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Identification of a Novel Heparin Binding Domain in RHAMM and Evidence That It Modifies HA Mediated Locomotion of *ras*-Transformed Cells

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Abstract We have previously reported that the hyaluronan (HA) receptor RHAMM (Receptor for HA Mediated Motility) [Turley et al., 1991] contains two HA binding motifs located within a 35 amino acid region of its C-terminus end [Yang et al., 1993] and that HA stimulation of the motility of ras-transformed fibroblasts is mediated via its interaction with RHAMM. Here we show that RHAMM also contains binding sites for heparin (HP) and that interaction of HP with these sites can regulate the locomotion of *ras*-transformed fibroblasts. At low concentrations (0.01 mg/ml), HP inhibited HA-induced locomotion of *ras*-transformed cells in a manner independent of RHAMM. At higher, but still physiological concentrations (0.1 mg/ml), HP alone stimulated cell locomotion and this stimulation appeared to be RHAMM-dependent as it was blocked by anti-RHAMM antibodies. Other related glycosaminoglycans such as chondroitin sulfate and dermatin sulfate had no effect on cell motility. In ligand blotting assays, GST-RHAMM fusion protein was shown to bind biotin-labelled HP and this binding was displaceable with unlabelled HP. In similar ligand binding analyses conducted with truncations of RHAMM fusion protein, the HP binding region was found to be localized in the same 35 amino acid segment of RHAMM that contains the two HA binding domains. Synthetic peptides corresponding to these HA binding domains were retained on and bound effectively to an HP-Sepharose affinity column. Fusion proteins generated by linkage of these peptides to the non-HP binding amino terminus of RHAMM conferred HP binding capacity to the genetically engineered proteins. Conversely, deletion of the HA binding domains of RHAMM resulted in fusion proteins devoid of HP binding activity. The relative affinities of RHAMM for HA and HP, as determined by competition and transblot assays as well as quantification of binding at various salt concentrations, indicated that RHAMM had lower affinity for HP than that for HA. These results demonstrate the existence of a new HP binding motif that has biological relevance to cell locomotion.

Key words: hyaluronan, heparin, RHAMM, locomotion, glycosaminoglycan binding domains

Glycosaminoglycans (GAGs) are negatively charged polysaccharides that play a role in diverse biological processes including growth control, cyto-differentiation, and cell locomotion [Laurent and Fraser, 1986; Brecht et al., 1986; Fraser and Laurent, 1989; Turley, 1989; Turley et al., 1990, 1991]. The two GAGS, hyaluronan (HA) and heparin (HP) and the homologous heparin sulfate proteoglycan form of HP found in tissues are particularly important regulators of cell locomotion [Turley et al., 1985, 1991; Majack and Clowes, 1984; Azizkhan et al., 1980].

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It has been noted that HA accumulation in tissues is correlated with the migration of, for example, neural crest cells [Derby and Pintar, 1975], heart cushion cells [Markwald et al., 1978], chondrocytes [Toole et al., 1989], mammary carcinoma cells [Schor et al., 1989], *ras*-transformed fibroblasts [Turley et al., 1991], smooth muscle cells [Boudreau and Rabinovitch, 1991], and endothelial cells [Kumar and West, 1990; Bannerjee and Toole, 1993]. Promotion of cell motility by HA appears to be mediated via its interaction with specific cell surface receptors, two of which, namely, CD44 and RHAMM (Receptor for HA-Mediated Motility), have been cloned and sequenced [Haynes et al., 1991; Hardwick et al., 1992]. Detailed studies of RHAMM indicate that it is critically involved in the locomotion capability of *ras*-transformed fi-

broblasts [Turley et al., 1991; Hardwick et al., 1992] and smooth muscle cells [Boudreau et al., 1991; Savani et al., in revision] as well as the increased motility of fibroblasts following stimulation by TGF-β_1 [Samuel et al., in press]. Although HP is found free in sera [Horner, 1975] and is most noted for its anticoagulant activity, it has been shown to stimulate the migration of bovine capillary endothelial cells [Azizkhan et al., 1980] and to inhibit that of vascular smooth muscle cells [Majack and Clowes, 1984]. When associated with acidic fibroblast growth factor, however, HP was reported to accelerate dermal wound closure, a process that involves cell migration [Thomas et al., 1987; Klein-Soyer et al., 1989]. While HP is known to interact with several proteins, the identity of those responsible for its effects on locomotion has not been characterized.

HA is composed of alternating non-sulfated N-acetyl glucosamine and glucuronic acid units [Laurent and Fraser, 1986], while HP is composed of alternating N-acetyl glucosamine and iduronic acid/glucuronic acid residues that are modified by various degrees of sulfation [Lindahl, 1990].

We observed that the HA binding domains described in our studies of the receptor RHAMM resembled HP binding motifs reported in other proteins [Ehrlich et al., 1992; Kost et al., 1992; Pratt and Church, 1992; Pratt et al., 1992; Sobel et al., 1992]. Thus, both HA and HP interact with motifs containing clusters of basic amino acids. We demonstrate here that HP binds with relatively high affinity to the HA binding domains of RHAMM, although not as strongly as does HA itself. This HP/RHAMM interaction appears to be relevant since concentrations of HP that promote locomotion, although 10-fold higher than motility-induced responses to HA, are still within its physiological ranges [Horner, 1975]. These results suggest that HP/RHAMM interactions may regulate cell locomotion under conditions such as wound repair or vascular remodelling where localized HP concentrations are high [Hyslop and de Nucci, 1993]. We further noted that HP at low concentrations inhibits fibroblast locomotion via a RHAMM independent pathway consistent with effects observed in other cells such as smooth muscle cells [Majak and Clowes, 1984]. Thus, our data suggest that HP regulates HA-mediated locomotion in a concentration-dependent manner.

EXPERIMENTAL PROCEDURES

Cell Culture

The C3 10T½ cells generated by transfection of H-*ras* and *neo*^{R} genes has previously been described [Egan et al., 1987]. C3 fibroblasts maintained at 37°C and in 5% CO_2 on plastic tissue culture dishes (Corning Glassworks, Corning, NY) were grown in alpha-modified Eagles media (α-MEM) supplemented with 10% fetal calf serum (FCS) (Hyclone, Gibco, BRL, Burlington, ON) and 10 mM HEPES (Sigma Chemical Co., St. Louis, MO), pH 7.2. When the cultures reached 80% confluency, the cells were subcultured by treatment with 0.25% trypsin/2 mM EDTA. For motility experiments, 10^5 cells were plated into 25 cm^2 tissue culture flasks (Corning Glassworks, Corning, NY) and maintained for 12 h as above. The media was then aspirated, the cells were rinsed with Hanks Balanced Salt Solution (HBSS) (Gibco, BLL, Burlington, ON), fresh, serum-free α-MEM containing 4.0 μg/ml transferrin (Gibco BLL, Burlington, ON) and 2.0 μg/ml insulin (Sigma Chemical Co., St. Louis, MO) (defined medium) was added, and the cells were maintained in this media for another 12 h before being used for locomotion assays.

Cell Locomotion in Response to HP and Neutralizing Antibodies

Cell locomotion was recorded using a computerized timelapse image analysis system (Image-1, Universal Imaging Corporation, Westchester, PA) that measures nuclear displacement. During the filming period, cells were maintained at 37°C in defined media at physiological pH. The effect of HP on cell locomotion was analyzed after addition of various concentrations of HP (10^{-13}–10^{-9} M) to the culture medium. For each experiment, 30 cells were tracked every 10 min for a 1 h period. Three tests at each concentration were conducted resulting in a total of 90 cells examined at a particular dose of HP. A concentration of 0.1 μg/ml found to be optimal for stimulating locomotion was used in the antibody neutralization experiments, wherein we aimed to determine whether antibody to RHAMM could nullify the effect of HP on motility. Antiserum (rabbit antibody against RHAMM-GST fusion protein, 10 μg/ml) was added to the culture media of C3 fibroblasts 5 min prior to the addition of HP (0.1 μg/ml). The locomotion of these cells and control cultures treated with buffer only were recorded as above.

To ascertain whether HP has an influence on HA stimulated locomotion, various concentrations of HP (0.001–0.1 μg/ml, calcium salt, Sigma Chemical Co., St. Louis, MO) or HP-leo (99% pure) were added to C3 cultures immediately prior to the addition of HA (0.01 μg/ml, Healon, Pharmacia, Uppsala, Sweden), and cell motility was recorded as above. Groups of fibroblast cultures treated with HA or HP alone or that received no treatments were analyzed as controls. Three trials of each group for a total of 90 cells per group were examined as above.

Construction and Expression of RHAMM cDNA

The RHAMM cDNA reading frame was amplified with Polymerase Chain Reaction [PCR, Sambrook et al., 1989] using two oligonucleotides as primers; one complimentary to the translation initiation region (nucleotides 1–22), creating a BamHI site linked to nucleotide 1 (5'GT GGA TCC ATG CAG ATC CTG ACA GAG AGG C), the other to the region 280 bp after the translation stop codon (nucleotides 1685–1706) creating an EcoRI site linked to nucleotide 1706 (5'AAT GAA TTC CTT TGG TGA TGA ACA GCA GT). The full length cDNA in Bluescript was used as a template. The reaction was carried out at 94°C (30 s), 54°C (30 s), and 72°C (90 s) for 25 cycles. The PCR product (1.7 Kb) was digested with proteinase K (50 μg/ml) in 0.5% SDS at 37°C for 30 min. DNA was extracted from the mixture with an equal volume of phenolchloroform (1:1), followed by an equal volume of chloroform and then precipitated with ethanol. The PCR product was doubled digested with EcoRI and BamHI and purified by agarose gel electrophoresis. The EcoRI-BamHI DNA fragment was ligated to an EcoRI and BamHI opened pGEX-2T [Eaton et al., 1986; Smith and Johnson, 1988] (Pharmacia). The ligation mixture was transformed into *Escherichia coli* HB101 [Sambrook et al., 1989]. Colonies were streaked on a master LB/amp agarose plate and a replica was prepared by overlaying a Nylon Hybond N^+ membrane on the LB/amp agar plate. Both plates were incubated at 37°C for 3 h. The Hybond N^+ membrane was screened with RHAMM cDNA [Hardwick et al., 1992] as a probe. The hybridization was carried out at 65°C in a solution containing 6 × SSC (1 M NaCl, 0.1 M Na_3 citrate), 5 × Denhardt's solution, 0.5% SDS and 20 μg/ml denatured salmon sperm DNA overnight as described [Sambrook et al., 1989]. The membrane was washed at 65°C with 2 × SSC, 1 × SSC, then 0.1 × SSC (30 min for each washing) and exposed to Kodak X-Omat film as described [Sambrook et al., 1989]. Positive colonies, which indicated the presence of a RHAMM cDNA insert, were recovered from the master plate and the presence of a correct RHAMM cDNA insert was confirmed by restriction endonuclease digestion and subsequent sizing of the resulting inserts with agarose gel electrophoresis.

Plasmid-containing colonies were grown in 5 ml LB/amp medium at 37°C overnight. Isopropylthio-β-D-galactoside (IPTG, 0.1 mM) was added to induce the production of fusion protein. The cultures were grown for another 90 min and the cells were harvested by centrifugation and resuspended in 1 ml 50 mM Tris-Cl, pH 8.0, containing 2 M urea and 1% Triton X-100. Cells were disrupted by sonication and centrifuged at 15,000*g* for 20 min. The supernatants that contained fusion proteins were recovered and the cell-free extract was subjected to SDS-PAGE.

Biotin-Labelling of HP and HA

Heparin (HP) and HA (1.5 mg each, Sigma products, calcium salt) were dissolved in 1 ml of 0.1 M sodium borate buffer, pH 8.8 [Hoare et al., 1993]. The mixture was incubated for 4 h with 4 mg N-hydroxysuccinimide biotin dissolved in 50 μl dimethyl sulfoxide at room temperature followed by a 10 min incubation with 300 μl 1 M ammonium chloride at room temperature. The solution was dialyzed against PBS, pH 7.4, and then PBS containing 30% glycerol. The resulting biotin-labelled HP and HA could be stored at 4°C for several months at a concentration of 2 μg/ml.

Transblot Assays

Proteins were fractionated on 10% SDS-PAGE and electroblotted onto nitrocellulose membranes in a buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol, pH 8.3. The remaining protein binding sites of the membrane were blocked in a buffer containing 10 mM Tris-HCl, pH 8.0, 150 mM NaCl (TBS), 0.05% Tween 20 (TBST), and 5% skim milk powder (TBSTS) for 1 h at room temperature. The blocked membranes were then incubated overnight at 4°C with biotin-labelled HP or biotin-labelled HA (2 μg/ml) in TBSTS containing either anti-RHAMM antibody [A^{268}, Hardwick et al., 1992, 10 μg/ml], 1.5 mg of HA or HP, or

0.15, 0.5, 1.0, 1.5, or 2.0 M NaCl. The membranes were washed for 4 × 10 min with TBST then incubated for 1 h with avidin-horseradish peroxidase (HRP) (1:1,000) diluted in TBSTS. The membrane was again washed with TBST for 4 × 10 min and rinsed with TBS. Bound biotinylated HA or HP was visualized with chemiluminescence using an ECL kit (Amersham, Oakville, Ontario, Canada) according to the manufacturer's instructions. The optical densities of the resultant bands were measured using the Bio-Rad 620 Video Densitometer (BioRad, Richmond, CA).

In an ELISA assay, HA was coated on immunoglobulin multiwell plates at pH 9.0. RHAMM fusion protein (10 μg/ml) was added to the plates and incubated for 1 h. The relative amount of bound RHAMM was detected with the monoclonal antibody 3T3-5 [Turley et al., 1991]. The bound antibody was quantified with alkaline phosphatase-anti mouse IgG. Increasing concentrations of HA or HP were added after the addition of RHAMM protein to displace protein from bound HA on the plate and the remaining protein was quantified as above.

To obtain binding isotherms, nitrocellulose membranes containing 10 μg of RHAMM fusion protein were incubated in TBSTS with biotinylated HA (10^{-6}, 5×10^{-7}, 10^{-7}, 5×10^{-8}, 10^{-8}, 10^{-9}, and 10^{-10} M) or biotinylated HP (2×10^{-6}, 10^{-6}, 2×10^{-7}, 10^{-7}, 2×10^{-8}, 2×10^{-9}, and 2×10^{-10} M), processed as above and the optical densities were obtained. The molecular weights of the GAGs were estimated from gel filtration to be 250 kDa (HA), and 16 kDa (HP). The amount of labelled HA or HP bound to RHAMM was estimated by extrapolation from densitometric values on a standard curve generated from density measurements of increasing amounts of biotinylated GAG dot-blotted onto nitrocellulose membranes. All binding was virtually eliminated with 50-fold excess labelled GAG and therefore was taken to represent specific binding.

Synthetic Peptide Binding Assays

Peptides with sequences corresponding to the two HA binding domains of RHAMM [Yang et al., 1993], K^{401}Q K I K H V V K L K (peptide$^{aa401-411}$), and K^{423}L R S Q L V K R K (peptide$^{aa423-432}$) were synthesized and HPLC purified by Cost Scientific (San Diego, CA). The synthetic peptides were dissolved in PBS containing 0.2 M NaCl at a concentration of 5 mg/ml. To evaluate non-specific binding, several randomized peptides and peptides encoded in other regions of the RHAMM cDNA were utilized. These included randomized peptide$^{401-411}$ (L K Q K K V K K H I V), randomized peptide$^{423-432}$ (Q S K R L K K R V L), peptide$^{125-145}$ (L K Q K K V T A Q S L R D V T A Q L E S V), peptide$^{269-288}$ (E S T N Q E Y A R M V Q D L Q N R S T L), peptide A (Y K Q K I K H D D K L K), and peptide B (Y K Q K I K H V V L L L). Peptide A and B represent RHAMM sequence 401–411 in which specific amino acids were altered to obliterate their ability to HA bind [Yang et al., 1994].

Heparin-Sepharose CL-6B (1 g, Pharmacia) was rehydrated in PBS containing 0.15 M NaCl and then transferred to eight Colex tubes (1 ml gel/tube). Four milligrams of each synthetic peptide were added to each of the above tubes. The mixture was incubated for 1 h at room temperature and then washed with PBS containing 0.2 M NaCl. The peptides were eluted with 3 ml PBS containing 2 M NaCl. The absorbances of the original peptide solution and of the eluted peptides were determined at 214 nm as a measure of the amount of peptide bound to the gels.

Genetic Manipulation of HP Binding Domains

Two oligonucleotides encoding the known HA binding domains of RHAMM (aa$^{401-411}$ and aa$^{423-432}$, 5′ AAA CAA AAA ATC AAA CAT GTT GTG AAA TTG AAA, and 5′ AAA CTG CGA TCT CAG CTT GTT AAA AGG AAA, respectively), were aligned by PCR to a cDNA encoding the non-HA binding amino terminus of RHAMM (aa$^{1-238}$) [Yang et al., 1993]. The recombinant cDNAs were obtained with PCR using the 2 primers and a primer mimicking nucleotides 1–22 that contained a BamHI site linked to nucleotide 1. Both PCR products were digested with EcoRI and BamHI and purified by agarose gel electrophoresis. The resulting cDNAs were inserted into pGEX-2T and transformed into *Escherichia coli* HB 101 [Eaton et al., 1986; Smith and Johnson, 1988]. The correct inserts were confirmed by restriction endonuclease digestion of selected clones. Cell lysates containing fusion proteins were prepared by sonication and fractionated on SDS-PAGE, then transblotted onto nitrocellulose membranes and visualized with either polyclonal antibody to RHAMM fusion protein or with biotin-labelled GAG. The fusion, non-recombinant RHAMM peptide$^{aa1-238}$, which does not bind to HA or to HP, was used as a control.

Deletion of HA-Binding Domains in RHAMM

RHAMM cDNA from nucleotides 1–1230 was amplified with PCR using two oligonucleotides as primers; one complimentary to the translation initiation region (nucleotide 1–22) creating a BamHI site linked to nucleotide 1, the other complimentary to nucleotide 1206–1230 creating a SacI site which was in the same reading frame as the SacI site in RHAMM cDNA [Hardwick et al., 1992]. In this way, the HA binding domain$^{aa423-432}$ was completely deleted while the other HA binding domain$^{aa401-411}$ was partially deleted. Two basic amino acids shown to be critical to HA binding (K^{405} and K^{409}) in the remaining domain were mutated to completely abolish the HA binding ability of this region [Yang et al., 1994]. The PCR product was doubly digested with BamHI and SacI. The pGEX-2T containing the complete RHAMM cDNA [Yang et al., 1993] was also doubly digested with BamHI and SacI. Plasmid-containing fragments (5.3 kb) were recovered by using a Prep-A-Gene Kit as above. The fragment was ligated to the BamHI-SacI 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 BamHI + SacI. Those clones containing a correct BamHI-SacI insert were used to make fusion proteins. Undeleted RHAMM fusion protein and HB101 lysate were used as controls.

RESULTS

Heparin Binds to RHAMM

As HA and HP are related molecules, we considered the possibility that HP could bind directly to RHAMM. To test this, GST-RHAMM fusion protein was subjected to SDS-PAGE electrophoresis and transblotted to a nitrocellulose membrane. Immunoblotting with a polyclonal antibody to GST-RHAMM fusion protein identified the presence of RHAMM fusion protein (Fig. 1A). Ligand-blotting assays using biotin-labelled HA or biotin-labelled HP were performed to determine the GAG binding properties of the fusion protein. Both biotin-labelled HA and HP bound to the RHAMM fusion protein (Fig. 1A).

To test the specificity of this interaction, two experiments were performed. In one, anti-RHAMM antibody was used to block the HP/RHAMM interactions in a ligand blotting assay. Preincubation of the transblotted protein with

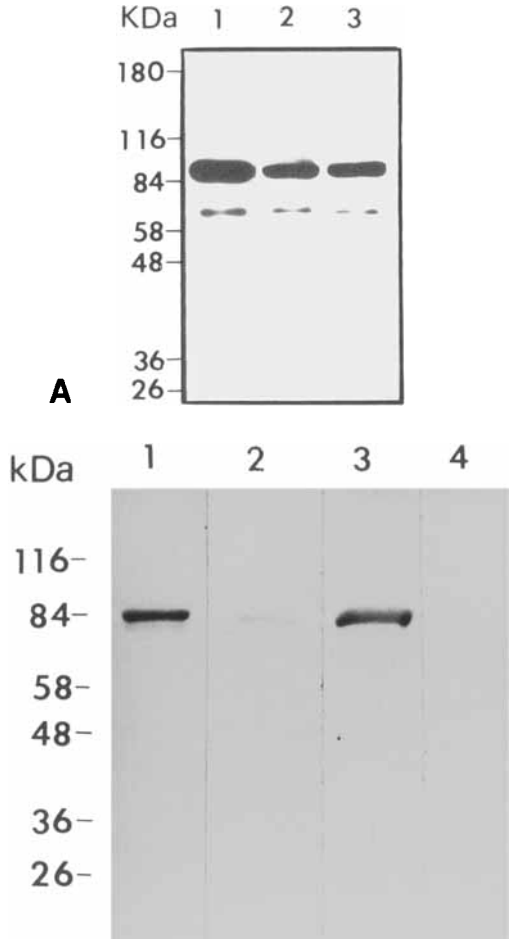

Fig. 1. The interaction of GST-RHAMM fusion protein with biotin-labelled HA and heparin. **A:** Cell lysates containing RHAMM fusion protein were fractionated on SDS-PAGE, and then transblotted onto a nitrocellulose membrane. RHAMM was visualized with polyclonal antibody to RHAMM peptide$^{aa125-145}$ (lane 1), biotin-labelled HA (lane 2) and biotin-labelled HP (lane 3). Both BHP and BHA bound RHAMM. **B:** Cell lysates containing RHAMM-GST fusion protein were separated on SDS-PAGE and processed for HP binding. Membrane 1 was incubated with TBST solution alone (lane 1). Membrane 2 was preincubated with RHAMM antibody (A^{268}) in TBST (lane 2). Membrane 3 was incubated with preimmune antiserum (lane 3) and were then incubated with biotin-labelled HP and developed as described in Experimental Procedures. Membrane 4 was stained with avidin-horse radish peroxidase (HRP) only as a negative control (lane 4). The antibody specifically blocked the interaction between HP and RHAMM (lane 2).

this antiserum prevented biotin-labelled HP binding to RHAMM whereas preincubation with preimmune antisera had no effect (Fig. 1B). Further, an excess of unlabelled heparin effectively prevented binding of biotinylated HP to RHAMM in transblot assays indicating that the interaction is specific (Fig. 2). Unlabelled HP also competed with biotinylated HA for binding

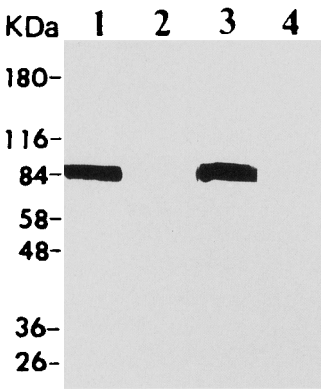

Fig. 2. Competitive inhibition assays. Cell lysates containing GST-RHAMM fusion protein were fractionated on SDS-PAGE and transblotted onto a nitrocellulose membrane. Lanes 1–2 were incubated with biotin-labelled HA; lanes 3–4 were incubated with biotin-labelled HP; lanes 1 and 3 were controls and Lanes 2 and 4 contained 1.5 mg unlabelled HP/ml in the staining solution to inhibit binding of biotin-labelled HA and HP to RHAMM. Excess unlabelled glycosaminoglycan successfully competed with biotinylated-HA and -HP for binding to RHAMM. Blots were developed with an ECL kit as described in Experimental Procedures.

to RHAMM fusion protein, suggesting the two GAG could be interacting with RHAMM at the same site (Fig. 2).

HP Binding Domain of RHAMM Occurs in the Sequences $aa^{401-411}$ and $aa^{423-432}$

We previously reported that the HA binding domains occur within $aa^{401-432}$ of the RHAMM protein [Yang et al., 1993]. To determine whether the same domains were involved in HP-binding to RHAMM, we prepared fusion proteins expressed from increasingly truncated RHAMM cDNAs. These truncations resulted in the following protein fragments: $peptide^{aa1-136}$, $peptide^{aa1-238}$, $peptide^{aa1-339}$, $peptide^{aa1-397}$, $peptide,^{aa1-435}$, and the complete, untruncated $protein^{aa1-476}$. These fusion proteins were fractionated on SDS-PAGE and transblotted onto nitrocellulose membranes, and then reacted with anti-GST antibody to establish that the expressed proteins were of the predicted size (Fig. 3A). Transblotting assays using biotin-labelled HP as a ligand revealed that only the $peptide^{aa1-435}$ and the complete GST-RHAMM fusion $protein^{aa1-476}$ bound biotin-labelled HP (Fig. 3B) and biotin-labelled HA (Fig. 3C). These results localized the HP binding domain to $peptide^{aa397-435}$ of RHAMM, the same region containing the HA binding domains [Yang et al., 1993].

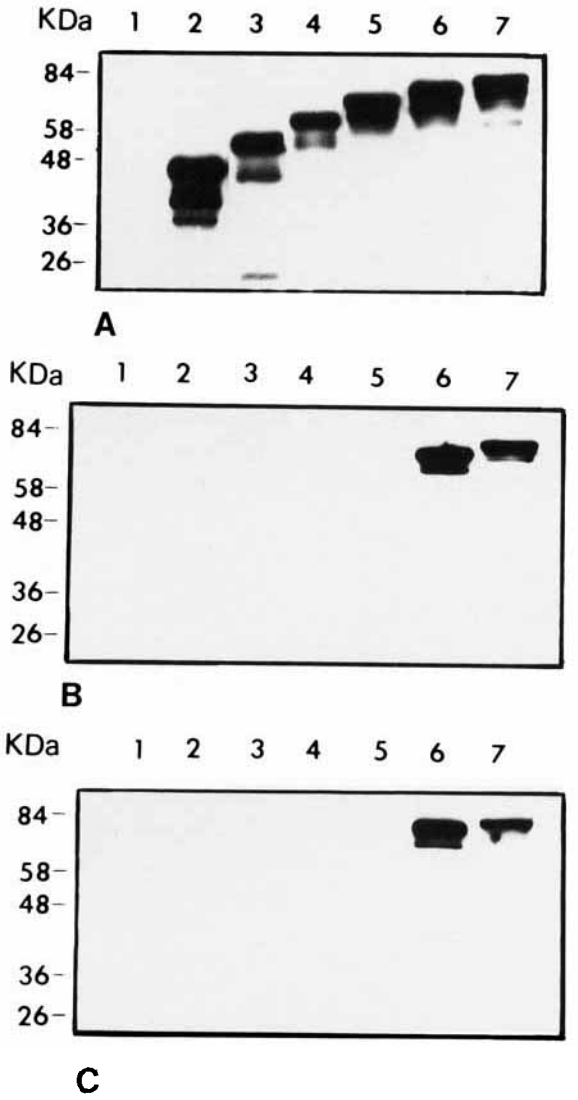

Fig. 3. Staining of fusion proteins from different truncated RHAMM cDNAs. RHAMM cDNA was truncated with different restriction endonucleases and expressed as a fusion protein with pGEX-2T. Electrophoresed proteins were obtained from bacterial lysates only (lane 1), RHAMM $peptide^{aa1-136}$ (lane 2), RHAMM $peptide^{aa1-238}$ (lane 3), RHAMM $peptide^{aa1-339}$ (lane 4), RHAMM $peptide^{aa1-397}$ (lane 5), RHAMM $peptide^{aa1-435}$ (lane 6), and complete RHAMM (lane 7). Cell lysates were fractionated on SDS-PAGE, transblotted onto nitrocellulose membranes and stained with anti-GST antibody (**A**), biotin-labelled heparin (**B**), or biotin-labelled HA (**C**).

To test the possibility that HA and HP share the same binding domains of RHAMM, peptides which mimic the HA binding domains of RHAMM, ($peptide^{aa401-411}$ and $peptide^{aa423-432}$) were synthesized and tested for their ability to bind to HP-Sepharose. The results (Fig. 4) show that $peptide^{aa401-411}$ and $peptide^{aa423-432}$ both bind in approximately 2-fold greater amounts of HP-Sepharose than do control peptides. This bind-

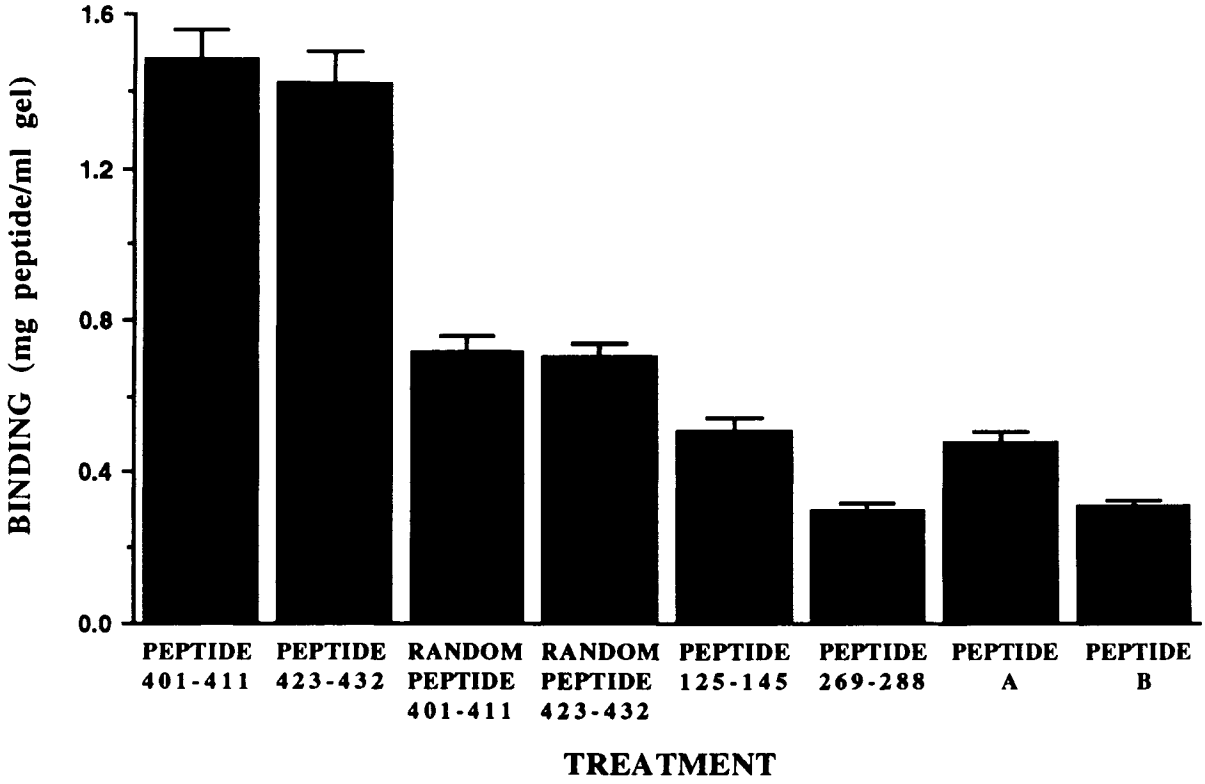

Fig. 4. Binding of synthetic peptides to HP-Sepharose. HP-Sepharose (Pharmacia) gel was prepared according to the manufacturer's instructions and peptides were applied to derivatized gels. Unbound peptides were removed by washing the gel with PBS containing 0.2 M NaCl and bound peptides were eluted with PBS containing 2 M NaCl. The amount of eluted peptide was determined by measuring an O.D. value (214 nm). The results indicated that peptide$^{aa401-411}$ and peptide$^{aa423-432}$ bound to HP-Sepharose with much higher affinity than all control peptides (see description in Experimental Procedures).

ing represents 40% of the amount of peptide applied to the column.

To confirm the above observation, oligonucleotides encoding peptide$^{aa401-411}$ and peptide$^{aa423-432}$ were aligned to a truncated cDNA encoding the N-terminus of RHAMM (peptide$^{aa1-238}$, Fig. 5A) that does not bind either HA or HP. The resulting recombinant cDNAs were expressed as GST-fusion proteins and the recombinant polypeptides containing the previously identified HA-binding domains were also found to bind biotin-labelled HP (Fig. 5B, lane 6 and lane 7). These results indicate that HP and HA interact with the same domains in RHAMM.

Peptide$^{aa401-411}$ and Peptide$^{aa423-432}$ Are the Only HP Binding Domains in RHAMM

We next sought to determine whether domains aa$^{401-411}$ and aa$^{423-432}$ identified above as HP binding domains were the only binding sites for this glycosaminoglycan in RHAMM. These two motifs were therefore deleted and mutated (Fig. 6A), a process shown to abolish the HA binding ability of RHAMM [Yang et al., 1994]. Undeleted RHAMM fusion protein was used as a control (Fig. 6B, lanes 2 and 4). The presence of the fusion proteins was identified by Western blot assays using an antibody to GST (Fig. 6B, lanes 1–2) and then assayed for binding to biotin-labelled HP as described in Experimental Procedures (Fig. 6B, lanes 3–4). The deleted/mutated fusion protein lost its ability to bind HP (Fig. 6B, lane 3) implying that aa$^{401-411}$ and aa$^{423-432}$ are the only HP binding domains in RHAMM.

Binding Affinity and Capacity of RHAMM for HA Is Greater Than for HP

An estimate of the affinity of HP for RHAMM was obtained by three approaches. First, increasing concentrations of unlabelled HA and HP were used in ELISA assays to displace RHAMM fusion protein already bound to HA (Fig. 7). In the second approach, increasing concentrations of NaCl were used to inhibit binding of biotinyl-

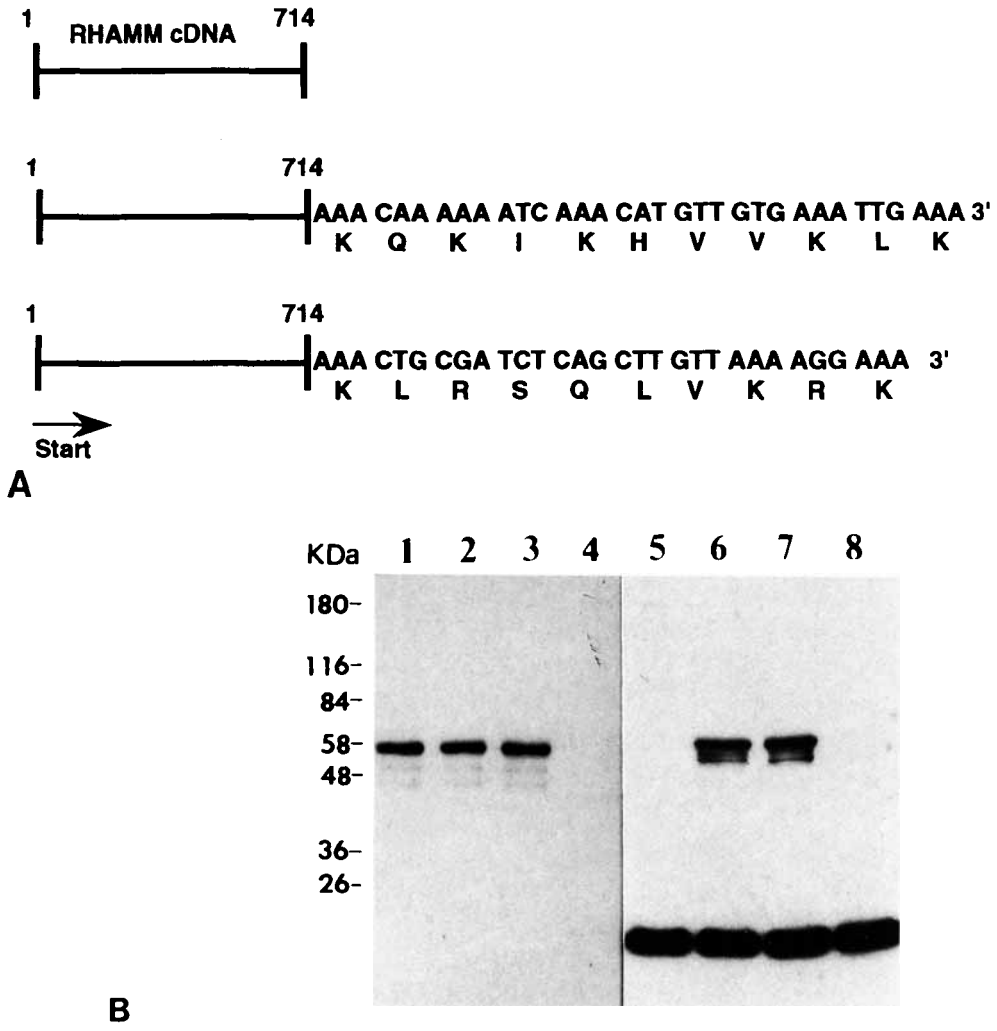

Fig. 5. Genetic manipulation of RHAMM peptide$^{aa1-238}$ to create HP binding domains. **A:** Oligonucleotides encoding peptide$^{aa401-411}$ and peptide$^{aa423-432}$ of RHAMM were each aligned by PCR to a cDNA encoding the N terminal RHAMM polypeptide (peptide$^{aa1-238}$) that does not bind heparin, and the resulting recombinant cDNAs were expressed in pGEX-2T to produce a chimeric fusion polypeptides. **B:** Cell lysates containing chimeric fusion polypeptides were fractionated on SDS-PAGE, transblotted onto a nitrocellulose membrane and visualized with 1) polyclonal antibody to RHAMM-GST fusion protein (lanes 1–4), and 2) biotin-labelled HP (lanes 5–8). The fusion non-recombinant peptide$^{aa1-238}$ (lanes 1 and 5) and bacterial lysate (lanes 4 and 8) were used as controls. The results show that the linkage of peptide$^{aa401-411}$ (lanes 2 and 6) or peptide$^{aa423-432}$ (lanes 3 and 7) to peptide$^{aa1-238}$ create heparin binding sites in the recombinant polypeptides.

ated HP to transblotted RHAMM fusion protein (Fig. 8). Finally, the binding of increasing molar amounts of biotinylated HA and HP to RHAMM were quantified using the transblot assay (Fig. 9). Higher molar concentrations of unlabelled HP than unlabelled HA were required to displace RHAMM from HA, suggesting a higher affinity of HA for RHAMM relative to HP (Fig. 7). Heparin was also displaced from RHAMM by lower salt concentrations than those required to displace HA which is consistent with a lower affinity interaction between HP and RHAMM than between HA and RHAMM (Fig. 8). A binding isotherm derived from the transblot assay indicated that HA bound to RHAMM with slightly greater capacity than did HP (Fig. 9).

HA and HP Regulate Cell Locomotion

To examine the locomotory responses of C3 fibroblasts to HA and HP, as well as two other related negatively charged molecules, namely, chondroitin sulfate and dermatan sulfate, these

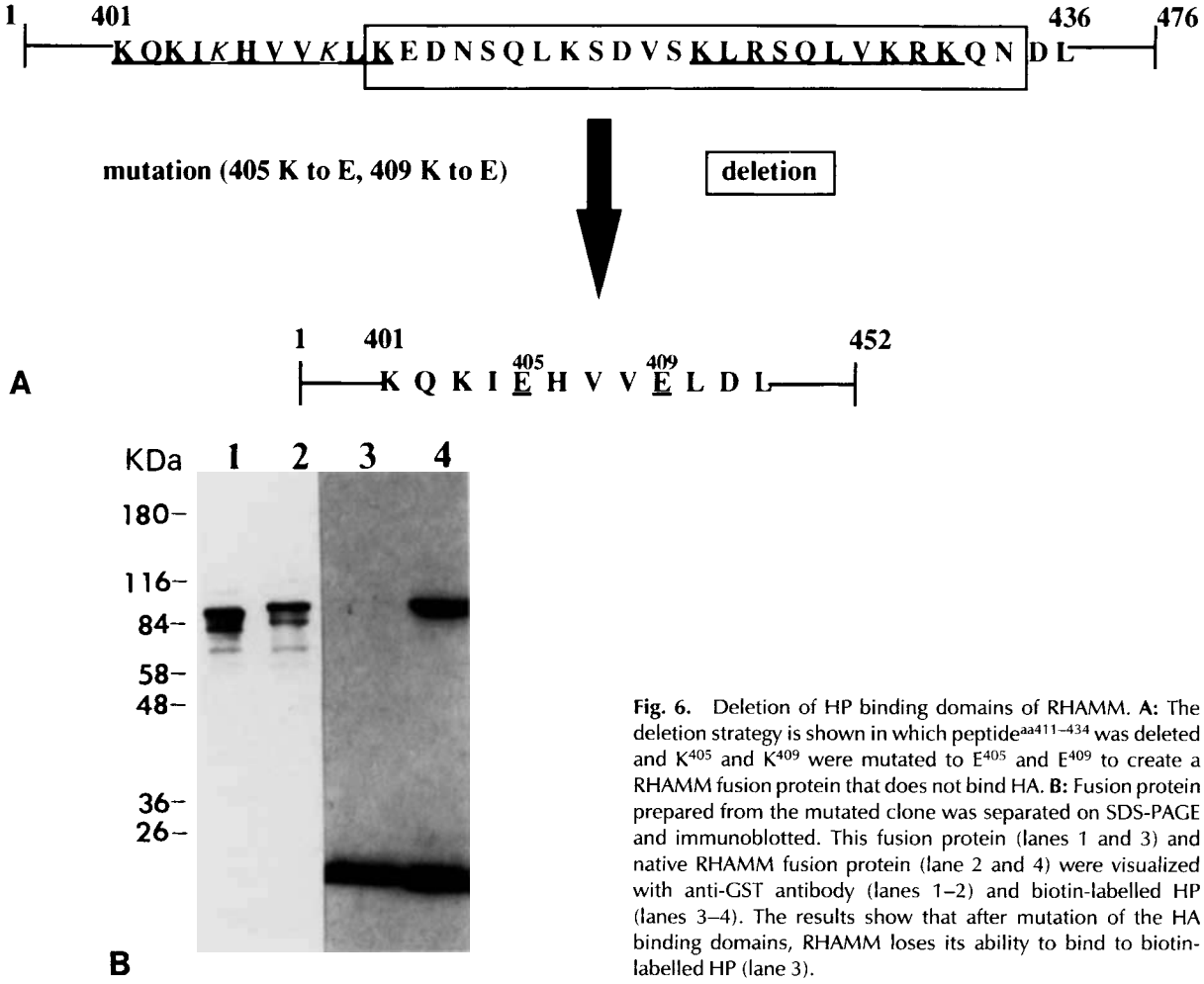

Fig. 6. Deletion of HP binding domains of RHAMM. **A:** The deletion strategy is shown in which peptide$^{aa411-434}$ was deleted and K^{405} and K^{409} were mutated to E^{405} and E^{409} to create a RHAMM fusion protein that does not bind HA. **B:** Fusion protein prepared from the mutated clone was separated on SDS-PAGE and immunoblotted. This fusion protein (lanes 1 and 3) and native RHAMM fusion protein (lane 2 and 4) were visualized with anti-GST antibody (lanes 1–2) and biotin-labelled HP (lanes 3–4). The results show that after mutation of the HA binding domains, RHAMM loses its ability to bind to biotin-labelled HP (lane 3).

GAGs were added to cell cultures and random cell locomotion was measured. The locomotory response of *ras*-transformed fibroblasts to HA has previously been noted to be concentration dependent [Turley et al., 1991] with optimal responses of these C3 cells occuring in the range of 0.001–0.01 μg/ml of HA. The locomotory response to HP was also concentration dependent (Fig. 10A), but 0.1 μg/ml of HP was required to cause a 1.5-fold increase in locomotion (Fig. 10A), while low concentrations of HP (0.001–0.01 μg/ml) had no significant effect on cell motility when added alone. However, HP at 0.001 μg/mL abrogated the stimulatory effect of HA on locomotion (Fig. 10B). These actions of HA and HP were specific in that chondroitin sulphate and dermatin sulfate had no effect on locomotion (Fig. 10C). To determine if HP might mediate its locomotory effects via the HA receptor RHAMM, anti-RHAMM antibody (A^{268}), previously reported to block HA binding to the receptor [Savani et al., in revision] was added to the cells prior to HP and the effect on locomotion was analyzed. The stimulatory effect of higher concentrations of HP was inhibited by anti-RHAMM antibody (Fig. 10C). The antibody also inhibited basal locomotion. These results indicate that the two glycosaminoglycans, HA and HP, regulate transformed fibroblast locomotion, and that they likely do so via specific GAG-RHAMM interactions.

DISCUSSION

This study demonstrates that HP regulates *ras*-transformed fibroblast locomotion with the precise effect depending upon the concentration of HP used. The stimulatory effect of high concentrations of HP appear to be mediated by RHAMM. We have identified the HP binding site in RHAMM and have shown it to be identical to the site bound by HA, thus defining a new heparin binding site. HP binds to these sites with 10- to 100-fold lower affinity than HA, as estimated by displacement assays. Consistent

with this lower affinity, 10-fold higher concentrations of HP were required to stimulate fibroblast locomotion. These concentrations are within physiological ranges that occur during wounding and vascular remodelling. Indeed, HP is released from most cells at sites of wound injury and occurs in μg levels in both tissues and serum [Horner, 1975]. These results predict that in circumstances where HP levels are high, this glycosaminoglycan may stimulate locomotion via a RHAMM-mediated pathway and that it may also be a potential physiological regulator of cell locomotion, particularly of vascular cells.

Heparin has been reported to exert either a stimulatory or inhibitory effect on cell locomotion depending upon the cell type examined [Clowes and Clowes, 1986; Norrby and Sorbo, 1992; Azizkhan et al., 1980; Majack and Clowes, 1984]. We have also noted that the effect of HP on cell locomotion is concentration-dependent such that stimulation of motility occurs at higher concentrations relative to that required for stimulation by HA. At lower concentrations, HP inhibits HA-mediated increases in the locomotion of *ras*-transformed fibroblasts and is there-

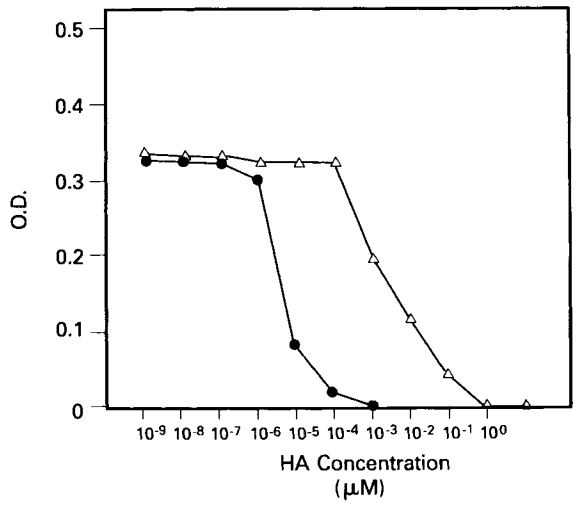

Fig. 7. Displacement of RHAMM fusion protein from HA coated wells; 1 μg/ml HA was absorbed to immunobilon wells and GST-RHAMM protein was then incubated in these wells, in the presence of increasing concentrations of unlabelled HA (●) or HP (△). Remaining bound RHAMM was detected with 3T3-5 monoclonal antibody specific to RHAMM and alkaline phosphatase labelled anti-mouse-IgG. The MW_E of HA was determined by gel filtration to be predominantly 250 kDa, while HP was shown to be 16 kDa. The results show that HA displaced RHAMM with a greater ability than HP.

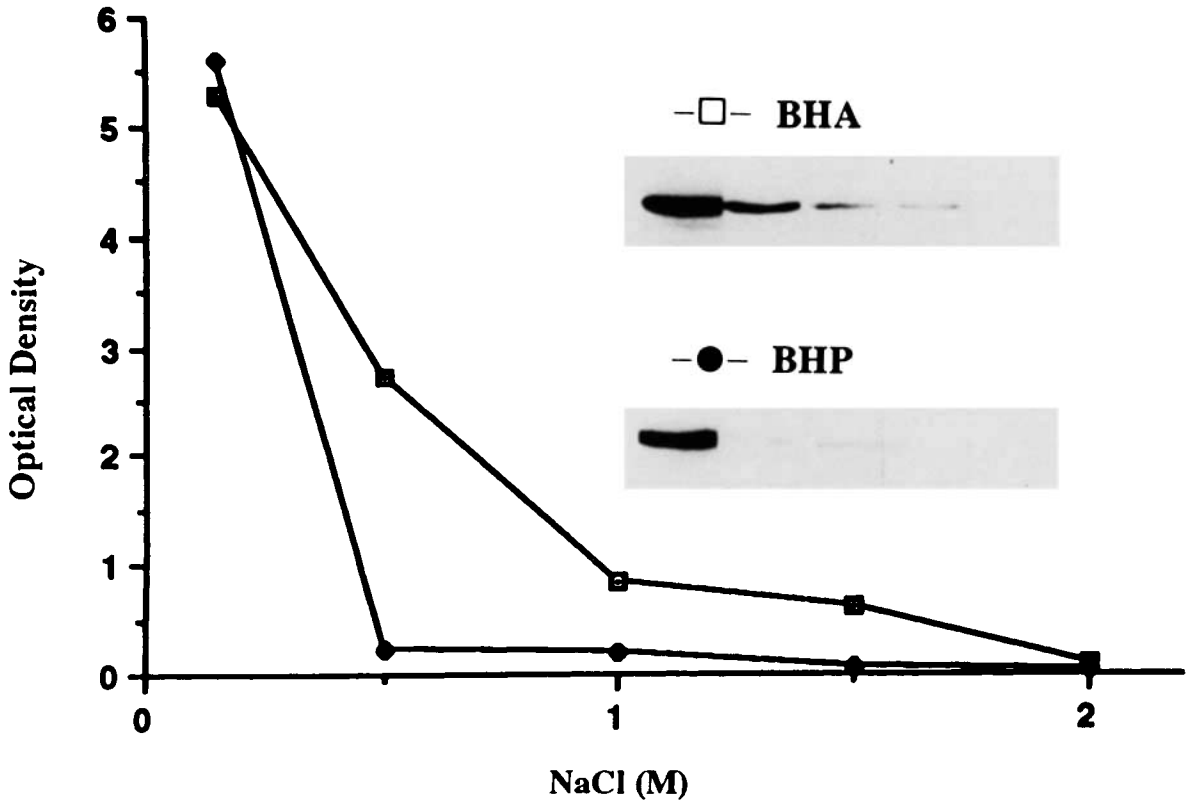

Fig. 8. The effect of salt concentration on the interaction between biotinylated glycosaminoglycans and RHAMM fusion protein. GST-RHAMM fusion protein was electrophoresed on SDS-PAGE, transblotted onto nitrocellulose and incubated with biotinylated HA (□) or HP (●) with increasing NaCl concentrations. The results indicate that binding of biotinylated HP to RHAMM was reduced by lower concentrations of salt than was the binding of BHA.

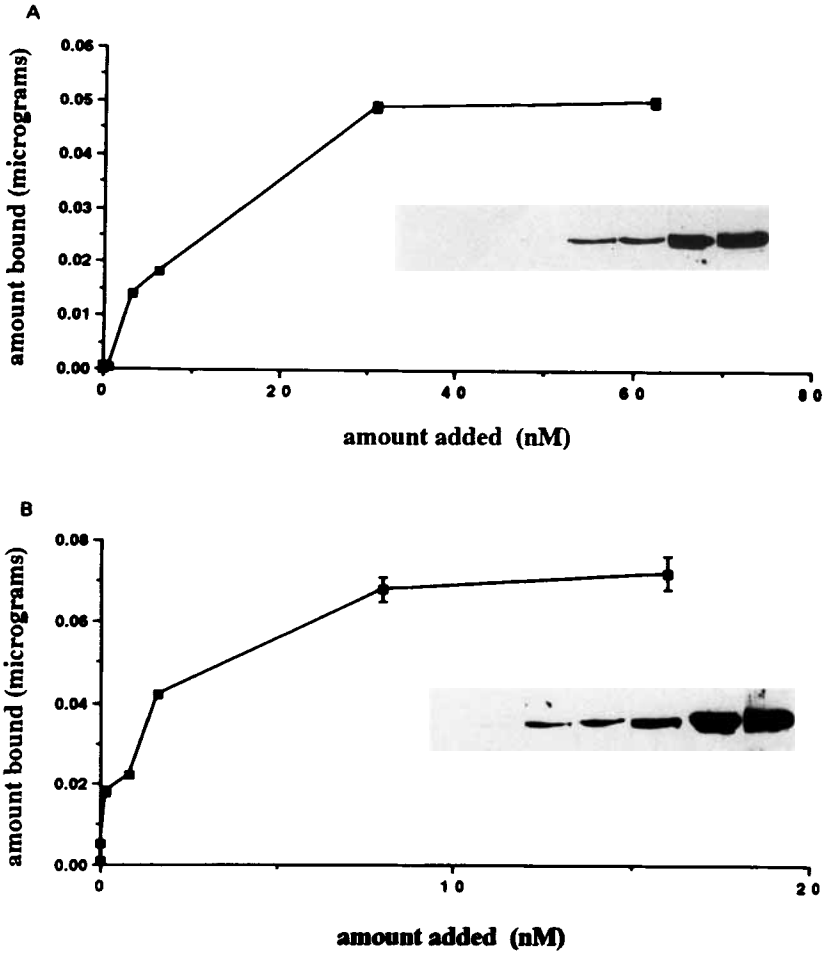

Fig. 9. Binding isotherm of increasing amounts of biotinylated glycosaminoglycans to RHAMM fusion protein. Increasing amounts of biotinylated HP (**A**, 0–80 nM) and biotinylated HA (**B**, 0–20 nM) were used to probe 10 μg RHAMM fusion protein separated by SDS-PAGE and transblotted onto nitrocellulose membranes. The amount (nM) of labeled glycosaminoglycans was extrapolated from a standard curve as described in Experimental Procedures. Biotinylated HA bound with greater capacity to RHAMM than did biotinylated HP and binding of both glycosaminoglycans to RHAMM was saturable.

fore identified here as a potentially important regulator of HA-promoted motility. HP, at low concentrations may inhibit motility by a RHAMM-independent pathway such as inhibition of protease release or oncogene function, while it is likely able to interact with RHAMM sufficiently well to promote locomotion at higher concentrations.

Interactions of HP with several proteins has been well defined and it has been consistently observed that clusters of basic amino acids are critical components of HP binding domains within these proteins [Gandrille et al., 1990; Kost et al., 1992; Pratt and Church, 1992; Ehrlich et al., 1992; Pratt et al., 1992]. Evidence has been presented that a consensus sequence BBXXBBBXXBB (where Bs are a basic amino acids and Xs are any amino acid) represents one requirement for HP binding [Sobel et al., 1992]. Further, a randomly rearranged sequence of this consensus motif within proteins decreases their binding affinity to HP [Sobel et al., 1992], suggesting that specific spacing of basic amino acids in the motif is critical for HP binding. The two sequences in RHAMM that are shown here to bind to both HA and HP, namely, KQKIKITV-VKLK and KLRSQLVKRK, share no homology with other HP binding proteins nor do they conform to consensus HP binding motifs reported to date [Gandrille et al., 1990; Kost et al., 1992; Pratt and Church, 1992; Ehrlich et al., 1992; Pratt et al., 1992; Sobel et al., 1992], suggesting the occurrence of novel domains that are able to interact with HP. Specific charge spacing within these newly identified domains is also important since randomized peptides composed of amino acids in these two domains show decreased HP-binding.

Because the transblot assay used here is based on detection of GAG binding peptides to denatured, reduced proteins, which do not reflect interactions under physiological conditions, our results must be considered approximate. All three approaches taken to assess the binding affinities between RHAMM and HP or HA uniformly gave a lower affinity of HP/RHAMM compared with HA/RHAMM interactions and this is consistent with a previous report that partially purified RHAMM binds with HA with greater affinity than HP [Turley and Moore, 1984]. Nevertheless, the interaction of RHAMM with HP is still significant and compares favourably with the affinity range of other ECM receptor interactions [Ruoslahti, 1991]. It is likely therefore that HP/RHAMM interactions are biologically significant.

It is interesting that neither chondroitin sulphate nor dermatan sulfate interact with RHAMM [Hoare et al., 1993] and that neither of these agents exerts an influence on cell motility. Although their structures are related to those of HA and HP, their helical configuration in solution differs from the confirmations attained by HA and HP and may contribute to the differential binding preferences to RHAMM. The characterization of the domains responsible for binding to HA has been analyzed using site-directed mutagenesis and peptide mimicry [Yang et al., 1994]. These domains interact with HP at a relatively high affinity under physiological conditions and since HA also exhibits high affinity for RHAMM, small changes in the relative concentrations of the two GAGs would allow for tight regulation of their effects on cell motility. Our data suggests a possible role for HP as a negative regulator of HA-induced locomotion in several conditions involving cell locomotion, as occurs, for example, when HP is released during wound healing [Carr, 1979; Clowes and Karnovsky, 1977] and in restenosis after balloon angioplasty [Cassells, 1992].

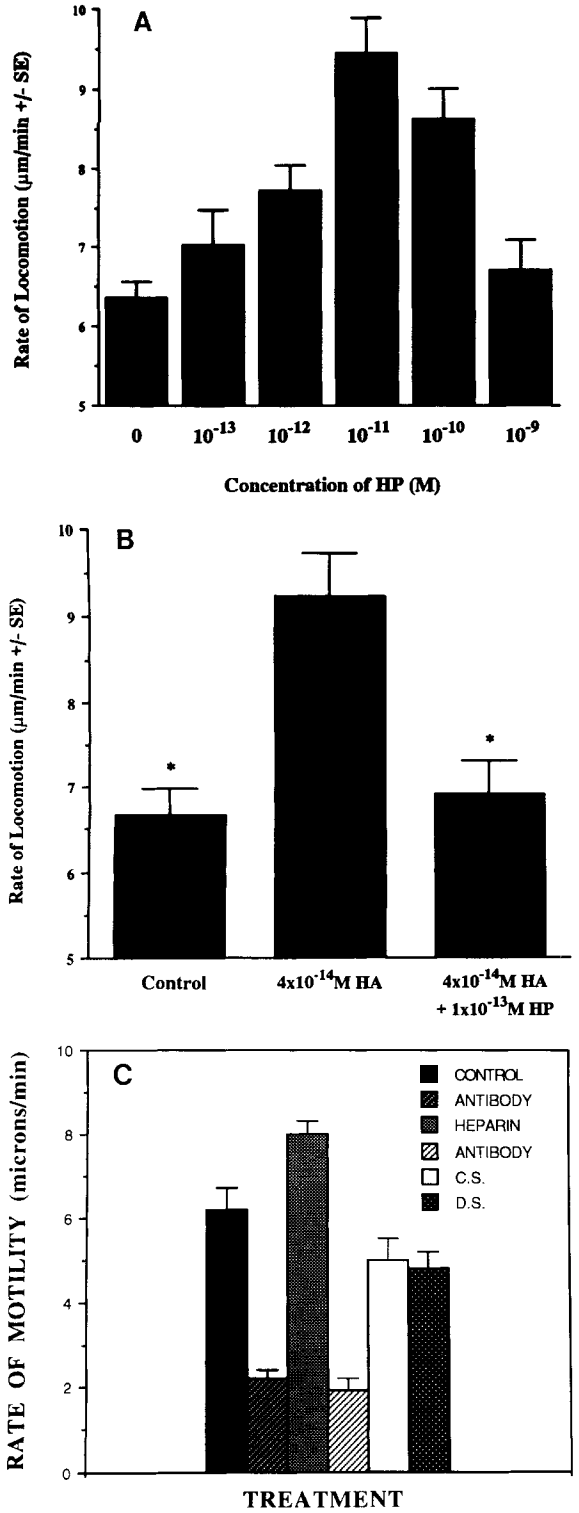

Fig. 10. Stimulation of cell locomotion by HP. **A:** *ras*-transformed fibroblasts (C3) were incubated with increasing concentrations of HP (10^{-13}–10^{-9} M) and random locomotion was measured by timelapse cinemicrography. HP concentrations of 10^{-11} M and 10^{-10} M significantly stimulated cell locomotion ($P < .001$, Student's *t*-test). **B:** Locomotion of C3 fibroblasts was analyzed in the presence of 10 ng/ml of HA with or without the presence of heparin (10^{-13} M) or in the absence of GAG (control). HA at a concentration of 4×10^{-14} M stimulated cell locomotion, and this increase was neutralized in the presence of heparin (10^{-13} M). Asterisks indicate significant differences ($P < .001$, Student's *t*-test). **C:** Antibody (rabbit antibody to RHAMM-GST fusion protein, 20 μg/ml) was added to the culture media of *ras*-transformed C3 cells 5 min prior to the addition of HP (10^{-11} M), and the antibody nullified the effect of HP on cell locomotion. The antibody also significantly inhibited base levels of C3 cell motility ($P < 0.001$ Student's *t*-test). Neither chondroitin sulfate (CS, 10^{-11} M) nor dermatan sulfate (DS, 10^{-11} M) affected cell locomotion.

In summary, we demonstrate that both HP and HA regulate *ras*-transformed fibroblast locomotion and that they do so by interacting with the GAG receptor RHAMM. Both HA and HP bind to the same two ten amino acid domains within RHAMM and are likely to be important physiological regulators of cell locomotion.

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