

# Molecular Cloning of a Novel Hyaluronan Receptor That Mediates Tumor Cell Motility

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**Abstract.** A cDNA encoding a unique hyaluronan receptor has been molecularly cloned from a  $\lambda$ GT11 3T3 cDNA expression library. Immunoblot analyses of cell lysates, using antibodies to peptides encoded in the cDNA, specifically react with a 58-kD protein. This protein is regulated by the mutant *H-ras* gene in cells containing a metallothionein promoter *H-ras* hybrid gene. Further, antibodies to peptide sequences encoded in the cDNA block the increase in locomotion resulting from induction of the mutant *H-ras* gene in this cell line. In a transblot assay, the bacterially expressed protein binds to biotinylated hyaluronan. Anti-

bodies to peptides encoded in the cDNA react in immunoblot assays with the 58- and 52-kD proteins of a novel hyaluronan receptor complex previously implicated in cell locomotion. Furthermore, antibodies specific to the 58- and 52-kD proteins, which block *ras*-induced locomotion, also cross-react with the expressed, encoded protein. The gene product described here appears to be a new type of hyaluronan receptor that is involved in cell locomotion. It is named RHAMM, an acronym for receptor for hyaluronan-mediated motility.

THE transforming oncogene *H-ras* has been reported to promote cell locomotion (17), although the regulatory mechanisms remain unknown. Several observations suggest that when this oncogene promotes locomotion, the mechanisms are complex and involve, at least, the release of autocrine motility factor(s) (14, 20), growth factors (14), and the glycosaminoglycan hyaluronan (HA)<sup>1</sup> (20, 34). In particular, HA appears to function as an autocrine mechanism for stimulating maximal locomotion in *ras*-transformed cells (34). Further, it is also required for the ability of an autocrine motility-stimulating factor to promote breast carcinoma cell locomotion (20). We have shown that HA-promoted, *ras*-transformed cell locomotion requires the presence of a novel hyaluronan receptor complex termed HARC (34). This complex of proteins occurs at the cell surface or is released as soluble proteins of 72, 68, 58, and 52 kD (32). The complex is tightly regulated in vitro (30) and expressed on the leading lamellae and perinuclear region only on rapidly locomoting cells (29, 31). Both polyclonal and monoclonal antibodies (pAbs and mAbs, respectively) prepared against this complex block cell locomotion regulated by mutant *ras* (34). In

a recent study, we have shown that these blocking mAbs are specific to the 58- and 52-kD proteins, that these proteins are isoforms of each other, and, further, that these proteins are the HA-binding component of HARC (Turley, E. A., K. Hoare, and V. Cripps, manuscript submitted for publication).

We have used the blocking antibodies specific to the 58- and 52-kD HARC proteins to screen a  $\lambda$ GT11 3T3 cDNA expression library. We describe here the molecular cloning of a cDNA encoding a unique protein that is regulated by mutant *H-ras*, that is essential for the locomotion of these *ras*-transformed cells, and that binds to HA. Further, antibodies to peptides encoded in the cDNA cross-react with the 58- and 52-kD proteins of HARC, while conversely blocking mAbs to the 58-kD HARC protein cross-react with the expressed, encoded protein. This is the first molecularly characterized protein identified as a requirement for the locomotion of *ras*-transformed cells. Further, our data suggest that this novel protein encodes a new HA receptor. It is therefore referred to by the acronym RHAMM for receptor for HA-mediated motility.

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1. *Abbreviations used in this paper:* HA, hyaluronan; HARC, hyaluronan receptor complex; pAb, polyclonal antibody; RHAMM, receptor for HA-mediated motility.

## Materials and Methods

### Antibodies

pAbs and mAbs to HARC were prepared as described previously (31, 32, 34). All antibodies were purified by affinity chromatography on HARC-

Sepharose and/or protein A-Sepharose. mAb 3T3-3, which blocks fibroblast locomotion, was originally used to screen the cDNA library. mAb 3T3-5 is shown here to recognize proteins which occur in two isoforms (58 and 52 kD) in SDS immunoblots of lysates of *ras*-transformed fibroblasts and has previously been shown to block HA-promoted locomotion of these fibroblasts (34). mAb 3T3-7, which was used for immunofluorescence, reacts specifically with the 52–58-kD protein of HARC, as shown here by SDS immunoblot analyses, but does not block HA-promoted locomotion (34). Synthetic peptides mimicking two amino acid sequences in the deduced sequence (peptide I, nucleotide sequence 372–435 [corresponding to a portion of the repeated sequence] and peptide II, nucleotide sequence 804–864 [Fig. 1]) were produced by the Peptide Analysis Core of the University of Alabama Cancer Center, Birmingham, AL. The peptides were purified by HPLC and characterized by amino acid composition analyses. Antibodies to the peptides were raised in rabbits as described (31, 32). These antibodies were purified by affinity chromatography on columns of the appropriate peptides coupled to CNBr-activated Sepharose and the specificity of purified antipeptide antibodies to the respective peptide was confirmed by dot blot assays. Preimmune antibodies were obtained from each rabbit or mouse before immunization, purified as described above and used as controls.

### Cloning and DNA Sequencing

A 3T3-cDNA library in  $\lambda$ gt11 was obtained from Clontech (Palo Alto, CA) and initially screened with both pAb and mAb (3T3-3) to HARC. One clone, which gave a positive signal upon repeated screening with the antibodies, had a 1.9-kb insert that was subcloned into pUC18 and M13. A restriction map was constructed using the enzymes (Un. States Biochem. Corp., Cleveland, OH) indicated in Fig. 1. Nucleotide sequences were determined by the dideoxy chain termination method using Sequenase I and Sequenase II kits (Un. States Biochem. Corp.). Digestion of the insert with *AccI* yielded a 0.7 kb fragment which was radiolabeled with a Random priming kit (Bethesda Research Laboratories, Bethesda, MD) and was used to rescreen the cDNA library at high stringency, resulting in the isolation of a 2.9-kb insert. The insert was isolated after digestion of the  $\lambda$ phage with *EcoRI* and ligated into pUC18 and M13 for sequencing. Sequence analysis and comparisons were conducted using IBI/Puskell sequence analysis software and NBRF database (version 18; reference 4). Additional homology searches were conducted with NBRF (6/90) and EMBL (5/90) using FASTA and word search programs in GCG software.

### Locomotion Assays

A 10T1/2 cell line containing an inducible EJ-*ras* metallothionein-neomycin hybrid gene (designated 212; reference 26) was used for quantitating the effect of both pAb to synthetic peptide and mAb to HARC on cell locomotion in response to 0.001  $\mu$ g/ml of HA as previously described (34). Antibodies (mAb 3T3.5, 1  $\mu$ g/ml, or pAb peptide II, 1  $\mu$ g/ml) were added to zinc-induced (24 h) cells for 0.5 h, and then exposed to 0.001  $\mu$ g/ml of HA and filmed for a total of 2 h in the presence of both HA and either of the above antibodies. To assess specific effects of the antibodies, they were preincubated for 1 h at 4°C with 50  $\mu$ g of HARC proteins before their addition to locomotion assays as above. Addition of these proteins by themselves had no effect on cell locomotion (results not shown). Data were analyzed with a Dynacell program (Carl Zeiss, Inc., Thornwood, NY) that used Fourier analysis of cell locomotion to derive a cell motility index (17).

### Immunofluorescence of Live Cells

Cells were grown on glass coverslips for 24 h after their subculture so that monolayers were subconfluent. Cells were then incubated with either  $\text{Ca}^{++}$ ,  $\text{Mg}^{++}$ -free Hanks' solution or 0.25% trypsin in  $\text{Ca}^{++}$ ,  $\text{Mg}^{++}$ -free Hanks' solution for 3 min. Primary antibody (pAb II, nucleotide sequence 804–864, or rabbit IgG) was then added at 1  $\mu$ g/ml in defined DMEM to cultures and incubated for 2–3 h at 37°C. Media was gently aspirated from cultures which were then washed and incubated with rhodamine-labeled goat anti-rabbit IgG (Sigma Chem. Co., St. Louis, MO; 1:100 dilution) for 1 h at room temperature. Cells were washed again, and then lightly fixed in freshly made 3% paraformaldehyde. Fixed monolayers were viewed on both an IM35 microscope (Carl Zeiss, Inc.) equipped with epifluorescence and also on a Nikon confocal laser scanning microscope. For confocal microscopy, only the first 3  $\mu$ m of the culture media-facing surface of cells was photographed.

### Immunofluorescence of Fixed Cells

Double immunofluorescence studies of zinc-induced 212 cells were performed as described (30, 31) using mAb 3T3-7 to HARC and pAb to peptide II (nucleotide sequence 804–864). Monolayers were fixed in freshly prepared 3% paraformaldehyde for 10 min, and then incubated with 0.1 M glycine to quench autofluorescence. Fixed monolayers were washed, and then incubated with 1  $\mu$ g/ml of the above antibodies overnight at 4°C. The monolayers were again washed, and then incubated with fluorescein-labeled goat anti-mouse IgG (to detect mAb 3T3-7) and rhodamine-labeled goat anti-rabbit IgG (to detect pAb to peptide I). These were purchased from Sigma Chem. Co. and used at 1:1,000-fold dilution. Processed monolayers were examined with an IM35 microscope (Carl Zeiss, Inc.) equipped with epifluorescence utilizing nonoverlapping filters of 510–560 nm (for rhodamine) and 450–490 nm (for fluorescein). Lack of bleedthrough was confirmed by examination of single immunofluorescence samples with both filters (data not shown).

### SDS Immunoblots

Immunoblot assays were conducted on isolated soluble HARC proteins (32) or cell lysates prepared from H-*ras*-transfected fibroblasts exposed to either zinc sulfate or buffer alone for 24 h. The cells were treated with lysis buffer containing 25 mM Tris, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 0.15 M NaCl, 1 mM EDTA, and protease inhibitors (32). Proteins were fractionated by SDS-PAGE on 12.5% polyacrylamide gels (32) and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Rockville Center, NY). Additional protein binding sites were blocked with 0.5% defatted milk in TBS (34) and the membrane was incubated with primary antibodies (1  $\mu$ g IgG/ml of TBS containing 0.5% defatted milk) for 12 h at 4°C on a gyratory shaker (Stovall, Greensboro, NC). The mAb to p21 *ras* (pan-*ras*-10) was purchased from Du Pont Co., Wilmington, DE. The washed membranes were incubated with HRP-labeled goat IgG as a secondary antibody for 20 to 60 min at room temperature. After washing, the membranes were developed with peracid followed by luminol and an enhancer following the instructions of the manufacturer (ECL; Amersham Corp., Arlington Heights, IL). The oxiaid product was detected by immediately exposing with Kodak X-Omat Xac-5 film.

### Isolation of RNA and Northern Assays

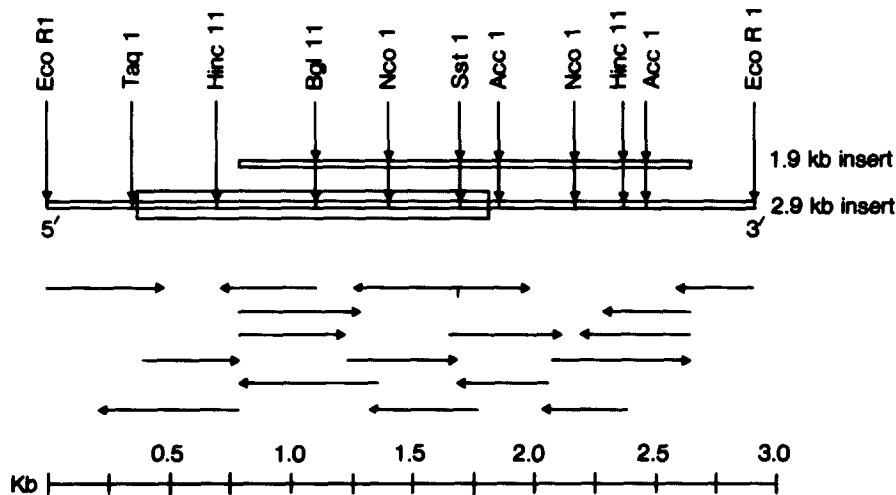
Confluent cultures of *ras*-transfected cells were exposed to zinc sulfate or to buffer alone (34) for 24 h. Total RNA was extracted according to Choy et al. (6). 60  $\mu$ g of RNA was electrophoresed on 1% agarose gels and transblotted onto Nytran Nylon membranes. The mRNA transcript for the encoded protein was detected using  $^{32}\text{P}$ -labeled 1.7-kb fragment of the cDNA that contained the open reading frame. Blots were reprobated with  $^{32}\text{P}$ -glyceraldehyde-3-phosphate dehydrogenase cDNA to control for RNA loading.

### Expression of RHAMM in Bacteria

Oligonucleotide primers corresponding to the second initiation codon and the stop codon were prepared and used to amplify the complete open reading frame by polymerase chain reaction. The second initiation codon was chosen since the size of the protein generated by *N*-glycanase digestion of the 58-kD protein closely matched this (Turley, E. A., K. Hoare, and V. Cripps, manuscript submitted for publication). Polymerase chain reaction generated a 1.3-kb DNA fragment which was cloned into the PGEX-2T expression vector (22) and transformed into *Escherichia coli* (JM101). Induction of protein expression in cultures of transformed *E. coli* with 0.2 mM isopropyl- $\beta$ -D-thiogalactopyranoside resulted in the production of an insoluble recombinant glutathione-S-transferase fusion protein. Insoluble fusion protein was solubilized by the addition of 4 M urea, 0.05 M Tris, 1 mM EDTA, 1% Triton X-100, pH 8.0, followed by gradual removal of the urea by dialysis into 2 M urea, 0.05 M Tris, 1 mM EDTA, 1% Triton X-100, pH 8.0, and then PBS, 1% Triton X-100, pH 7.4. Solubilized fusion protein was subsequently purified by affinity chromatography on glutathione-agarose (22).

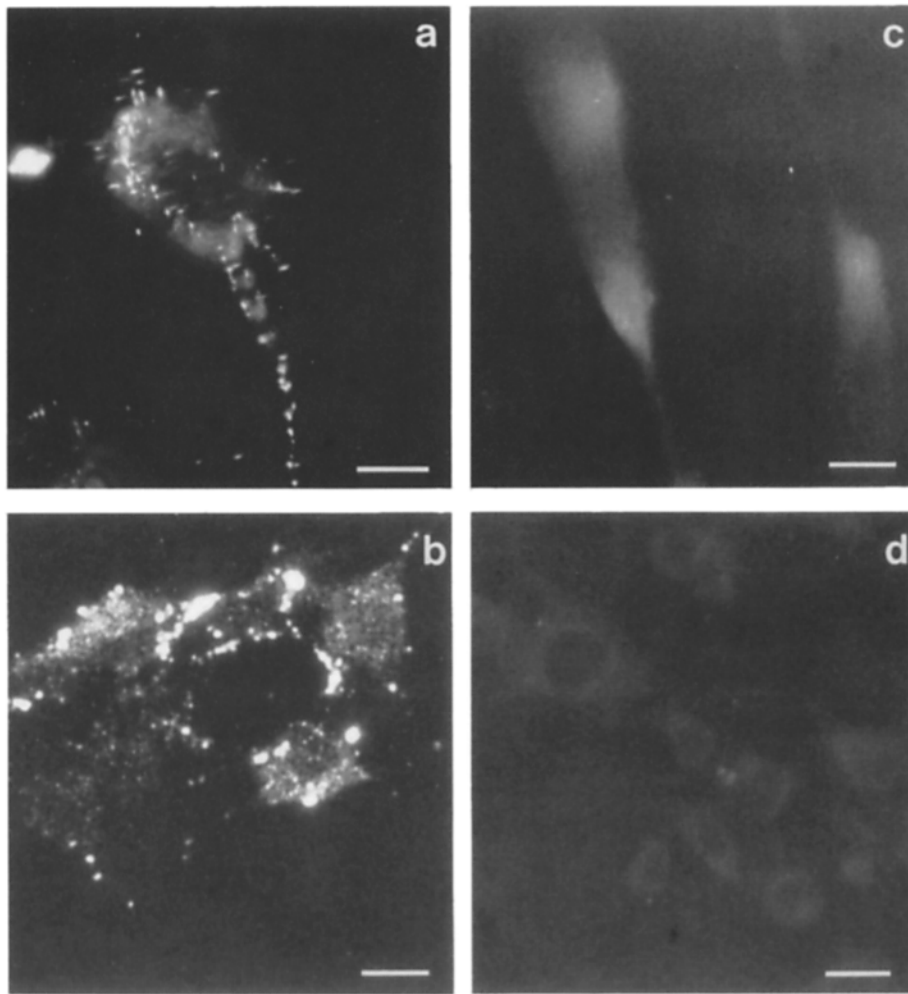
### HA Binding Assays

Lysates (25  $\mu$ g) from induced bacteria containing either the parental PGEX-2T vector or the PGEX-2T vector with RHAMM DNA were electrophoresed on 10% SDS-PAGE and transblotted onto nitrocellulose membranes.



<b>ATG</b>	Gln	Ile	Leu	Thr	Glu	Asp	Leu	Ala	Leu	Glu	Arg	Gln	Glu	Tyr	Glu	Lys	Leu	Gln	57
CAA	AAA	GAA	TTC	Gln	Ser	Gln	Ser	Leu	Leu	Gln	Gln	Glu	Lys	Glu	Leu	Ser	Ala	Arg	114
CTC	GAG	CAG	CAG	Leu	Cys	Ser	Phe	Gln	Glu	Glu	<b>Met</b>	Thr	Ser	Glu	Lys	Asn	Val	<b>Phe</b>	171
<b>AAA</b>	<b>GAA</b>	<b>GAG</b>	<b>CTA</b>	<b>AGC</b>	<b>GCG</b>	<b>CTG</b>	<b>GCT</b>	<b>GAG</b>	<b>TTG</b>	<b>GAT</b>	<b>GGC</b>	<b>GTC</b>							228
CAG	ACT	GAA	AGC	Leu	Val	Lys	Gln	Leu	Glu	Glu	Thr	Lys	Ser	Thr	Ala	Glu	Gln	285	
CTC	ACC	CCG	CTC	GAC	Aen	Leu	Leu	Arg	Glu	Lys	Glu	Val	Glu	Leu	Glu	Lys	His	Ile	342
GCT	CCT	CAA	GCC	Ala	Ile	Leu	Ile	Ala	Gln	Glu	Lys	Tyr	Asn	Asp	Thr	Ala	Gln		399
<b>AGT</b>	<b>CTG</b>	<b>AGG</b>	<b>GTC</b>	<b>GTC</b>	<b>ACT</b>	<b>GCT</b>	<b>CAG</b>	<b>TTG</b>	<b>GAA</b>	<b>ACT</b>	<b>GTG</b>	<b>CAA</b>	<b>GAG</b>	<b>AGG</b>	<b>TAG</b>	<b>AAT</b>	<b>GAC</b>	<b>AGA</b>	456
<b>GCA</b>	<b>CAG</b>	<b>AGT</b>	<b>CTG</b>	<b>AGC</b>	<b>GTC</b>	<b>AGT</b>	<b>GCT</b>	<b>CAG</b>	<b>TTG</b>	<b>GAA</b>	<b>AGT</b>	<b>GAG</b>	<b>CAA</b>	<b>GAG</b>	<b>AGG</b>	<b>TAG</b>	<b>AAT</b>		513
<b>GAC</b>	<b>ACA</b>	<b>GCA</b>	<b>CAG</b>	<b>AGT</b>	<b>GTC</b>	<b>AGC</b>	<b>GAG</b>	<b>GTC</b>	<b>ACT</b>	<b>GCT</b>	<b>CAG</b>	<b>TTG</b>	<b>GAA</b>	<b>AGT</b>	<b>GAG</b>	<b>GAA</b>	<b>CAG</b>	<b>AGC</b>	570
<b>TAC</b>	<b>AAT</b>	<b>GAC</b>	<b>ACA</b>	<b>GCA</b>	<b>CAG</b>	<b>ACT</b>	<b>CTG</b>	<b>AGG</b>	<b>GAG</b>	<b>GTC</b>	<b>ACT</b>	<b>GCT</b>	<b>CAG</b>	<b>TTG</b>	<b>GAA</b>	<b>AGT</b>	<b>GTC</b>	<b>CAA</b>	627
<b>GAG</b>	<b>AGC</b>	<b>TAC</b>	<b>AAT</b>	<b>GAC</b>	<b>ACA</b>	<b>GCA</b>	<b>CAG</b>	<b>ACT</b>	<b>CTG</b>	<b>AGC</b>	<b>CAC</b>	<b>GTG</b>	<b>AGT</b>	<b>GCT</b>	<b>GTC</b>	<b>TTG</b>	<b>GAA</b>	<b>AGC</b>	684
TAT	TAT	TAT	TAT	TAT	TAT	TAT	TAT	TAT	TAT	TAT	TAT	TAT	TAT	TAT	TAT	TAT	TAT	TAT	741
CAA	GAA	AAA	GTA	GCT	ATG	GCT	GAA	AAA	AGT	GTA	GAA	GAT	GTT	CAA	CAG	CAG	CAG	Ile	798
ACA	GCT	<b>GAG</b>	<b>AGC</b>	<b>ACA</b>	<b>AAT</b>	<b>CAA</b>	<b>GAA</b>	<b>TAT</b>	<b>GCA</b>	<b>AGG</b>	<b>ATG</b>	<b>GTT</b>	<b>CAA</b>	<b>GAT</b>	<b>TTG</b>	<b>CAG</b>	<b>AGC</b>	<b>AGA</b>	855
<b>TCA</b>	<b>ACC</b>	<b>TTA</b>																	912
ACT	ASP	Leu	Lys	Aen	Leu	Arg	Gln	Gln	Asp	Glu	Asp	Phe	Arg	Lys	Gln	Leu	Glu	969	
GAG	AAA	GCA	AAA	ACA	GCA	GAG	AAA	GAA	AAT	GTA	ATG	ACA	GAA	TTA	ACC	ATG	GAA	1026	
ATT	Aen	Lys	Trp	GCT	Leu	Tyr	TAT	Glu	Glu	Tyr	Glu	Lys	Thr	Lys	Pro	Phe	Gln	1083	
CAA	CTC	GAT	GCC	TTT	GAA	GCC	GAG	AAA	CAG	GCA	TTG	TTG	AAT	GAA	CAT	GAT	GCT	1160	
CAG	CAG	CAG	CTA	Aen	Lys	Ile	Arg	Asp	Ser	Tyr	Ala	Gln	Leu	Leu	Gly	His	Gln	1197	
CTA	AGG	CAA	AAA	ATC	AAA	CAT	GTT	CTC	AAA	TTG	AAA	GAT	GAA	AAT	AGC	CAA	CTC	1254	
TCC	GAG	CTC	TCA	AAA	CTC	GCA	TCT	CAG	CTT	GTT	Lys	Arg	Lys	CAA	AAT	GAG	CTC	1311	
CTT	GAG	GGA	GAA	TTA	GAT	AAA	GCT	CTC	GCC	ATC	AGA	CAC	TTT	ASP	CCT	TCC	TCC	1368	
TTT	TCT	GAT	GCA	TCT	AGC	GAG	AAT	TTT	ACT	CCA	TTA	AAA	GAA	GCC	AAC	CCA	AAC	1422	
Cys	TGC	TGA																	

Figure 1. Restriction map and sequence of the complete 2.9-kb cDNA clone encoding the 52-58 HARC protein(s). Blocking pAbs and mAbs to HARC were prepared and used to screen a  $\lambda$ gt11 3T3 cDNA expression library (Clontech). A restriction map was constructed using the enzymes (Un. States Biochem. Corp.) indicated in the restriction map. The open reading frame of the clones is boxed. The sequencing strategy is shown below the cDNA clones. Both antibodies to peptides encoded in the cDNA (*underlined sequences*) and a radiolabeled ACCI fragment of the 1.9-kb insert were used to isolate the 2.9 complete cDNA. The amino acid sequences are shown above the DNA sequences. Two possible initiation codons are indicated with highlighted letters. Potential N-glycosylation sites are marked with asterisks and possible signal sequences are underlined with broken lines. The stop codon is indicated by highlighted letters. The sequence data are available from EMBL/GenBank/DBJ under accession No. X-64550.



**Figure 2.** Immunofluorescent localization of encoded protein to the surface of live cells. To demonstrate whether or not encoded protein localized to the cell surface, H-*ras*-expressing cell monolayers were processed with (a) pAb to peptide II. (b) As in a but photographed with a confocal microscope. (c) Cells were treated lightly with trypsin before staining with primary antibody. (d) Cells were incubated with nonimmune rabbit IgG. Primary antibodies were visualized with RITC-conjugated goat anti-rabbit IgG and localized RHAMM to the cell surface. Trypsin treatment abolished staining and samples incubated with nonimmune sera did not exhibit staining. Bars: (a and c) 17.2  $\mu\text{m}$ ; (b) 38  $\mu\text{m}$ ; (d) 43  $\mu\text{m}$ .

Nitrocellulose was blocked by incubating with 5% defatted milk in PBS for 1 h followed by incubation in the presence of biotinylated HA (Turley, E. A., K. Hoare, and V. Cripps, manuscript submitted for publication). Nitrocellulose was washed for 1 h in TBS containing 0.05% Tween and bound HA was detected by incubation with streptavidin-HRP (1:1,000 dilution; Sigma Chem. Co.), followed by visualization with chemiluminescence (ECL; Amersham Corp.).

## Results

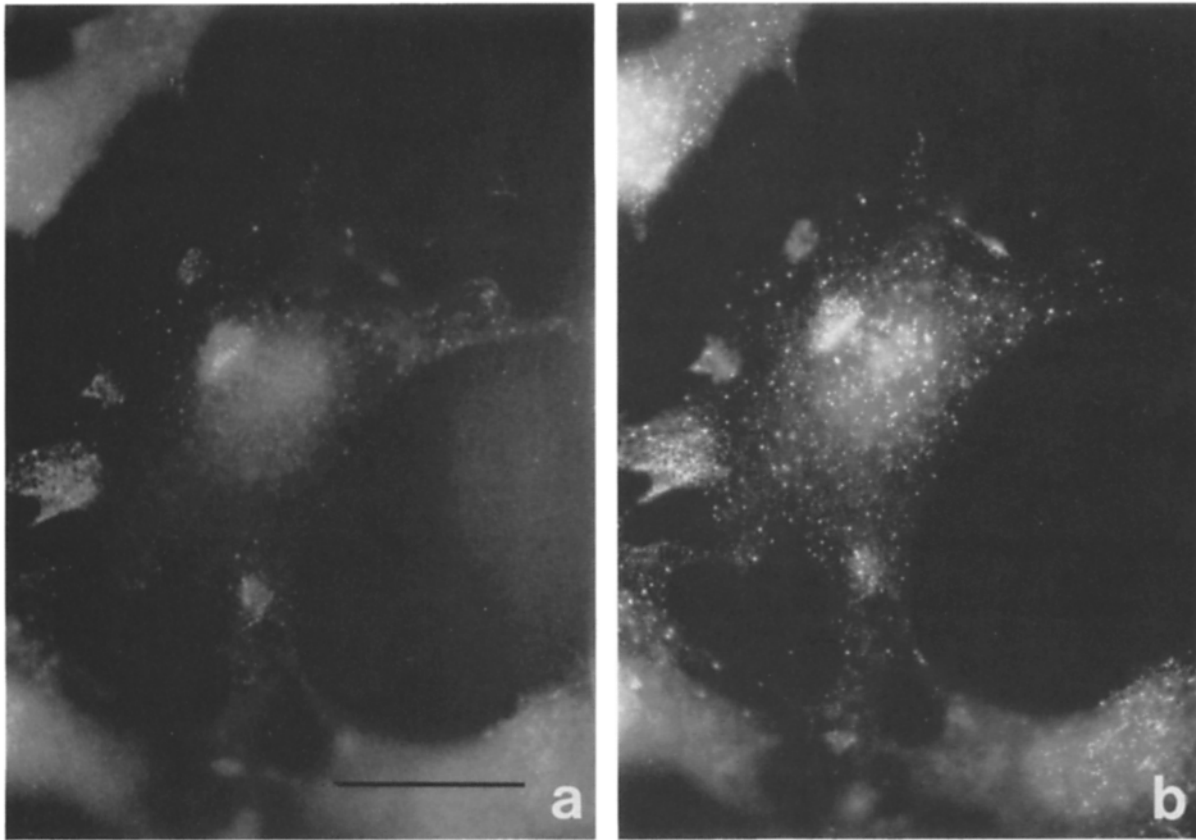
### Isolation of a cDNA Encoding RHAMM

A clone containing a 1.9-kb insert was isolated by screening a 3T3 cell cDNA expression library in  $\lambda\text{gt}11$  with both a mAb (designated 3T3-3) and a pAb to HARC. Sequencing of the insert revealed an open reading frame corresponding to a 340-amino acid residue of a COOH-terminal protein segment that did not include an initiation codon (Fig. 1). Additional clones coding for the same protein were isolated by rescreening the library with a 0.7-kb radiolabeled *AccI* restriction fragment of the 1.9-kb cDNA and with pAb to synthetic peptides mimicking segments of the deduced sequence (peptides I and II; Fig. 1). A clone containing a 2.9-kb insert was positive in both screenings and was further characterized (Fig. 1). Restriction mapping and sequencing of this insert demonstrated that it contained a complete open reading frame and the original 1.9-kb cDNA sequence in its central region (Fig. 1). The sequence was unique and did not bear

significant homology to other proteins registered in NBRF or EMBL data banks or to factors known to be involved in *ras*-regulated locomotion (14). Like previously characterized proteins such as p53 (36), it contained two possible initiation codons, encoding proteins of either 52.2 or 46.7 kD, respectively (Fig. 1). The encoded protein was rich in glutamic acid, lysine, glutamine, and leucine. It had a  $\text{P}_i$  of 5.2, was hydrophilic, and most of the polypeptide was predicted to occur as an alpha helix by Chou-Fasman analyses (5). The most notable feature of the deduced sequence was a 21-amino acid stretch (which corresponded to peptide I, underlined in Fig. 1) that was repeated five times near the  $\text{NH}_2$  terminus. The predicted protein contained eight putative *N*-glycosylation sites, five of which were concentrated within the repeated motif. The protein also contained clusters of positively charged amino acids throughout the open reading frame. It did not encode a hydrophobic sequence long enough to span the plasma membrane and possible signal sequences following either initiation codon were weak (Fig. 1).

### The Encoded Protein Occurs at the Cell Surface

The encoded protein occurred at the cell surface as demonstrated by positive immunofluorescent staining for RHAMM using live cells (Fig. 2, a, and b). Further, the majority of staining occurred on cell processes and at the media surface of cells as demonstrated by optically "sectioning" cells using a confocal microscope. Staining using pAb to peptide II en-



**Figure 3.** Immunofluorescent localization of encoded protein relative to HARC proteins. Double immunofluorescence studies of zinc-induced fibroblasts were performed using (a) mAb 3T3-5 which specifically reacted with 52-58-kD proteins of HARC and (b) pAb to peptide II (nucleotide sequence 804-864). The mAb was detected with FITC anti-mouse IgG and the pAb was detected with RITC anti-rabbit IgG. Both antibodies were strikingly localized in the ruffles and processes of zinc sulfate-induced fibroblasts. Preimmune sera showed no immunofluorescence (data not shown). Bar, 16  $\mu$ m.

coded in the cDNA (nucleotide sequence 804-864) was abolished by light trypsin treatment (Fig. 2 c). Fluorescence-activated cell sorter analysis of these cells showed quantifiable staining providing further evidence of a cell surface localization (data not shown). In fixed cells, where lamellae were well-preserved, the encoded protein was seen to strikingly accumulate in the ruffles and processes of H-*ras*-transformed cells (Fig. 3). Staining also occurred intracellularly in the perinuclear region (data not shown). This distribution is typical of molecules that regulate cell locomotion (1, 14). It is further noted that the pAb to peptide I colocalized precisely with mAb to the 58-kD HARC protein (Fig. 3).

#### **Mutant H-*ras* Gene Regulates Expression of RHAMM**

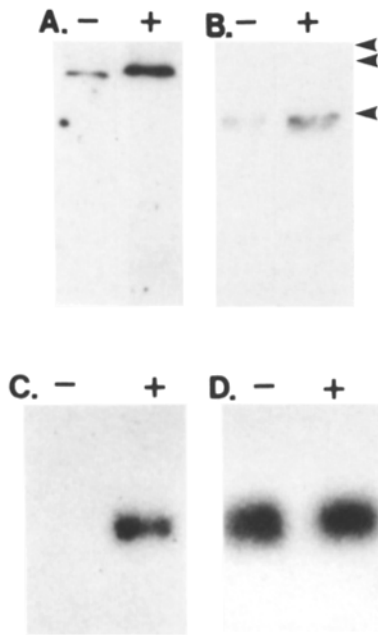
Zinc sulfate induction of a metallothionein-regulated mutant H-*ras* gene transfected into 10T1/2 fibroblasts (212 cells; reference 26) increased the expression of 58-kD proteins (Fig. 4 A; +, -) detected by pAb to peptide II (nucleotide sequence 804-864, Fig. 1). p21 *ras* proteins were also increased upon induction (Fig. 4 B, +, -). An mRNA transcript of 5.2 kb was detected using a cDNA probe encoding the open reading frame of the 2.9-kb cDNA insert and was increased 24 h after zinc induction of p21 H-*ras* expression (Fig. 2 c). Northern blots were reprobed with glyceraldehyde-3-phosphate dehydrogenase cDNA for a loading control (Fig. 4 D).

#### **Antibodies to Peptides Encoded in the RHAMM cDNA Block Locomotion**

Direct evidence for a role of the encoded protein in H-*ras*-regulated cell locomotion was demonstrated in experiments designed to test whether antibodies to the encoded protein inhibited cell locomotion. As noted previously (34), induction of the mutant H-*ras* gene with zinc sulfate activated an HA-dependent motility mechanism in mutant H-*ras*-transfected fibroblasts (Fig. 5). Antibodies to peptide II (nucleotide sequence 804-864, Fig. 1) specifically inhibited *ras*-regulated locomotion (Fig. 5).

#### **Bacterially Expressed RHAMM Binds to HA**

An insert containing the open reading frame from the second initiation codon was expressed in bacteria as a glutathione-S-transferase fusion protein. The fusion protein was analyzed as described in Materials and Methods and separated from bacterial proteins by electrophoresis on 12.5% SDS-PAGE. RHAMM was identified with mAb 3T3-5, specific to the 52- and 58-kD HARC proteins, on immunoblots as a 75-kD protein (Fig. 6). About 26 kD is due to the presence of the glutathione-S-transferase peptide with the remaining 45-50 kD representative of the recombinant RHAMM peptide. The molecular mass of the recombinant peptide is therefore in agreement with that observed for the deglycosylated 58-kD protein isolated from fibroblasts (Turley, E. A., K. Hoare,



**Figure 4.** Expression of encoded protein is regulated by the mutant *H-ras* oncogene. Cell lysates (*A* and *B*) were prepared from buffer- (–) and zinc sulfate-treated (+) fibroblasts transfected with mutant *H-ras* under the control of a metallothionein promoter (26). Transblots from SDS-PAGE were probed with (*A*) pAb to peptide II (nucleotide sequence 804–864) or (*B*) RAS-10 antibody. Protein standards are marked by arrowheads and include, from the top of the gel, phosphorylase b (97.4 kD), BSA (68 kD), and trypsin inhibitor (21.5 kD). Induction of the mutant *H-ras* gene increased expression of the encoded protein. RNA (*C* and *D*) was isolated from buffer- (–) and zinc sulfate-treated (+) *H-ras*-transfected fibroblasts. RNA was hybridized with a <sup>32</sup>P-labeled cDNA fragment containing the open reading frame (*C*). Blots were reprobed with H-glyceraldehyde-3-phosphate dehydrogenase cDNA to control for RNA loading (*D*). The induction of the mutant *ras* gene with zinc sulfate increased the expression of a 5.2-kb mRNA transcript.

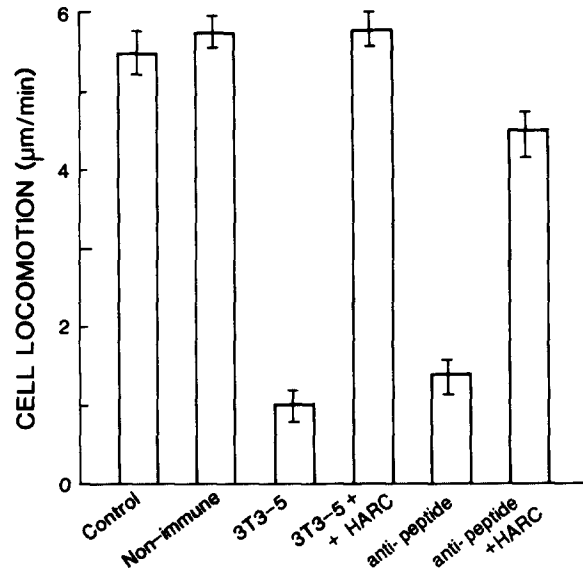
and V. Cripps, manuscript submitted for publication) as well as that deduced from the nucleotide sequence. This protein specifically bound to biotinylated HA and was competed with excess labeled HA (Fig. 6). Bacterial lysates that contained plasmids without the insert did not bind HA (Fig. 6).

#### **RHAMM Is Antigenically Related to the 58- and 52-kD HARC Proteins**

The encoded protein was shown to be antigenically related to the 52–58 kD of HARC (32) which are the HA-binding proteins of this complex (Turley, E. A., K. Hoare, and V. Cripps, manuscript submitted for publication). Thus, in transblot immunoassays and using purified HARC proteins as substrate (Fig. 7 *A*), PabI cross-reacted with the 58- and 52-kD proteins (Fig. 7 *C*). The blocking mAb 3T3-5 also cross-reacted with these proteins (Fig. 7 *B*).

#### **Discussion**

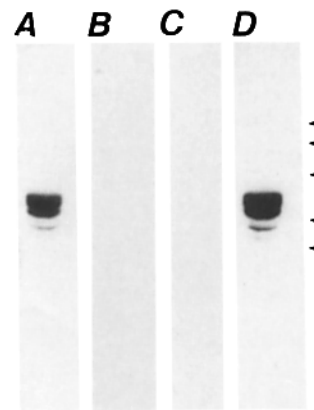
Oncogenic transformation by both *src* and activated *ras* genes have been reported to promote synthesis of HA (21, 32, 34) and the growth of many human tumors is accompanied by elevated levels of this glycosaminoglycan in the serum or in tissue surrounding the tumor (19, 25, 27). Tumor



#### **TREATMENT**

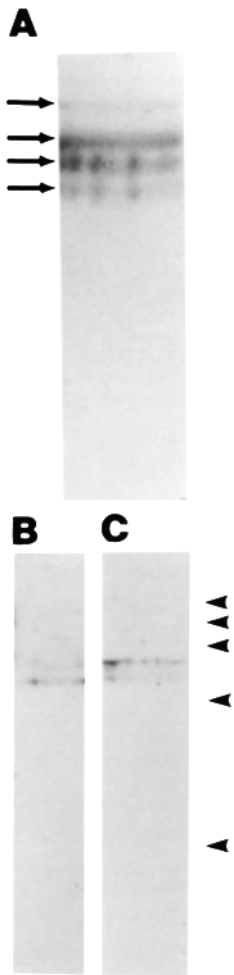
**Figure 5.** The encoded protein regulates *ras*-promoted cell locomotion. Mutant *H-ras*-transfected cells (26) induced with zinc sulfate were filmed by video timelapse and analyzed with a Dynacell program (Carl Zeiss, Inc.). The effect of nonimmune sera, mAb 3T3-5 to HARC and pAb to peptide II (nucleotide sequence 804–964) on HA-promoted locomotion was analyzed. Both mAb 3T3-5 to HARC and pAb to peptide II inhibited cell locomotion in response to HA. This effect was reversed by the addition of excess HARC proteins. Nonimmune sera had no effect on locomotion relative to controls. Values represent the mean + SEM. *n* = 50 cells.

cells often show increased responsiveness to HA-stimulating factors (4, 13) and, recently, the increase in locomotion of a *ras*-transformed cell line has been shown to be mediated by HA (34). We have isolated and characterized a cDNA



**Figure 6.** Bacterially expressed RHAMM binds to biotinylated HA. Lysates from induced bacteria plasmids with either an insert encoding RHAMM (lanes *A*, *B*, and *D*) or no insert (lane *C*) were electrophoresed on SDS-polyacrylamide gels and transblotted. Biotinylated HA was added to the blot in the absence (lanes *A* and *C*) or presence of 100-fold excess unlabeled HA (lane *B*). Bound HA was then detected with streptavidin-HRP and visualized with chemiluminescence. Transblots of bacterial lysates containing the insert were also incubated with monoclonal antibody 3T3-5 (lane *D*) to the 58- and 52-kD HARC proteins. These results show that HA bound to the expressed protein and binding was competed with unlabeled HA. mAb 3T3-5, which blocks locomotion, specifically recognized the expressed protein. The standards are marked with arrowheads and include  $\alpha_2$  macroglobulin (180 kD);  $\beta$ -galactosidase (116 kD); fructose-6-phosphate kinase (84 kD); pyruvate kinase (58 kD); and fumarase (48.5 kD).

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**Figure 7.** Immunoreactivity of anti-peptide antibody to the encoded protein. HARC proteins were purified from spent culture media, and then fractionated by SDS-PAGE and transblotted. HARC proteins were visualized with (lane A) India ink and had molecular masses of 72, 68, 58, and 52 kD (arrows). The 52–58-kD proteins reacted specifically with (lane B) mAb 3T3-5 (blocker of locomotion) to HARC, and (lane C) pAb to peptide I (nucleotide sequence 372–435). Molecular mass protein standards are marked by arrowheads and include from the top of the gel, myosin (200 kD); phosphorylase b (97.4 kD); BSA (68 kD); ovalbumin (43 kD); and trypsin inhibitor (21.5 kD).

clone from a  $\lambda$ GT11 cDNA expression library prepared from 3T3 cells that encodes a 48- or 52-kD protein, depending upon the initiation codon used. This protein is unique, occurs on the cell surface, is regulated by the H-*ras* oncogene, and mediates locomotion of *ras*-transformed cells responding to HA. Furthermore, in transblot assays it specifically binds to HA. Its unique structure and HA-binding properties indicate that it is a new HA receptor. Further, its role in cell locomotion predicts that it will play an important role in developmental, disease, and repair processes. Based on these two functional properties, this protein is referred to as RHAMM, an acronym for receptor for HA-mediated motility.

The encoded protein appears to be identical or related to the 58- and 52-kD proteins of an HARC originally isolated by one of us (32). These proteins have been shown to be involved in locomotion (2, 33, 34) and, recently, to also bind to HA (Turley, E. A., K. Hoare, and V. Cripps, manuscript submitted for publication). Thus, pAb specific to the encoded protein cross-reacts with the 52, 58-kD HARC protein. Conversely, a battery of monoclonal antibodies that are specific for the 52- and 58-kD HARC proteins cross-react with bacterially expressed RHAMM. These observations, combined with (a) the ability of the HARC proteins to reverse the blocking effect of pAb specific to the encoded protein on cell locomotion (Fig. 5), (b) the precise colocalization of the two antigens, (c) their common regulation by the H-*ras* oncogene, and (d) their ability to bind to HA and to mediate

*ras*-regulated locomotion provide strong evidence that the gene product isolated is identical to the HARC protein.

The HARC is released into the supernatant media (27) and contains several components that can be recovered by HA affinity purification, including 72-, 68-, 58-, and 52-kD proteins (32). Interestingly, the 52-kD HARC protein is not observed in cell lysates but only in the released form. The cell surface form of the complex, which is held together by as yet unidentified mechanisms, appears to be transmembrane (29). Ultrastructural studies reported elsewhere localize HARC (29) to the cell surface but the cDNA encoding RHAMM does not encode a long enough hydrophobic region to allow it to span the membrane. Either RHAMM is associated with an unidentified transmembrane component of, for instance, the HARC complex, or two forms of RHAMM exist: a soluble and membrane-associated form. The first prediction would resemble the organization observed for some animal lectins (1) and the structure that is predicted for an elastin-laminin receptor complex in which the laminin and elastin-binding component binds to a transmembrane receptor (18, 35).

Although the cDNA encoding the 56-kD protein does not contain a strong signal sequence, it is clearly located at the cell surface, as demonstrated by immunofluorescent staining of live cells with antibodies specific to RHAMM. Although somewhat unusual, a similar lack of signal sequence has been noted for other cell surface receptors including lymphocyte Fc receptor for IgE (10), transferrin receptor (15), liver asialoglycoprotein receptor (9), and the high-affinity laminin receptor (18, 35). It is of course possible that we have cloned a soluble form of RHAMM and that a membrane form also exists. We will further investigate these possibilities.

Previous to this report, several HA-binding proteins, including CD44 (3, 23, 24), hyaluronectin/versican (11), aggrecan (7), and link protein (8, 16) have been described. These proteins share a homologous region containing the HA binding domain (8). The predicted sequence of the cDNA reported here is unrelated to these molecularly characterized HA-binding proteins and the encoded protein therefore likely represents a new type of HA receptor. It is perhaps relevant that the 21-amino acid repeat motif identified from nucleotide sequence 372–684 (Fig. 1) contains a series of amphipathic alpha helices (5). Similar structures may be important in the binding of proteins of the clotting cascade to a related glycosaminoglycan, heparin (12).

In summary, we have characterized a novel hyaluronan receptor that is directly involved in tumor cell locomotion. The regulation of its expression by the *ras* oncogene predicts that it plays an important role in oncogenesis and possibly morphogenesis.

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