

Hyaluronan and the Hyaluronan Receptor RHAMM Promote Focal Adhesion Turnover and Transient Tyrosine Kinase Activity

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Abstract. The molecular mechanisms whereby hyaluronan (HA) stimulates cell motility was investigated in a C-H-*ras* transformed 10T 1/2 fibroblast cell line (C3). A significant ($p < 0.001$) stimulation of C3 cell motility with HA (10 ng/ml) was accompanied by an increase in protein tyrosine phosphorylation as detected by anti-phosphotyrosine antibodies using immunoblot analysis and immunofluorescence staining of cells. Tyrosine phosphorylation of several proteins was found to be both rapid and transient with phosphorylation occurring within 1 min of HA addition and dissipating below control levels 10–15 min later. These responses were also elicited by an antibody generated against a peptide sequence within the HA receptor RHAMM. Treatment of cells with tyrosine kinase inhibitors (genistein, 10 μ g/ml or herbimycin A, 0.5 μ g/ml) or microinjection of anti-phosphotyrosine antibodies inhibited the transient protein tyrosine phosphorylation in response to HA as well as prevented HA stimulation of cell motility. To determine a link

between HA-stimulated tyrosine phosphorylation and the resulting cell locomotion, cytoskeletal reorganization was examined in C3 cells plated on fibronectin and treated with HA or anti-RHAMM antibody. These agents caused a rapid assembly and disassembly of focal adhesions as revealed by immunofluorescent localization of vinculin. The time course with which HA and antibody induced focal adhesion turnover exactly paralleled the induction of transient protein tyrosine phosphorylation. In addition, phosphotyrosine staining colocalized with vinculin within structures in the lamellapodia of these cells. Notably, the focal adhesion kinase, pp125^{FAK}, was rapidly phosphorylated and dephosphorylated after HA stimulation. These results suggest that HA stimulates locomotion via a rapid and transient protein tyrosine kinase signaling event mediated by RHAMM. They also provide a possible molecular basis for focal adhesion turnover, a process that is critical for cell locomotion.

COMPONENTS of the extracellular matrix (ECM)¹ play a fundamental role in the process of cell migration and hyaluronan (HA), an ECM glycosaminoglycan, has been particularly well studied in this context. HA has been shown to promote cell motility in vitro (Bernanke and Markwald, 1979; Boudreaux et al., 1991; Hadden and Lewis, 1991; Hakansson et al., 1980a,b; Hardwick et al., 1992; Huszar et al., 1990; Kornovski et al., 1994; Savani, R. C., C. Wang, B. Yang, M. G. Kinsella, T. N. Wight, R. Stern, and E. A. Turley, manuscript submitted for publication; Stamenkovic et al., 1991; Turley et al., 1991; West and Kumar, 1989) and to contribute to the motile responses of cells during wound repair (Toole et al., 1984; Weigel et al., 1986, 1989), tumor invasion (Iozzo, 1985; Pauli et al., 1983;

Toole, 1982; Turley, 1984), and tissue morphogenesis (Copp and Bernfield, 1988a,b; Toole et al., 1984, 1989). Like other ECM components that bind to specific receptors on cells (Aznavorian et al., 1990; McCarthy et al., 1985, 1986; Mensing et al., 1985; Yusa et al., 1989), HA interacts with cell surface receptors, which govern locomotion (for review see Turley, 1992). Thus, it has been shown that the HA receptor, RHAMM, mediates the motility of *ras*-transformed fibroblasts (Turley et al., 1991; Hardwick et al., 1992) and that the HA receptor CD44 is associated with lymphocyte homing (Stamenkovic et al., 1991) and tumor cell migration on HA substrata (Hart et al., 1991; Thomas et al., 1992). The molecular mechanisms underlying HA regulation of cell locomotion are unknown, but interactions between ECM elements and cell surface receptors have been linked to adhesion and signal transduction events that are required to initiate a motility response (for review see Lester and McCarthy, 1992; Ruoslahti, 1992; Starkey, 1990). Similarly, we have proposed (Turley, 1989a,b; Turley, 1992) that HA-receptor interactions trigger a signal transduction cascade that is responsible for orchestrating key features of locomoting cells, namely lamellae formation and ruffling (unpublished results;

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1. *Abbreviations used in this paper:* ECM, extracellular matrix; FAK, focal adhesion kinase; HA, hyaluronan; PTK, protein tyrosine kinase; RHAMM, receptor for hyaluronic acid mediated motility.

Abercrombie et al., 1970, 1977; Goldman et al., 1976; Harris, 1973; Izzard and Lochner, 1980).

The formation of cellular protrusions in the course of cell locomotion involves reorganization of actin networks at the leading edge of cells and constant formation/disassembly of close contacts and focal adhesions at the cell margin (Burrage et al., 1988). Although formation of focal adhesions is necessary for cell locomotion, these structures when present in a stable state appear to impede locomotion and there are fewer, less developed focal adhesions in motile cells. Focal adhesions may also harbor a composite of signal transduction mechanisms such that they may receive information from the ECM and serve as a point of convergence for signals from other pathways (see Gingell and Owens, 1992; Lo and Chen, 1994; Zachary and Rozengurt, 1992). This possibility is consistent with the presence of regulatory molecules such as protein kinase C (Jaken et al., 1989; Woods and Couchman, 1992), a calcium-dependent protease (Beckerle et al., 1987) and tyrosine kinase oncoprotein (Rohrschneider, 1980; Rohrschneider and Gentry, 1984; Rohrschneider and Najita, 1984) in focal adhesions as well as with the posttranscriptional modifications these structures have been reported to undergo (Beckerle, 1990; DeClue and Martin, 1987; Freed et al., 1989; Glenney and Zokas, 1989; Hirst et al., 1986; Litchfield and Ball, 1986; Pasquale et al., 1986; Sefton et al., 1981; Shaw et al., 1990; Tapley et al., 1989; Turner et al., 1989; Werth et al., 1983; Werth and Pastan, 1984). Furthermore, growth factors and tumor promoters affect focal adhesion organization (for review see Burrage et al., 1988) and perhaps most interestingly, a specific focal adhesion tyrosine kinase (FAK), pp125^{FAK} (Schaller et al., 1992) has been identified. In view of the foregoing base of information, identification of the factors that regulate focal adhesion assembly/disassembly and the characterization of the molecular sequelae leading to their turnover will likely contribute substantially to the understanding of mechanisms whereby diverse stimuli promote cell locomotion.

In this study, C-H-*ras*-transformed 10T1/2 fibroblasts were used as a model of metastatic cells to investigate signal transduction processes in response to HA-induced locomotion since these cells use HA to maintain a highly motile phenotype (Turley et al., 1991). We demonstrate that HA initiates locomotion in these cells via a protein tyrosine phosphorylation pathway, that HA regulates focal adhesion turnover, and that these effects are mediated by the HA receptor RHAMM. Our results provide the first direct evidence for the proposal that ECM-cell interactions evoke rapid intracellular signals that directly lead to locomotion. We suggest that focal adhesion turnover is an immediate result of the HA signal transduction pathway and that regulation of this pathway by HA is one of the events essential for cell locomotion to occur.

Materials and Methods

Cell Culture

As previously described (Egan et al., 1987), the CIRAS-3 (C3) cell line is derived from a 10T1/2 cell line transfected with the H-*ras* and *neo*^R genes. The cells were maintained at 37°C in 5% CO₂ on plastic tissue culture dishes (Corning, Corning, NY) in alpha-MEM growth media (GIBCO BRL, Gaithersburg, MD) supplemented with 10 mM Hepes, pH 7.4 (Sigma Chemical Co., St. Louis, MO) and 10% FCS (Hyclone Labs, Inc., Logan, UT). Cells were subcultured in 0.25% trypsin (Difco Bactotrypsin)/2 mM

EDTA (Sigma) when cultures reached 80% confluence. All experiments were performed on cells with low passage numbers (P9-P15) and at 24 h after subculture, a time when cells were locomoting rapidly and expressing elevated levels of HA receptors (data not shown). For motility studies, 10⁵ cells were placed into 25 cm² tissue culture flasks (Corning) containing growth media and maintained as above. After 12 h of growth, the media was aspirated, the cells were rinsed with HBSS (GIBCO BRL), and fresh serum-free medium containing 4.0 µg/ml transferrin (human, GIBCO BRL) and 2.0 µl/ml insulin (bovine, Sigma) was added to the flasks. The cells were maintained in this defined medium for another 12 h after which the media was changed to fresh defined media before the cell motility analysis. Cells were treated as above for all remaining experiments except as follows: for phosphorylation and immunoprecipitation analysis, 5 × 10⁵ cells were plated on 100-mm tissue culture dishes (Corning); for immunofluorescence, 10⁵ cells were plated on sterile coverslips in 60-mm dishes; and for microinjection, 10⁵ cells were plated on 60-mm dishes. To maintain cells at physiological pH during microinjection, an altered defined media containing 25 mM Hepes (instead of 10 mM Hepes) and 1.8 g/L NaHCO₃ (instead of 3.6 g/L NaHCO₃) was added before injection. In cases where cells were plated on a fibronectin-coated surface, coverslips or culture dishes were incubated with human plasma fibronectin (50 µg/ml; Collaborative Biomedical/Becton Dickinson Labware, Bedford, MA) overnight at 4°C before plating of cells.

Addition of HA and Antibody

Varying dilutions of Healon® (0.1 ng/ml–1 µg/ml; rooster comb hyaluronan; Pharmacia LKB Biotechnology, Uppsala, Sweden) were added directly to the culture medium of C3 cells in order to test appropriate concentrations required to stimulate cell motility. A concentration of 10 ng/ml was found to be optimal and was used in all HA stimulation experiments. Similarly, antisera generated against a peptide corresponding to a sequence in RHAMM (amino acids 125–145, QEKYNDTAQSLRDVTAQLESV; Hardwick et al., 1992) was added to C3 fibroblasts and a 1:100,000 dilution was found to be optimal for stimulating locomotion. The same dilution of preimmune serum was used as control.

Treatment of C3 Fibroblasts with Tyrosine Kinase Inhibitors

Stock solutions of genistein and herbimycin A (GIBCO BRL) were prepared in DMSO and stored at –20°C. Concentrations of 10 µg/ml (37 µM) of genistein or 0.5 µg/ml (0.87 µM) of herbimycin A (Akiyama et al., 1987; Uehara et al., 1989) were added directly to the cell culture medium. Timed studies indicated that a pretreatment time of 10 min for genistein or 4 h for herbimycin A was required to inhibit the HA-induced locomotory responses (data not shown). For controls, cells were treated with DMSO for the same time periods.

Locomotion Analysis

Cell locomotion was recorded using a computerized timelapse image analysis system (Image-1, Universal Imaging Corporation, Westchester, PA) capable of measuring random locomotion by nuclear displacement. During the filming period, cells were maintained in defined medium (described above) at physiological pH on a heated stage (37°C). For each experiment, a minimum of 30 cells were tracked either every 20 min for a 10-h period or every 10 min for a 1–2-h period. At least three trials of each experimental group were observed for a total of 90 cells per group. The results were expressed as means (µm/h) ± SEM.

Microinjection

Twenty-four hours after subculture, C3 fibroblasts prepared as described above were microinjected with anti-phosphotyrosine antibody (rabbit polyclonal; Upstate Biotechnology Incorporated [UBI], Lake Placid, NY) or control antibody (rabbit IgG; Sigma). Antibodies (2 mg/ml) were dissolved in injection buffer (150 mM KCl, 2 mM MOPS, pH 7.0; Cooper et al., 1988) containing 1 mg/ml BSA and Lucifer yellow dye, and then backloaded into micropipettes (pulled to 1 µm tip diameter using a model PD-5 microelectrode puller; Narishige Scientific Instrument Lab., Tokyo, Japan). Cells were microinjected using an Eppendorf microinjector (model 5242; Eppendorf, Hamburg, Germany) and Leica micromanipulator on a Zeiss fluorescence microscope. After microinjection, cells were allowed to recover for 30 min before addition of HA and commencement of motility analysis.

Cell Lysis and Immunoblot Analysis

C3 cell cultures were exposed to HA (10 ng/ml), anti-RHAMM, or control treatments for various time periods at 37°C, and then placed on ice. The culture media was removed, the plates rinsed with cold PBS (2.7 mM KCl, 1.1 mM KH₂PO₄, 138 mM NaCl, 8.1 mM Na₂HPO₄; pH 7.4) containing 250 μ M sodium orthovanadate and the cells were lysed with ice cold RIPA lysis buffer (25 mM Tris, pH 7.2, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 0.15 M NaCl, 1 mM EDTA) containing 1 μ M leupeptin, 1,000 KU/ml Aprotinin, 1 μ g/ml PMSF, and 1 mM sodium orthovanadate (all chemicals from Sigma). Lysates were scraped into microcentrifuge tubes and after 10 min on ice were centrifuged at 13,000 rpm for 15 min at 4°C (Hereaus Biofuge 13, Baxter Diagnostics Corporation, Mississauga, Ontario). Protein concentrations of the supernatants and BSA standards were determined using the DC protein assay (Bio-Rad Laboratories, Rockville Center, NY) and duplicate samples containing 100 μ g of protein each along with prestained molecular weight markers (Sigma) were separated by SDS-PAGE (10% gel; Laemmli, 1970). The proteins on the gels were either electrophoretically transferred to nitrocellulose membranes (Bio-Rad) or stained with Coomassie blue to check for equal loading. Additional protein-binding sites on the nitrocellulose membranes were blocked with 5% defatted milk in TBS (50 mM Tris HCl, pH 7.4, 200 mM NaCl), and then the membranes were incubated with anti-phosphotyrosine mAb (1 μ g/ml 4G10 [UBI] in 1% defatted milk/TBS) for 2 h at room temperature on a rotator (Nutator; Becton Dickinson and Company, Parsippany, NJ). The membranes were washed four times in 0.05% Tween-20/TBS before incubation with peroxidase-conjugated goat anti-mouse secondary antibody (1:5,000 dilution in 1% milk/Tween-TBS; Sigma) for 1 h at room temperature. After washing, blots were developed using the ECL Western blotting detection system (Amersham International plc, Amersham, UK) according to manufacturer's instructions. To establish antibody specificity, parallel blots were probed with anti-phosphotyrosine that had been preincubated with 200 μ M phosphotyrosine (Sigma) for 1 h. To further check for equal loading, blots were stripped (stripping buffer: 100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7, at 50°C for 30 min), blocked, and reprobed using an anti-vinculin mAb [1:200; Sigma] (data not shown). Densitometry of the blots was performed on a Bio-Rad Video Densitometer (model 620) and analyzed using the 1-D Analyst II software.

Immunoprecipitation

C3 fibroblasts were prepared, stimulated with HA, and lysed as above. Each sample (500 μ g of protein) was incubated with anti-p125^{FAK} mAb (10 μ g/ml; UBI) and goat anti-mouse IgG (5 μ g/ml; Sigma) for 1 h at 4°C by mixing end-over-end. To precipitate, 100 μ l protein G-Agarose (GIBCO BRL) was added to each tube and the samples were mixed end-over-end for another 30 min at 4°C. The beads were pelleted by brief centrifugation at 13,000 rpm and washed three times with RIPA buffer. The proteins were released from the beads by boiling in Laemmli sample buffer then subjected to SDS-PAGE and anti-phosphotyrosine immunoblotting as above. To assess whether equal quantities of FAK were immunoprecipitated from control and treated samples, the blots were stripped and reprobed with purified anti-FAK (10 μ g/ml antibody 2A7; a generous gift from J. T. Parsons, University of Virginia, Charlottesville, VA).

Immunofluorescent Staining

C3 fibroblasts were grown on untreated or fibronectin-coated glass coverslips for 24 h as described above. The cells were exposed to HA (10 ng/ml), anti-RHAMM or control treatment for 1 min, 5 min, 15 min, 30 min or 1 h. At the appropriate time periods, the media was aspirated, the cells rinsed with PBS, and then fixed with 3% paraformaldehyde (Sigma)/PBS for 10 min. Cells were washed three times for 10 min with wash solution (10% FCS/PBS containing 0.02% sodium azide), permeabilized with 0.2% Triton X-100/PBS for 5 min, and washed three more times. For single immunofluorescence studies, the fixed cells were incubated either at 4°C overnight or at 37°C for 2 h with anti-phosphotyrosine mAb (5 μ g/ml; 4G10, UBI), anti-vinculin mAb (1:50; Sigma), or appropriate IgG controls (Sigma) in wash solution. After washing five times, coverslips were incubated with goat anti-mouse TRITC (1:300; Sigma) for 3 h. For double immunofluorescence studies, the cells were simultaneously incubated with anti-vinculin and a rabbit polyclonal anti-phosphotyrosine (10 μ g/ml; UBI) primary antibodies and with goat anti-mouse TRITC and goat anti-rabbit FITC secondary antibodies as above. After washing, the coverslips were mounted in no-fade mountant (10 mM p-phenylenediamine, 118 mM Tris-

HCl, pH 7.4, 90% glycerol) onto glass slides and sealed with nail polish. Observations and photomicrographs were obtained with a Zeiss Axiovert 35M fluorescent microscope using epifluorescence.

Results

Exogenous HA Stimulates Locomotion of C3 Fibroblasts

HA has previously been reported to stimulate the locomotion of *ras*-transformed fibroblasts (Hardwick et al., 1992; Turley et al., 1991). Similarly, the response of the *ras*-transformed 10T1/2 cell line (Egan et al., 1987) used here (C3 cells) was presently found to be concentration dependent (data not shown) with maximal stimulation of motility occurring at 10 ng/ml of HA. Two hours after addition of HA, C3 cells reached a peak locomotion rate of 18.8 μ m/h, which was significantly different ($p < 0.001$) from that of control untreated cells (Fig. 1).

Exogenous HA Stimulates Tyrosine Phosphorylation in C3 Fibroblasts

C3 cells respond to HA with an increase in protein tyrosine phosphorylation as determined by immunoblot analysis (Fig. 2) and immunofluorescent staining (Fig. 3) with anti-phosphotyrosine antibodies. The phosphorylation response was both rapid and transient; it occurred within 1 min of HA addition (10 ng/ml) and decreased within the subsequent 5–15 min (Figs. 2 and 3). In Fig. 2 *a*, multiple protein bands (185, 125, 115, 85, 75, 65, 60, 56, 50, 40, and 38 kD) in cell lysates are seen to be tyrosine phosphorylated and many of these show a transient increase over controls after 1 min of HA stimulation (Fig. 2 *b*). As well, after 15 min of HA stimulation (Fig. 2 *a*, lane 5), the relative levels of tyrosine phosphorylated proteins drop below control levels (Fig. 2, *a* and *b*) indicating that a secondary dephosphorylation event fol-

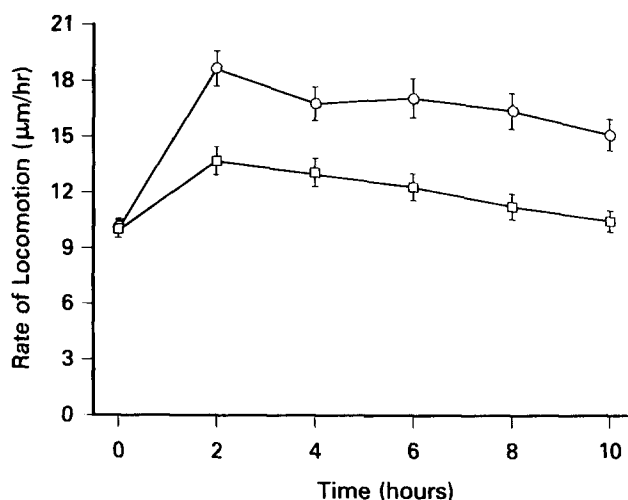


Figure 1. Locomotion of C3 cells with (○) or without (□) the addition of HA (10 ng/ml). C3 cells, 24 h after subculture, were analyzed by computer timelapse image analysis for 10 h in the presence (○) or absence (□) of HA. Within 2 h of HA addition, cells were locomoting maximally, after which locomotion decreased slightly. Values remain elevated over the controls, for the remaining 8 h. Values represent the mean \pm SEM. $n = 90$ cells.

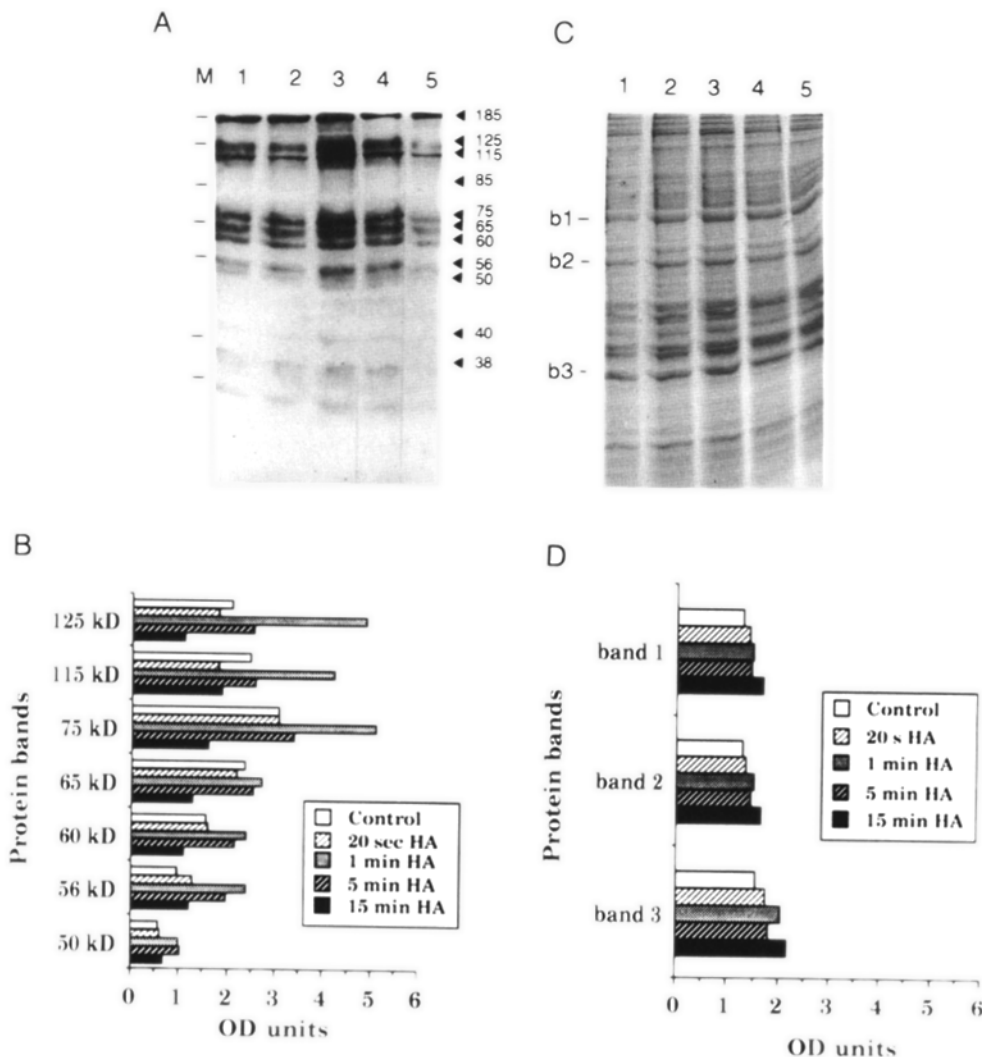


Figure 2. Effect of HA addition on tyrosine phosphorylation. C3 cells were incubated in the absence (lane 1) or presence (lanes 2–5) of 10 ng/ml of HA for 20 s (lane 2), 1 min (lane 3), 5 min (lane 4), or 10 min (lane 5) before cell lysis with RIPA buffer. Equal protein concentrations from the lysates were electrophoresed on SDS-PAGE gels and either stained with Coomassie blue (C) or transferred to nitrocellulose, immunoblotted using an antiphosphotyrosine mAb (4G10, UBI) and developed with ECL (A). Densitometric analysis (B) reveals increased phosphotyrosine in bands at 125, 115, 75, 60, 56, and 50. Densitometry of three bands (b1, b2, and b3) from the Coomassie stain is shown (D). The molecular weight markers (M) are marked at 190, 125, 88, 65, 56, 38, and 33.5 kD.

lows the initial phosphorylation. Anti-phosphotyrosine recognition of all protein bands above is completely eliminated by preincubation of the antibody with phosphotyrosine (data not shown). In addition, densitometric analysis of a Coomassie blue stained parallel gel shown in Fig. 2, c and d demonstrates equal loading of cell lysates.

Fig. 3 (a–c) shows anti-phosphotyrosine immunofluorescence of representative control cells demonstrating the variations of staining observed in these experiments. Immunofluorescence revealed a transient increase and a distinct pattern of anti-phosphotyrosine antibody staining after HA stimulation (Fig. 3, d–f). After 1 min of HA treatment, an increase in staining generally occurred along the advancing lamellae, that was often amorphous (Fig. 3 d) or in punctate regions. As well, the punctate regions of staining become larger than seen in control cells (Fig. 3, a–c). Although difficult to quantitate, in all experiments over 40% of the 1 min HA stimulated cells exhibited the high intensity of punctate staining observed in Fig. 3 d while less than 5% of control treated cells showed a similar intensity of staining. Consistent with Western blot analysis, staining decreased to below control levels by 15 min (Fig. 3, g and h).

Anti-RHAMM Mimics HA Responses

Antibodies generated against RHAMM have previously been shown to block the motility of *ras*-transformed cells (Hardwick et al., 1992; Turley et al., 1991). However, low concentrations of an anti-peptide antibody to RHAMM was found here to mimic HA in that it stimulated both cell locomotion (Fig. 4 A) and tyrosine phosphorylation (Fig. 4, B and C). The time course and staining patterns observed in these experiments are similar to those seen above in response to HA; locomotion reached a maximum in the first 2 h after antibody addition and tyrosine phosphorylation peaked after 1 min, but decreased below control levels by 15 min after antibody stimulation. In Fig. 4 C, representative cells were chosen to display the variations in staining patterns observed. As noted after HA stimulation, protein tyrosine phosphorylation occurred primarily in the leading lamellae as plaque-like and amorphous staining.

Tyrosine Kinase Inhibitors Block HA-mediated Motility

To determine whether inhibitors of protein tyrosine kinases

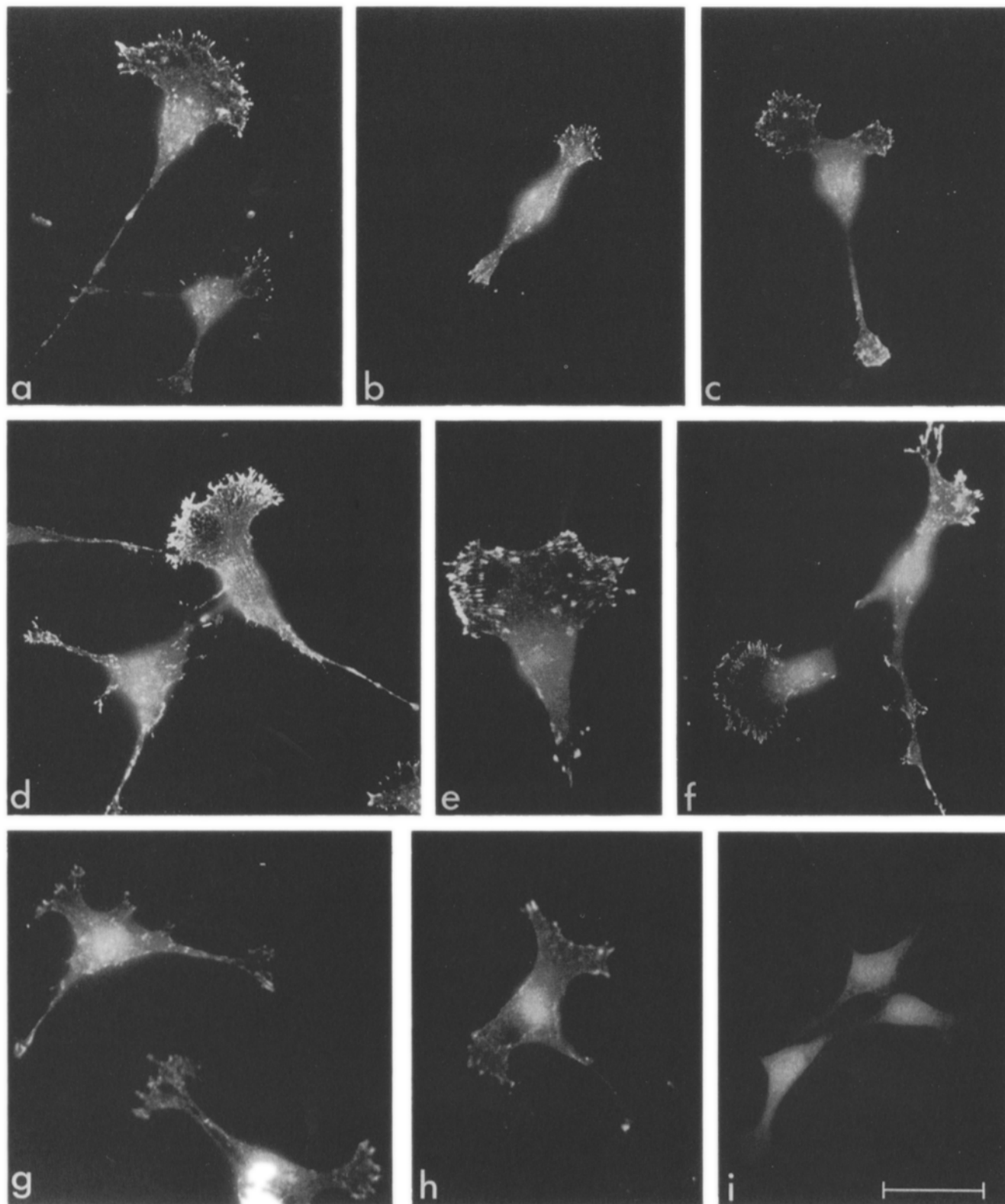


Figure 3. Immunofluorescence localization of phosphotyrosine in HA-stimulated cells. C3 fibroblasts were incubated in the absence (*a-c*) or presence of 10 ng/ml HA for 1 min (*d-f*), or 15 min (*g* and *h*) before fixation and staining with anti-phosphotyrosine. Cells exposed to HA for 1 min show an increased staining for phosphotyrosine that drops below control levels by 15 min. Mouse IgG control is shown (*i*). Size bar represents 25 μ m.

effectively block HA stimulated locomotion, the two inhibitors genistein and herbimycin A were used. Genistein (10 $\mu\text{g/ml}$), added to cell cultures 10 min before HA stimulation, abrogates the HA stimulated tyrosine phosphorylation (Fig. 5, *A* and *B*). Both inhibitors block protein tyrosine phosphorylation as determined by Western blot analysis (Fig. 5, *A* and *B*) and immunofluorescence (data not shown).

Addition of genistein to C3 fibroblasts had no effect on basal motility rate of control cells (Figs. 1 and 6), indicating that it is non-toxic. However, genistein pretreatment of C3 cells prevents the HA-induced increase in motility (Fig. 6). Addition of herbimycin A (0.5 $\mu\text{g/ml}$; 4 h pretreatment) to C3 cells similarly results in inhibition of HA-mediated motility (Fig. 7).

Cells were stimulated with HA just before the addition of genistein to determine whether an initial burst of protein tyrosine phosphorylation is sufficient for enhancement of cell locomotion by HA. This experimental paradigm does not inhibit a locomotory response to HA (Fig. 8), indicating that only subsequent protein tyrosine phosphorylation events are necessary for increased locomotion elicited by HA (Fig. 8).

Microinjection of Anti-Phosphotyrosine Blocks HA-mediated Motility

To further assess the role of protein tyrosine phosphorylation in HA-mediated cell locomotion, C3 fibroblasts were microinjected with either an anti-phosphotyrosine polyclonal antibody or a control antibody. After recovery from injection for 30 min, cells were treated with HA and their motility was recorded for 2 h. The controls responded to HA with a significant ($p < 0.01$) increase in locomotion (injected, unstimulated control cells locomote at 10.4 ± 1.1) (Fig. 9, *A* and *B*), while the motility of the anti-phosphotyrosine-injected cells was inhibited to below basal level (Fig. 9 *C*).

HA Promotes Reorganization and Phosphorylation within Focal Adhesion Sites

Locomoting *ras*-transformed fibroblasts, like other motile cells, exhibit few poorly developed focal adhesions (Takahashi et al., 1986; Fig. 10) and display vinculin staining that localizes to heavily stained belts within the lamellae, to contact points in the trailing end of cells, and to punctate regions in the cell body and lamellapodia that do not resemble the more developed focal contacts seen in stationary normal fibroblasts. To facilitate the study of focal contacts in *ras*-transformed cells, C3 cells were plated on fibronectin-coated surfaces which are known to promote focal adhesion assembly even in transformed cells (see Burridge et al., 1988). Under these conditions, the cells exhibited a somewhat more flattened appearance, but remained responsive to HA and stimulatory anti-RHAMM antibody (data not shown). Anti-vinculin immunofluorescence staining of these cells plated on fibronectin (Fig. 10, *a-c*) revealed the presence of mainly small punctate focal contacts as well as vinculin staining within the lamellae of cells. However, immediately after HA addition (1 min; Fig. 10, *d-f*), the vinculin staining increased, appearing as well defined plaques within the lamellapodia and along the leading edge of the cells, typical of focal adhesions of less motile or normal cells. This appearance of focal adhesions is transient and by 15 min after HA addi-

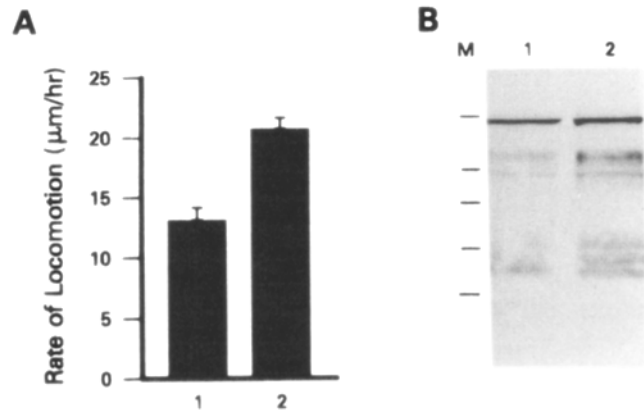


Figure 4. Anti-RHAMM mimics HA responses. (*A*) Anti-RHAMM (lane 2) or control preimmune (lane 1) serum was added to C3 fibroblasts and the motility recorded for 2 h. Anti-RHAMM, like HA, addition results in a significant increase ($p < 0.001$) in cell locomotion. (*B*) Anti-RHAMM (lane 2) or preimmune (lane 1) serum was added to C3 cells for 1 min, followed by cell lysis, SDS-PAGE, and anti-phosphotyrosine immunoblotting. Anti-RHAMM, like HA, stimulated an increase in tyrosine phosphorylation of several protein bands. Markers (*M*) are marked at 211, 119, 98, 80.6, and 64.4. (*C*) C3 cells were treated with preimmune serum (*a-c*) for 1 min, or anti-RHAMM for 1 min (*d-f*), or 15 min (*g* and *h*) before fixation and indirect immunofluorescent staining with anti-phosphotyrosine mAb. Like HA treatment, anti-RHAMM treatment results in a transient increase in phosphotyrosine staining that drops below control levels by 15 min after stimulation. Mouse IgG control shown (*i*). Size bar represents 25 μm .

tion (Fig. 10, *g* and *h*) there is a loss of staining and cells begin to more closely resemble control cells. The rapid appearance/disappearance of distinct focal adhesions corresponds to the transient stimulation of HA-induced protein tyrosine phosphorylation. Cells treated with anti-RHAMM also exhibit this induced focal adhesion turnover in an identical timecourse (data not shown).

Double staining of the cells with anti-vinculin mAb and polyclonal anti-phosphotyrosine mAb show that when cells are maintained under standard culture conditions without fibronectin, phosphotyrosine colocalizes with vinculin staining in the lamellae of locomoting cells (Fig. 11). Colocalization of vinculin and phosphotyrosine at the front of the cells suggests that tyrosine phosphorylation is associated with immature, newly formed adhesion sites at the leading lamellae of the cells. Similar results were obtained when cells were maintained on a fibronectin substrate.

Focal Adhesion Kinase, *pp125^{FAK}*, Is Phosphorylated/Dephosphorylated with HA Stimulation

Because HA stimulation results in both an increase in the specific tyrosine phosphorylation of a 125-kD protein (the size of FAK), since FAK is localized to focal adhesions, and since integrins have been shown to mediate an increase in FAK phosphorylation (Burridge et al., 1992; Guan and Shalloway, 1992; Hanks et al., 1992; Kornberg et al., 1992; Lipfert et al., 1992), we examined the effect of HA addition on the tyrosine phosphorylation of this kinase. In contrast to reports of integrin-activated cells, where a prolonged stimulation of FAK phosphorylation occurs (Burridge et al., 1992;

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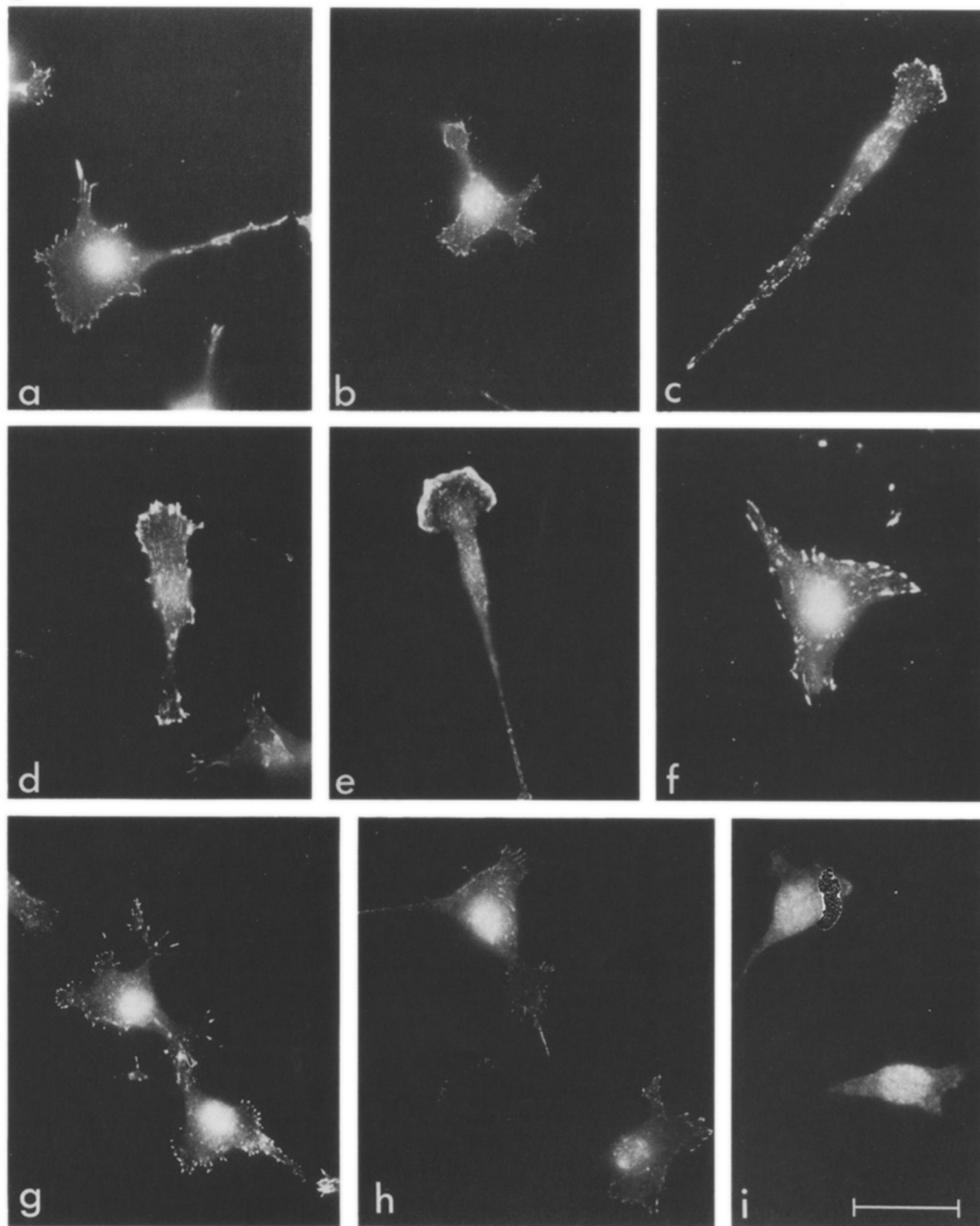


Figure 4.

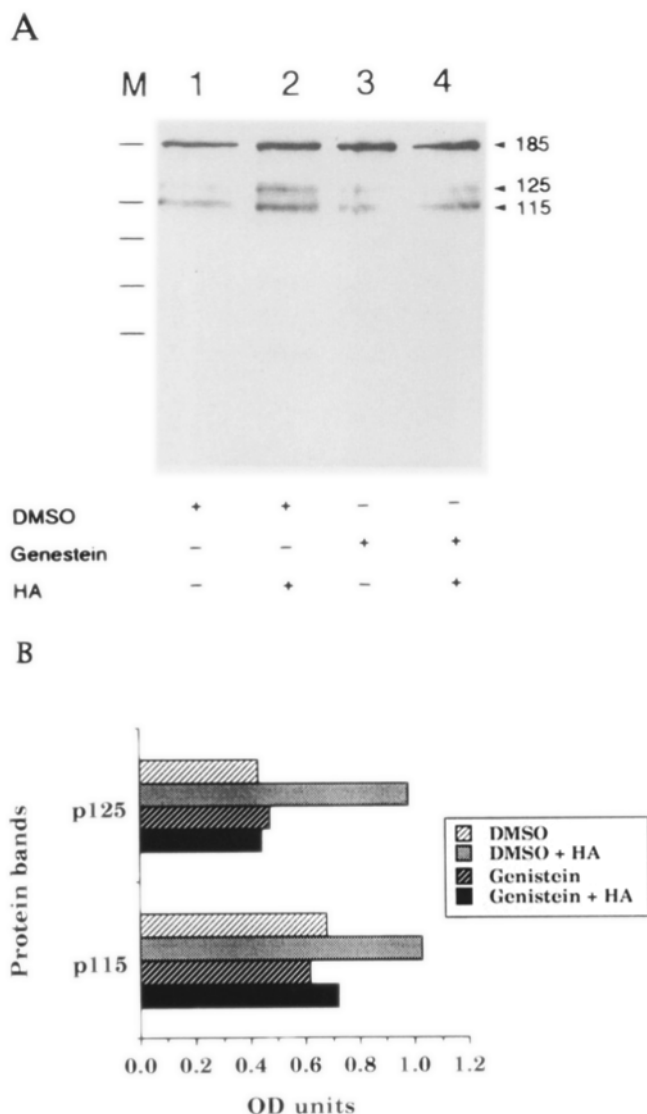


Figure 5. Protein tyrosine kinase inhibitors diminish HA-mediated tyrosine phosphorylation as detected by anti-phosphotyrosine immunoblotting. (A) C3 fibroblasts were incubated with genistein (10 $\mu\text{g/ml}$; lanes 3 and 4) or DMSO for controls (lanes 1 and 2) for 10 min before incubation with (lanes 2 and 4) or without (lanes 1 and 3) HA (10 ng/ml) for 1 min. Lysates were subjected to SDS-PAGE and anti-phosphotyrosine immunoblot analysis. Genistein addition diminishes the HA-stimulated phosphorylation of the bands at 125 and 115 kD, as seen by densitometric analysis (B). The molecular weight markers (M) are marked at 211, 119, 98, 80.6, and 64.4 kD.

Hanks et al., 1992; Kornberg, et al., 1992; Lipfert et al., 1992), HA treatment results in only a transient increase in FAK phosphorylation (1 min; Fig. 12) and this is followed by a clear decrease in phosphorylation of this kinase (5 min; Fig. 12). The timecourse of FAK phosphorylation/dephosphorylation coincides with the HA stimulated assembly/disassembly of adhesion plaques within the C3 fibroblasts. Although this data supports a role for FAK in HA promoted focal adhesion assembly/disassembly, the kinase could not be detected in the focal adhesions of these cells by immunofluorescent staining (data not shown).

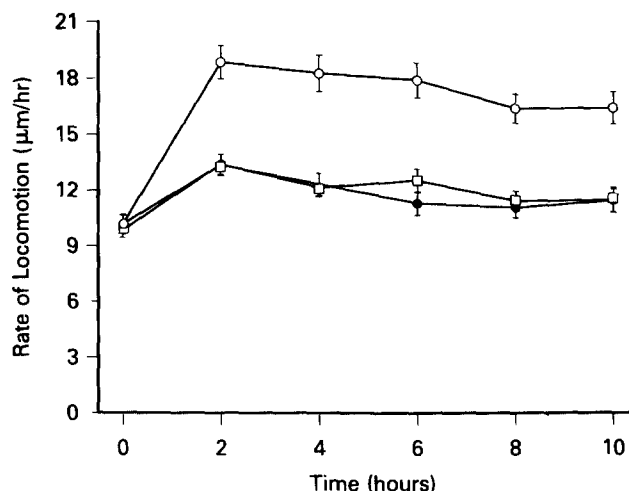


Figure 6. Genistein inhibits HA-mediated locomotion. C3 cells, 24 h after subculturing, were treated with genistein (10 $\mu\text{g/ml}$; \square , \bullet) or control treatment (\circ), and then the motility was recorded with (\circ , \bullet) or without (\square) HA (10 ng/ml) addition. The genistein + HA- (\bullet) treated cells locomote basally, in contrast to the control treatment + HA (\circ) cells. Values represent the mean \pm SEM. $n = 90$ cells.

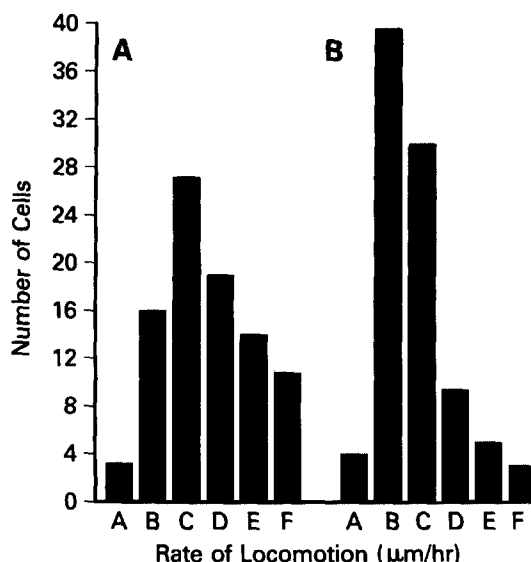


Figure 7. Herbimycin A inhibits HA-promoted motility. C3 cells, 24 h after subculturing, were treated with control treatment (A) or Herbimycin A (0.5 $\mu\text{g/ml}$; B) for 4 h before HA (10 ng/ml) addition. The cell motility was recorded for 2 h using computer timelapse image analysis and the motility rates were grouped accordingly (A, 0-2.0; B, 2.1-4.0; C, 4.1-6.0; D, 6.1-8.0; E, 8.1-10.0; F, ≥ 10.1 $\mu\text{m/min}$). It is apparent that a large population of the cells pre-treated with herbimycin A (B) have a lower motility rate than the HA-treated controls (A). $n = 90$.

Discussion

HA is emerging as a critical regulator of the locomotion of fibroblasts (Bernanke and Markwald, 1979; Hardwick et al., 1992; Turley et al., 1991), epithelial cells (Hadden and Lewis, 1991), white cells (Hakansson et al., 1980, a,b; Stamenkovic et al., 1991), smooth muscle cells (Boudreaux et al., 1991; Savani, R. C., C. Wang, B. Yang, M. G. Kinsella, T. N.

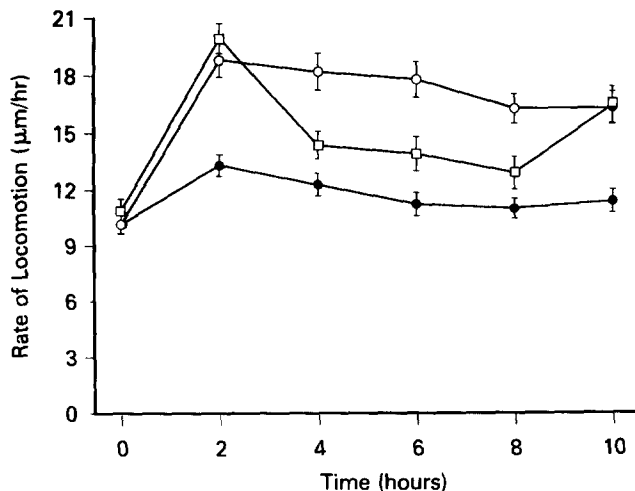


Figure 8. The effect of genistein added after HA stimulation. C3 cell cultures were treated with 10 ng/ml HA (○, □, ●) before or after genistein (10 μg/ml) addition. The sample treated with HA only (○) illustrates a typical HA-mediated locomotory response. Genistein added 10 min before HA completely inhibits the HA-promoted locomotion increase (●), but genistein addition 10 min after HA addition does not affect the sharp increase in locomotion (□). However the post-HA genistein-treated cells do not have a high maintained rate of locomotion. Values represent the mean ± SEM. *n* = 90 cells.

Wight, R. Stern, and E. A. Turley, manuscript submitted for publication), sperm (Huszar et al., 1990; Kornovski et al., 1993), and vascular endothelial cells (Banerjee and Toole, 1992; West and Kumar, 1989). Moreover, HA is required in the process whereby motility factors (Schor et al., 1989) and

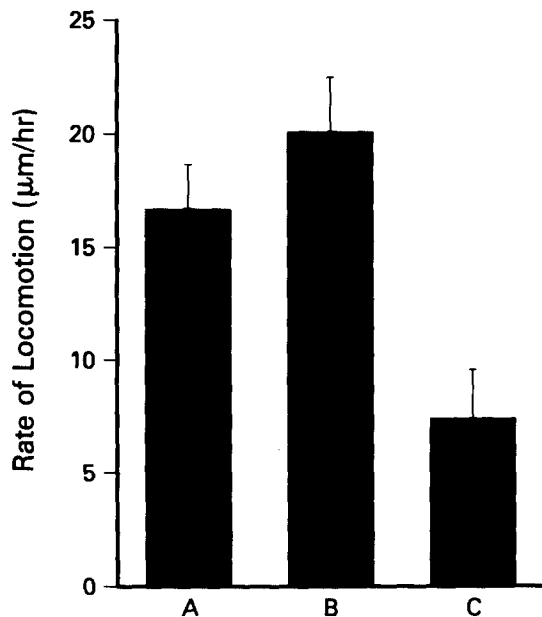


Figure 9. Microinjection of antiphosphotyrosine antibodies inhibits HA-stimulated motility. C3 fibroblasts were microinjected with anti-phosphotyrosine antibodies or rabbit IgG control injection before HA (10 ng/ml) addition and motility was recorded for 2 h. Uninjected control cells (A) and cells injected with rabbit IgG (B) respond to HA with increased locomotion but cells injected with anti-phosphotyrosine antibodies (C) do not. Values represent the mean ± SEM.

growth factors such as TGF-β (Samuel et al., 1993) regulate locomotion in vitro. Although virtually nothing is known about the molecular mechanisms underlying HA action on cell motility, its weakly adhesive physicochemical properties have been considered to contribute to cell detachment during locomotion. We provide evidence here that HA/receptor interactions, like ECM/integrin interactions (Juliano and Haskell, 1993), result in cell signaling events culminating in profound effects on cell behavior. The C-H-ras fibrosarcoma (C3) cells are a rapidly locomoting cell line that requires HA/RHAMM interactions to maintain their highly motile phenotype. Here we show that HA initiates cell locomotion in C3 cells via a RHAMM-induced protein tyrosine kinase (PTK) pathway, that inhibition of this PTK activity abolishes the HA locomotory response, and that an initial, short-lasting phosphorylation event is required for the stimulation of motility to occur. These results clearly implicate the regulation of protein tyrosine phosphorylation in ECM-driven cell locomotion. We also show that the transient assembly of focal adhesions results from HA signaling and identify HA as the first extracellular ligand to regulate focal adhesion turnover concomitant with enhanced locomotion. We further identify pp125^{FAK} as a protein target of the HA signaling pathway. Perhaps most importantly, and unlike the effect of other ECM components, the effect of HA on protein tyrosine phosphorylation is transient, such that the burst of tyrosine phosphorylation is rapidly dampened to below control levels, and focal adhesions disappear as rapidly as they appear. This transient event suggests that HA may also regulate phosphatases or perhaps other signal transduction pathways that are involved in focal adhesion disassembly.

The HA:RHAMM signaling pathway is clearly complex in nature and can be divided into two phases. In the first phase, HA:RHAMM interactions mediate both rapid and transient protein tyrosine phosphorylation and focal adhesion turnover, with the brief tyrosine phosphorylation and focal adhesion assembly (after 1 min of stimulation) giving way to a more long term dephosphorylation and disassembly (within 15 min of stimulation). Further, if HA is added before the inhibitor genistein, stimulation occurs indicating that only the rapid and transient burst in tyrosine phosphorylation in the first phase is sufficient for stimulating the subsequent motility. This first phase may be critical for bringing together regulatory proteins within focal adhesion sites. In the second phase, cell locomotion increases significantly and peaks within the first 2 h of HA or anti-RHAMM stimulation. Thus, elevated cell motility correlates with protein tyrosine dephosphorylation and focal adhesion disassembly. This appears initially to be at odds with our observation that treatment of fibroblasts with tyrosine kinase inhibitors, genistein or herbimycin A, or with microinjected anti-phosphotyrosine antibodies inhibits HA-mediated motility. Since the inhibitors did not further enhance the locomotory/dephosphorylation phase, as one might expect, the initial organization of focal adhesions and concomitant protein tyrosine phosphorylation must be required for subsequent locomotory events. The molecular basis for this requirement is not yet clear. As a result, there is an interesting turn of events in which an initial burst of tyrosine phosphorylation triggers a later cell motility/dephosphorylation phase.

Such an association between protein tyrosine phosphorylation and downstream locomotory behavior has been noted

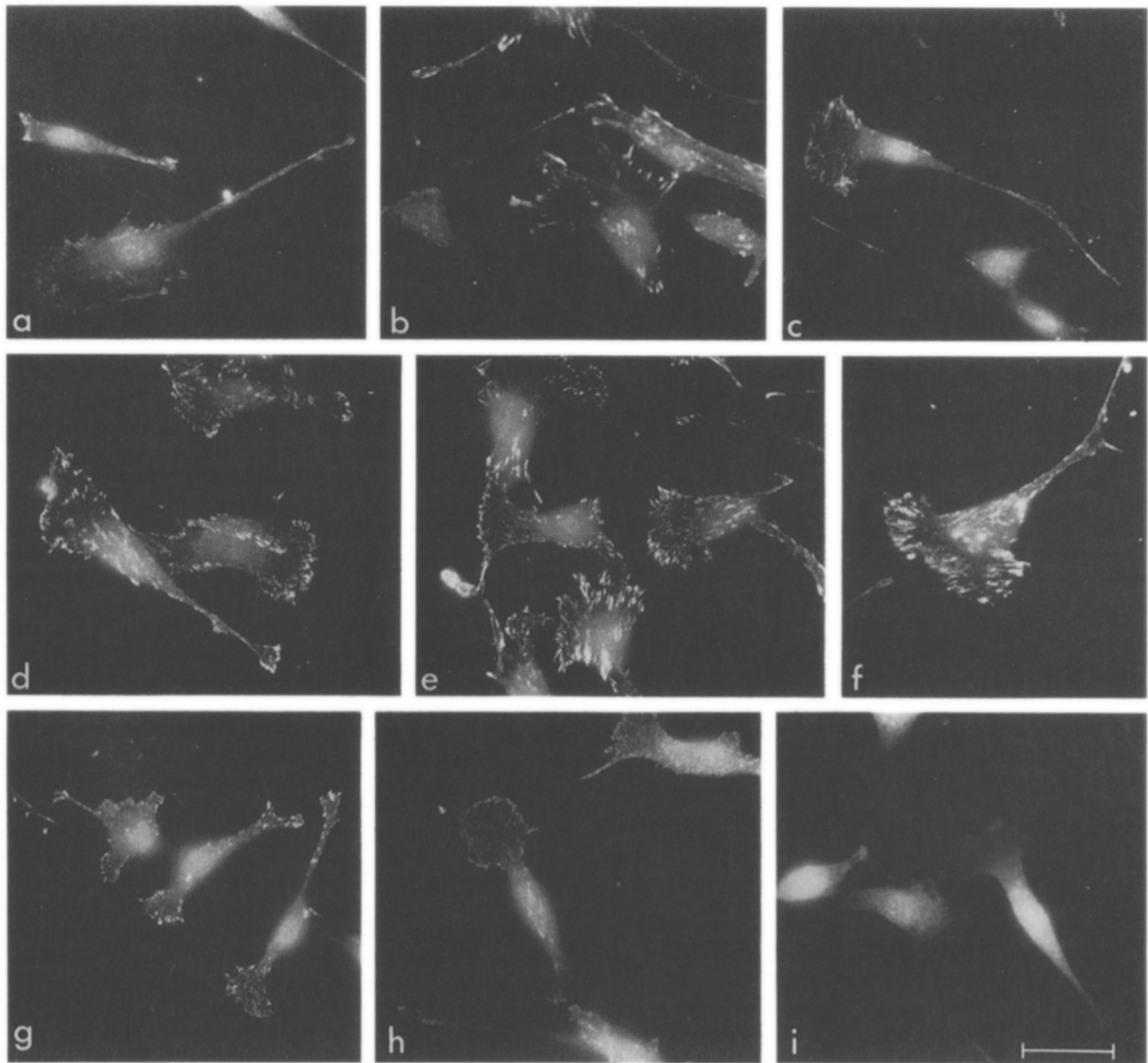


Figure 10. Immunofluorescent localization of vinculin after HA stimulation. C3 fibroblasts were incubated in the absence (a–c) or presence of 10 ng/ml HA for 1 min (d–f) or 15 min (g–i) before fixation and staining with anti-vinculin. Control cells have a few vinculin containing plaques. However, with HA stimulation, there is an increased number of vinculin containing plaques then a disappearance of punctate vinculin staining. Mouse IgG control is shown (i). Size bar represents 26 μ m.

previously. PTK activity has been implicated in the ligand-induced membrane ruffling of human epidermoid carcinoma cells (Izumi et al., 1988) and in neutrophil migration induced by the chemotactic factor fMet-Leu-Phe (Gaudrey et al., 1992). As well, other chemotactic factors and ECM molecules that regulate cell locomotion also promote protein phosphorylation (Bottaro et al., 1991; Ferrel and Martin, 1989; Mueller et al., 1992; Nakamura and Yamamura, 1989; Segall and Gerisch, 1989; Zigmund, 1989). In particular, the cell motility triggered by scatter factor is mediated by the tyrosine kinase receptor c-Met (Komada and Kiamura, 1993; Weidner et al., 1993). Like HA:RHAMM signaling, c-Met activation and tyrosine phosphorylation results in cell motility that occurs more downstream. Al-

though these studies predict that protein phosphorylation is essential for ligand-induced locomotion, the targets of these protein kinase cascades have remained elusive. It is likely that ligand binding and signal transduction, such as ligand regulated PTKs, target the cytoskeleton and result in changes in cytoskeletal organization which is necessary for cell locomotion. Since focal adhesion turnover is required for cell locomotion, these adhesion sites are also likely targets for factors that stimulate locomotion. Cycles of attachment and detachment, which are a prerequisite to lamellae extension and hence cell motility, are likely to be mediated by close contacts and point focal adhesions (Burridge et al., 1988). Excessive development of mature focal adhesions, regulated in part by ECM/integrin interactions (see Burridge et al.,

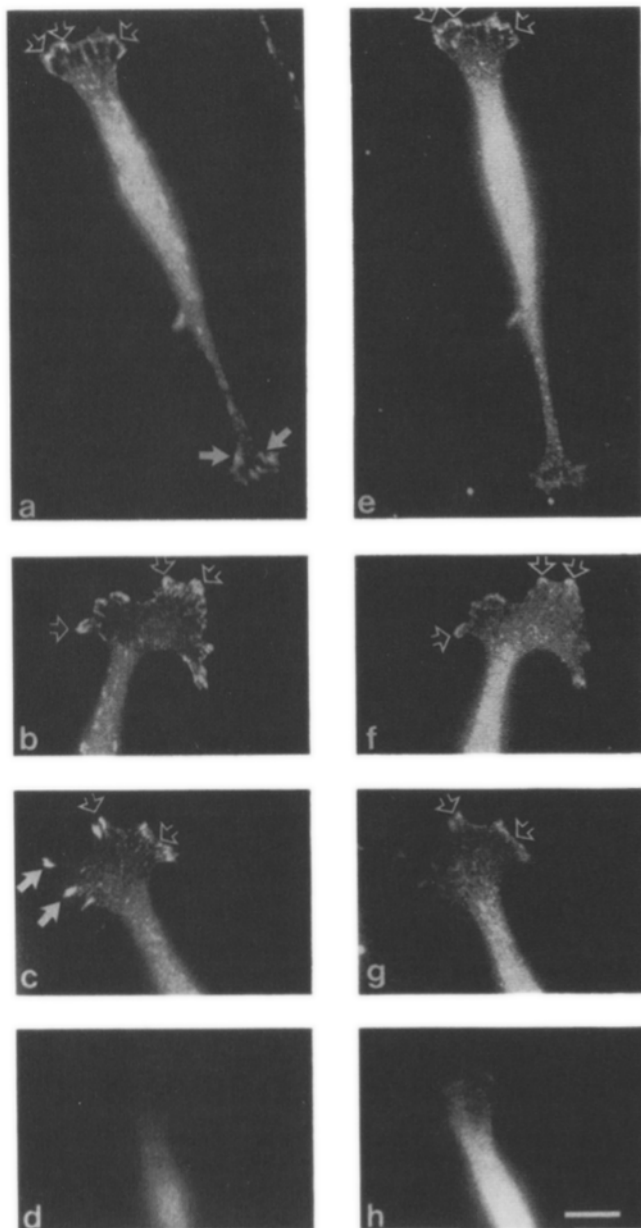


Figure 11. Double immunofluorescence for vinculin and phosphotyrosine after HA stimulation. C3 fibroblasts were incubated with 10 ng/ml HA for 1 min before fixation and staining with mouse anti-vinculin (*a*, *b*, and *c*) and rabbit anti-phosphotyrosine (*e*, *f*, and *g*). Vinculin and phosphotyrosine staining colocalize in some (*open arrows*) but not all (*solid arrows*) areas. Mouse IgG (*d*) and rabbit IgG (*h*) controls are shown. Size bar represents 8.5 μ m.

1988), impedes locomotion. On the other hand, factors that promote extensive focal adhesion disassembly such as tenascin also inhibit locomotion (Chiquet-Ehrismann et al., 1988; Lightner and Erickson, 1990; Murphy-Ullrich et al., 1991; Sage and Bornstein, 1991). In contrast, factors like HA that promote rapid turnover of focal adhesions allow for rapid locomotion. Strong detaching mechanisms provided by tenascin may be necessary for rear detachment, while a more subtle detaching/attaching mechanism under the influence of HA may be required for lamellae extension.

Reports indicate that focal adhesion structure and function

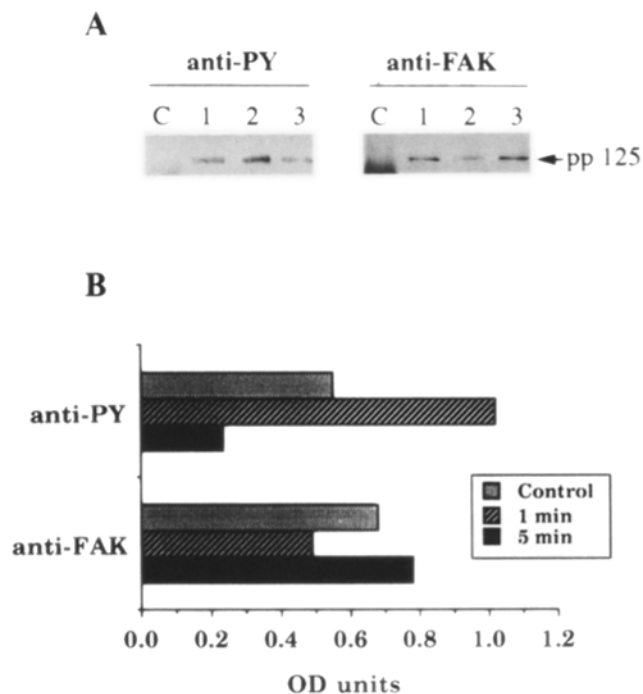


Figure 12. The effect of HA addition on the tyrosine phosphorylation of pp125^{FAK} (*A*). C3 fibroblasts were incubated in the absence (lane 1) or presence of HA (10 ng/ml) for 1 min (lane 2) or 5 min (lane 3) before cell lysis. Immunoprecipitation was performed with anti-FAK or mouse IgG control (lane C), followed by SDS-PAGE and anti-phosphotyrosine (4G10) immunoblotting and stripping and reprobing with anti-FAK. (*B*) Densitometry of both blots reveals that after HA addition, there is a transient increase followed by a decrease in pp125^{FAK} phosphorylation.

are regulated by changes in protein tyrosine phosphorylation (Burrige et al., 1992; Guan and Shalloway, 1992; Hanks et al., 1992; Schaller et al., 1992) and that these structures contain tyrosine phosphorylation sites in both normal (Maher et al., 1985) and transformed cells (Comoglio et al., 1984). In addition, focal adhesion disassembly is promoted by TPA (Schliwa et al., 1984), which activates protein kinase C, and some viral oncogenes, while their assembly has been correlated with localized protein tyrosine phosphorylation in response to ECM/integrin interactions (Burrige et al., 1992; Guan et al., 1991; Kornberg et al., 1991). These observations in conjunction with our results support a role for tyrosine phosphorylation in the assembly and maintenance of focal adhesions in spreading and stationary cells, as well as a role for transient phosphorylation in initiating focal adhesion turnover required for cell locomotion. A potentially important regulator of focal adhesions, and, as shown here a target for HA-induced signaling, is the newly characterized focal adhesion kinase. Tyrosine phosphorylation of FAK in response to extracellular matrix proteins has been associated with increased kinase activity, cell adhesion and focal adhesion assembly (for review see Zachary and Rozengart, 1992). The coincidental occurrence noted here of tyrosine phosphorylation of FAK and focal adhesion assembly, as well as the dephosphorylation of FAK coincident with the loss of focal adhesions in response to HA, is consistent with a role for this protein in focal adhesion turnover in HA sig-

naling as well. However, the possibility that FAK plays a passive role in HA signaling cannot at this point be ruled out.

Although RHAMM possesses no intrinsic kinase activity, a complex associated with RHAMM contains an HA-stimulated PTK (Turley, 1989a) that is antigenically related to pp60^{c-src} (Turley, 1989b). In addition, RHAMM also colocalizes both with cytoskeletal elements on locomoting chick heart fibroblasts (Turley et al., 1990) and with pp60^{c-src} on mouse fibroblasts (unpublished observation). Since members of the src family kinases are known to associate with several membrane proteins (see Bolen et al., 1991 and references therein), it is possible that the src kinase, or another member of the src family, could be associated with RHAMM and thus be involved in the HA triggered tyrosine phosphorylation. In this light, connections between src family kinases, the cytoskeleton and cell motility have been reported; pp60^{c-src} has been found to be associated with cytoskeletal proteins (Hamaguchi and Hanafusa, 1987), adhesion plaques, and focal adhesions (Nigg et al., 1982; Rohrschneider, 1980; Rohrschneider et al., 1982; Shriver and Rohrschneider, 1981a,b). As well, overexpression of c-src in endothelial cells increases their rate of locomotion (Bell et al., 1992). Activated src substrates include several cytoskeletal or cytoskeletal-associated proteins as well as p125^{FAK} (Davis et al., 1991; Glenney and Zokas, 1989; Kanner et al., 1991a,b; Matten et al., 1990; Pasquale et al., 1986; Powell and Glenney, 1987; Reynolds et al., 1992; Rothberg et al., 1992; Schaller et al., 1992; Sefton et al., 1981; Turner et al., 1990; Wu and Parsons, 1993). In fact, among the tyrosine phosphorylated src substrates, p130, p120, p110, p210, p125, p118, p85, p185/p64 (Kanner et al., 1990), six have similar molecular weights to the presently observed proteins phosphorylated in response to HA stimulation. Further investigations are expected to indicate whether there is a connection between the src family proteins and HA/RHAMM.

In summary, we have shown that HA/RHAMM interactions result in rapid tyrosine phosphorylation that leads to locomotion in *ras* transformed fibroblasts. We propose that a target of this signaling pathway is focal adhesions and the focal adhesion kinase, FAK. These results further identify, for the first time, hyaluronan as an important regulator of focal adhesion turnover.

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