

TGF- β_1 Stimulation of Cell Locomotion Utilizes the Hyaluronan Receptor RHAMM and Hyaluronan

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Abstract. TGF- β is a potent stimulator of motility in a variety of cell types. It has recently been shown that hyaluronan (HA) can directly promote locomotion of cells through interaction with the HA receptor RHAMM. We have investigated the role of RHAMM and HA in TGF- β -stimulated locomotion and show that TGF- β triggers the transcription, synthesis and membrane expression of the RHAMM receptor and the secretion of HA coincident with the induction of the locomotory response. This was demonstrated by both incubating cells with exogenous TGF- β_1 and by stimulating the production of bioactive TGF- β_1 in tumor cells transfected with TGF- β_1 under the control of the metallothionein promoter. TGF- β_1 -induced locomotion was suppressed by antibodies that prevented HA/RHAMM interaction, using polyclonal antibodies

to either RHAMM fusion protein or RHAMM peptides, or mAbs to purified RHAMM. Peptides corresponding to the HA-binding motif of RHAMM also suppressed TGF- β_1 -induced increases in motility rate. Spontaneous locomotion of fibrosarcoma cells was blocked by neutralizing secreted TGF- β with panspecific TGF- β antibodies and by inhibition of TGF- β_1 secretion with antisense oligonucleotides. Polyclonal anti-RHAMM fusion protein antibodies and peptide from the RHAMM HA-binding motif also suppressed the spontaneous motility rate of fibrosarcoma cells. These data suggest that fibrosarcoma cell locomotion requires TGF- β , and the pathway by which TGF- β stimulates locomotion uses the HA receptor RHAMM and HA.

ALTHOUGH the factors that control cell locomotion are poorly understood, it is becoming clear that some cells can produce autocrine motility factors and cytokines (Liotta and Schiffman, 1988; Stoker and Gherardi, 1991) through which they regulate chemokinesis and chemotaxis. Transforming growth factor betas (TGF- β s)¹, of which there are three mammalian isoforms, are 25-kD homodimeric peptides that act as multifunctional regulators of mesenchymal, endothelial, and epithelial cells (Roberts and Sporn, 1989; Barnard et al., 1990; Massagué et al., 1990). TGF- β has been implicated in a variety of physiological and pathological processes in which cell locomotion is essential such as embryogenesis (Heine et al., 1987), wound repair (Pierce et al., 1989), inflammation (Allen et al., 1990; Khalil et al., 1990, 1991), and tumor invasion (Welsh et al., 1991; McCarthy and Turley, 1992; Samuel et al., 1992).

TGF- β , in its role as a regulator of cell motility is a powerful chemoattractant that promotes both chemokinesis and chemotaxis in a variety of cell types (Postlethwaite et al.,

1987; Wahl et al., 1987; Reibman et al., 1991; Samuel et al., 1992). In neutrophils and macrophages, TGF- β has been observed to be a potent chemoattractant (Wahl et al., 1987; Fava et al., 1991; Reibman et al., 1991), and it has been suggested that the chemotactic potency of TGF- β for macrophages may be relevant for diseases that are characterized by inflammation and fibrosis (Khalil and Greenberg, 1991; Fava et al., 1991). TGF- β may be involved in the initial recruitment of macrophages and the migration of fibroblasts seen in these lesions. Postlethwaite and co-workers (1987) have shown that picogram levels of TGF- β_1 are sufficient to enhance chemokinesis and chemotaxis of normal fibroblasts.

While the mechanism through which TGF- β regulates locomotion is poorly understood, studies in neutrophils indicate that TGF- β_1 triggers protein synthesis and actin assembly that are required for chemotaxis but, unlike conventional chemoattractants, it does not act via common signal transduction pathways (Reibman et al., 1991). In many cell types, TGF- β promotes hyaluronan (HA) synthesis (Toole et al., 1989; Heldin et al., 1989), while HA is important in fibrosarcoma cell locomotion following transformation by *H-ras* (Turley et al., 1991) and in mammary carcinoma cell locomotion (Schor et al., 1989). HA is a repeating disaccharide of *N*-acetyl-glucosamine and *D*-glucuronic acid that is

1. *Abbreviations used in this paper:* DM, defined medium; ECL, enhanced chemiluminescence; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, hyaluronan; TGF- β , transforming growth factor beta.

not sulfated, unlike other glycosaminoglycans. HA accumulates at sites of embryonic cell migration (for review see Toole et al., 1989), and coincides with the appearance of macrophages and fibroblasts in wound repair responses in the lung (Bray et al., 1991; Nettelbladt et al., 1989). Increased synthesis of HA is seen in melanoma and carcinoma cell lines both in vivo and in vitro, and HA synthesis correlates with increased invasiveness and metastatic ability of these tumors (Turley and Tretiak, 1985; Kimata et al., 1983; Toole et al., 1979). HA can directly promote locomotion and accumulates in the extending lamellae of motile cells (Turley et al., 1991). Several HA binding proteins have been identified but only two cell membrane receptors, CD44 and RHAMM, have been cloned (for reviews see Toole, 1990; Turley, 1991). Recently, we have shown that the production of RHAMM and HA is required for tumor cell locomotion (Hardwick et al., 1992). RHAMM, a 58-kD glycoprotein, is a novel HA receptor that bears no primary sequence homology to other known HA-binding proteins. RHAMM accumulates at the tips of advancing lamellipodia in locomoting cells and polyclonal antibodies directed to peptides of the deduced amino acid sequence block HA-stimulated locomotion (Hardwick et al., 1992). In the present study, we have examined the mechanism by which TGF- β stimulates locomotion and show that HA/RHAMM signalling is required for cell motility.

Materials and Methods

Cell Lines

The properties of the C1, C3, and C17.18 cell lines have been described previously (Samuel et al., 1992). In brief, C1 and C3 were obtained when the parental 10T1/2 fibroblasts were transfected with a plasmid which contains the 6.6-kb fragment of T-24-H-ras and neo^R gene. C17.18 was obtained when the parental C1 fibroblasts were transfected with pPK9A, which contained a complete TGF- β 1 cDNA under the control of the metallothionein promoter. Furthermore, this cDNA was subjected to site-directed mutagenesis of cysteines 223 and 225 in the propeptide in order to allow for secretion of bioactive TGF- β 1 (Samuel et al., 1992). All cell lines were maintained at 37°C on 150 mm plastic tissue culture plates (Falcon Plastics, Cockeysville, MD) in culture medium containing α -MEM (Flow Laboratories, Mississauga, Ontario) supplemented with antibiotics and 10% (vol/vol) FCS (GIBCO BRL, Gaithersburg, MD). A serum-free medium called defined medium (DM) was also used which contained 4.0 μ g/ml transferrin and 2.0 μ g/ml insulin in α -MEM.

Antibodies

Panspecific neutralizing anti-TGF- β antibody (R&D Systems, Inc., Minneapolis, MN) was used at a concentration of 500 μ g/ml as suggested by the manufacturer. Monoclonal and polyclonal antibodies to RHAMM used in this study have been described previously (Turley and Torrance, 1985; Turley et al., 1987; Turley et al., 1991; Hardwick et al., 1992). The antibodies include murine monoclonals MSV 10-4 and 10-6 that were raised against purified RHAMM protein, rabbit anti-RHAMM peptide^{aa266-288} (R3.2), and rabbit anti-RHAMM fusion protein antibody all of which can block HA-mediated locomotion. Rabbit anti-RHAMM peptide^{aa124-145} antibody, which did not block locomotion, and normal murine IgG served as controls.

Northern Blotting

Cells were plated onto 150 mm tissue culture plates containing 10 ml α -MEM (Flow Laboratories) supplemented with antibiotics and 10% (vol/vol) FBS (Hyclone Laboratories, Logan, UT). At \sim 90% confluence, cells were washed with Hanks medium and 2.0 ml of phosphate buffered 0.1% trypsin-EDTA solution was added. Cells were subsequently cultured in DM. At \sim 60% confluence, C3 cells were exogenously stimulated with

TGF- β 1 (10 ng/ml) (R & D Systems, Inc.) or its secretion induced by the addition of zinc sulfate (100 μ M) to cultures of C17.18. After appropriate incubation times, cells were washed with PBS (three times) and then removed with trypsin solution. A rapid RNA extraction method was used to prepare total cellular RNA (Gough, 1988), which was subjected to electrophoresis through 1% formaldehyde-agarose gels followed by transfer to Nytran nylon membranes (Schleicher and Schuell, Keene, NH). Blots were prehybridized and hybridized as described previously (McClarty et al., 1987; Hurta et al., 1991), using a ³²P-labeled 1.9-kb EcoRI fragment from the RHAMM cDNA containing plasmid DHI (Hardwick et al., 1992). Blots were washed, and autoradiography was performed as previously described (Hurta et al., 1991). Loading was monitored with a plasmid containing the glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) labeled by nick translation. Densitometric analysis of appropriate autoradiograms was carried out using a gel scanning spectrophotometer (DU-8; Beckman Instruments, Palo Alto, CA) and the one-dimensional Bio-Rad program (Bio-Rad Laboratories, Cambridge, MA).

Western Blot Assays

Western blot analyses were conducted using lysates of C3 and C17.18 fibroblasts prepared by treating cells 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 (Turley et al., 1987). Proteins were resolved on 10% SDS-PAGE gels (Laemmli, 1970) and transferred to nitrocellulose membranes at 175 V for 1 h at 15°C. The nitrocellulose was blocked by incubation for 1 h with 5% nonfat powdered milk in TBS (20 mM Tris, 130 mM NaCl, pH 7.4) and incubated with primary antibody (R3.2; 1:1,000 dilution in TBS containing 0.05% Tween-20 and 1% skim milk) for 12 h at 4°C on a gyratory shaker. The blot was then washed three times (10 min) with TBS containing 0.05% Tween-20 (TTBS), incubated with HRP-conjugated goat anti-rabbit IgG (1:5,000 in TTBS containing 1% skim milk) for 1 h, and then washed with TTBS. Blotting was visualized by the enhanced chemiluminescence (ECL) Western blotting detection system (Amersham, Mississauga, Ontario) following manufacturers' directions. Quantitation was performed on a videodensitometer (model 620; Bio-Rad Laboratories).

Cellular Immunofluorescence and Flow Cytometry

C3 and C17.18 fibroblasts were cultured in 10% FCS and then switched to defined medium for between three and eight cell divisions before initiating treatment with either TGF- β 1 or ZnSO₄. After stimulation for defined time periods, the cells were lifted from the petri dishes using 2.5 mM EDTA in HBSS and washed once with HBSS (GIBCO-BRL) containing 10% FCS. The viability of cells was determined by Trypan blue exclusion and was between 85 and 90%. An aliquot of 2 \times 10⁶ cells was then incubated with a 1/50 dilution of R3.2 antibody to peptide 268-288 encoded in the RHAMM cDNA (Hardwick et al., 1992) in a total volume of 200 μ l of HBSS/10% FCS for 30 min on ice. At the end of the incubation, the cells were washed once in cold HBSS/10% FCS and resuspended in a 1/100 dilution of fluorescein-conjugated goat anti-rabbit IgG antibody (Sigma Immunochemicals, St. Louis, MO) in the same buffer, incubated, and washed as above. The cells were then fixed in 400 μ l of 1% paraformaldehyde in PBS and analyzed using an EPICS Model 753 flow cytometer (model 753; Epics, Inc., New York) (500 mW, 488 nm). Fluorescence was detected at 525 nm. Forward and 90° light scatter measurements were used to establish gates for intact, viable fibroblasts. Single parameter, 255 channel, log integral green fluorescent histograms were obtained, each based on 5 \times 10³ gated events.

Membrane immunofluorescence of C3 and C17.18 cells for photomicrography was performed as described in our earlier publications (Turley and Torrance, 1985; Turley and Auersperg, 1989) using the R3.2 polyclonal antibody to peptide^{aa268-288} of RHAMM. Monolayers were fixed in freshly prepared 3% paraformaldehyde for 10 min. Fixed monolayers were then incubated with antibody (1:40 dilution in PBS) overnight at 4°C. The monolayers were again washed then incubated with fluorescein-labeled goat anti-rabbit IgG (1:1,000 dilution in PBS). Processed monolayers were viewed with a Zeiss IM35 microscope (Carl Zeiss, Oberkochen, Germany) equipped with epifluorescence using nonoverlapping filters of 450-490 nm.

Cell Motility Analysis

An image analysis system capable of quantifying cell motility as nuclear displacement from a sequence of digitalized or time-lapsed images was used (Image 1; Universal Imaging Corporation, Westchester, PA). Aliquots of

5×10^3 cells were placed onto a 25 cm² tissue culture flask (Falcon Laboratories) and cultured as described above. Cells were tracked 24-h after subculturing every 10 min for 27 h and a minimum of 30 cells were examined in each experiment.

Quantification of Hyaluronan Secreted into Medium

Cell lines were cultured in 150 mm tissue culture plates (Falcon Laboratories) with DM at 37°C with or without TGF- β_1 . At appropriate time intervals, this conditioned medium was removed, centrifuged to remove cell debris, placed in siliconized polypropylene tubes with protease inhibitors and then frozen at -80°C as described by Danielpour et al. (1988). Conditioned medium obtained from above-mentioned cell lines was used to quantitate the amount of HA being secreted by C3 and C17.18 with or without TGF- β_1 . This was done by using the Pharmacia HA Test (Pharmacia, Sweden) according to manufacturers' directions.

Antisense Oligonucleotides

Phosphorothioate oligodeoxynucleotides were prepared on a DNA synthesizer (380B; ABI Adv. Biotechnologies, Inc., Columbia, MD). Sequences used were as follows: TGF- β_1 antisense, CGGAGGGGTAC-GGCGGA where only the sequence GGGGTACG used phosphorothioates and the remaining bases were phosphodiester; TGF- β_1 sense, GCCTCCCCATGCGGCCCT where only the sequence CCCCCATGC used phosphorothioates. This mixture of phosphorothioates and phosphodiester was the most effective and least toxic of the sequences that we evaluated. The oligonucleotides were added directly to subconfluent cells at the indicated times.

Peptides of the Hyaluronan Binding Domains

A peptide corresponding to one of the HA binding domains of RHAMM (Yang et al., 1993) was prepared on a peptide synthesizer (Applied Biosystems, Foster City, CA). The peptide from the second domain of RHAMM at amino acids⁴²³⁻⁴³² (KLRSQVLRK) or randomized peptide⁴²³⁻⁴³² (QSKRLKRVL) were added directly to subconfluent cells at a concentration of 10 μ g/ml in the presence or absence of TGF- β .

Results

Induction of Locomotion by TGF- β_1

The rate of locomotion of subconfluent C3 fibrosarcoma cells was determined by timelapse image analysis of random locomotion at increasing concentrations of TGF- β_1 in serum-free DM. We observed a maximal rate of C3 locomotion in the presence of TGF- β_1 between 3 and 10 ng/ml (Fig. 1 A), from 5 to 11 h after addition of TGF- β_1 to culture medium (Fig. 1 B). At 30 ng/ml and higher, locomotion decreased (Fig. 1 A). No discernible increases in rates of locomotion over the controls were observed at the lower concentrations of TGF- β_1 (0.1-0.5 ng/ml). Cells cultured in 10 ng/ml TGF- β_1 increased then decreased their motility rate with time after subculture so that locomotion had dropped to the rate of cells incubated without TGF- β by 24 h, but not to the initial locomotion rate (Fig. 1 B). Without added TGF- β_1 , the motility rate increased progressively over 24 h to 26 μ m/min or 2.4-fold over the rate measured within the first 2 h of plating the cells (Fig. 1 B).

To determine whether TGF- β could induce motility through autocrine stimulation, we also examined the C17.18 fibrosarcoma cells, which were transfected with an expression vector in which the complete coding region of TGF- β_1 was placed under the control of the metallothionein promoter (Samuel et al., 1992). Cys 223 and 225 in the TGF- β_1 propeptide had been converted to serines, mutations that result in dissociation of the propeptide and secretion of bioactive TGF- β_1 (Samuel et al., 1992). After zinc sulphate

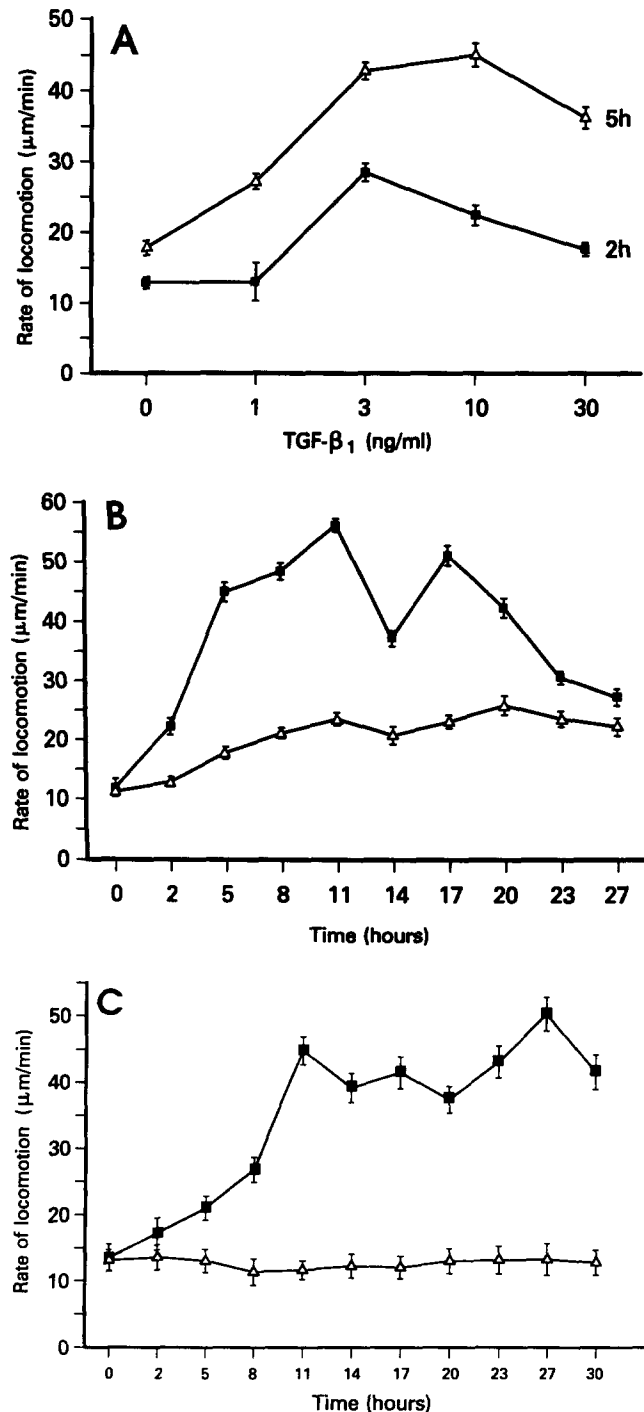


Figure 1. Locomotion rate of TGF- β_1 -stimulated cell lines. (A) C3 fibrosarcoma cells were subcultured and at 50 to 75% confluence TGF- β_1 was added at the doses indicated. Time lapse image analysis recorded the motility rate of 30 cells at 2- (■) and 5-h (Δ) intervals. (B) Locomotion rate of C3 cells incubated in 10 ng/ml TGF- β_1 (■) or control medium (Δ) over 27 h of image recording. (C) C17.18 has been transfected with the plasmid pPK9A that contains the complete open reading frame of TGF- β_1 with cysteine to serine mutations in positions 223 and 225 of the propeptide, and is under the control of the metallothionein promoter. The addition of ZnSO₄ to the culture medium induces synthesis and secretion of bioactive TGF- β_1 within 2 h (Samuel et al., 1992). Image analysis of locomotion rates were recorded at time intervals following addition of 100 μ M ZnSO₄ or diluent to the culture medium of subconfluent cells.

(ZnSO₄) treatment of C17.18, TGF-β₁ mRNA was induced sixfold and bioactive TGF-β was increased 9- to 14-fold (Samuel et al., 1992). This cell line also became more motile after addition of ZnSO₄ to subconfluent monolayers with increased locomotion detected as early as 2 h after TGF-β₁ induction. Motility rates reached maximal levels by 8–10 h (Fig. 1 C).

RHAMM and Hyaluronan Synthesis Are Stimulated by TGF-β₁

It has previously been shown that TGF-β₁ increased HA production (Toole et al., 1989; Heldin et al., 1989), and HA

could itself mediate locomotion through an interaction with the HA receptor RHAMM (Turley, 1991). We examined the regulation of RHAMM and HA by TGF-β₁ using subconfluent C3 cells cultured in defined medium in the presence or absence of TGF-β₁. At designed time intervals, both mRNA and cell protein samples were prepared. Increased RHAMM mRNA levels were seen as early as 2 h after TGF-β stimulation (Fig. 2 A). Total cellular RHAMM protein was elevated in cells exposed to 3 to 30 ng/ml TGF-β₁ with maximal response occurring at a concentration of 10 ng/ml for 8 h (Fig. 2 B), which was similar to the locomotory response to TGF-β₁ (Fig. 1, A and B). Over a 24-h time course, increased RHAMM protein expression was first detected 1 h after stimulation with TGF-β₁ rising to near maximum levels by 4 h. A gradual but much smaller increase in RHAMM expression was also observed in C3 cells cultured without added TGF-β₁ reaching maximal expression at 24 h relative to controls at the initiation of the culture (Fig. 2 C). Induction of TGF-β₁ secretion by ZnSO₄ in C17.18 also resulted in an elevation in RHAMM mRNA expression (Fig. 3 A) for up to 8 h poststimulation which diminished somewhat at later times. RHAMM mRNA in uninduced C17.18 cells was essentially unchanged over 24 h of culture. Corresponding changes in protein levels were detected in ZnSO₄-induced cells, with maximal RHAMM expression fivefold over controls by 8-h postinduction (Fig. 3 B).

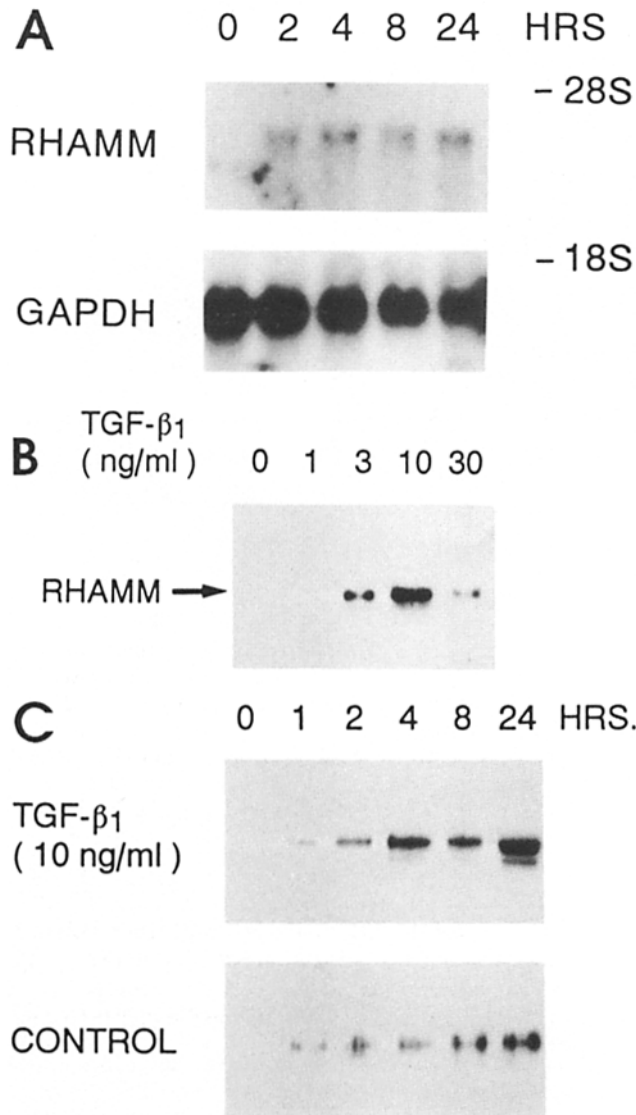


Figure 2. RHAMM mRNA and protein synthesis is enhanced by TGF-β₁. C3 cells were incubated in TGF-β₁, and RHAMM mRNA or protein levels were determined as described in methods. (A) Northern analysis was conducted on cells incubated in 10 ng/ml of TGF-β₁ at the times indicated. (B) Cells incubated in increasing concentrations of TGF-β₁ were analyzed by Western blotting after 8 h. (C) C3 cells were incubated in 10 ng/ml TGF-β₁ and sampled at the times for Western analysis. GAPDH is shown in (A) as a control for RNA loading.

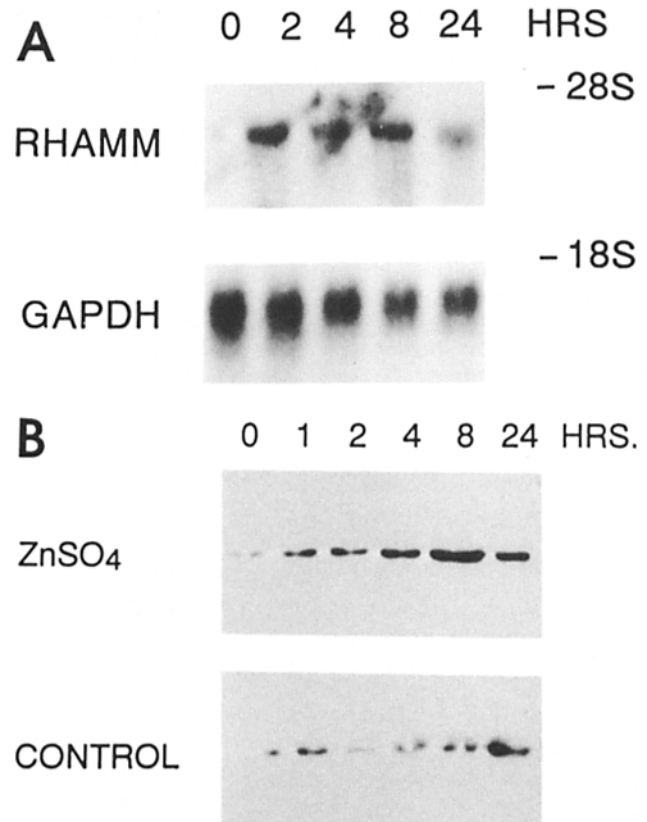


Figure 3. Autocrine stimulation of RHAMM mRNA and protein synthesis following activation of TGF-β₁ secretion. C17.18 was stimulated with 100 μM ZnSO₄ and RHAMM RNA (A) and protein (B) levels were determined at the indicated time intervals. GAPDH was used as a loading control.

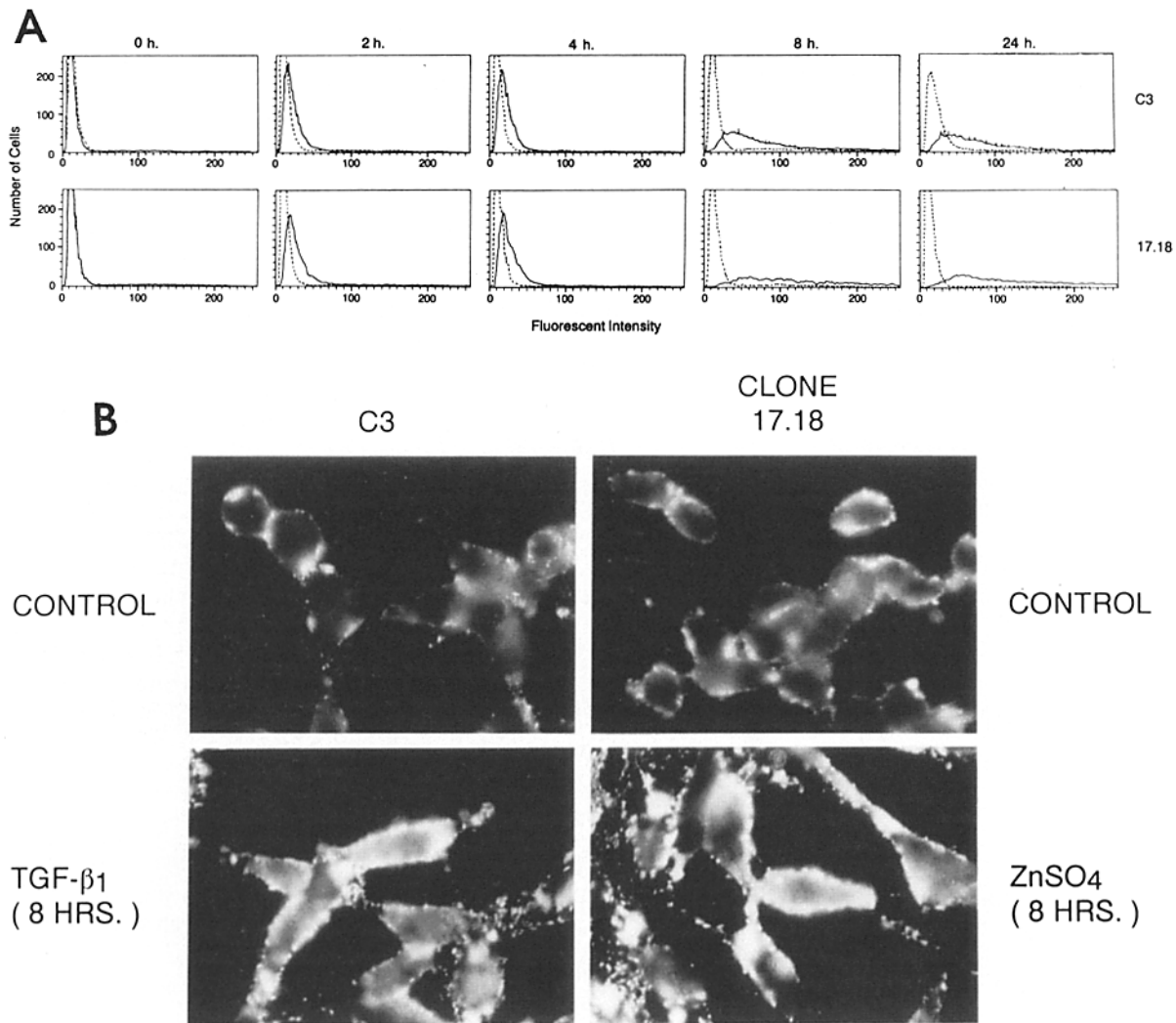


Figure 4. TGF- β_1 increases RHAMM expression on the cell membrane. (A) C3 cells were incubated with (solid lines) or without (dashed lines) 10 ng/ml TGF- β_1 , and C17.18 cells were incubated in the presence (solid lines) or absence (dashed lines) of 100 μ M ZnSO₄ for the time periods indicated. The cells were stained with rabbit anti-RHAMM peptide²⁶⁸⁻²⁸⁸ antibody and fluorescein-labeled goat anti-rabbit antibody, then analyzed on a Coulter counter (Epics, Inc.) as described in Materials and Methods. All experiments included two controls which were not significantly different from the time 0 h in cells treated with anti-RHAMM peptide²⁶⁸⁻²⁸⁸ antibody; normal IgG as first antibody, and fluorescent second antibody alone. (B) C3 and C17.18 cells were incubated and stained as described in A. Shown are fluorescence photomicrographs of the cells following 8 h of stimulation or controls incubated for an equivalent period in medium.

Increased RHAMM protein was detected on the cell surface by flow cytometry within 2 to 4 h after TGF- β_1 stimulation of C3 cells or ZnSO₄ treatment of C17.18 cells using a polyclonal rabbit anti-RHAMM peptide 268-288 antibody and fluoresceinated goat anti-rabbit antibody (Fig. 4 A). Peak expression was reached between 8 and 24 h, corresponding to the times noted for maximal production of RHAMM protein as detected by immunoblot assays (Figs. 2 C and 3 B). Fluorescent photomicrography of cells stimulated for 8 h confirmed that most of the RHAMM protein was expressed at the cell surface (Fig. 4 B).

C3 cells synthesized and released HA into the medium in large amounts when cultured in 10 ng/ml TGF- β_1 . A transient increase in HA secretion in both TGF- β_1 -treated cells and control was seen in the first 2 h of culture. However, the untreated control C3 cells gradually decreased secretion,

while TGF- β_1 -treated cells continued to produce HA at a relatively stable level thereafter of about 1 μ g/ml (Fig. 5 A). Increased HA secretion by C17.18 was also observed after TGF- β_1 induction by ZnSO₄ and remained higher than controls over the next 24 h (Fig. 5 B).

Inhibition of Cell Motility with Anti-TGF- β Antibody and Anti-Sense TGF- β_1 Oligonucleotides

C3 cells secrete increased amounts of bioactive TGF- β_1 (Schwarz et al., 1990). The hypothesis that this secreted TGF- β_1 autostimulates locomotion was tested by treating C3 cells in subconfluent culture with a panspecific TGF- β neutralizing antibody (500 μ g/ml). A 90% reduction in the rate of locomotion was observed within 5 h and then remained very low over the next 20 h (Fig. 6). This antibody

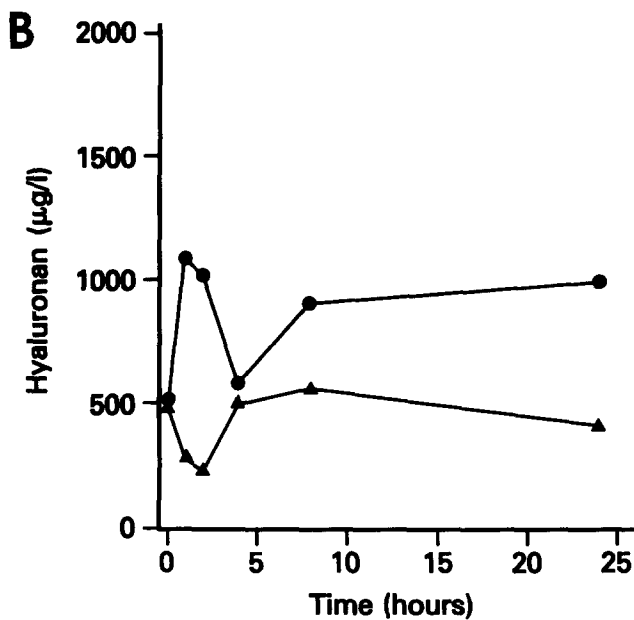
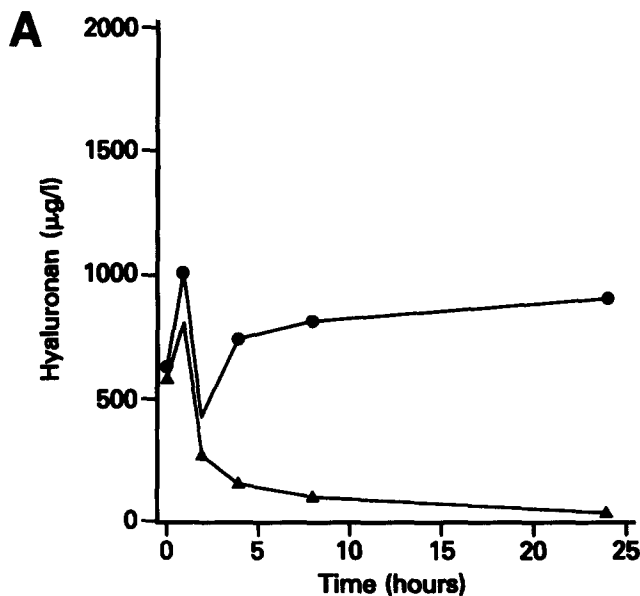


Figure 5. TGF- β_1 stimulates HA synthesis in locomoting cells. After subculture, C3 cells (A) were cultured in TGF- β_1 and culture medium sampled at the time intervals indicated. TGF- β_1 secretion in C17.18 cells (B) was initiated with ZnSO₄ and medium assayed for hyaluronan at increasing time in culture. Conditions were identical to those used to induce cell locomotion shown in Fig. 1.

was also able to significantly decrease locomotion when it was added to cultures of C3 cells containing 3 ng/ml TGF- β_1 (not shown).

To further test this hypothesis, an anti-sense TGF- β_1 oligonucleotide was synthesized based on the murine TGF- β_1 sequence spanning the initiation codon from -9 to +10 (see Materials and Methods). Either the antisense or sense oligonucleotide was added at a concentration of 0.8 μ M to subconfluent C3 cells. A decrease in locomotion of 45% of sense or untreated controls was observed by 8 to 10 h (Fig. 7). Examination of culture supernatants indicated that the

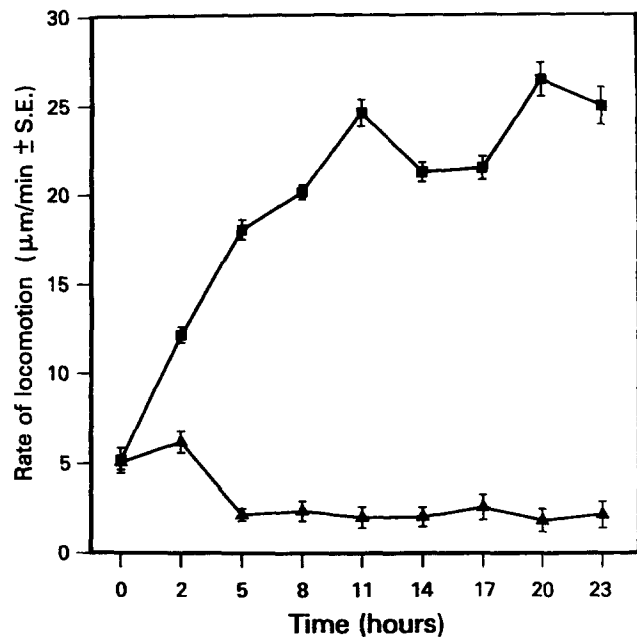


Figure 6. Inhibition of spontaneous locomotion of fibrosarcoma cells by anti-TGF- β_1 antibodies. C3 cells were cultured in the presence of pan specific rabbit anti-TGF- β antibody (500 μ g/ml) (\blacktriangle), or normal rabbit IgG (500 μ g/ml) (\blacksquare), and locomotion analyzed over time.

antisense oligonucleotides reduced TGF- β_1 secretion to about 50% of sense and untreated controls (Fig. 7), which was about equivalent to the inhibition of cell locomotion.

Inhibition of TGF- β_1 -Induced Locomotion by Anti-RHAMM Antibodies

We hypothesized that the TGF- β_1 -induced locomotion was mediated through HA stimulation of the RHAMM receptor. To test this directly, we used a panel of monoclonal anti-RHAMM antibodies as well as an anti-RHAMM peptide (268-288) and anti-RHAMM fusion protein polyclonal antibody, which have been shown to inhibit HA-induced locomotion (Turley et al., 1991; Hardwick et al., 1992). Antibody or normal IgG was added to subconfluent C3 cells after 8 h of stimulation with 10 ng/ml TGF- β_1 . Before the addition of antibody, C3 locomotion progressively increased to an average velocity of $46.9 \pm 1.9 \mu\text{m}/\text{min}$. Within 30 min of the addition of 1 μ g/ml of either RHAMM monoclonal or polyclonal antibodies, a reduction in the rates of locomotion to between 3.66 ± 0.8 and $11.09 \pm 1.4 \mu\text{m}/\text{min}$ (Fig. 8) was observed, with the polyclonal anti-RHAMM fusion protein antibody being the most effective. Controls included normal mouse IgG and polyclonal anti-RHAMM peptide 124-145 antibody, which do not block HA-induced locomotion (Hardwick et al., 1992). Identical results were obtained when ZnSO₄-stimulated C17.18 cells were treated with these antibodies (not shown).

We observed that C3 cells in culture in the absence of added TGF- β progressively increased locomotion (Fig. 1 A) as well as RHAMM protein expression (Fig. 2 C). This increase may have been due to autocrine stimulation of RHAMM as a result of the synthesis and secretion of TGF-

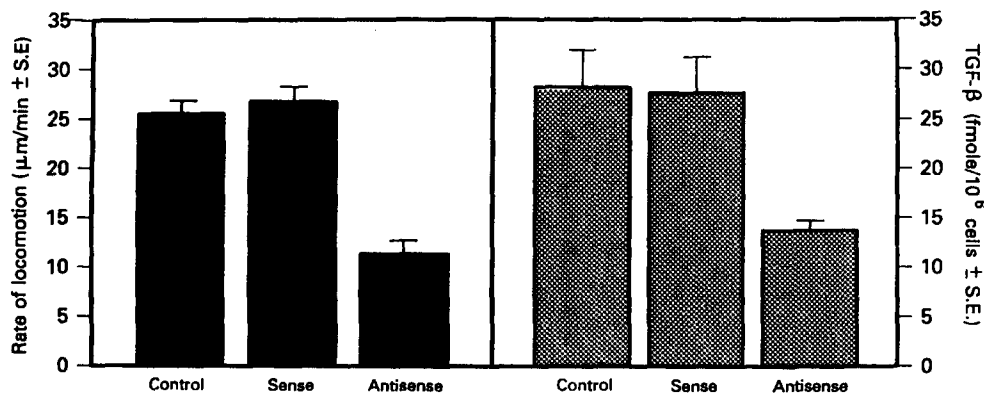


Figure 7. Inhibition of fibrosarcoma locomotion and TGF- β secretion with TGF- β_1 antisense oligonucleotides. C3 cells treated for 8 h with 0.8 μ M of anti-sense TGF- β_1 or sense TGF- β_1 oligonucleotides or left untreated. Anti-sense (CGGAGGGGGTACGGC-GGGA) and sense (GCCTCC-CCCATGCGGCCCT) oligonucleotide sequences were taken from the -9 to +10 positions spanning the initiation codon of TGF- β_1 . Locomotion rate (\pm SE) was tracked

by image analysis on 30 cells in each condition. Culture supernatants from cells treated with oligonucleotides or culture medium for 24 h were collected and assayed for TGF- β by the CCL64 bioassay (Khalil et al., 1989). The TGF- β levels were determined from four independent cultures in two experiments.

β_1 by C3 cells (Schwartz et al., 1990). This was tested by treating C3 cells, after 24 h in culture, with the anti-RHAMM fusion protein antibody in the absence of added TGF- β_1 . Within 2 h of addition of antibody, the rate of locomotion decreased significantly from 24.5 ± 3.1 to 7.5 ± 1.3 μ m/min indicating that spontaneous locomotion of these cells could be blocked by an antibody that interferes with HA/RHAMM interaction.

Inhibition of TGF- β -Induced and Spontaneous Fibrosarcoma Motility by Peptide^{aa423-432} from the Hyaluronan-Binding Domain of RHAMM

RHAMM contains two discrete HA binding domains located near the carboxyl terminus characterized by clusters of basic amino acids (Yang et al., 1993). Peptides mimicking either of the two domains inhibit HA binding to RHAMM (Yang

et al., 1993). We have tested the hypothesis that peptide corresponding to amino acid⁴²³⁻⁴³² of RHAMM will block TGF- β_1 -induced and spontaneous motility of the C3 fibrosarcoma, presumably by interfering with the ability of HA to bind RHAMM. C3 cells were cultured, at subconfluence as described in Materials and Methods, in the presence or absence of 10 ng/ml of TGF- β_1 . At the initiation of the culture, either peptide^{aa423-432}, or scrambled peptide controls, was added at 10 μ g/ml. Complete abrogation of TGF- β_1 -induced cell migration by RHAMM binding domain peptide, but not the scrambled peptide, was observed over a tracking period of 8 h (Fig. 9). Spontaneous locomotion of C3 cells was also suppressed by the peptide mimicking the HA binding domain. Cell locomotion increased from 10.8 μ m/min seen at the initiation of the culture to 28.3 μ m/min after 24 h of culture and was inhibited to 14.7 μ m/min by the RHAMM peptide (Fig. 9). The scrambled peptide had no effect on spontaneous tumor locomotion.

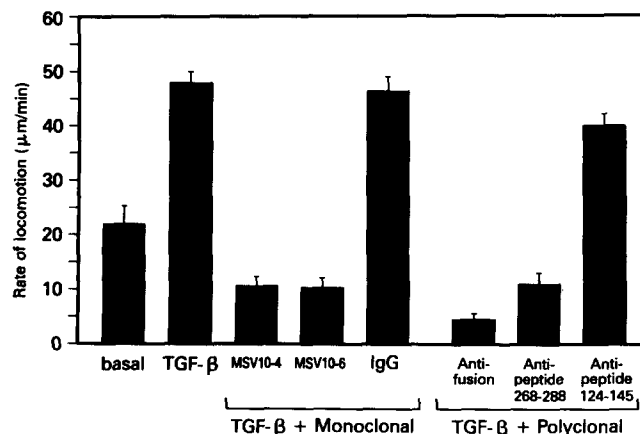


Figure 8. Anti-RHAMM monoclonal and polyclonal antibodies block TGF- β_1 induced locomotion. C3 cells were stimulated with 10 ng/ml TGF- β_1 for 8 h in the presence or absence of antibodies. Monoclonal anti-RHAMM antibodies MSV 10-4 and MSV 10-6 are compared to normal murine IgG. Rabbit antibody to RHAMM peptide²⁶⁸⁻²⁸⁸ and anti-RHAMM fusion protein antibody, which have been shown to block HA-induced locomotion, were compared to anti-RHAMM peptide¹²⁴⁻¹⁴⁵ polyclonal antibody that is inactive (Hardwick et al., 1992).

Discussion

The evidence presented supports the hypothesis that the participation of the HA receptor RHAMM is necessary for TGF- β_1 -mediated locomotion. The addition of TGF- β_1 to cell cultures, or induction of TGF- β_1 secretion, rapidly stimulated the synthesis of RHAMM message and protein, surface expression of RHAMM, HA synthesis, and cell locomotion. Antibodies that inhibited HA/RHAMM signaling and peptides that blocked HA binding to RHAMM prevented TGF- β_1 -induced increases in the rate of locomotion, and the spontaneous locomotion of these cells. Furthermore, blocking experiments with either neutralizing anti-TGF- β antibody or antisense oligonucleotides to TGF- β_1 decreased cell locomotion indicating that TGF- β_1 was essential for spontaneous fibrosarcoma cell motility. From these data, we hypothesize that TGF- β_1 -induced locomotion requires the participation of the HA receptor RHAMM. TGF- β_1 likely activates the RHAMM receptor through increased synthesis of HA since peptides and antibodies that interfere with HA binding to RHAMM blocked TGF- β_1 -induced motility. Additional evidence that RHAMM may directly control cell motility comes from the recent analysis

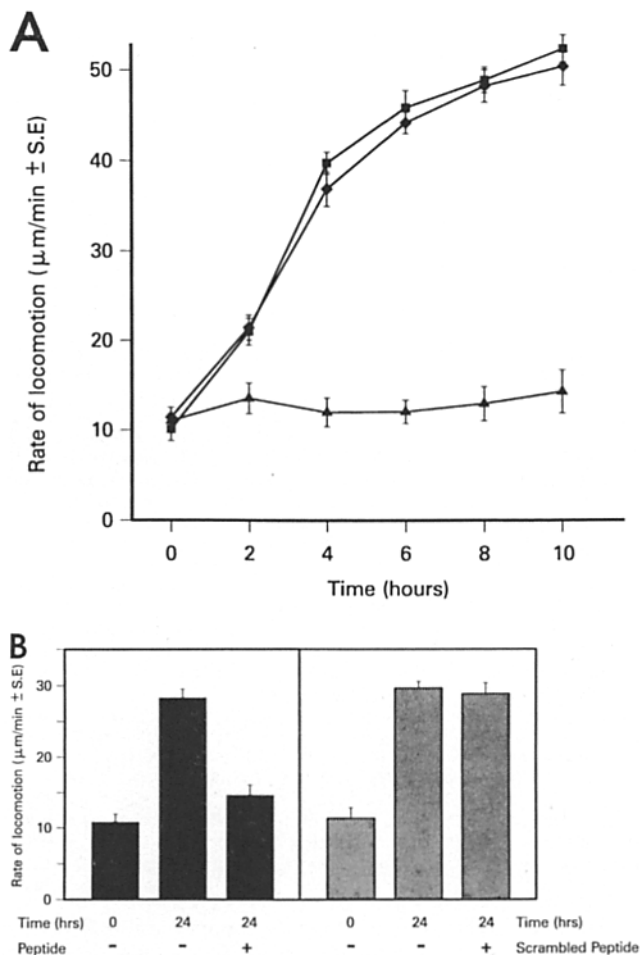


Figure 9. Inhibition of TGF- β_1 -induced and spontaneous locomotion by peptides mimicking the HA-binding domains of RHAMM. (A) C3 fibrosarcoma cells were cultured at subconfluence in the presence of 10 ng/ml of TGF- β_1 (■). Peptide⁴²³⁻⁴³² K-L-R-S-Q-L-V-K-R-K (▲) corresponding to the second HA-binding domain of RHAMM (Yang et al., 1993), or the scrambled sequence Q-S-K-R-L-K-K-R-V-L (◆) was added to the cultures at a concentration of 10 μ g/ml with the TGF- β_1 , and cell locomotion was analyzed every 2 h. (B) C3 cells were cultured in the absence of TGF- β_1 . The cells were tracked for 2 h at the initiation of the culture (0 h) and between 22 and 24 h of culture (24 h). Then, either peptide (left) or scrambled peptide (right) was added to the culture and the motility rate analyzed for 2 h.

of fibroblasts transfected with a genomic clone of RHAMM. These cells showed both enhanced membrane expression of the receptor and an increased cell locomotion rate (Yang, B., G. Curpen, C. Wong, A. H. Greenberg, and E. A. Turley, manuscript submitted for publication).

The initial stimulation of motility by TGF- β was closely tied to increased levels of RHAMM mRNA and protein, and expression of RHAMM at the cell surface. Furthermore, a decrease in locomotion observed at TGF- β_1 concentrations of 30 ng/ml or greater, was associated with lower RHAMM protein synthesis suggesting that down-regulation of cell motility was linked to the decreased availability of RHAMM. However, this may not be the only way in which locomotory responses to TGF- β_1 can be down-regulated as a progres-

sive decline in locomotion was observed after prolonged (>24 h) TGF- β_1 stimulation in the presence of stable or increasing RHAMM expression (Fig. 2 B). The decline in locomotion rate may come from either rapid neutralization of TGF- β_1 in tissue culture, or from other actions of TGF- β_1 that may suppress cell movement.

TGF- β_1 stimulates HA synthesis, and HA accumulates in the extending lamellae of locomoting tumor cells and can directly promote locomotion (Turley et al., 1991). However, TGF- β induces the synthesis of other proteoglycans including chondroitin/dermatan sulfate (Chen et al., 1987; Bassols and Massagué, 1988), heparan sulfate (Nugent et al., 1992), as well as biglycan and versican (Kähäri et al., 1991). TGF- β is inactivated by binding to the proteoglycan decorin (Yamaguchi et al., 1990). Since TGF- β reduces the synthesis of decorin in fibroblasts (Kähäri et al., 1991) it is unlikely that decorin is inactivating TGF- β_1 and modulating the locomotory response. Glycosaminoglycans such as chondroitin/dermatan sulfate and heparan sulfate along with HA are important modulators of cellular recognition, adhesion, and motility (Höök et al., 1984). The possibility therefore exists that proteoglycans induced by TGF- β_1 , other than HA, participate in the locomotory response either indirectly by affecting adhesion, or directly by interfering with HA interaction with RHAMM. Recent data indicate that heparan sulfate binds with high affinity to the RHAMM receptor and effectively inhibits HA-binding and locomotion (Yang, B., and E. A. Turley, manuscript submitted for publication) consistent with other reports that heparan sulphate inhibits cell motility (Klein-Soyer et al., 1989). Thus, TGF- β_1 may ultimately downregulate motility by inducing heparan sulfate synthesis.

TGF- β also regulates the expression of the heterodimeric extracellular matrix integrin receptors (Heino et al., 1989) that have been implicated in the control of cell adhesion and locomotion (Hynes, 1992). In fibroblasts and fibrosarcomas, as well as other cell types, TGF- β_1 enhances the expression of the β_1 integrin subunit and several α integrin subunits (Heino et al., 1989). By substituting intracellular domains of different α subunits, Chan et al. (1992) have recently shown that the α_4 integrin was important for rhabdomyosarcoma cell migration. Migration on various extracellular matrices can be blocked by antibodies to the β_1 subunit, while antibodies and peptides for different α subunits blocked migration on certain types of matrix substrates (Yamada et al., 1990). Because the integrin receptors are important in cellular interaction with extracellular matrix and in focal adhesion formation, TGF- β -induced increases in expression likely effect locomotion, although this has not been demonstrated directly. How the RHAMM and integrin receptors might interact to achieve the coordinated responses of sequential adhesion and deadhesion that cells require to locomote is not known.

Although HA may promote locomotion by interacting with cell surface receptors (Toole, 1990; Turley, 1992), it also binds to a number of proteins that are extracellular such as link protein (Goetinck et al., 1987; Neame et al., 1986), versican (Krusius et al., 1987), hyaluronectin (Delpech and Halavent, 1981; Perides et al., 1989), and aggrecan (Doerge et al., 1987). Once HA secretion is stimulated by TGF- β , it may interact with the extracellular binding proteins that could enhance or inhibit its interaction with membrane

receptor RHAMM. The other major HA-binding cell surface receptor is CD44. One isoform, CD44H, binds HA while the CD44E isoform does not (Culty et al., 1990; Stamenkovic et al., 1989). CD44H but not CD44E, when expressed in melanoma cells, increased migration on HA-coated substrate (Thomas et al., 1992). Although this suggests a role for CD44 in cell locomotion, it is not clear what is the relative contribution of CD44 and RHAMM to the locomotory response. It is possible that both HA receptors are required but may have different functions in adhesion and locomotion. However, at this point we only have evidence to suggest that RHAMM participates in the TGF- β -induced locomotory response.

Many human and animal tumors secrete elevated levels of TGF- β or have increased mRNA or protein expression (Anzano et al., 1985; Dalal et al., 1993; Derynck et al., 1987; Schwarz et al., 1990), and in human and experimental tumors, TGF- β_1 is detected at high levels in metastases in vitro and in vivo (Dalal et al., 1993; Schwarz et al., 1990; Perotti et al., 1991). Tumors that are highly metastatic are also often highly motile (Paratin et al., 1988, 1989). TGF- β_1 has been found to stimulate the motility and invasion of tumors of both mesenchymal and epithelial origin such as murine fibrosarcoma (Samuel et al., 1992), human pulmonary adenocarcinoma (Mooradian et al., 1992), and murine breast carcinoma (Welsh et al., 1991). Our observation that the spontaneous motility of fibrosarcoma cells can be blocked by inhibiting TGF- β_1 synthesis or neutralizing its activity following secretion is direct evidence that the motility of malignant fibrosarcoma cells may be regulated through the production of TGF- β .

TGF- β is only one of many cytokines that promote motility, and it does not promote motility in all cell types. Locomotion of some cells, such as those of endothelial origin, is suppressed by TGF- β but is enhanced by bFGF (Sato and Rifkin, 1988; Madri et al., 1988). The FGF family, IGF, PDGF family, TGF- α , TNF- α , CSF family, IL-8, and interferons, in addition to complement and some matrix proteins, induce locomotion of many normal and transformed cells of epithelial and mesenchymal origin (as reviewed in Stoker and Gherardi, 1991). It is therefore likely that these cytokines are also used to promote locomotion by autocrine or paracrine stimulation, similar to TGF- β . Recent evidence from this laboratory suggests that macrophage chemotaxis induced by endotoxin activated serum (C5a), as well as IL-8-induced neutrophil chemotaxis, are inhibited by blocking HA/RHAMM interaction (Shi, Y., R. Savani, and E. A. Turley, manuscript in preparation). It remains to be seen whether the RHAMM receptor will also be utilized in other types of cytokine-induced motility.

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