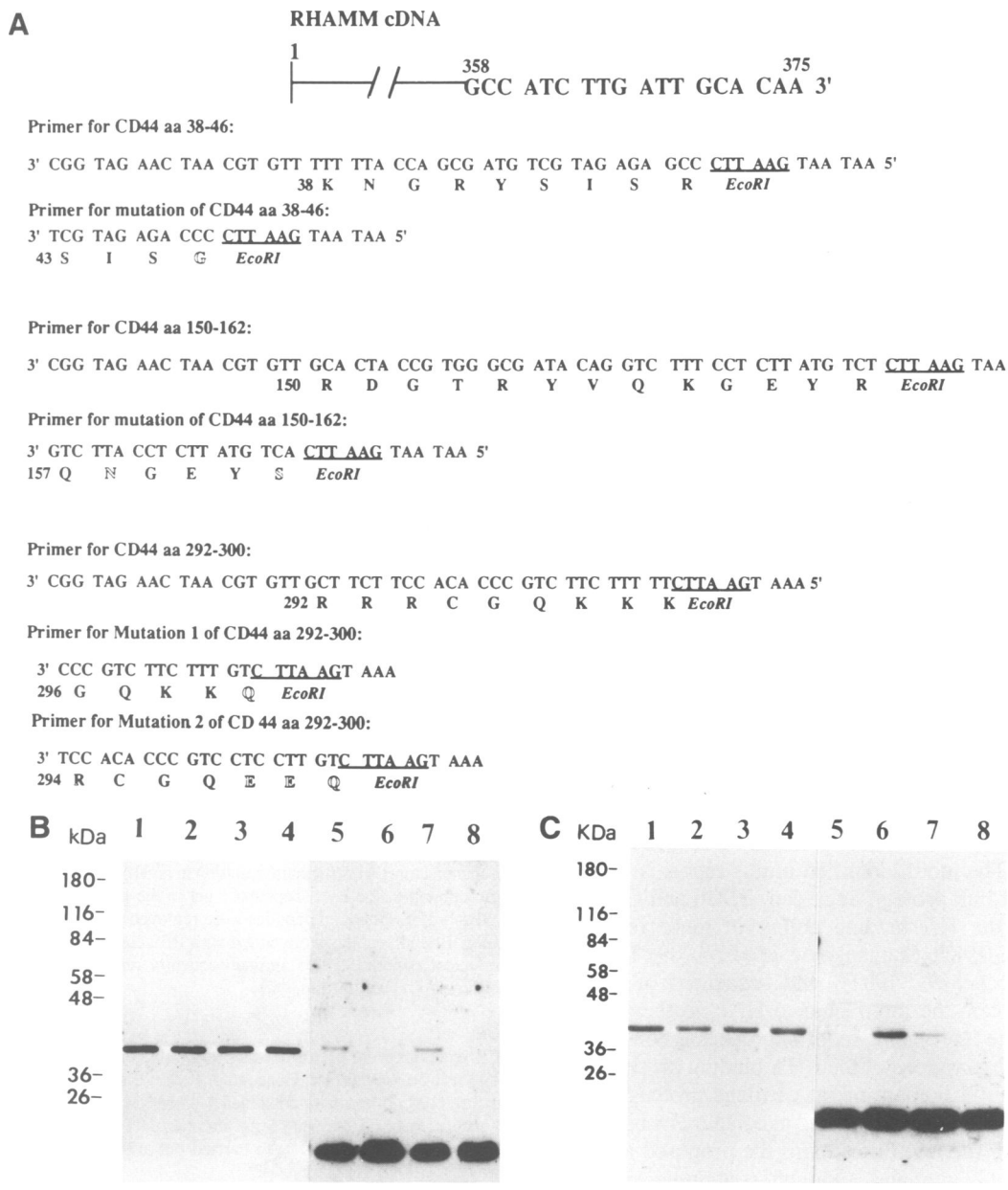

Fig. 9. Identification of HA binding domains in CD44. **(A)** The strategy for designing chimeric peptides of RHAMM and CD44 is outlined. The diagram shows that three domains of CD44, aa 38–46, 150–162 and 292–300, were each aligned by PCR to the 5′ region of RHAMM cDNA (nt 1–375) which encodes a non-HA binding polypeptide. Each of these domains was then mutated as diagrammed in the strategy. **(B)** Lanes 1–4 represent fusion protein visualized with anti-RHAMM antibody and lanes 5–8 represent fusion protein reacted with biotinylated HA. Fusion protein containing aa 38–46 of CD44 bound to biotin-labelled HA weakly (lane 5). Mutation of R46 to G abolished HA binding activity (lane 6). Fusion protein containing aa 150–162 of CD44 also bound to biotin-labelled HA weakly (lane 7). The mutation of K158 and R162 to N and S respectively abolished HA binding activity (lane 8). **(C)** Fusion proteins were prepared from selected clones containing the appropriate and mutated inserts of aa 292–300. Western blot assays were carried out using cell lysates. Expression of fusion proteins was identified by anti-RHAMM antibody (lanes 1–4) and their ability to bind to HA was assayed using biotinylated HA (lanes 5–8). Lanes 1 and 5 represent fusion protein obtained from RHAMM cDNA nt 1–375; lanes 2 and 6 represent fusion protein containing the CD44 domain (aa 292–300); lanes 3 and 7 represent mutation I of aa 292–300, lanes 4 and 8 represent mutation II of aa 292–300. Results show that the mutation of the basic amino acid K300 severely reduced HA binding activity while mutations of K298, K299 and K300 to E, E and Q respectively abolished HA binding activity of the chimeric CD44–RHAMM polypeptide.

experiments suggest that internal residues cannot vary by more than one amino acid without a detrimental effect to binding (Figure 2A and data not shown). Recombinant experiments also indicate that the presence of an acidic acid residue within the seven amino acid stretch reduces binding. RHAMM contains several $B(X_7)B$ motifs in addition to the two HA binding domains characterized here, but the others all contain varying numbers of acidic amino acid residues within the motifs and do not bind to HA as determined by the deletion experiments described here (Figure 1). Acidic amino acid residues are likewise generally not present in the HA binding motifs of other proteins. Interestingly, substitution of the critical arginine or lysine residues with a histidine abolishes HA binding, suggesting that mere positive charge is not sufficient to establish HA–protein interactions. It is noteworthy that none of the motifs found

in other HA binding proteins contains a histidine residue as the flanking basic amino acids (Table I).

The molecular constraints in HA–protein interactions that require the above characteristics are not known. However, HA is able to form an extended polymer that can exist as an α helix of defined dimensions (Laurent and Fraser, 1992). It is possible that the above configurations of the motif are required to fit these dimensions. The spacing of the carboxyl group of the uronic acid of HA is probably critical for an ionic interaction to occur. Whatever model is derived will have to take into account that the minimum binding unit of HA for CD44 and RHAMM is a hexasaccharide, while that of such extracellular HA binding proteins as link and aggrecan is a decasaccharide. Clearly, NMR studies are required to clarify further the manner in which HA–B(X_7)B interactions occur.

Proteolytic mapping of link protein has been used to localize its HA binding domain to the tandem repeat loops (B regions) (Goetinck *et al.*, 1987). This region is homologous in most HA binding proteins and this homology has been used to predict accurately the HA binding capability of several recently identified proteins (for reviews, see Turley, 1992; Toole, 1990). However, the properties of these domains that are required for interaction with HA have remained elusive. Some proteins that bind HA with high affinity such as hyaluronidase (Hope *et al.*, 1993) and RHAMM do not contain regions that are homologous to B regions of link protein, and link sequences within the B loops that bind to HA are not homologous at the primary amino acid level with other HA binding proteins (Goetinck *et al.*, 1987; Table I). The motif identified in this report is found in the B loops of link protein, aggrecan, TSG6 and CD44, consistent with the HA binding ability of these regions (Goetinck *et al.*, 1987; Stamenkovic *et al.*, 1989; Doege *et al.*, 1991; Lee *et al.*, 1991), and sequences of these proteins that contain the motif bind to HA. Furthermore, the presence of the HA binding motif in proteins is consistent with published observations of their HA binding properties. For instance, the GI domain of rat cartilage proteoglycan core protein, previously demonstrated to contain HA binding activity (Doege *et al.*, 1987), contains the proposed motif. In contrast, the G2 domain, although containing similar amounts of basic amino acids (9% in G1 and 8% in G2), does not bind HA (Doege *et al.*, 1987; Fosang and Hardingham, 1989) and likewise does not contain the defined motif. The motif occurs between aa 2319 and 2327 in versican (Zimmerman and Ruosolahti, 1989), and within the B region at the amino-terminus of CD44, both of which have previously been shown to bind HA. Our data also predict that two additional HA binding sites exist for HA in CD44, one outside of the B loop (aa 150–162) and one in the cytoplasmic domain (aa 292–300) (for sequence, see Stamenkovic *et al.*, 1989). A recent report confirms that aa 150–162 of CD44 indeed bind to HA (Peach *et al.*, 1993). Mutational analyses indicate that all of these sites require B(X_7)B spacing of basic amino acids.

The definition here of an HA binding motif does not explain all of the known binding characteristics of HA binding proteins. Thus, link protein, aggrecan and CD44 bind HA poorly when they are chemically reduced (Toole, 1990), suggesting that a three-dimensional structure influences their binding to HA. This is not the case for RHAMM where reduction of this protein does not impair its HA binding ability (Hoare *et al.*, 1993). Further, not all isoforms of CD44 bind HA although all of them contain the B loop and the binding motif described here. Recent studies suggest that modification of the cytoplasmic region has profound effects on the HA binding capability of this protein in an as yet undefined manner (Lesley *et al.*, 1992).

In summary, in this report we identify and characterize a HA binding motif that is found in all HA binding proteins that have been molecularly characterized to date. This motif is defined as B(X_7)B where B is either of the basic amino acids arginine or lysine and X is any non-acidic amino acid. We further show that this motif is present in sequences of RHAMM, link protein and CD44, and is responsible for the binding of these sequences to HA.

Materials and methods

Materials

Biotin-labelled HA was prepared by dissolving HA (1.5 μg/μl) in 0.1 M sodium borate, pH 8.8, incubating it with *N*-hydroxysuccinimide biotin (50:1, HA:Biotin) prepared in DMSO (7.5 μg/μl) at room temperature for 4 h and reacting the resulting products with 15 μl of 1 M ammonium chloride for 10 min. The reacted solution was dialyzed against PBS, pH 7.2, and then PBS containing 20% glycerol. The biotin-labelled HA was stored at 4°C.

Peptides were synthesized at the Manitoba Institute of Cell Biology. The purity of synthetic peptides was determined on HPLC by measuring optical densities (OD) at 280 and 214 nm. Oligonucleotides were synthesized in the Department of Physiology, University of Manitoba. Oligonucleotides longer than 30 bases were purified on HPLC.

Hyaluronan–Sepharose affinity chromatography

HA (Sigma) was coupled to HA–Sepharose 4B (Pharmacia) according to the manufacturer's instructions, resulting in an HA–Sepharose gel. Peptides were applied to the HA–Sepharose gel in the presence of 0.15 M NaCl in PBS buffer. Unbound peptides were removed by washing with the same buffer. Bound peptides were eluted with PBS containing 1.5 N NaCl. The amount of peptide applied and subsequently removed was quantified by measuring OD at 280 nm.

PCR

PCR reactions were performed as previously described (Sambrook *et al.*, 1989) using a standard kit (Gene Amp, Perkin Elmer–Cetus). The reaction mixture (100 μl) normally contained 1.25 nM $MgCl_2$, 75 μM dNTP, 1 μg of each primer (in excess), 5 ng template DNA and 1 U of Taq DNA polymerase. All reactions were carried out at 94°C (30 s), 54°C (30 s) and 72°C (90 s) for 25 cycles.

DNA purification and ligation

DNA products from the PCR mixtures were purified using a Prep-A-Gene DNA Purification kit (Bio-Rad). The volume of the reaction mixture was brought to 1 ml by adding 25 μl Prep-A-Gene matrix and 875 μl binding buffer (50 mM Tris, 1 mM EDTA, 6 M NaCl, pH 7.5) according to the manufacturer's manual. After washing twice in binding buffer and three times in wash buffer (20 mM Tris, 4 mM EDTA, 0.4 M NaCl, pH 7.4 containing 50% ethanol, v/v), the DNA was eluted in 100 μl H_2O. The DNA was digested with two appropriate restriction endonucleases, purified as described above and eluted in 50 μl H_2O. The DNA was ligated to plasmids as described by Sambrook *et al.* (1989) using DNA ligase (Boehringer Mannheim).

Transformation, DNA preparation and digestion with restriction endonucleases

Competent bacteria were prepared as described by Sambrook *et al.* (1989) with the following modifications. Bacteria (*Escherichia coli* HB101 or XL1-Blue), stored in glycerol, were grown in 1 ml LB/amp (Luria Bertani medium with 100 μg/ml ampicillin) medium at 37°C overnight. The resulting culture was brought to 10 ml with LB/amp medium and Mg_2SO_4 was added to a concentration of 10 mM. The culture was incubated at 37°C for 2 h with constant agitation. The cells were chilled on ice for 20 min, pelleted at 1000 *g* for 5 min at 4°C, resuspended in 3 ml 50 mM $CaCl_2$, chilled on ice for 20 min, pelleted at 1000 *g* for 5 min at 4°C, resuspended in 500 μl 50 mM $CaCl_2$ and put on ice for transformation. Transformation

was carried out exactly as previously described (Sambrook *et al.*, 1989). The transformed mixtures were spread on three LB/amp agar plates for HB101 or three LB/amp agar plates containing isopropyl-β-D-thiogalactoside (IPTG, 0.2 mM) and X-gal (2 mg/plate) for XL1-Blue. The plates were incubated at 37°C overnight. Twelve white colonies of XL1-Blue or 12 randomly picked colonies of HB101 were streaked on LB/amp agar plates, incubated at 37°C overnight, inoculated into 1 ml LB/amp medium in microfuge tubes then incubated at 37°C overnight with shaking at 280 r.p.m.

DNA was prepared from the above 1 ml culture as described by Sambrook *et al.* (1989) with the following modifications. The cells were sedimented and resuspended in 100 μl of Solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris−Cl, pH 8.0) and with 200 μl 0.2 M NaOH containing 1% SDS (Sambrook *et al.*, 1989). Cells from each tube were lysed with 100 μg lysozyme dissolved in 10 μl Solution I at room temperature for 10 min. 150 μl of Solution III [3 M potassium acetate containing 11.5% glacial acetic acid (Sambrook *et al.*, 1989)] were added to each lysate and put on ice for 15 min. Genomic DNA and bacterial debris were sedimented at 10 000 *g* for 15 min. Plasmid DNA in the supernatant was precipitated with 0.6 vols of isopropyl alcohol at room temperature for 10 min and then centrifuged at 10 000 *g* for 15 min. The DNA was resuspended in 30 μl TE buffer (10 mM Tris, 1 mM EDTA; pH 8.0) and purified with a Prep-A-Gene kit (Bio-Rad, Richmond, CA) using 5 μl Prep-A-Gene matrix washed twice with binding buffer and twice with wash buffer (see 'DNA purification and ligation' section above). The purified DNA was eluted in 50 μl H_2O.

DNA samples were doubly digested with two restriction endonucleases (i.e. *Bam*HI+*Sac*I, *Bam*HI+*Eco*RI, *Bam*HI+*Hin*dIII or *Hin*dIII+*Eco*RI) and electrophoresed on an agarose gel to select those clones containing the correct inserts. For experiments using Bluescript, the correct insert was recovered for further use by the Prep-A-Gene DNA purification kit. For experiments using pGEX-2T (Eaton *et al.*, 1986; Smith and Johnson, 1988), the clones containing the correct insert were used for the preparation of fusion proteins as described below.

Fusion protein preparation

Colonies containing the correct insert in pGEX-2T were grown in 200 μl LB/amp medium at 37°C overnight. 800 μl LB/amp medium and IPTG were then added to the cultures to a final concentration of 0.1 mM IPTG for 2 h at 37°C to induce the biosynthesis of fusion proteins (Towbin *et al.*, 1979). Cells were harvested by centrifugation at 5000 *g* for 5 min, resuspended in 200 μl 50 mM Tris−Cl, pH 8.0, containing 2 M urea, 1% Triton X-100 and 1 × protease inhibitors (Hardwick *et al.*, 1992) and disrupted by sonication. Bacterial debris was pelleted by centrifuging at 10 000 *g* for 20 min and the supernatants containing the extracted fusion protein were recovered for Western blot assays.

Western blot and transblot assays

These assays were performed as previously described with the following modifications (Towbin *et al.*, 1979; Timmons and Dunbar, 1990). Proteins were separated on 10% SDS−PAGE and transblotted onto a nitrocellulose membrane in a Tris buffer containing 25 mM Tris, 192 mM glycine and 20% methanol at pH 8.3. Additional protein binding sites on the membrane were blocked in 10 mM Tris−Cl, pH 8.0, containing 150 mM NaCl, 0.05% Tween 20 and 5% skim milk powder (TBSTS) for 1 h. The blocked membrane was incubated with primary antibody diluted in TBSTS for 1 h at room temperature or overnight at 4°C. The membrane was washed with TBST (TBSTS without milk powder) extensively and then incubated with an appropriate second antibody diluted in TBST. Antibody binding was visualized with a chemiluminescence method (Isacsson and Watermark, 1974; Roswell and White, 1978) (ECL kit, Amersham, used according to the manufacturer's instructions) that uses luminal and hydrogen peroxide to visualize bound antibody.

Strategy for the deletion of HA binding domains in RHAMM

RHAMM cDNA (nt 1−1230) was amplified with PCR using two oligonucleotides as primers to create a protein in which aa 411−434 were deleted (see Figure 1A). One primer was complementary to the translation initiation region (nt 1−22) and was designed to create a *Bam*HI site at nt 1. The other primer was complementary to the 3′ end of the cDNA (nt 1206−1230) and was designed to create a *Sac*I site following nt 1230 (Figure 1A). The resulting PCR product encoded RHAMM in which the HA binding domain II (aa 423−432) was completely deleted and the HA binding domain I (aa 401−411) was partially deleted (Figure 1A). The two basic amino acids (K405 and K409) of domain I, shown to contribute to HA binding, were both mutated to E (Figure 1A). The resulting PCR product (Figure 1B, lane 2) of this mutated and deleted RHAMM cDNA was doubly digested with *Bam*HI and *Sac*I. The plasmid-containing fragment (5.3 kb, Figure 1B, lane 3) was recovered by using the Prep-A-Gene kit as above. The *Sac*I fragment was ligated to the *Bam*HI−*Sac*I fragment of the PCR product and transformed into *E.coli* HB101. DNA samples prepared from randomly picked clones were confirmed to contain correct inserts by restriction with *Bam*HI+*Sac*I (Figure 1B, lane 4). Those clones containing a correct *Bam*HI−*Sac*I insert were then used to make fusion proteins. Undeleted RHAMM fusion protein and HB101 lysate were used as positive and negative controls, respectively, in these experiments.

Genetic alteration of HA binding domain II (aa 423−432)

To assess the role of both basic and acidic amino acids on the binding of domain II to HA, six oligonucleotides containing mutated nucleotides (Figure 2A) were prepared and then linked by recombinant techniques to the amino-terminus (nt 1−720) of RHAMM, which does not bind HA. These amino-terminal fragments of RHAMM containing a mutated HA binding domain II were prepared using each of six primers. They were: (i) a primer complementary to the translation initiation region of RHAMM (nt 1−22) that created a *Bam*HI site linked to nt 1, and (ii) one of six primers containing altered nucleotide(s) linked to an *Eco*RI site (listed in Figure 2A). These primers were designed to encode an altered amino acid as shown in the strategy outlined in Figure 2B. The PCR products from each pair of the primers were doubly digested with *Bam*HI+*Eco*RI and ligated to *Bam*HI- and *Eco*RI-opened pGEX-2T. The ligation mixtures were transformed into *E.coli* HB101. DNA samples were prepared from transformed colonies, doubly digested with *Bam*HI+*Eco*RI and electrophoresed on agarose gel to confirm that correct inserts were present in the plasmids. Mutated protein was prepared from bacterial lysates obtained from those colonies containing the correct inserts.

Site-directed mutagenesis of domains I and II in the RHAMM cDNA

Mutation of specific basic amino acids in domains I and II of RHAMM was accomplished in two steps, generating RHAMM mutated in both HA binding domains. In the first step, site-directed mutations were confined to basic amino acids K405 and K409 which were both altered to E. A *Hin*dIII ligation site was created between the HA binding domains. Four primers were used in PCRs to generate this altered RHAMM cDNA as two segments which were then ligated at the *Hin*dIII site. These included 5′-GTGGATCCATGCAGATCCTGACAGAGAGGC and 3′-TAGCTTGTACAACACCTTAACTTCGAAAAT for the first segment containing mutated domain I and 5′-TAAAAGCTTGAAAATAGCCAACTCAAA and 3′-TGACGACAAGTAGTGGTTTCCTTAAGTAA for the second segment. Both segments were ligated into Bluescript with appropriate restriction enzymes and ligation mixtures were transformed into *E.coli* XL1-Blue. DNA was prepared from white colonies and doubly digested with *Bam*HI+*Hin*dIII or *Hin*dIII+*Eco*RI to select the clones containing the appropriate inserts. The inserts were recovered with a Prep-A-Gene kit, doubly ligated into *Bam*HI- and *Eco*RI-opened Bluescript and a proper insert containing the mutated cDNA was selected and amplified. The insert was then expressed in pGEX-2T as above. Fusion protein was prepared and analyzed for its HA binding ability in a transblot assay.

Using the RHAMM cDNA that was mutated in the first HA binding domain as described above, the second HA binding domain was altered so that the basic amino acids predicted to be critical for binding were mutated. K430, R431 and K432 were changed to N, W and E respectively, using a primer (3′-CAATTAACCCTTGTTTTACTCGAGTCTGAA) complementary to HA binding domain II together with a primer complementary to the amino-terminal translation initiation region (nt 1−22) to create a modified RHAMM cDNA using PCR. The mutated RHAMM cDNA was doubly digested with *Bam*HI+*Sac*I, inserted into *Bam*HI- and *Sac*I-opened pGEX-2T and transformed into *E.coli* HB101. DNA samples prepared from randomly picked clones were confirmed to contain correct inserts by restriction digestion with *Bam*HI+*Sac*I. Those colonies containing a correct *Bam*HI−*Sac*I insert were used to make fusion proteins as described above. Unmutated RHAMM fusion protein and HB101 lysate were used as controls.

Generation of artificial HA binding domains and chimeric link protein and CD44 peptides

To determine the role of basic amino acids in HA binding, we designed four artificial peptides and tested their ability to bind to HA using the transblot method (Hoare *et al.*, 1993). This was carried out by linking the artificial domains to the amino-terminus of RHAMM (aa 1−125) using the PCR methods described before (Yang *et al.*, 1993). Briefly, the primer complementary to the translation initiation region of the RHAMM cDNA (Hardwick *et al.*, 1993) was used in combination with four primers. These were 3′-CGGTAGAACTAACGTGTTGCTCCTCCACCACCCCCGCCTCCATCCTTAAGTAAA, generating an artificial domain, RGGGGGGGR; 3′-CGGTAGAACTAACGTGTTGCTCCTCCACCATCCCCGCCTCCATCCTTAAGTAAA, generating an artificial domain, RGGGRGGGR; 3′-CGGTAGAACTAACGTGTTGCTCCTCCACCATCCCCGCCTGCA-

TCCTTAAGTAAA, generating an artificial domain, RGGGRGGRR; and 3'-CGGTAGAACTAACGTGTTGCTCCTCCACCATCCCCGTCTGCA-TCCTTAAGTAAA, generating a domain RGGGRGRRR. Fusion proteins containing these four domains were prepared as above and then assayed for their ability to bind HA in the transblot assay. In the same way, link protein aa 316–325 were combined with RHAMM aa 1–125 (see Figure 8A). CD44 aa 38–46, aa 150–162 and aa 292–300 were also linked to RHAMM aa 1–125 (see Figure 9A). Mutations (R46 to G in aa 38–46, K158 and R162 to N and S respectively in aa 150–162, K300 to Q in aa 292–300, and K298, K299 and K300 to E, E and Q respectively in aa 292–300) were generated as described in 'Genetic alteration of HA binding domain II (aa 423–432)' described above and in Figure 9A.

DNA sequencing

The mutated DNAs generated in the above sections were sequenced using a CircumVent Thermal Cycle Sequencing Kit from Biolabs according to the manufacturer's instructions to confirm the occurrence of the correct mutations.

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