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 λGT11 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. Antibodies 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) (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 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 λGT11 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.

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-
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Cells were grown on glass cover slips for 24 h after their subculture so that 804-864, or rabbit IgG) was then added at 1/xg/ml in defined DMEM to Ca ++, Mg ++-free Hanks' solution or 0.25% trypsin in Ca ++, Mg ++-free Hanks' solution. The cultures were washed and incubated with fluorescein-labeled goat anti-rabbit IgG (to detect mAb 3T3-7) and rhodamine-labeled goat anti-rabbit IgG (to detect mAb to peptide II). 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 Centre, NY). Additional protein binding sites were blocked with 0.5% defatted milk. The membranes were preincubated for 1 h with 1/xg/ml of TBS containing 0.5% defatted milk, and then exposed to 0.001/xg/ml of HA and/or R2 vector, 1/xg/ml of TBS containing 0.5% defatted milk, and then PBS, 1% Triton X-100, pH 7.4. Solubilized fusion proteins were subsequently purified by affinity chromatography on glutathione-Sepharose and/or protein A-Sepharose. mAb 3T3-3, which blocks fibroblast growth factor receptors, was chosen since the size of the protein generated by N-glycanase digestion of the fusion protein was approximately 35,000 Da.

Cloning and DNA Sequencing

A 3T3-cDNA library in agt11 was obtained from Clonetech (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 word search programs in GCG software. Additional homology searches were conducted with NBRF (6/90) and EMBL (5/90) using FASTA 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–neonycin 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 0.01/xg/ml of HA as previously described (34). Antibodies (mAb 3T3-3, 1/xg/ml, or pAb peptide II, 1/xg/ml) were added to zymosan cells for 0.5 h, and then exposed to 0.001/xg/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, the cultures were preincubated for 1 h at 4°C with 50/μg 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 Ca ++, Mg ++-free Hanks' solution or 0.25% trypsin in Ca ++, Mg ++-free Hanks' solution for 3 min. Primary antibody (pAb II, nucleotide sequence 804–864, or rabbit IgG) was then added at 1/xg/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 under 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 μm 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, or rabbit IgG) was then added at 1/xg/ml in DMEM to Ca ++, Mg ++-free paraformaldehyde for 10 min, and then incubated with 0.1 M glycine to quench autofluorescence. Fixed monolayers were washed, and then incubated with 1/xg/ml of the above antibodies overnight at 4°C. The monolayers were then washed again, 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 II). 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 single immunofluorescence samples with both filters (data not shown).

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. Houré, 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 (IM100). Induction of protein expression in cultures of transformed E. coli with 0.2 mM isopropyl-β-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 μg) from induced bacteria containing either the parental PEGX-2T vector or the PGEX-2T vector with RHAMM DNA were electrophoresed in 10% SDS-PAGE and transblotted onto Nitrocellulose membranes.
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 λgt11 3T3 cDNA expression library (Clonetech). 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 sequences (boxed). The numbers indicate the position of the last nucleotide of each cDNA. The restriction map and sequence data are available from EMBL/GenBank/DDBJ under accession No. X-64550.
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 λgt11 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 pI 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 NH₂ 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-

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*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 μm; (b) 38 μm; (d) 43 μm.
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 μm.

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,
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 immunosassays and using purified HARC proteins as substrate (Fig. 7 A), Pab I 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 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...
Clone from a λGT11 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 transfact assays it specifically binds to HA. Its unique structure and HA-binding properties indicate that it is a new HA receptor. It is 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 amphiphatic 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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