Hyaluronan and a Cell-associated Hyaluronan Binding Protein Regulate the Locomotion of Ras-transformed Cells

E. A. Turley, L. Austen, K. Vandeligt, and C. Clary

Department of Pediatrics, Health Sciences Center, Manitoba Institute of Cell Biology, University of Manitoba, Winnipeg, MB, Canada R3E OV9

Abstract. Hyaluronan (HA) and one of its cell binding sites, fibroblast hyaluronan binding protein (HABP), is shown to contribute to the regulation of 10T1/2 cell locomotion that contain an EJ-ras-metallothionein (MT-1) hybrid gene. Promotion of the ras-hybrid gene with zinc sulfate acutely stimulates, by 6–10-fold, cell locomotion. After 10 h, locomotion drops to two- to threefold above that of uninduced cells. Several observations indicate increased locomotion is partly regulated by HA. These include the ability of a peptide that specifically binds HA (HABR) to reduce locomotion, the ability of HA (0.001–0.1 μg/ml), added at 10–30 h after induction to stimulate locomotion back to the original, acute rate, and the ability of an mAb specific to a 56-kD fibroblast HABP to block locomotion. Further, both HA and HABP products are regulated by induction of the ras gene. The effect of exogenous HA is blocked by HABR, is dose-dependent and specific in that chondroitin sulfate or heparan have no significant effect. Stimulatory activity is retained by purified HA and lost upon digestion with *Streptomyces* hyaluronidase indicating that the activity of HA resides in its glycosaminoglycan chain. Uninduced cells are not affected by HA, HABR, or mAb and production of HA or HABP is not altered during the experimental period. These results suggest that ras-transformation activates an HA/HABP locomotory mechanism that forms part of an autocrine motility mechanism. Reliance of induced cells on HA/HABP for locomotion is transient and specific to the induced state.

HYALURONAN has been implicated in a variety of disease, homeostatic, and developmental processes (1, 14). Although the manner in which it exerts its effects is not clear, an increasingly common paradigm is a correlation of an increased but transient production of hyaluronan (HA)[1] that coincides with rapid cell migration during processes such as inflammation, wound repair (50, 51), tumor invasion (15, 30, 35, 38), and the morphogenesis of a variety of tissues (4, 5, 35, 36). It has been proposed that HA promotes cell locomotion, in part, by interacting with the cell surface (7, 38). Hyaluronan binds to the cell surface via several mechanisms: ionically as a coat, by a covalently attached integral protein (12, 14, 35), and by specific-binding sites that have been proposed to include integral glycoproteins (6, 17, 21–23, 26, 45, 47). One of these proteins, termed fibroblast hyaluronan binding protein (HABP) (41, 45) has been particularly implicated in the regulation of cell locomotion (40, 41). This protein is concentrated in the lamellae and protrusions of rapidly locomoting cells but is reduced or absent on slowly moving or stationary cells (42). Reconstitution of stationary embryonic fibroblast monolayers with HABP renders them responsive to HA as detected by increased random locomotion (42).

This paper demonstrates that HA promotes the locomotion of 10T1/2 fibroblasts that have been stably transfected with a mutant ras-metallothionein hybrid gene (212 cell line; 37) only when the mutant gene is induced with heavy metal. Responsiveness to HA is transitory and mediated by HABP as demonstrated by antibody-blocking experiments. These results provide evidence for the proposal that endogenous HA production forms part of an autocrine mechanism for stimulating tumor cell locomotion and also provides direct evidence implicating HABP as a mediator of HA-promoted locomotion. The 212 cell line will be a useful model to study the molecular mechanisms of HA-HABP-driven locomotion and to dissect the role of activated ras in this process. The implications for the role of HA and HABP in disease, homeostatic, and developmental processes that involve cell locomotion are discussed in light of these findings.

Materials and Methods

Cell Culture

The 212 cell line was a kind gift of Dr. T. Haliotis (Cancer Research Laboratories, Queens University, Kingston, Canada) and is a 10T1/2 cell line that has been transfected with EJ-ras gene linked to a neomycin construct and metallothionein (MT-1) promoter (37). Cells were routinely maintained in DMEM plus 10% FCS (complete medium) containing geneticin G-418 (Sigma Chemical Co., St. Louis, MO; 1 mg/ml) for selection of the neomycin construct. For experimental analysis, cell cultures

1. *Abbreviations used in this paper*: HA, hyaluronan; HABP, hyaluronan binding protein; HABR, hyaluron binding fragment of aggrecan.

were subcultured at a dilution of 1:10 and cultured for 24–48 h before the addition of zinc sulfate or buffer alone. This allowed for ~50% culture confluence. At this degree of confluence, uninduced cells locomoted slowly. However, for stock cultures, cells were routinely subcultured at a 1:50 dilution as described by Trimble et al. (37). The promotion of the ras gene after the addition of zinc sulfate was confirmed by immunoprecipitation of p21ras and by Northern blot analysis of ras message (24; and results not shown). Zinc sulfate did not affect the locomotion of either the parent (10T1/2) cell line or a line that had been transfected with the neomycin MT-1 promoter construct alone (data not shown).

Time-Lapse Cinemicrography

Time-lapse cinemicrography was conducted as described previously (10) using nuclear displacement as a measure of random locomotion. Results obtained with this method were confirmed with a program ("Dynacell"; Zeiss, Oberkochen, FRG) that is based upon Fourier analysis of cell motility incorporating such parameters as net translation, undulation, ruffling, cell shape changes, and pseudopodal extension, quantified together as a motility index (29). For routine displacement assays, cell motility was monitored on a microscope (model IM35; Zeiss) equipped with a 10X objective and a video camera. During the filming period, cells were maintained in either DMEM alone or complete media (DMEM + 10% FCS) that had been depleted of HA by chromatography of media on an HABR-Sepharose affinity column. HA removal was monitored by measuring levels of this polymer with a Pharmacia Kit (Pharmacia Fine Chemicals, Piscataway, NJ; see below). All cultures were maintained at pH 7.2. Locomotion was observed at varying times after the induction of the mutant-hybrid gene with zinc sulfate and filmed for 1-2-h periods. At least 100 cells were observed for each assay and the results were expressed as means (μm/min) ± SEM.

Addition of Glycosaminoglycans

HA was obtained from several biological sources including human umbilical cord (type I; Sigma Chemical Co.), rooster comb (Healon; Pharmacia Fine Chemicals), and bacteria (Diagnostic Products Corp., Los Angeles, CA). The molecular weight for each preparation was disperse but ranged among 40,000 (bacterial), 1 million (umbilical cord), and 10 million (Healon). All HA preparations were tested for the presence of contaminating protein and other glycosaminoglycans. HA from the human umbilical cord preparation contained variable amounts of protein as well as chondroitin sulfate A and C (43). HA obtained from bacteria was contaminated with variable amounts of protein but contained no other glycosaminoglycans; rooster comb preparations did not contain detectable protein or other glycosaminoglycans. To purify HA preparations free of possible contaminating growth factors and endotoxin that might affect cell locomotion, the following procedures were used. HA (1 mg/ml) was chromatographed on a Sephadex G-200 column (1 cm × 60 cm; flow rate 1 ml/h) in 2.0 M NaCl buffer at pH 3.0 described for removing growth factors from the related glycosaminoglycan, heparin (19). These conditions will also remove an HA-bound growth factor (16). HA, eluted in the void volume, was detected by a carbazole reaction (2) then chromatographed on an affinity column (toxigel; Pharmacia Fine Chemicals) to adsorb endotoxin. The purified HA did not contain detectable protein as judged by absorption at 280/260 nor did it affect [^{3}H]thymidine incorporation in a serum-starvation culture assay (9). An effect would be predicted to occur if HA was complexed with a growth factor that could be slowly released or if, conversely, HA bound to growth factors present in growth media to render them inactive. Heparin (pig mucosal lining) and chrondroitin sulfate (type A and C; shark cartilage) were purchased from Sigma Chemical Co. To digest HA to tetrasaccharides, *Streptomyces* hyaluronidase (100 IU/ml; Miles Scientific Div., Naperville, IL) was added to a 100 μg/ml solution of Healon(R) at 37°C for 48 h in a Na_2CO_3 buffer at pH 5.1 (39).

Addition of Antibodies and HABR

Monoclonal antibodies were prepared against HABP by injecting mice with 50 μg of HABP, linked to keyhole lymphocyte hemacyanin, three times over a 4-wk period. 2 wk after the final injection, mice were primed by daily injection of 5 μg of underivatized HABP for 5 d. The spleen was then removed and hybridomas were prepared by fusing spleen cells with NS-1 cells using 50% polyethylene glycol. Cells were plated onto peritoneal macrophage feeder layers in HAT medium. Positive colonies were cloned three to four times by limiting dilution and HABP binding was assessed with an ELISA (45). Clones were injected into mice and ascites obtained were again tested for a positive reaction to HABP with an ELISA.

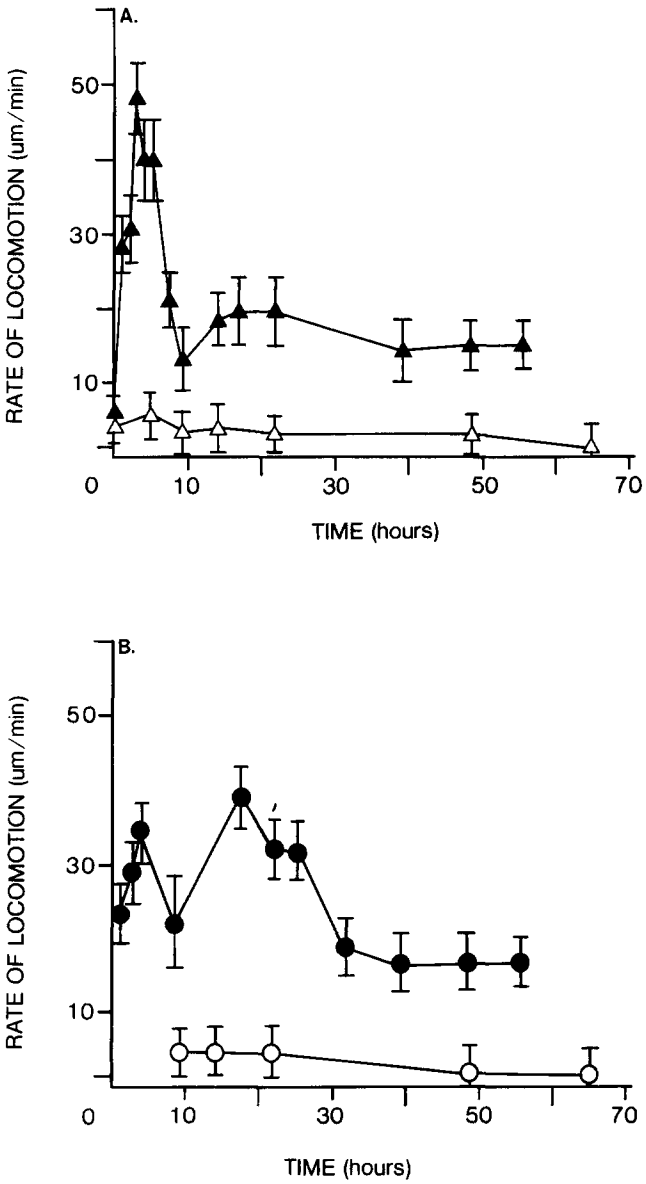

Figure 1. (*A*) The locomotion of 212 cells with (▲,●) or without (△,○) zinc induction of the mutant ras-hybrid gene. (*B*) The effect of HA on the locomotion of zinc induced (●) and uninduced (○) 212 cells. Random cell translocation was calculated from displacement of nuclei in a 1-h filming period. (*A*) Within several hours of mutant gene induction, cells began to translocate more rapidly reaching a peak of locomotory activity at 4–6 h after gene induction. Thereafter, locomotion dropped to ~15 μm/min, a value that was two- to threefold higher than uninduced cells. This rate was sustained for the remainder of the filming period. (*B*) HA (.001 μg/ml) had no effect on cell locomotion between 0–10 h or 30–60 h after gene induction (●). However, this glycosaminoglycan promoted locomotion of cells between 10–30 h after mutant gene induction. HA (○) had no effect on locomotion of uninduced cells. Values represent the mean ± SEM. n = 100 cells.

All monoclonal antibodies isolated from ascites were IgG_1 and were purified on both protein-A Sepharose and HABP-Sepharose affinity columns (45). Of the 13 monoclonal antibodies isolated, three were shown to block the locomotion of fibroblasts. One, 3T3-5, was used in this study to probe 212 cell responses to HA. This antibody was added for 1/2 h before the beginning of the 2-h filming period at dilutions of 1:100 (10 μg/ml).

To assess specificity of antibodies, HABP (10–200 μg/ml) was added to

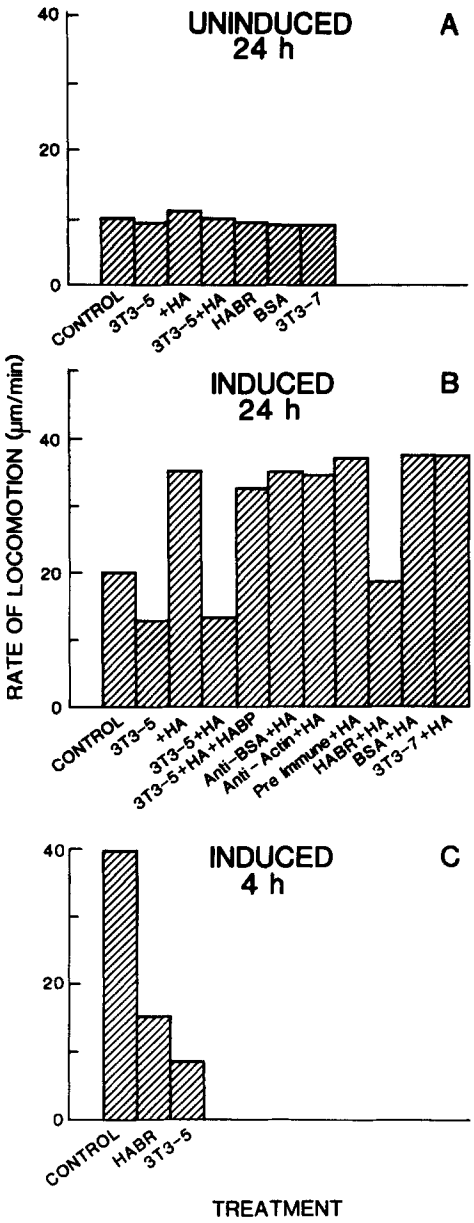

Figure 2. The effect of antibodies to HABP and of HABR upon 212 cell locomotion. Uninduced 212 cells (*A*), 212 cells induced with zinc for either 24 h (*B*), or for 4 h (*C*), were exposed to either monoclonal antibodies to fibroblast HABP (3T3-5, 3T3-7), other antibodies or to HABR for 1/2 h before filming their locomotory behavior. The effect of these reagents on rate of locomotion in the presence or absence of HA (.001 μg/ml) or HABP (20–200 μg/ml) was calculated from cell displacement during a 2-h filming period. Uninduced cells (*A*) did not respond to antibodies or to HABR. The locomotion of cells that had been induced for either 24 or 4 h was reduced by both antibodies to HABP and by HABR in the presence or absence of HA. The effect of HABP antibodies was reversed by preincubation of antibody with excess HABP. Antiactin, anti-BSA, and the 3T3-7 monoclonal antibody to HABP had no effect on HA-promoted cell locomotion. Values represent the mean. n = 100 cells.

neutralize the antibody in sample locomotion assays. HABP added by itself did not, as noted previously (42) have an effect on cell locomotion.

Antibodies to BSA (20 μg/ml; Sigma Chemical Co.) and actin (20 μg/ml; Sigma Chemical Co.) were used as controls. Nonimmune mouse sera was either purified as described for antibodies to HABP or added as antisera at a dilution of 1:40. The proteolytic fragment of cartilage aggrecan that contains the HA-binding region (HABR) of this molecule (15), was purchased from Pharmacia Fine Chemicals and used at concentrations calculated to bind 10 μg/ml of HA. HABR, like the antibodies, was added 1/2 h before the beginning of the filming period.

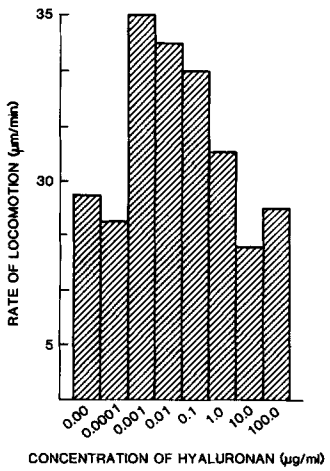

Figure 3. The effect of varying concentrations of HA on induced 212 cell locomotion. 212 cells were sparsely subcultured and induced with zinc for 24 h. Cells were then exposed to increasing concentrations of HA (umbilical cord) and the rate of locomotion was calculated during a 2-h filming period. Cells moved most rapidly in response to 0.001–0.1 μg/ml HA. Values represent the mean. n = 100 cells.

Measurement of HA Release

212 cells were subcultured at a 1:10 dilution in 60-mm tissue culture dishes (Nunc, Roskilde, Denmark) containing HA-depleted growth medium described above. After 24 h, the mutant-hybrid gene was induced, and medium was isolated at 1, 2, 4, 6, 12, 16, 24, 48, 72, and 96 h. Media was also collected at these times in uninduced cultures. The HA present in the media was quantitated using HA Test 50 (Pharmacia Fine Chemicals). All samples were corrected for background using HA-depleted medium: these values were generally below the limits of detection (5 μg/liter). Results were quantified relative to HA standards prepared in this laboratory. Linearity was achieved between 10–100 μg HA/liter. Precision was determined by SEM and judged to be, on average, <10% of the mean.

Immunofluorescence

Induced and uninduced 212 cells were grown on glass coverslips, fixed for 10 min in freshly prepared 3% paraformaldehyde then incubated in 1.0 M glycine to reduce unreacted aldehyde groups (40). Cultures were incubated with mAb to HABP (3T3-7; 1 μg/ml in DMEM + 10% calf serum) for 2 h

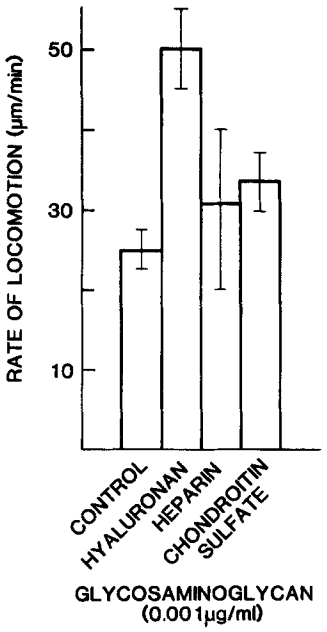

Figure 4. The effect of additional glycosaminoglycans on induced 212 cell locomotion. 212 cells were sparsely subcultured and the mutant hybrid gene was induced with zinc sulfate for 24 h. Cells were then exposed to 0.0001–100 μg/ml of glycosaminoglycan. Data presented in this figure included 0.001 μg/ml only, but results were similar to the other concentrations that were examined. The rate of locomotion was calculated from a 2-h filming period. Statistical analysis of data indicated that only HA-stimulated cell locomotion ($p < 0.05$). Values represent the mean ± SEM. n = 100 cells.

Table I. The Effect of Purified and Unpurified HA from Different Biological Sources on Zinc-induced 212 Cell Locomotion

Treatment	*Rate of locomotion*
	(μ/min ± SE)
Umbilical cord HA	44 ± 1.2
Purified umbilical cord HA	42 ± 3.4
HEALON[R]	40 ± 2.3
Bacterial HA	45 ± 5.6
Tetrasaccharides of HA	15 ± 1.9

Cultures of 212 cells were induced with zinc sulfate for 24 h and then exposed to the above treatments for an additional 2 h for analysis of cell locomotion. Umbilical cord HA was purified by gel filtration in 2 M NaCl at pH 3.0 to remove putative growth factors and chromatographed on a toxigel column to remove possible endotoxin contamination. Values represent means ± SEM. n = 100 cells.

at room temperature or for 24 h at 4°C. Excess antibody was removed by washing and monolayers were incubated with Fab' RITC-anti-mouse IgG (Sigma Chemical Co., 1:1,000 dilution) for 1 h at room temperature. Excess antibody was removed by washing and cells were examined with epifluorescence.

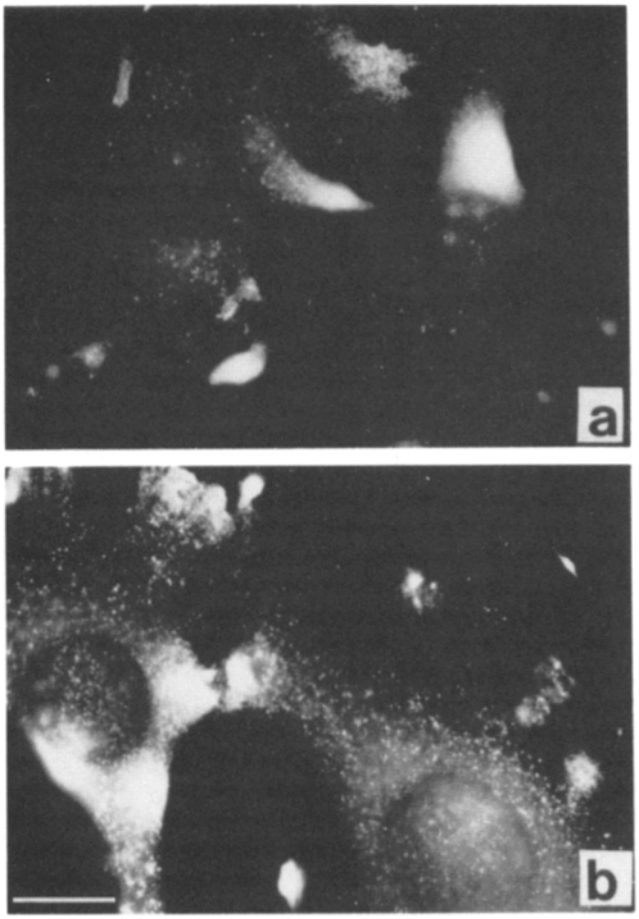

Figure 5. Immunofluorescent localization of HABP in (*a*) uninduced and (*b*) zinc-induced 212 cells. HABP was located on 212 cells by indirect immunofluorescence 24 h after induction of mutant hybrid gene or exposure to buffer alone. HABP was present in both cell populations, particularly in the cell processes. Between 4–24 h after induction, increased amounts of HABP were observed. This occurred as both amorphous and granular staining over the cell body. Bar, 2.8 μm.

Immunoprecipitation Assays

After subculture, 212 cells were grown for 24 h in DMEM + 10% FCS and exposed to 30 μM zinc sulfate or buffer alone together with Trans[^{35}S]methionine/cysteine (100 μCi/ml; specific activity is 1,100 Ci/ml; ICN Radiochemicals, Irvine, CA) for an additional 24 h. The cell layer was extracted with lysis buffer (10 mM Na_2HPO_4, 154 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate, 1 μM leupeptin (Sigma Chemical Co.); Aprotinin, 1,000 KU/ml, and phenylmethyl sulfonamide, 1 μg/ml (Sigma Chemical Co.) and lysates were spun at 25,000 *g* (DuPont Co., Sorvall Instruments Div., Newton, CT) for 60 min (24). To normalize lysates for protein content, a sample was precipitated with 10% TCA and a constant amount of radioactivity was used for immunoprecipitation assays. Samples were incubated with the 3T3-7 mAb to HABP (0.1 μg/ml) or preimmune sera for 2 h on ice. The antibody was adsorbed for 2 h with anti-mouse IgG-*Staphylococcus aureus* (10% vol/vol). Adsorbed antibody was washed in lysis buffer and then electrophoresed on 12.5% SDS-PAGE. Immunoprecipitated proteins were detected by autoradiography.

Results

Induction of the Mutant Ras-Hybrid Gene-promoted Cell Locomotion

Within 1–2 h after induction of the EJ-ras hybrid gene with zinc sulfate, an acute increase in the random locomotion of 212 cells was observed (Fig. 1 *A*). The rate of locomotion peaked between 2–5 h at ~48 μm/min. By 10 h, this rate had dropped to 15–20 μm/min (Fig. 1 *A*). In contrast, the locomotion of uninduced 212 cells was ~5 μm/min, eight- to ninefold less than the maximum rate of induced cells. Locomotion of uninduced cells gradually decreased to <1 μm/min by 72 h after subculture (Fig. 1 *A*).

The increase in locomotion resulting from induction of the mutant ras-hybrid gene occurred before promotion of [^{3}H]thymidine incorporation (data not shown). Several observations suggested that the increase in cell locomotion resulting from zinc induction of the mutant hybrid gene was regulated in part, by the glycosaminoglycan, HA, and by fibroblast HABP (Figs. 1 and 2).

Exogenous HA-stimulated Locomotion of Induced Cells

The addition of HA to 212 cells 24 h after zinc induction of the mutant hybrid gene-stimulated locomotion approximately threefold (Figs. 1 *B*, 2, and 3). This rate of locomotion approximated the rate observed after initial induction of the mutant gene (Fig. 1, *A* and *B*). Addition of HA before or after this time frame had no effect. Further, HA had no effect on locomotion of uninduced cells (Fig. 1 *B*).

A response to HA was concentration dependent with maximal stimulation occurring between 0.001–0.1 μg/ml (Fig. 3). HA was specific in its ability to promote the locomotion of induced 212 cells in that neither heparin nor chondroitin sulfate were statistically effective (Fig. 4). Several observations also suggested that the effect of HA on cell locomotion was not because of the presence of contaminating factors possibly associated with HA preparations (Table I). Complete digestion of HA to tetrasaccharides with *Streptomyces* hyaluronidase, under conditions that controlled for protease activity, abolished the stimulatory effect of HA on cell locomotion. HA, purified to remove both growth factors and endotoxin, retained its ability to promote locomotion. Consistent with this, HA did not influence [^{3}H]thymidine incor-

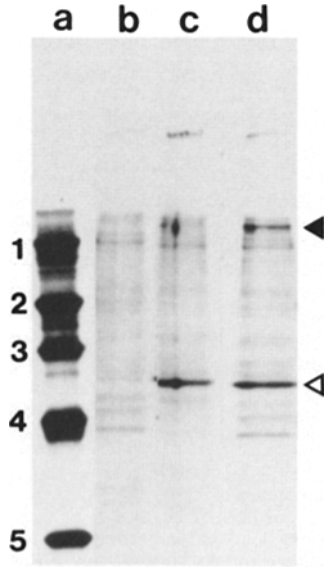

Figure 6. Immunoprecipitation of HABP from lysates of (*c*) uninduced and (*d*) zinc-induced 212 cells. 212 cells were grown to subconfluence and labeled with [^{35}S]methionine for 24 h. HABP was immunoprecipitated with mAb 3T3-5 from cell lysates (as described in Materials and Methods), resolved on SDS-PAGE, and processed for autoradiography. Zinc-induced cells contained more of an HABP complex (*closed arrowhead*; and see reference 40) than uninduced cells. A smaller protein was also resolved in both uninduced and zinc-induced cells that corresponds to nonglycosylated HABP (*open arrowhead*). Lysates immunoprecipitated with preimmune sera (*b*) did not contain significant amounts of either protein band. (*a*) Methylated ^{14}C standards include (*1*) myosin (200 kD); (*2*) phosphorylase b (97.4 kD); (*3*) BSA (69 kD); (*4*) ovalbumin (46 kD); and (*5*) carbonic anhydrase (30 kD).

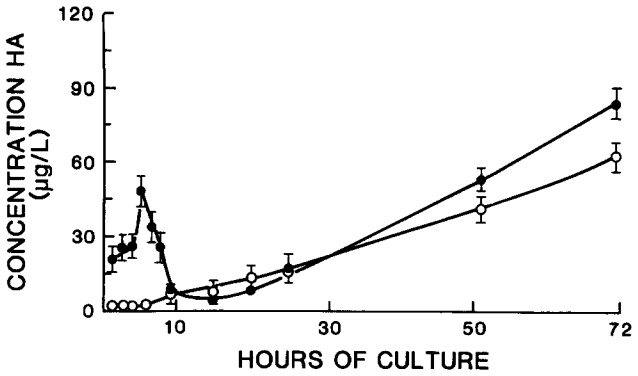

Figure 7. HA production of 212 cells. 212 cells were sparsely subcultured and maintained in complete medium after zinc induction (●) or no treatment (○) for up to 72 h. At regular time intervals, medium was collected and analyzed for the presence of HA using the Pharmacia Test kit (Pharmacia Fine Chemicals). The accumulation of HA in media transiently increased between 0–10 h after mutant hybrid gene induction, dropped and then gradually increased again. The transient increase in HA production was not observed for uninduced 212 cells. Rather after 10 h, HA gradually accumulated with time in culture. Values represent the mean ± SEM. $n = 4$.

poration (data not shown). HA isolated from such divergent species as mammals and bacteria also displayed similar stimulatory activities (Table I).

Fibroblast HABP Mediated the Effect of HA on Induced Cell Locomotion

HABR, added at levels calculated to bind 10 μg HA, as predicted from an HA test 50 kit (Pharmacia Fine Chemicals) reduced the locomotion of induced 212 cells at 4 h after zinc induction (Fig. 2). Further, the addition of HABR with exogenous HA negated the stimulatory activity of this glycosaminoglycan (Fig. 2 *B*). HABR had no effect on uninduced cells (Fig. 2 *A*).

Several observations implicated HABP in the increased locomotion of zinc-induced cells. A neutralizing mAb (3T3-5), specific to a 56-kD HABP (Hardwick, C., H. P. Hohn, M. Hook, E. Taylor, K. Vandeligt, L. Austen, and E. Turley, manuscript submitted for publication), blocked responsiveness to HA (Fig. 2 *B*). The mAb had no effect on the zinc-induced cells activated for longer than 36 h (data not shown) or on the locomotion of uninduced cells (Fig. 2 *A*). HABP, added together with mAb (3T3-5), abolished the antibodies' inhibitory activity. Preimmune mouse sera, anti-BSA or anti-actin had no effect on cell locomotion (Fig. 2 *B*).

Consistent with an involvement of HABP in responses of HA, expression of this protein was increased between 4–36 h after promotion of the mutant-hybrid gene as indicated by both immunofluorescence (Fig. 5) and immunoprecipitation assays (Fig. 6) using an mAb (3T3-7, a non-blocking antibody) that is specific to the HABP. As noted previously (49), HABP occurred within a complex of proteins (HA receptor complex) that electrophoresed at the stacking–separating gel interface. Assays also detected a protein of MW_E 48–49 kD that is believed to correspond to the nonglycosylated form of HABP (Hardwick, C., H. P. Hohn, M. Hook, E. Taylor, K. Vandeligt, L. Austen, and E. Turley, manuscript submitted for publication).

Increased Locomotion of Induced Cells Correlated with Increased HA Production

An increase in the production of HA occurred transiently between ~1–12 h after zinc induction of the mutant hybrid gene, paralleling the transient increase in cell locomotion (compare Figs. 1 and 7) and was similar to the concentration of exogenous HA required to stimulate induced 212 cells (Fig. 3). Production dropped to undetectable levels (<0.005 μg/ml) by 10 h (Fig. 3) but gradually increased to 0.09 μg/ml 24–72 h after zinc induction.

The production of HA by uninduced 212 cells was undetectable during the first 18 h but rose gradually to 0.06 μg/ml by 72 h after subculture: HA production was always lower than that of induced cells (Fig. 7).

Discussion

Expression of a ras oncogene has previously been shown to correlate with increased cell locomotion (28, 48). We show here that acute induction of this mutant gene activates an autocrine HA-HABP-mediated locomotory mechanism. Data indicate that this mechanism is specific to the induced state, transient, and accounts for ~50% of the observed ras-induced increase in locomotion. With time however, it is disassembled as indicated by a reduction in autocrine production of HA, followed by a loss of responsiveness to both exogenous HA and antibodies to HABP.

Several characteristics of the 212 cell locomotion occurring in response to HA are noteworthy. Firstly, HA-HABP-driven locomotion is associated with the initiation of rapid locomotion. A similar paradigm has been observed in vivo where increased production of HA is associated with the onset of locomotion (15, 35, 38, 50). Secondly, HA and a functional HABP are transiently produced, as has been ob-

served in vivo (27, 31). The diminishing reliance of induced 212 cells on HA, as reflected by their lack of responsiveness to this polymer, correlates with insensitivity of cells to the mAb specific for HABP (data not shown). Both decreases in HABP expression, which occur with chronic induction of the ras gene, and functional alterations of this binding site may contribute to insensitivity to HA.

The ability of antibodies to HABP to block locomotion is consistent with several previous observations. In most cell types, expression of HABP correlates with rapid locomotion (40, 41) and this protein is localized to areas of cells that are relevant to locomotion, such as ruffles and lamellipodia (40, 41). Polyclonal antibodies to HABP block HA-promoted migration of embryonic ductus aorta smooth muscle cells into collagen gels (3). Conversely, reconstitution of confluent contact inhibited chick heart fibroblasts with HABP (42) permits stimulation by HA.

In 212 cells, changes in expression of HABP does not completely correlate with their lack of response to HA. Uninduced 212 cells do express some HABP, but do not respond to either HA or mAb to HABP. Lack of response is unlike other fibroblasts we have studied, where expression of HABP correlates strongly with response to these regulators. Rather, they resemble embryonic vascular endothelial cells which express small amounts of HABP but do not respond to HA or anti-HABP (3). Possibly either a threshold amount of HABP is required to affect locomotory responses (45) or HABP can, as eluded to above, occur in "functional" and "nonfunctional" states.

Perturbation of the HA-driven locomotion by either HABR or mAb-HABP does not completely abolish ras-induced increases in motility. Cells clearly use additional mechanisms for locomotion that are also regulated by the mutant hybrid gene and these alternate mechanisms appear to predominate with chronic induction of the gene.

The molecular mechanisms by which HA-HABP interactions promote cell locomotion are not clear. Hyaluronan has long been proposed to function in the locomotory cycle as a detaching factor because of its physicochemical properties (7, 14, 38, 50). However, both the low concentration at which this glycosaminoglycan promotes locomotion (at approximately the K_D for HABP; 45) and a previous observation that HA can promote protein phosphorylation (39) suggest additional molecular mechanisms. The last possibility is consistent with the observation that HABP is complexed with a protein kinase at the cell surface (39) and with the ability of chemotactic factors (33, 52) and other extracellular matrix molecules (12, 25, 49) involved in cell locomotion to promote protein phosphorylation.

The mechanisms by which the ras gene might regulate HA/HABP are unknown, but activation of the ras gene has been associated with release of autocrine mobility factors (18, 33, 34) and growth factors, both of which may promote HA production (11, 44). Other factors such as HA-stimulating activities (HASA), present in serum (8, 44) may also be controlled by the ras gene.

In summary, an oncogene-transformed fibroblast model that relies upon HA and HABP for locomotion is described. These results implicate HA and fibroblast HABP as important regulators of cell locomotion during oncogenic transformation and are consistent with a recent report noting that HA is required for tumor cells to respond to a tumor motility factor (52). This model will be useful in dissecting the molecular mechanisms by which HA and one of its cell binding sites, HABP, acts to promote cell locomotion.

The authors thank Dr. M. Hook and M. Hollenberg for critical reading of the manuscript.

This work was supported by the National Cancer Institute of Canada and Medical Research Council of Canada grants to E. Turley.

Received for publication 23 May 1990 and in revised form 12 November 1990.

References

1. Bansal, M. K., and R. M. Mason. 1986. Evidence for rapid metabolic turnover of hyaluronate synthetase in swarm rat chondrosarcoma chondrocytes. *Biochem. J.* 236:515–519.
2. Bitter, T., and H. M. Muir. 1963. A modified carbazole reaction. *Anal. Biochem.* 4:330–334.
3. Boudreaux, N., E. A. Turley, and M. Rabinovitch. 1990. Fibronectin, Hyaluronan and a Hyaluronan binding protein contribute to increased ductus arteriosis smooth muscle cell migration. *Dev. Biol.* In press.
4. Copp, A. J., and M. Bernfield. 1988*a*. Glycosaminoglycans vary in accumulation along the neuraxis during spinal neurulation in the mouse embryo. *Dev. Biol.* 130:573–582.
5. Copp, A. J., and M. Bernfield. 1988*b*. Accumulation of basement-membrane associated hyaluronate is reduced in the posterior neuropore region of mutant (curly tail) mouse embryos developing spinal neural tube defects. *Dev. Biol.* 130:583–590.
6. Crossman, M. V., and R. M. Mason. 1990. Purification and characterization of a hyaluronan-binding protein from rat chondrosarcoma. *Biochem. J.* 266:399–406.
7. Culp, L. A. 1976. Molecular composition and origin of substrate-attached material from normal and virus-transformed cells. *J. Supramol. Struct.* 5:239–255.
8. Decker, M., E. S. Chiu, C. Dollbaun, A. Moiin, J. Hall, R. Spendcove, M. T. Longaker, and R. Stern. 1989. Hyaluronic acid stimulatory activity in sera from the bovine fetus and from breast cancer patients. *Cancer Res.* 49:2499–3505.
9. Edwards, D. R., and D. T. Denhardt. 1985. A study of mitochondrial and nuclear transcription with cloned cDNA probes. *Exp. Cell Res.* 157:127–143.
10. Erickson, C. A., and E. A. Turley. 1983. Substrata formed by combinations of extracellular matrix components alter neural crest cell motility *in vitro*. *J. Cell Sci.* 61:299–323.
11. Erickson, C. A., and E. A. Turley. 1987. The effects of Epidermal Growth Factor on Neural Crest Cells in Tissue Culture. *Exp. Cell Res.* 169:267–279.
12. Ferrell, J. E., and G. S. Martin. 1989. Tyrosine-specific protein phosphorylation is regulated by glycoprotein 11b and 111a in platelets. *Proc. Natl. Acad. Sci. USA.* 86:2234–2238.
13. Deleted in proof.
14. Hook, M., L. Kjellen, S. Johansson, and J. Robinson. 1984. Cell-surface glycosaminoglycans. *Annu. Rev. Biochem.* 53:847–869.
15. Iozzo, R. V. 1985. Proteoglycans: structure, function and role in neoplasia. *Lab. Invest.* 53:373–396.
16. Keating, A., E. Turley, S. Saragosa, and C. Nijuen. 1990. Effect of glycosaminoglycans on haematopoietic progenitor cells: compartmentalization of GMCSF by hyaluronic acid. *Exp. Hematol (NY).* In press.
17. Laurent, T. C., and J. R. E. Fraser. 1986. The properties and turnover of hyaluronan. Functions of the proteoglycans. *Ciba Found. Symp.* 124: 9–29.
18. Liotta, L. A., R. Mandler, G. Murano, D. A. Katz, R. K. Gordon, P. K. Chiang, and E. Schiffmann. 1986. Tumor cell autocrine motility factor. *Proc. Natl. Acad. Sci. USA.* 83:3303–3306.
19. Lobb, R. R., and J. W. Fett. 1984. Purification of two distinct growth factors from bovine neural tissue by heparin-affinity chromatography. *Biochemistry.* 23:2960–2966.
20. Longaker, M. T., M. R. Harrison, J. C. Langer, T. M. Crombleholme, M. Decker, E. D. Verrier, R. Spendlove, and R. Stern. 1989. Studies in fetal wound healing: I. A factor in fetal serum that stimulates deposition of hyaluronic acid. *Pediatr. Surg. Int.* 24:793–798.
21. McCarthy, M. T., and B. P. Toole. 1989. Membrane-associated hyaluronate-binding activity of chondrosarcoma chondrocytes. *J. Cell. Physiol.* 141:191–202.
22. McGary, C. T., R. H. Raja, and P. H. Weigel. 1989. Endocytosis of hyaluronic acid by rat liver endothelial cells. *Biochem. J.* 257:875–884.
23. Moczar, M., M. F. Poupon, and E. Moczar. 1990. Hyaluronate-binding proteins in weakly and highly metastatic variants of rat rhabdomyosarcoma cells. *Clin. & Exp. Metastasis.* 8:129–140.
24. Myrdal, S. M., and N. Auersperg. 1985. p21 ras heterologous localization in transformed cells. *Exp. Cell Res.* 159:441–450.

25. Nakamura, S. I., and H. Yamamura. 1989. Thrombin and collagen induce rapid phosphorylation of a common set of cellular proteins on tyrosine in Human Platelets. *J. Biol. Chem.* 264:7089–7091.
26. Newell, P. C., G. N. Europe-Finner, N. V. Small, and G. Liu. 1988. Inositol phosphates, G-proteins and ras genes involved in chemotactic signal transduction of Dictyostelium. *J. Cell. Sci.* 89:123–127.
27. Orkin, R. W., W. Knudson, and B. P. Toole. 1985. Loss of hyaluronate-dependent coat during myoblast lesion. *Dev. Biol.* 107:527–530.
28. Partin, A. W., J. T. Isaacs, B. Trieger, and D. S. Coffey. 1988. Early cell motility changes associated with an increase in metastatic ability in rat prostatic cancer cells transfected with the v-Harvey-ras oncogene. *Cancer Res.* 48:6050–6053.
29. Partin, A. W., J. S. Schoemiger, J. C. Mohler, and D. S. Coffey. 1989. Fourier analysis of cell motility: correlation of motility with metastatic potential. *Proc. Natl. Acad. Sci. USA.* 86:1254–1258.
30. Pauli, B. U., D. E. Schwartz, E. J. Thonar, and K. E. Kuettner. 1983. Tumor invasion and host extracellular matrix. *Cancer Metastasis Rev.* 2:129–152.
31. Prehn, P. 1983. Synthesis of hyaluronate in differentiated teratocarcinoma cells: characterization of the synthase. *Biochem. J.* 211:181–189.
32. Schor, S. L., A. M. Schor, A. M. Grey, J. Chen, G. Rushton, M. E. Grant, and I. Ellis. 1989. Mechanisms of action of the migration stimulating factor produced by fetal and cancer patient fibroblasts: Effect on hyaluronic acid synthesis. *In Vitro Cell. & Dev. Biol.* 25:737–745.
33. Segal, J. E., and G. Gerish. 1989. Genetic approaches to cytoskeleton function and the control of cell motility. *Curr. Opin. Cell Biol.* 1:44–50.
34. Stoker, M., and M. Perryman. 1985. An epithelial scatter factor released by embryo fibroblasts. *J. Cell Sci.* 77:209–224.
35. Toole, B. P. 1982. Developmental role of hyaluronate. *Connect. Tissue Res.* 10:93–100.
36. Toole, B. P., S. F. Munaim, S. Welles, and C. B. Knudson. 1989. Hyaluronate-cell interactions and growth factor regulation of hyaluronate synthesis during limb development. Biology of Hyaluronan. *Ciba Found. Symp.* 143:138–149.
37. Trimble, W. S., P. W. Johnson, N. Hozumi, and J. C. Roder. 1986. Inducible cellular transformation by a metallothionein-ras hybrid oncogene leads to natural killer cell susceptibility. *Nature (Lond.).* 321:782–784.
38. Turley, E. A. 1984. Proteoglycans and cell adhesion: their putative role during tumorigenesis. *Cancer Metastasis Rev.* 3:325–339.
39. Turley, E. A. 1989. Hyaluronic acid stimulates protein kinase activity in intact cells and in an isolated protein complex. *J. Biol. Chem.* 264:8951–8955.
40. Turley, E. A., and J. Torrance. 1985. Localization of hyaluronate and hyaluronate binding protein on motile and non-motile fibroblasts. *Exp. Cell Res.* 161:17–28.
41. Turley, E., and N. Auersperg. 1989. A hyaluronate binding protein transiently codistributes with $p21^{k-ras}$ in cultured cell lines. *Exp. Cell Res.* 181:340–348.
42. Turley, E. A., P. Bowman, and M. A. Kytryk. 1985. Effects of hyaluronate and hyaluronate binding proteins on cell motility and contact behavior. *J. Cell Sci.* 78:133–145.
43. Turley, E. A., C. A. Erickson, and R. P. Tucker. 1985. The retention and ultrastructural appearance of various extracellular matrix molecules incorporated into three-dimensional hydrated collagen lattices. *Dev. Biol.* 109:347–369.
44. Turley, E. A., M. D. Hollenberg, and R. M. Pratt. 1985. Effect of epidermal growth factor/urogastrone on glycosaminoglycan synthesis and accumulation *in vitro* in the developing mouse palate. *Differentiation.* 28:279–285.
45. Turley, E. A., D. Moore, and L. J. Hayden. 1987. Characterization of hyaluronate binding proteins isolated from 3T3 and murine sarcoma virus transformed 3T3 cells. *Biochemistry.* 26:2997–3005.
46. Turley, E. A., S. Roth, and J. A. Weston. 1989. A model system that demonstrates interactions among extracellular matrix macromolecules. *Connect. Tissue Res.* 23:221–235.
47. Underhill, C. B., S. J. Green, P. M. Lomoglio, and C. Tarne. 1987. The hyaluronate receptor is identical to a glycoprotein of M_r 85,000 (SP85) as shown by a monoclonal antibody that interferes with binding activity. *J. Biol. Chem.* 262:13142–13146.
48. Volk, T., B. Geiger, and A. Raz. 1984. Motility and adhesive properties of high and low metastatic neoplastic cells. *Cancer Res.* 44:811–824.
49. Watson, S. P., B. Reep, B. T. McConnell, and E. G. Lapetina. 1985. Collagen stimulates [^{3}H]inositol triphosphate formation in indomethacin-treated human platelets. *Biochem. J.* 226:831–837.
50. Weigel, P. H., G. M. Fuller, and R. D. LeBoeuf. 1986. A model for the role of hyaluronic acid and fibrinogen in the early events during the inflammatory response and wound healing. *J. Theor. Biol.* 119:219–234.
51. Weigel, P. H., S. J. Frost, R. D. LeBoeuf, and C. T. McGary. 1989. The specific interaction between fibrinogen and hyaluronan: possible consequences in haemostasis, inflammation and wound healing. Biology of Hyaluronan. *Ciba Found. Symp.* 143:248–264.
52. Zigmund, S. H. 1989. Cell locomotion and chemotaxis. *Curr. Opin. Cell Biol.* 1:80–86.