Requirement of the Hyaluronan Receptor RHAMM in Neurite Extension and Motility as Demonstrated in Primary Neurons and Neuronal Cell Lines

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The recently cloned and characterized hyaluronan (HA) receptor RHAMM (receptor for HA-mediated motility) has been shown to play a critical role in mechanisms underlying the motile capacity of a variety of peripheral cell types. Similarities in molecular processes that govern cell locomotion and growth cone migration prompted us to investigate whether RHAMM also contributes to neurite migration *in vitro*. In immunohistochemical studies of PC12 cells, NG108-15 cells and a neuroblastoma/spinal cord neuronal hybrid cell line (NSC-34 cells) as well as rat and human primary neurons, a punctiform RHAMM labeling pattern was detected in cell bodies, along processes, and at growth cones. By Western blot analysis, the cell lines expressed major RHAMM forms with apparent MW of 60, 75, and 116 kDa. Treatment of NG108-15 cells with dibutyryl-cAMP led to a clear increase in immunolabeling for RHAMM and enhanced expression of the 60 and 75 kDa forms. A polyclonal anti-RHAMM antibody that interferes with HA/RHAMM interaction significantly reduced neurite migration of each cell type examined, while another directed against a RHAMM repeat sequence thought to promote RHAMM receptor aggregation significantly stimulated neurite migration of NSC-34 and rat primary neurons. Different monoclonal anti-RHAMM antibodies had differential inhibitory actions on neurite movement. Low concentrations (ng/ml) of a peptide corresponding to an HA binding domain within RHAMM inhibited neurite migration. These results are the first to implicate RHAMM in the mediation of neurite motility and migration and to point to the potential importance of HA in this process.

[Key words: hyaluronan receptors, RHAMM, neurite migration, PC12 cells, NG108-15 cells, extracellular matrix]

Molecules of the extracellular matrix (ECM) and their receptors regulate adhesive interactions and signaling events in a number of cellular processes including growth and motility. Studies fo-

cused on, for example, fibronectin, laminin, vitronectin, collagen, and proteoglycans together with their receptors such as the integrins, high-affinity laminin/elastic receptor, and CD44 indicate that they are critical to tissue repair and embryogenesis. Numerous reports on the integrin and other classes of ECM receptors and their various ligands have also demonstrated important roles in neurite extension and neuronal cell migration (Bixby and Harris, 1991; Venstrom and Reichardt, 1993). Despite the presence of several hyaluronan (HA) binding proteins including hyaluronectin, versican/GHAP, and CD44 as well as high levels of the ECM component HA in the CNS (Werz et al., 1985; Delpech et al., 1989; Picker et al., 1989; Toole, 1990; Bignami et al., 1991, 1993a,b; Turley, 1991; Akiyama et al., 1993; Perides et al., 1993), little is known about HA function in neural tissue. Indeed, HA is generally considered to serve as a space-filling molecule that provides a watery environment enabling interactions between other cellular components (Margolis and Margolis, 1989). However, HA stimulates locomotion of several cell types *in vitro* (Toole, 1990; Laurent and Fraser, 1992; Turley, 1992) and may support the motility of neurons along morphogenetic migratory pathways (Bignami et al., 1991; Reichardt and Tomaselli, 1991). Since cell locomotion through tissue shares mechanistic commonalities with neurite migration through the ECM (Devoto, 1990), proteins that mediate the actions of HA on cell motility may also contribute to neurite migration and examination of HA receptors in neurites may clarify the role of HA in this process.

A logical candidate protein to examine is the protein RHAMM (receptor for hyaluronic acid-mediated motility). The amino acid sequence predicted from murine RHAMM cDNA (Hardwick et al., 1992) is unrelated to that of any other HA binding protein (Stamenkovic et al., 1989) and its increased expression in highly motile tumor cells, in fibroblasts, smooth muscle cells, and macrophages migrating in response to tissue injury suggests that it is fundamental to the process of cell locomotion (Turley et al., 1991; Savani et al., 1993). Moreover, RHAMM is found primarily at protruding lamellae of migrating cells (Turley and Torrance, 1985) and its expression is regulated by motility-promoting factors including serum and cytokines such as TGF β 1 (Samuel et al., 1993). Further, antibodies as well as peptides mimicking HA-binding domain sequences of RHAMM block HA-induced cell locomotion (Turley et al., 1991; Hardwick et al., 1992; Samuel et al., 1993). Most dramatic is that overexpression of RHAMM gene products in transfected 10T1/2 cells results in loss of contact inhibition and significantly increases cell locomotion without alterations of growth rates, which indicates that this gene is a critical regulator of cell contact and

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motile behavior (Yang et al., 1994). In view of the above, we have begun to investigate possible roles of this receptor in growth cone extension and motility. To explore a diversity of cell types in which RHAMM may be involved in neurite migration, RHAMM and the effects of anti-RHAMM reagents were examined in cultures of human and rat primary neurons as well as in several established neuronal cell lines.

Materials and Methods

Preparation of cultures. The three neuronal cell lines utilized in the present study were NSC-34 cells, NG108-15 cells, and PC12 cells. NSC-34 cells (provided by Dr. N. R. Cashman, Montreal Neurological Institute, McGill University, Montreal) comprise various subclones of a mouse–mouse hybridoma cell line produced by fusion of motoneuron-enriched primary spinal neurons with N18TG2 neuroblastoma cells. In addition to a multipolar neuron-like morphology, their neuronal properties include induction of myotube twitching when cocultured with mouse myotubes, expression of choline acetyltransferase and neurofilament proteins, and generation of action potentials (Cashman et al., 1992). In addition, they exhibit properties of adhesion to S-laminin similar to that of motoneurons (Hunter et al., 1991). NSC-34, PC12, and NG108-15 cells were passaged at least once after thawing and maintained in 75 cm² tissue culture flasks (Falcon) in DMEM1 [Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 2.5 gm/liter sodium bicarbonate, 5 gm/liter glucose, and 1% antibiotic-antimycotic solution (Sigma Chemical Co., St. Louis, MO)] in a 5% CO₂/95% air incubator at 36°C. Medium was changed twice weekly. Three to four days before cells were to be used for experiments, they were washed with HBSS and incubated for 2–3 min in 0.25% trypsin in HBSS at 36°C. Following the addition of an equal volume of DMEM1, adherent cells were washed off the bottom of the flask by pipetting and the suspension was centrifuged at 100 × *g* for 6 min. After aspiration of the supernatant and resuspension in DMEM1, the cells were replated into Primaria 35 mm culture dishes at a density, determined by microscopic inspection of aliquots, which yielded cultures of approximately 10–25% confluence 24 hr postplating. The PC12 and NG108-15 cell cultures were each divided into two groups. One group of PC12 cell cultures were treated daily for 3 d with nerve growth factor (NGF) (50 ng/ml) and one group of NG108-15 cell cultures were treated daily for 2–3 d with dibutyl-cyclic AMP (db-cAMP) (1 mM).

For preparation of fetal rat brainstem/spinal cord neurons, uteri were removed from timed-pregnant (E16–E18) Sprague–Dawley rats sacrificed by CO₂ narcosis, and washed in two or three changes of HBSS, and 8–12 embryos were aseptically removed and rinsed in several changes of HBSS at room temperature. Brainstem/spinal cords dissected from embryos were placed into a 35 mm dish containing ice-cold HBSS, stripped of meninges, washed in fresh cold HBSS, and finely minced with a sterile razor blade. Tissue pieces were transferred to a 15 ml centrifuge tube containing cold (10°C) HBSS, allowed to settle, washed several times in room temperature HBSS, and then incubated for 20 min at 36°C in 5 ml of 0.5% trypsin (Sigma Chemical Co.) in HBSS containing 50 KU/ml of DNase I type II-S (Sigma Chemical Co.) with gentle swirling every 4–5 min. The pieces were allowed to settle for 2–3 min, 3–4 ml of solution was removed, and 2 ml of MEM1–Eagle's Minimum Essential Medium (Sigma Chemical Co.) containing N2 supplements (GIBCO), 5% fetal bovine serum (Hyclone), 5% equine serum (Hyclone), 10 mM HEPES buffer, 6 gm/liter glucose, and 2.4 gm/liter sodium bicarbonate were added to neutralize the trypsin. The pieces were triturated 10–20 times with a flame-polished Pasteur pipette, the larger pieces were allowed to settle, and 1 ml of supernatant was transferred to a 15 ml centrifuge tube. After addition of 1 ml of MEM1 to the undissociated tissue pieces, the trituration procedure was repeated with pipettes of increasingly narrower bore until the tissue was largely dissociated. The cell suspension was brought to a volume of 15 ml with MEM1, plated into 100 mm Primaria (Falcon) culture dishes, and kept in a 5% CO₂/95% air incubator at 36°C for 3–4 hr to allow attachment of non-neuronal cells. The medium containing unattached cells was collected from the dishes, centrifuged at 100 × *g* for 6 min at 20°C, the pellet was resuspended in 25 ml of MEM1, diluted for appropriate seeding density, and 0.25 ml aliquots were plated either in Primaria (Falcon) 35 mm tissue culture dishes for tracking of neurite migration or onto poly-D-lysine-coated 12 mm round Carolina Micro Coverglasses (Carolina Biological) for immunohistochemical staining. Cultures were

maintained in a 5% CO₂/95% air incubator at 36°C for 2–3 d prior to use in experiments. Coverslips were coated by applying 0.2 ml of a 50 mg/ml solution of poly-D-lysine (30–70 kDa; Sigma Chemical Co.) in sterile 0.1 M boric acid/NaOH buffer (pH 8.4) to each coverslip. They were then dried at 36°C for 1–2 hr, rinsed in calcium and magnesium-free Hanks' Balanced Salt Solution (HBSS), and air dried.

Cultures of human fetal neurons were prepared from fetal brain tissue obtained from 12–16-week-old aborted fetuses. Brain tissues were obtained with approval from both Hospital and University ethics committees and with written consent from women undergoing elective termination of pregnancy. Tissues were placed into a sterile petri dish, healthy pieces of cortex were collected in 10 ml of medium A consisting of RPMI-1640 with 2.0 gm/liter sodium bicarbonate and 1% antibiotic-antimycotic solution (Sigma Chemical Co.), and blood vessels were stripped away. Tissues were then placed into a 50 ml centrifuge tube containing 10 ml of medium A, dissociated by trituration, and centrifuged at 200 × *g* for 10 min. The pellet was resuspended in 15 ml of medium B consisting of Opti-MEM with 2 ml/liter of N2 supplement (GIBCO), 2.4 gm/liter sodium bicarbonate, 5% fetal bovine serum, and 1% antibiotic-antimycotic solution (Sigma Chemical Co.), and 5 ml of the cell suspension was added to 75 cm² flasks containing 20 ml of medium B. The cells were grown in a 5% CO₂/95% air incubator at 36°C for at least 4 weeks. After the first 3 weeks in culture, the medium was changed and successive changes were then made weekly. When numerous well-developed neurons with extensive neurite growth and nonvacuolated cytoplasm were seen growing over a glial cell layer, the flasks were hand shaken and the medium containing dislodged neurons was pooled in 50 ml centrifuge tubes and centrifuged at 200 × *g* for 10 min. The pellet was resuspended in 4–6 ml of medium B and the suspension was plated into 35 mm Primaria (Falcon) tissue culture dishes as a concentration of 1 ml/dish. The neurons were maintained in culture for 1–2 weeks until taken for immunohistochemistry or neurite tracking experiments.

Anti-RHAMM antibodies and RHAMM peptides. Two polyclonal and a variety of monoclonal anti-RHAMM antibodies were utilized in the present study. A polyclonal antibody (designated A-268) was prepared against a peptide corresponding to the amino acid sequence 268–288 encoded in RHAMM cDNA (Hardwick et al., 1992). Another (designated A-124) was prepared against a peptide corresponding to the RHAMM amino acid sequence 124–144. As antibody A-268 has previously been shown to block fibroblast (Hardwick et al., 1992), macrophage (Savani et al., 1994a), and glial cell locomotion *in vitro* (Turley et al., 1994), while A-124 was found to stimulate locomotion of fibroblasts (Hall et al., 1993, 1994), both of these antibodies were tested for their effects on neurite migration. A total of 15 mouse monoclonal antibodies were prepared against native, purified RHAMM as previously described (Turley et al., 1991) and some of these have been reported to influence the motility of a variety of peripheral cell types (Turley et al., 1991, 1993; Pilarski et al., 1993) were also examined here. The specificity of these polyclonal and monoclonal antibodies was demonstrated by their detection of RHAMM fusion protein and their recognition of corresponding RHAMM isoforms in cell lines as determined by Western blots (Savani et al., 1994a), and abolition of this reactivity after preadsorption with RHAMM fusion protein (Hardwick et al., 1992). Moreover, as shown by truncation of recombinant RHAMM protein (Savani et al., 1994a), the function-blocking polyclonal anti-RHAMM antibody A-268 specifically recognizes the epitope on RHAMM corresponding to the synthetic peptide sequence against which it was raised. Further, antisera depleted of RHAMM antibodies by chromatography over a RHAMM fusion protein column no longer detected RHAMM on Western blots (not shown) or in fixed cultures. In addition, preimmune sera, control IgG, and scrambled peptide had no effect on cell locomotion or neurite migration. We have also found that affinity-purified antibodies A-268 and A-124 have the same effects on the motility of fibroblasts as anti-RHAMM serum (unpublished observations). We have firmly established that our antibodies against peptides derived from murine cDNA sequences react with rat RHAMM (Savani et al., 1994a).

A peptide corresponding to the sequence of the first HA binding domain (amino acids 401–411) of two such domains in RHAMM (Yang et al., 1993) was synthesized at the Manitoba Institute of Cell Biology and HPLC purified to 99% homogeneity. This peptide blocks cell motility *in vitro* (Samuel et al., 1993) and *in vivo* (unpublished observations) and was tested at various concentrations for effects on neurite migration. As a control procedure, the effects of a scrambled version of this peptide was examined in some cultures.

Immunohistochemistry. Randomly selected groups of 9–20 cultures of each of the cell types prepared as described above were taken for immunohistochemical staining at the same time that others were taken for analysis of neurite extension and motility. Cultures were fixed and stained essentially as previously described (Turley et al., 1994). Briefly, after aspirating the medium, cultures were rinsed for 1–2 min with 0.1 M sodium phosphate buffer, pH 7.4 (PB), containing 0.9% saline (PBS) and then fixed with 4% paraformaldehyde in 0.1 M PB for 30 min at room temperature. Following a wash with PBS, cultures were incubated with various primary antibodies diluted in PBS containing 0.3% Triton X-100 (PBS-T). For immunolocalization of RHAMM, all cultures were incubated for 48 hr at 4°C with a 1:300 dilution of a previously characterized rabbit polyclonal anti-RHAMM antibody (A-268) (Hardwick et al., 1992). Some rat primary neuronal cultures were simultaneously incubated with a 1:1,000 dilution of a monoclonal antibody against neurofilament protein (Sternberger Monoclonal Inc.) for colocalization analysis of this neuronal marker with RHAMM. Primary neuronal cultures were also taken for immunostaining with a 1:500 dilution of a rabbit anti-neuron-specific enolase (NSE) antibody (Chemicon) for assessment of the distribution and density of neurons in cultures prepared at different times. After the primary incubation, cultures were washed three times for 20 min with PBS-T and incubated for 1.5 hr at room temperature with Cy3-conjugated sheep anti-rabbit IgG (diluted 1:250) (Sigma Chemical Co.) for detection of RHAMM. For labeling neurofilament protein, cultures were incubated with biotinylated horse anti-mouse IgG (diluted 1:100) (Vector), washed for 20 min once with PBS-T and twice with PBS, and further incubated with fluorescein isothiocyanate (FITC)-conjugated streptavidin (diluted 1:100 in PBS) (Amersham) for 1.5 hr at room temperature. For detection of NSE, cultures were incubated with FITC-conjugated donkey anti-rabbit antibody (Vector) diluted 1:100. All cultures were then washed in PBS followed by a rinse in Tris-HCl buffer, coverslipped with antifade medium (Valnes and Brandtzaeg, 1985), and taken for microscopic examination. In control cultures, no labeling was seen after omission of primary antibodies. In double labeling studies, no Cy3 fluorescence was seen after omission of anti-RHAMM antibody and no FITC fluorescence was seen after omission of anti-neurofilament protein antibody, indicating the absence of primary and secondary antibody cross-reactions. In addition, labeling for RHAMM was greatly reduced in cultures incubated with antisera depleted of anti-RHAMM antibody by affinity chromatography on a column linked with RHAMM fusion protein followed by reconstitution of serum to original protein concentration (Hardwick et al., 1992). Fluorescence was examined on a Leitz Dialux 20 fluorescence microscope using a N2 Ploemopak filter cube (excitation 530–560 nm; long pass 580 nm) for Cy3 (excitation max 552 nm; emission max 568–574) and a L3 Ploemopak filter cube (excitation 450–490 nm; bandpass 500–540 nm) for viewing FITC. The N2/L3 combination gives negligible Cy3 fluorescence with L3 illumination and, conversely, no FITC fluorescence with N2 illumination. For comparisons of RHAMM immunofluorescence staining intensity with anti-RHAMM serum and antibody depleted serum, cultures were photographed at the same time on the same roll of film and prints were prepared under identical conditions. This was also the case for comparisons of staining in PC12 and NG108-15 cells cultured in the presence and absence of NGF in the case of the former and db-cAMP in the case of the latter.

Western blots. Due to the mixed cell types present in primary neuronal cultures and the resulting ambiguity in assigning particular forms of RHAMM to particular cells types, only NG108-15, PC12, and NSC-34 cells were biochemically analyzed. Cells grown to about 50% confluence were harvested and lysed with RIPA buffer (Lipfert et al., 1992) containing the protease inhibitors PMSF (5 mg/ml), leupeptin (1 mg/ml), and aprotinin (1 mg/ml). Lysate protein concentrations were determined with a DC protein assay kit (Bio-Rad) and proteins were separated on 10% SDS-polyacrylamide (PAGE) gels with 5–10 mg of total protein loaded per lane. Nitrocellulose membranes containing transblotted proteins were blocked with defatted milk, incubated with anti-RHAMM antibody (A-268) (Hardwick et al., 1992) at a dilution of 1:2000, and developed by chemiluminescence using luminol and H_2O_2 (Amersham).

Neurite motility analysis. Neuronal cell lines and primary neurons were analyzed for neurite extension and motility 48–72 hr after plating. Immediately prior to monitoring of cell processes, cultures received a medium change to α -MEM medium buffered with sodium bicarbonate and 24 mM HEPES, equilibrated for 30 min and placed on a microscope stage heated to 37°C. Video images were viewed with a Hamamatsu camera linked to a monitor, and randomly selected fields containing

neurites were recorded utilizing an image analysis program (IMAGE 1, Universal Imaging, NY). Images were obtained every 5–15 min over a 1–2 hr period, depending on rates of neurite migration assessed upon initial examination of each cell type. Rates of neurite migration were subsequently calculated by tracking changes in their location from frame to frame over the recorded period with the aid of the image analysis program precalibrated for spatial displacement measurements. Only individual neurites that grew unobstructed and remained free of contacts with other elements were chosen for movement analysis. At the various concentrations indicated in Results, either polyclonal or monoclonal anti-RHAMM antibodies, nonimmune IgG (1 mg/ml), antisera (1:50 dilution) from which anti-RHAMM antibody had been removed by affinity chromatography on a RHAMM fusion protein affinity column, RHAMM-derived HA binding domain peptides, or corresponding scrambled peptides were added to cultures 15–30 min prior to commencement of imaging. Data gathered from measurement of between 20 and 30 neurites per experiment for each cell type were analyzed for significance of the effects of reagents by Student's *t* test. Values marked with asterisks in the figures were significant at a level of $p < 0.01$ or greater.

Results

Immunofluorescence

The majority of NG108-15 cells maintained in the absence of db-cAMP for 3 d after plating had large, flattened processes that often extended one-half or, very occasionally, one to two cell body lengths. Cells treated with db-cAMP exhibited both thin as well as thick bulbous processes with up to five cell body-lengths. In both untreated (Fig. 1A) and treated cells (Fig. 1B–D), RHAMM was localized throughout the cell bodies and was present in most processes. Intensity of cell labeling was clearly greater in cultures to which db-cAMP had been added. Labeling consisted of aggregates of either round or irregularly shaped puncta, and an increased concentration of these after db-cAMP treatment was particularly striking along swellings and at the ends of processes (Fig. 1D). A much finer granular labeling was also seen evenly distributed in the large, short, flat processes of db-cAMP-treated NG108-15 cells (not shown).

After 3 d in culture in the absence of NGF, PC12 cells tended to be clustered in small colonies, had a rounded appearance, and were almost entirely devoid of processes. These cells treated with NGF for 2 or 3 d had a more flattened, irregular morphology, and most, though not all, cells extended either short or long processes. In non-NGF-treated cells, weak to moderate punctate immunostaining for RHAMM was seen throughout the cell bodies and punctiform sites of more intense staining were seen discretely localized around the periphery of many cells (not shown). Treatment of PC12 cells with NGF led to a slight increase in cell body staining, moderate to intense staining of cell processes, particularly at their extremities, and a loss of the peripheral punctate staining seen in untreated cells (Fig. 1F). Immunolabeling of both NG108-15 (Fig. 1E) and PC12 (Fig. 1G) cells was greatly reduced in cultures processed with anti-RHAMM serum eluted from a RHAMM fusion protein affinity column.

Shortly after plating, nearly all NSC-34 cells extended processes measuring about one cell body length, and numerous cells with multiple, short neurites were still evident 3 d after plating. Some cells had several thin, smooth, highly branched, and occasionally heavily varicose processes that measured up to 150 μ m in length. Cell bodies and both short and long processes were consistently immunopositive for neurofilament protein (not shown). Immunolabeling for RHAMM in these cells had much the same appearance and distribution as seen in NG108-15 cells. Punctate immunoreactivity was localized in cell bodies and pro-

Figure 1. Immunofluorescence detection of RHAMM in NG108-15 and PC12 cells. NG108-15 cells were cultured in the absence (*A*) or presence (*B–E*) of db-cAMP, and PC12 cells (*F,G*) in the presence of NGF. Both untreated (*A*) and db-cAMP-treated (*B–D*) NG108-15 cells contain RHAMM, but labeling intensity is greater in the latter. Punctiform aggregates of labeling for RHAMM are seen both in cell bodies (*C*, arrows) as well as along and at the ends of processes (*C* and *D*, arrowheads). In PC12 cells, staining is seen in cell bodies and is distributed along processes (*F*, arrows) that form after NGF treatment. Little labeling is seen with immune serum depleted of anti-RHAMM antibody affinity chromatography (*E*, *G*). Magnification: *A*, *B*, and *E*, 225 $\times$; *C*, 380 $\times$; *D*, 820 $\times$; *F* and *G*, 200 $\times$.

Figure 2. Immunofluorescence detection of RHAMM in NSC-34 cells (*A* and *B*) and in primary neurons (*C–F*) prepared from E18 rat brainstem/spinal cord. In NSC-34 cells, patchy or punctate immunolabeling for RHAMM is seen throughout cell bodies as well as in long processes (*A* and *B*, arrows). In primary neurons double-labeled for RHAMM and neurofilament protein, photomicrographs of the same field (*C* and *D*) show that RHAMM-positive cells (*C*, arrows) are also labeled for neurofilament protein (*D*, arrows). Thin processes emerging from RHAMM-positive neurons (*E*, arrow) and extending over long distances over the surface of astrocytes exhibit intermittent labeling for RHAMM (*E*, arrowheads). No labeling is seen with anti-RHAMM serum depleted of antibody (*F*). Magnification: *A* and *E*, 170 $\times$; *B* and *F*, 260 $\times$; *C* and *D*, 330 $\times$; *E*, 230 $\times$.

cesses, tended to be more concentrated in long compared with short processes (Fig. 2*A,B*) and was more abundant at swellings along varicose processes than along intervaricose segments (not shown). Labeling was eliminated in cells processed with serum depleted of anti-RHAMM antibody (not shown).

Primary neurons prepared from embryonic rat brainstem/spinal cord tissue had a healthy appearance by 3 d after plating as judged by their density, phase brightness, and elaboration of

numerous processes over either a bed layer of astrocytes or on exposed plastic or poly-D-lysine-coated substratum. Cells with this neuronal morphology were found to be immunopositive for NSE (not shown). Neurons exhibited a wide range of staining intensity for RHAMM (Fig. 2*B,C*) in view of which it was difficult to discern whether all primary neurons expressed this protein. However, in cultures double stained for both RHAMM (Fig. 2*C*) and neurofilament protein (Fig. 2*D*), cells stained for

Figure 3. Immunofluorescence detection of RHAMM in primary neurons prepared from human fetal brain. Phase contrast (*A*) and immunofluorescence labeling for NSE (*B*) in the same field illustrate that neurons and neuronal processes (*A*, arrows) growing on a bed of astrocytes are NSE positive (corresponding arrows in *B*). Photomontage at a focal plane above the bed layer of astrocytes shows punctate labeling for RHAMM in neuronal cell bodies (*C*, arrows) and their processes (*C*, arrowheads). Magnification: *A* and *B*, 130 $\times$; *C*, 520 $\times$.

the latter were consistently found to be immunopositive for RHAMM. RHAMM immunoreactivity was concentrated in cell bodies as well as in short neurites and relatively thick initial processes, at swellings along beaded processes and intermittently along thin processes that extended considerable distances from neuronal cell bodies (Fig. 2*E*). Due to their presence on the surface of astrocytes, it was difficult to ascertain fine features of RHAMM labeling in neurons. The underlying astrocytes were also immunopositive for RHAMM, and these displayed a labeling pattern similar to that previously reported (Turley et al., 1994). All labeling was reduced in cultures processed with control serum (Fig. 2*F*).

Human primary neurons, as shown by phase contrast optics (Fig. 3*A*) and by NSE immunolabeling in the same field (Fig. 3*B*), had much the same appearance as rat primary neurons. Labeling for RHAMM in these neurons was seldom distributed throughout the perikaryal compartment, but rather was sparse and finely punctate (Fig. 3*C*). Similar labeling of slightly lower intensity was evident along neuronal processes. Throughout of the plane of focus in Figure 3*C*, many but not all of the underlying non-neuronal cells displayed intense punctate staining for RHAMM. Due to a limited supply of these cultures and the demonstrated specificity of the anti-RHAMM antibody, staining with control anti-RHAMM serum was not conducted.

Western blots

Results of Western blot analyses of total protein lysates obtained from NG108-15 cells cultured for 3 d in the presence and absence of db-cAMP are shown in Figure 4. Nitrocellulose membranes containing transblotted proteins from untreated cells (Fig. 4*A*, lane 1) and cells treated with db-cAMP for 1 d (Fig. 4*A*, lane 2) and 2 d (Fig. 4*A*, lane 3) revealed three corresponding immunoreactive bands when probed with anti-RHAMM antibody A-268. Untreated cells produced a major RHAMM isoform of 75 kDa as well as forms having estimated molecular weights of 116 and 60 kDa. A very faint band was also detected at 48 kDa. In lysates of cells treated with db-cAMP, the levels of both the 75 and 60 kDa isoforms were elevated at both the posttreatment times examined. In lysates of cultured NSC-34 cells, Western blots probed with anti-RHAMM antibody A-268 revealed two major protein forms of RHAMM migrating with apparent MW of 116 and 75 kDa (Fig. 4*B*). Although run on a separate gel, these forms likely correspond to the 116 and 75 kDa RHAMM proteins detected in NG108-15 cells and, as in the case of the latter, the 75 kDa form was slightly more abundant in the NSC-34 cells. PC12 cells expressed the same forms of RHAMM seen in NSC-34 cells, except that the 75 kDa form was present in relatively greater abundance (Fig. 4*C*). There

Figure 4. Western blot analysis of RHAMM in NG108-15 and NSC-34 cells. Equal quantities of lysate proteins from NG108-15 (*A*), NSC-34 (*B*), and PC12 (*C*) cells were separated on polyacrylamide gels, transblotted, and probed with A-268 anti-RHAMM antibody. All three cell types produced RHAMM proteins of 116 and 75 kDa. NG108-15 cells produced an additional 60 kDa protein. Compared with NG108-15 cells cultured in the absence of db-cAMP (*A*, lane 1), enhanced expression of the 75 and 60 kDa RHAMM proteins was seen in these cells cultured in the presence of db-cAMP for 1 d (*A*, lane 2) and 2 d (*A*, lane 3). No differences in RHAMM levels were detected in NGF-treated (*C*, lane 1) and untreated (*C*, lane 2) cultures of PC12 cells.

appeared to be no differences in the levels of RHAMM in PC12 cells that were treated with NGF (Fig. 4*C*, lane 1) compared with those that were not treated (Fig. 4*C*, lane 2).

Neurite extension and motility

All three cell lines as well as primary rat and human fetal neurons were taken for examination of the effect of a polyclonal anti-RHAMM antibody on neurite extension and motility. This antibody at a dilution of 1:50, which was determined to be an optimal concentration for achieving motility inhibition of peripheral cell types *in vitro*, significantly reduced neurite migration of NG108-15 cells by threefold (Fig. 5*A*), PC12 cells by twofold (Fig. 5*B*), NSC-34 cells by fourfold (Fig. 5*C*), rat primary neurons by fourfold (Fig. 5*D*), and human fetal neurons by twofold (Fig. 5*E*). Analyses of the time-lapse images of control cultures receiving either no additions or treatments with preimmune serum, between which no significant differences in neurite migration were observed, indicated that net neurite extension was a dynamic process involving sequential periods of both extension and retraction. This was especially true of short neurites with one to two cell body lengths emanating from NSC-34 cells that, although exhibiting a similar net rate of overall elongation as the other cells examined, were, in fact, difficult to track due to their rapid extension from and equally rapid withdrawal into the cell body (not shown). Addition of the antibodies inhibited both extension and retraction so that processes appeared to be frozen in position. In these studies, although no attempt was made to determine the time course or rapidity of action of the reagents added to cultures, it appeared that their full effect was exerted within the 15–30 min preincubation before imaging was begun.

Neurite migration of NSC-34 cells and rat primary neurons were also assessed for the effects of monoclonal anti-RHAMM antibodies. With dilutions of all monoclonal antibodies of 1:50, it was found here that monoclonal antibody 3T3-3 significantly blocked ($p < 0.0001$) the migration of NSC-34 cell neurites by twofold (Fig. 6*A*), while neither antibody 3T3-7 nor 3T3-8 obtained from different hybridomas had any effect on the neurites of these cells. In contrast, antibody 3T3-7 effectively and significantly blocked neurite extension of rat primary neurons by eightfold (Fig. 6*B*), but other monoclonal antibodies (data not shown), as exemplified by antibody 3T3-6, had no such inhib-

itory actions. Since all of the monoclonal antibodies used here were of the IgG1 isotype, the nonblocking monoclonal antibodies served as isotype controls in these studies. That the differential responses of neurites to these antibodies were not simply due to differences in antibody titers was indicated by ELISA assays of IgG1, which showed similar titers for each of the ascites used. Moreover, antibodies exhibiting an inhibitory action on neurites of NSC-34 cells were found to be devoid of actions on those of rat primary neurons (Fig. 6) and, conversely, some that lacked effects on neurites of NSC-34 cells inhibited neurite movement of rat primary neurons (data not shown).

To determine further whether other antibody reagents against RHAMM have the same actions on neurite migration as they do on the locomotion of various peripheral cell types *in vitro*, we examined the effects of a polyclonal antibody (designated A-124) generated against a peptide corresponding to a region in the RHAMM sequence (amino acids 124–144) that is repeated five times within the molecule. This anti-repeat sequence antibody significantly stimulated neurite migration of both NSC-34 cells by twofold ($p < 0.0001$) at a dilution of 1:1000 (Fig. 7*A*) and rat primary neurons by two- to threefold at dilutions of 1:1000 and 1:5000 (Fig. 7*B*). The stimulatory action was lost at higher dilutions and, notably, was absent at lower dilutions where, instead, a nonsignificant inhibition of 40% compared with control was observed. No differences in neurite dynamics were observed between culture receiving no additions and those treated with A-124 preimmune serum.

To assess further the potential contribution of RHAMM/HA interactions to neurite migration, we determined the effect of a peptide with an amino acid sequence corresponding to one of the two HA binding domains within RHAMM. Addition of RHAMM peptide to cultures at concentrations of 25 ng/ml or greater significantly ($p < 0.001$) inhibited neurite migration of NSC-34 cells (Fig. 8*A*). Lower concentrations had no significant effect, while concentrations of 50 and 100 ng/ml abolished their movement nearly entirely. In contrast, rat primary neurons appeared to be much more sensitive to the inhibitory effects of the RHAMM peptide such that neurite migration was significantly ($p < 0.01$) reduced by twofold at concentrations as low as 1.5 ng/ml (Fig. 8*B*). However, the maximum degree of inhibition achieved with doses of peptide as high as 50 ng/ml did not reach that seen in the case of NSC-34 neurites. As controls

Figure 5. Polyclonal anti-RHAMM antibody inhibition of neurite migration in cultures of neuronal cell lines and primary neurons. Culture preparations indicated in A–E were treated with either control preimmune serum (solid bars) or polyclonal anti-RHAMM (A-268) antibody (hatched bars) and neurites were tracked for 1–2 hr by image analysis. Neurite migration was significantly inhibited in antibody-treated compared with control cultures (*, $p < 0.01$). Error bars represent SD of between 20 and 30 neurites tracked.

in these experiments, scrambled peptide added to NSC-34 and rat primary neuronal cultures at a concentration of 50 ng/ml was found to be devoid of actions on neurites when compared with cultures that received additions of vehicle solution alone.

Discussion

This report is the first documentation of the HA binding protein RHAMM in neurons and its involvement in neurite extension. We have shown that RHAMM is concentrated at the tips as well as along neurites, that anti-RHAMM antibodies exhibit domain-specific inhibition and stimulation of neurite migration in neuronal cultures, that a peptide mimicking an HA-binding domain in RHAMM inhibits neurite migration, and that RHAMM expression is increased in NG108-15 cells induced to sprout neurites in the presence of db-cAMP. These obser-

Figure 6. Monoclonal anti-RHAMM antibody inhibition of neurite migration in cultures of NSC-34 cells and rat primary neurons. Cultures received additions of control mouse IgG (solid bar, cont) or the anti-RHAMM antibodies indicated, and neurites were tracked for 1–2 hr by image analysis. Antibodies 3T3-3 and 3T3-7 significantly inhibited neurite migration in NSC-34 and primary neurons, respectively. Error bars represent SD of between 20 and 30 neurites tracked.

vations, together with our detection of RHAMM in CNS neurons (unpublished observations), suggest that RHAMM is a major neuronal HA receptor and that it may mediate functions of HA in the CNS.

Evidence for a causal link between RHAMM and neurite migration rests upon the ability of anti-RHAMM antibodies and HA binding domain peptides to alter migration. The sequence-specific polyclonal antibody A-268 found here to inhibit neurite extension was previously shown to reduce binding of HA to RHAMM and to block both random cell locomotion and chemotaxis (Hardwick et al., 1992; Savani et al., 1994a). It is plausible that since antibody A-268 inhibits HA/RHAMM interactions (Savani et al., 1994b), it blocks neurite extension by preventing interaction of HA with cell surface RHAMM. The cell-specific inhibition of neurite migration displayed by monoclonal anti-RHAMM antibodies raised against biochemically purified RHAMM is consistent with their similarly selective inhibitory actions on the motility of distinct T and B cell lineages, macrophages, and smooth muscle cells (Pilarski et al.,

Figure 7. Polyclonal anti-RHAMM antibody stimulation of neurite migration in cultures of NSC-34 cells and rat primary neurons. Cultures received additions of either control preimmune serum (solid bar, *cont*) or the indicated dilutions of an antibody against a repeat sequence domain in RHAMM, and neurites were tracked for 1–2 hr by image analysis. High concentrations of antibody (1:100 dilution) had no effect on neurite movement, while lower concentrations significantly (*, $p < 0.01$) stimulated migration. Error bars represent SD of between 20 and 30 neurites tracked.

1993; Turley et al., 1993; Savani et al., 1994a). For example, some of these antibodies block fibroblast locomotion, but have no effect on thymocyte migration (Hardwick et al., 1992; Pilarski et al., 1993). It is possible that these antibodies recognize cell-specific glycosylation patterns of RHAMM or RHAMM sequences, peptide mapping of which is expected to provide a basis for antibody selectivity. The stimulatory antibody A-124, directed against a repeated sequence near the amino terminus of RHAMM (Hardwick et al., 1992) and found here to increase neurite migration, was recently reported to enhance fibroblast locomotion and to promote signal transduction events when added to cultures at low concentrations (Hall et al., 1994). Since repeated sequences are often sites of protein/protein interactions, it is possible that antibody A-124 may aid receptor cross-linking, which results in signaling and consequent motility (Hall et al., 1994). Stimulatory antibodies have also been reported for adhesion molecules on lymphocytes and the autocrine motility factor receptor gp78 where these promote adhesion, signaling events, and cell locomotion (Nabi et al., 1992). With respect to RHAMM peptides, those mimicking either of the two HA binding domains of this protein were shown to inhibit the TGF β -augmented locomotion of fibroblasts and to prevent chemotactic responses of macrophages (Samuel et al., 1993; Savani et al., 1994a). The HA binding capacity of these peptides is

Figure 8. Inhibition of neurite migration by a peptide with amino acid sequence corresponding to one of the two HA binding domains of RHAMM. Cultures of NSC-34 cells and rat primary neurons were treated with either a buffer vehicle (*cont*), a scrambled version of the RHAMM peptide (*scram*), or various quantities (ng/ml of culture medium) of RHAMM peptide, and neurites were tracked for 1–2 hr by image analysis. Scrambled peptide had no effect on neurite migration as compared with vehicle-treated controls. RHAMM peptide significantly inhibited (*, $p < 0.01$) neurite movement of both cell types at each of the three concentrations employed. Error bars represent SD of between 20 and 30 neurites tracked.

presumably key to their inhibition of neurite migration, since peptides with mutated HA binding motifs (Yang et al., 1993) no longer inhibit, for example, fibroblast locomotion.

Although neurites grow on substratum coated with HA combined with appropriate growth factors (Walicke, 1988), they are either unaffected by, fail to grow, grow very poorly or actively avoid substratum coated with HA alone (Carbonetto et al., 1983; Saxod and Bizet, 1988; Verna et al., 1989). Similarly, peripheral cell types locomote poorly when plated on an HA substrate (Erikson and Turley, 1983; Tucker and Erikson, 1984). How can these already generally negative influences of HA on neurite extension be reconciled with the observed inhibition of this process following functional blockade of the HA receptor RHAMM? First, in view of the complex behavior displayed by cells and neurites grown on artificial ECM and cell adhesion factors (Reichardt et al., 1990; Bixby and Harris, 1991; Edelman, 1992), their behavior on a pure substrate, as in the case of HA, cannot fully predict the functional contribution of that component to cell or neurite migration when present in an ECM composite. Second, concentrations, relative proportions, and pericellular distributions of ECM components are likely im-

portant factors governing neurite migration. For example, the effect of HA on cell locomotion is concentration dependent and biphasic; motility is stimulated at low and inhibited at high concentrations (Bernanke and Markwald, 1979; Hakansson et al., 1980a,b; Hadden and Lewis, 1991; Turley et al., 1991) and a similar situation may hold for actions of HA on neurite migration. Third, the present studies were conducted with cells taken for analysis at specific times postplating. It remains to be determined whether RHAMM is equally involved in early rapid as well as sustained phases of neurite growth and migration (Smalheiser and Schwartz, 1987; Bixby and Jhabvala, 1990). And fourth, it would be premature to attribute antibody blockade of HA/RHAMM interactions as being singularly responsible for inhibition of neurite migration. Since HA binding domains are found in other ECM proteins including neurocan, versican/GHAP, hyaluronectin, and aggrecan (Hardingham and Fosang, 1992; Laurent and Fraser, 1992; Rauch et al., 1992), it is conceivable that interference of HA binding to RHAMM alters HA interactions with other HA binding proteins. Conversely, RHAMM has been shown to bind to heparin, laminin, and collagen type IV, albeit with lower affinity than it binds to HA (Turley et al., 1987). Thus, anti-RHAMM antibodies and RHAMM peptides may affect neurite migration, in part, by interfering with interactions of RHAMM with other ECM components shown to affect neurite outgrowth (Carbonetto et al., 1983; Bozyczko and Horwitz, 1986; Carri et al., 1988; Dow et al., 1993). Finally, it should be noted that antibody blockade of RHAMM function only partially inhibits neurite motility. This less than total inhibition, also found previously with fibroblasts, may be due to RHAMM-independent mechanisms involving cell surface receptors such as integrins, CD44, and proteoglycans that contribute to a basal rate of cell locomotion.

The present results on the RHAMM/ECM system are similar to the intensely studied integrin class of ECM receptors and their ligands (Bixby and Harris, 1991; Neugebauer et al., 1991; Ruoslahti, 1992; Venstrom and Reichardt, 1993) in that the functional integrity of each is required for normal neurite migration. In addition, the RHAMM gene is complex encoding two isoforms as well as alternatively spliced entities that target the protein to the cell surface or to the extracellular compartment (Hardwick et al., 1992; Yang et al., 1994). This complexity is analogous to the multiple domain structure of such ECM proteins as fibronectin (Limper and Roman, 1992) and such cell-specific adhesion molecule as NCAMs (Wallis and Walsh, 1992). Interference with the function of these specific domains or receptor subunits impairs adhesion, growth, and other properties of neurites, depending on the moiety targeted (Carri et al., 1988; Neugebauer et al., 1991; Doherty et al., 1992; Appel et al., 1993; Taylor et al., 1993). The present results are consistent with a similar existence of multiple RHAMM domains or isoforms that are differentially expressed and that may perform distinct cell-specific functions. Nevertheless, several differences are noteworthy. First, while anti-integrin antibodies differentially inhibit extension of neurites on the surface of some cell types as compared with ligand-coated substrata and, to some extent, cause previously extended neurites to detach and retract (Bozyczko and Horwitz, 1986; Tomaselli et al., 1987; Letourneau et al., 1988), anti-RHAMM antibodies inhibited equally well the migration of neurites on plastic and those dispersed on the surface of support cells, and only halt neurite movement rather than evoke their retraction. Second, unlike the mM concentrations of RGDS peptide required to interfere with integrin/fi-

bronectin interactions (Letourneau et al., 1988; Lin et al., 1993), HA binding domain peptide of RHAMM had inhibitory actions in the nanomolar range. And third, while integrins appear to be concentrated mainly in neurites (Letourneau et al., 1988), RHAMM is distributed both in the cell bodies and neurites.

The molecular mechanisms underlying the contribution of RHAMM to neurite growth are not known. Immunoreactivity within cell bodies and processes appeared to be localized largely to the plane of substrate attachment and consisted of distinct punctate deposits or patches, suggesting that RHAMM is targeted to discrete sites of cell/substrate interaction. Recently, we have reported that RHAMM regulates locomotion by a protein tyrosine phosphorylation cascade resulting in focal adhesion turnover and focal adhesion kinase (FAK) phosphorylation/dephosphorylation (Hall et al., 1994). Although FAK is abundant in brain (Andre and Becker-Andre, 1993) and some reports have implicated a role of focal adhesions in growth cones (Halegoua, 1987; Igarashi et al., 1990; Cypher and Letourneau, 1991), these have not been well studied in the context of neurite extension. Since focal adhesions are well known to regulate fibroblast locomotion *in vitro* (Burridge et al., 1988) and given the above functional links between these structures and RHAMM, further studies may aim to determine whether a similar relationship exists between RHAMM and focal adhesions in neurites.

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