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Rapid hyaluronan uptake is associated with enhanced motility: implications for an intracellular mode of action

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Abstract Texas red-labeled hyaluronan (TR-HA) is rapidly taken up in a CD44 independent manner into *ras*-transformed 10T1/2 fibroblasts, where it accumulates in both cell ruffles/lamellae, the perinuclear area, and the nucleus. HA does not accumulate in the cell ruffles/lamellae of parental 10T1/2 cells. Addition of HA to *ras*-transformed cells promotes their random motility but has no effect on 10T1/2 cell motility. 10T1/2 cells can be modified to take up HA into cell ruffles by exposure to phorbol ester or direct microinjection of HA into cells. Both treatments significantly stimulate 10T1/2 cell motility.

Key words: Hyaluronan; RHAMM; CD44; Internalization; Lamella; Nucleus

1. Introduction

RHAMM is an hyaladherin that occurs at several subcellular loci including the cell surface, cytoplasm and nucleus [1–3]. An intracellular as well as surface form of RHAMM regulates cell motility and controls the cell cycle [4,5]. Intracellular RHAMM forms appear to do so at least in part by co-associating with *erk* kinase [3] and controlling signaling through the *ras-erk* kinase cascade [3,6]. Interestingly, the hyaluronan (HA) binding domains of intracellular forms of RHAMM are required for activation of *erk* kinase by mutant active *ras* and by growth factors that activate *ras*, such as PDGF [3]. Several other hyaladherins that are located both at the cell surface and in the cytoplasm have also been described [7–10]. One of these, cdc37 or hsp50, like RHAMM, regulates cell cycle and motility and in its intracellular form binds to signaling proteins, such as CDK4 [7,11–13], *raf* and *src* [13–15]. Both the presence of these hyaladherins inside the cell and their requirement for HA binding domains in activation of signaling cascades [3] is puzzling since HA has traditionally been considered to regulate cell behavior by interacting with cell surface receptors [16–18] or by modifying the extracellular matrix [4,5].

HA is internalized for degradation by an endocytic pathway that requires CD44 function [19–21]. It is questionable, however, whether this pathway would allow any association of HA with the signaling modifying function of intracellular HA binding proteins. It is therefore intriguing that HA and other glycosaminoglycans/proteoglycans have previously been reported to occur intracellularly within cell structures that are not traditionally associated with the endocytic pathway. For instance, electron microscopic analysis using gold-labeled aggrecan to detect HA indicates the presence of this polysaccharide within caveoli and the nucleus [22,23], confirming earlier subcellular fractionation studies [24,25]. Careful confocal and functional analyses show the presence of related molecules, such as heparan sulfate and chondroitin sulfate proteoglycans, within the nucleus [26–28]. Neither the mechanisms by which glycosaminoglycans might reach these sites nor their function at these sites are currently understood or, indeed, generally focused upon. However, HA has been reported to bind to chromatin [24,25] and we previously proposed that intracellular HA may function to regulate key signaling pathways that impact on cell cycle and cell motility [1]. Others have shown that a highly sulfated glycosaminoglycan, intracellular heparin, regulates transcriptional activation [29–31].

In an ongoing attempt to assess the mechanisms by which the HA binding domains of RHAMM regulate *ras* signaling, we have assessed whether Texas red-labeled HA (TR-HA) can be taken up by the cell and accumulate within subcellular compartments, such as cell processes and the nucleus, sites that are relevant for MAP kinase signaling cascades [32]. We show here that TR-HA is taken up within minutes of its addition to *ras*-transformed cells rapidly accumulating both around and within the nucleus and cell lamellae by mechanisms that appear, at least in *ras*-transformed cells, to be independent of CD44-mediated uptake [19–21]. Addition of HA elicits an increase in random cell motility [33]. In contrast, the parent 10T1/2 cells do not show a rapid accumulation of HA into lamellae or the perinuclear area and, interestingly, do not respond to HA by increasing their rate of cell motility. However, treatment of 10T1/2 cells with phorbol ester, which facilitates HA uptake into these subcellular locations, or direct microinjection of HA into the cell cytoplasm significantly and specifically promotes their random motility. These results suggest that uptake mechanisms that allow intracellular accumulation of HA in multiple subcellular compartments exist and that HA may act here in diverse roles to control cell behavior.

2. Materials and methods

2.1. Cell culture

Murine 10T1/2 *ras*-transformed (C3) [34] or RHAMMv4-transfected cells [6] were maintained at 37°C in 5% CO_2 on 100 mm plastic tissue culture dishes (Nunclon) in DMEM (Gibco BRL) supplemented with 10% fetal calf serum (FCS) (Intergen) and 10 mM HEPES (Sigma), pH 7.3. Cells were routinely subcultured using 0.25% trypsin (Sigma) from 80% confluent cultures and passaged at a 1:10 dilution. In experiments which required a specific number of cells, a viable cell count was determined using trypan blue exclusion (0.4% in PBS) and a hemacytometer.

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2.2. Conjugation of HA with Texas red

700 kDa HA (Hyal Pharmaceutical Corp., Mississauga, Ont.) suspended in 20 mM MES (Sigma), pH 4.5, with 30% ethanol, was mixed with three-fold excess EDCI (Aldrich) as per the number of HA disaccharides. Texas red hydrazide (Molecular Probes) dissolved in DMF (Aldrich) at a molar ratio of probe to disaccharide of 1:10 was added and the mixture was shaken overnight at room temperature. Unconjugated material was then removed with dialysis in 10 000 molecular weight cut-off membranes (Pierce) against 75 mM NaCl and 40% ethanol for 4 days in the dark. The product was lyophilized for storage. Analysis of success of conjugation was conducted with gel permeation chromatography using refractive index and UV absorption to determine purity and molecular size range of the bioconjugate.

2.3. Immunofluorescence

Cells were seeded in DMEM (Gibco BRL) supplemented with 10% FCS (Intergen) at 60% confluence on sterile glass coverslips (VWR) in 35 mm tissue culture plates (Costar Corp.). After 8 h, the culture medium was aspirated, cells were rinsed and medium was replaced with serum-free DMEM containing 4 mg/ml transferrin (Gibco BRL) and 4 mg/ml insulin (Sigma) (defined medium) for 12 h. The cells were then exposed to 150 μg TR-HA conjugate in 1 ml of defined medium for varying durations (2 min to 12 h), rinsed twice in cold 5×PBS, and fixed in a solution of 2% paraformaldehyde and 1% cetylpyridinium chloride (CPC) in a 0.1 M Na-phosphate buffer pH 7.4 for 10 min at room temperature. Coverslips were then washed three times for 5 min each in 1×PBS, mounted with elvanol (PVA 15%, glycerin 30%), and viewed on a Zeiss Axiophot 100 confocal microscope. To ensure that confocal images represented internalized TR-HA, some cultures were exposed to TR-HA for 10 min, washed, then digested with *Streptomyces* hyaluronidase (1 IU/ml at 37°C for 1 h to remove TR-HA remaining on the cell surface that might interfere with confocal images), fixed and examined with a confocal microscope.

2.4. Digestion of TR-HA with hyaluronidase and competition with unlabeled HA

Digestion of 150 μg of TR-labeled HA prior to addition to cells was carried out in acetate buffer pH 5.0, using 15 TRU of *Streptomyces* hyaluronidase (Sigma) for 24 h at 37°C in a proteinase inhibitor buffer (25 mg/ml ovomucoid; 1.0 mg/ml pepstatin A; 18.6 mg/ml iodoacetic acid; 37 mg/ml EDTA; 17.4 mg/ml PMSF). The mixture was heat-inactivated at 56°C for 30 min to destroy enzyme activity. Control incubations (minus the hyaluronidase) were carried out under the same conditions. To assess specificity of uptake, cultures were also exposed to TR-labeled HA combined with excess (4 mg/ml) unlabeled HA.

2.5. Incubation of cells with peptides

150 μg TR-labeled HA was preincubated with 1 mg/ml of a peptide that mimics the HA binding domain I, RHAMM (peptide$^{aa423-432}$ [35]) or 1 mg/ml of a scrambled peptide of domain I which has been shown not to bind to HA [35], for 2 h at 37°C prior to cell treatment as above.

2.6. Anti-CD44 antibody blocking

Ras-transformed cells were preincubated for 30 min at 37°C with 10–50 μg/ml of KM201 (R&D Systems) monoclonal anti-CD44 Ab or KM114 (Pharmingen) monoclonal antibody diluted in 1 ml DMEM prior to addition of TR-labeled HA.

2.7. Microinjection analysis

10T1/2 fibroblasts were microinjected with 0.1 or 1.0 ng/ml HA (MW distribution 60 000–600 000 medical grade, Hyal Pharma, Mississauga, Ont., Canada) in PBS, together with luciferase yellow to detect microinjected cells. Control cells were microinjected with PBS alone or with either 1.0 ng/ml heparin (Sigma Chem. Co.) or 1.0 ng/ml chondroitin sulfate A and B (Sigma Chem. Co.). Cells were allowed to recover from microinjection for 2 h, then filmed on a heated stage for 1 h using Empix Northern Exposure image analysis program (Empix, Mississauga, Ont.) to quantify random motility. Microinjection was accomplished using an Eppendorf microinjection system.

2.8. Exposure of 10T1/2 fibroblasts to phorbol ester

Phorbol 12-myristate 13-acetate (PMA) was added at 100 nM in defined media to 10T1/2 cells for up to 4 h in the presence or absence of 50 μg/ml cycloheximide, added at the same time. Treated cells were exposed to unlabeled HA as above and analyzed for motility or to TR-HA and analyzed for uptake with confocal analysis.

2.9. Cell motility

Ras-transformed and parental 10T1/2 cells were plated at 40% confluence for 24 h and then serum starved for 24–48 h. HA (1 ng to 1 μg/ml) was added to cells and random locomotion of the cells was determined by image analysis 1 h later (Northern Exposure, Empix). Approximately 100 cells were analyzed per experiment.

3. Results

3.1. TR-labeled HA is rapidly taken up and targeted to the perinuclear area, cell processes and the nucleus of ras-transformed cells

TR-HA is internalized within subconfluent monolayers of *ras*-transformed cells minutes after its addition, affecting approximately 90% of cells and rapidly accumulating within their lamellae and nuclei (Fig. 1). Some general cytoplasmic and striking perinuclear accumulation is also observed. Optical sectioning of cells, from which cell surface-bound TR-HA had been removed with hyaluronidase, suggests an intracellular location of the TR-HA (Fig. 1). Interestingly, the acute appearance of TR-HA in *ras*-transformed cells appears punctate (Fig. 1). Nuclear localization is transient, and by 2 h after addition nuclear TR-HA is not detected (data not shown). *Streptomyces* hyaluronidase-digested TR-HA (Fig. 2a) or TR-HA mixed with excess unlabeled HA (Fig. 2b) do not show uptake of the TR label by cells, indicating that unlabeled HA competes effectively for the uptake of TR-HA and that the uptake of TR-HA requires larger than tetra- or hexa-

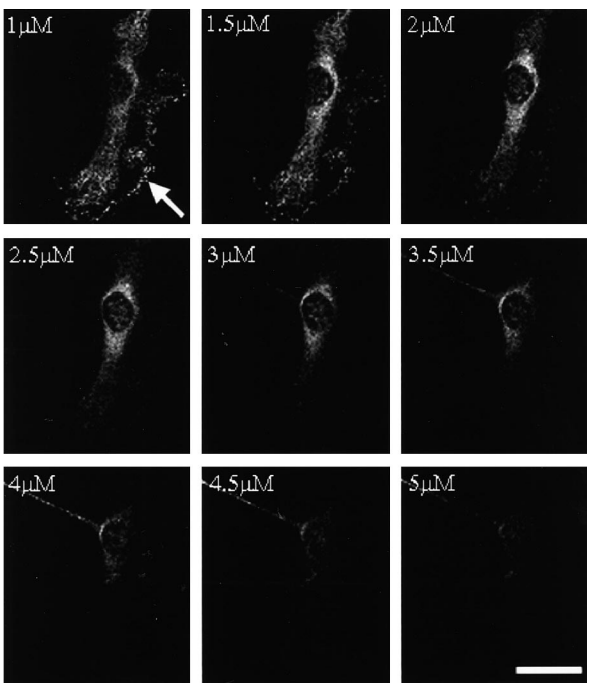

Fig. 1. Confocal analysis of *ras*-transformed 10T1/2 cells exposed to TR-labeled HA. Consecutive optical sections, beginning from the apical surface of the cell, show TR-HA in the cell processes (indicated by arrow), perinuclear area, and nucleus, 10 min after its addition in the culture medium. Bar = 10 μm.

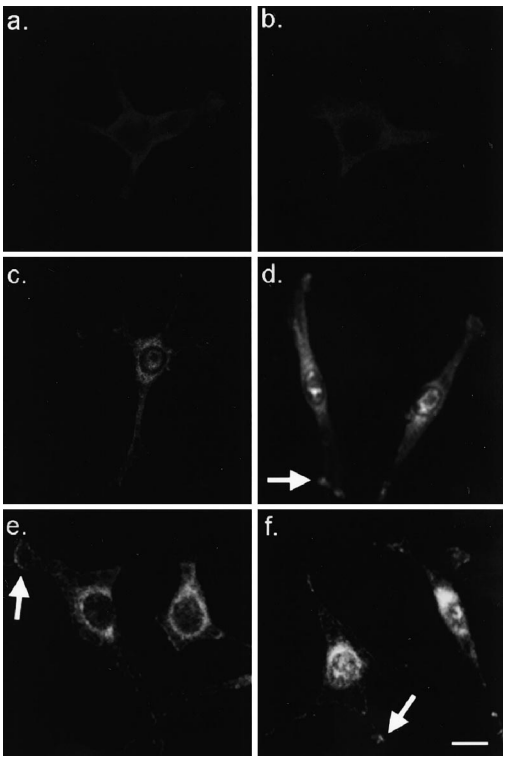

Fig. 2. Specificty of TR-HA uptake into *ras*-transformed 10T1/2 cells. a: TR-HA was digested with *Streptomyces* hyaluronidase prior to its addition to cells. b: TR-HA was mixed with 4 mg/ml unlabeled HA, prior to addition to cells. Both treatments abolish cellular uptake of TR-HA. c,d: Cells were preincubated for 30 min with anti-CD44 antibodies KM201 (c) and KM114 (d). Neither anti-CD44 antibody appeared to affect accumulation of TR-HA in cell processes or in the nucleus. e: TR-HA was preincubated for 2 h with 1 mg/ml of a peptide mimicking an HA binding domain of RHAMM [35]. The accumulation of HA within the nucleus is blocked, but HA is still taken up into cell processes and the perinuclear area. f: As in e but using a scrambled peptide, which was not able to bind to HA. This has no effect on the uptake processes. Bar = 10 μm. Arrows indicate TR-HA accumulation in cell processes.

saccharides. 80–90% of 10T1/2 cells also rapidly internalized TR-HA and like transformed cells accumulate this polysaccharide in the nucleus and in the cytoplasm (Fig. 3a). However, the staining pattern in the nucleus and cytoplasm was more diffuse than in *ras*-transformed cells, and 10T1/2 cells did not accumulate TR-HA as punctate accumulations in cell lamellae or in the perinuclear area (Fig. 3a).

3.2. Anti-CD44 blocking antibodies do not block rapid uptake of TR-HA in ras-transformed cells, but RHAMM peptides block localization of TR-HA to the nucleus

Anti-CD44 function blocking antibodies used at concentrations that have previously been shown to block endocytic uptake of HA [19,21,36] do not affect nuclear or lamellar localization of HA in *ras*-transformed cells (Fig. 2c,d). In contrast, peptides mimicking an HA binding domain of RHAMM [35] strikingly block accumulation of HA within the nucleus (Fig. 2e). Nevertheless, in the presence of these peptides, HA is still taken up into cell processes and in the perinuclear area in the presence of this peptide (Fig. 2e). A scrambled peptide control has no detectable effect on HA uptake (Fig. 2f). These results suggest that HA accumulation in the nucleus may require interaction with proteins, possibly hyaladherins that exhibit RHAMM-like HA binding motifs [7–9]. As well, these results suggest that the mechanisms directing HA to the nucleus and cell processes/cytoplasm are distinct.

3.3. Intracellular HA promotes cell motility

HA added to cell cultures promotes the locomotion of *ras* ($P < 0.05$, Fig. 3d), but not 10T1/2 cells (Fig. 3d) (used here at 10 ng/ml but a range from 1 ng to 100 μg was effective, data not shown). Addition of phorbol ester to 10T1/2 cells does not significantly increase cell motility by itself ($P = 0.5$, SEM) (Fig. 3d), but permits 10T1/2 cells to increase random locomotion in response to HA added to culture medium ($P < 0.001$, SEM) (Fig. 3d). This effect of phorbol ester is abolished by cycloheximide suggesting the process involves de novo protein synthesis. Interestingly, 10T1/2 cells treated acutely with phorbol ester now exhibit an intracellular accumulation pattern in the cytoplasm that resembles *ras*-transformed cells in that the TR-HA accumulation is enhanced in cell processes and in the perinuclear area (Fig. 3b). Furthermore, treatment of cells with PMA for 4 h (the exposure time to PMA when cells were analyzed for motility) appears to result in a further enhancement of TR-HA uptake (Fig. 3c).

Direct assessment of a role for enhanced cytoplasmic HA in cell motility is provided by microinjection of HA into the cytoplasm of 10T1/2 cells (Fig. 4a, 1.0 ng/ml). This significantly promotes random motility ($P < 0.0001$) (Fig. 4a). In contrast to HA, microinjection of either 1 ng/ml chondroitin sulfate (Fig. 4b) or 1.0 ng/ml heparin (Fig. 4c) has no significant effect on cell motility.

These results suggest that *ras* transformation and phorbol ester treatment both affect the subcellular distribution of TR-HA and they may also promote HA uptake, although no attempt was made here to quantify the amount of HA taken up by cells. This work, however, is ongoing.

4. Discussion

HA is ubiquitous in the extracellular matrix, and although its production is acutely upregulated following injury of most tissues, the function of this in the course of repair has remained elusive [37]. HA production and metabolism are often altered during tumorigenesis [38] and a causal role for this modification has recently been established by a study showing that HA accumulation around and within colorectal tumor cells is prognostic of poor outcome [39]. Consistent with the notion that HA directly regulates cell behavior, study of HA binding proteins termed hyaladherins has indicated that HA indeed contributes to the control of cell cycle and cell motility [1,3,4]. In spite of the importance of these observations, the molecular mechanisms by which HA directs these processes are not yet clear. HA regulates protein tyrosine phosphorylation cascades [1], signaling through growth factors [1,3], actin cytoskeleton assembly [1,33,40] and activity of MAP kinase cascades [3], all of which impact on cell motility and/or cell cycle [34,41]. Most of these actions of HA are considered to be initiated by HA/cell surface receptor interactions and, consistent with this possibility, many of the effects of HA on signaling cell motility can be blocked using antibodies to CD44, RHAMM, cdc37 or p68 [1,4,5,42]. Understanding the mechanisms of HA/cell surface interactions in signaling

has been complicated by the clear role of multiple cell surface-associated HA binding proteins [4], not all of which are transmembrane receptors and some of which (e.g. RHAMM) are only transiently expressed at the cell surface [1]. A further complication has arisen from the realization that intracellular HA binding proteins also exist [1]. Thus, the identification of intracellular forms of RHAMM [3], the known occurrence of cdc37 [43] and p68 [44] in the cytoplasm, their collective ability to bind to signaling molecules that regulate cell cycle and motility and the presence of additional, yet uncharacterized, cytoplasmic HA binding proteins have raised the possibility that intracellular HA may function in regulating aspects of cell behavior [1]. The ability of mutant forms of intracellular RHAMM that are impaired in HA binding to block activation of *erk* kinases [3,6] provides further, but still indirect, evidence for this possibility. Data presented here provide preliminary evidence that further supports this possibility by demonstrating that cells are able to accumulate HA within cell processes and the nucleus areas rich in intracellular hyaladherins. Further, these events correlate with promotion of cell motility by HA. The ability of HA, microinjected into cells that do not naturally take up HA into these compartments, to stimulate cell motility provides the first direct evidence of an effect of intracellular HA on cell behavior. Collectively, our results suggest that both HA/cell surface receptor and intracellular HA/protein interactions may be involved in regulation of cell motility. Our results further suggest that the uptake and intracellular targeting of HA is controlled by *ras* and PKC signaling pathways.

A conceptual problem with assessing the potential functions of intracellular HA is how this high molecular weight polysaccharide is delivered to the cytoplasm and to the nucleus. Recent cloning of HA synthases [45] predicted that these enzymes occur at the cell surface, with the UDP-sugar binding sites present on the synthase cytoplasmic face. Therefore, mechanisms must exist that allow extrusion of the large HA polymer to the extracellular milieu. These mechanisms may also permit, under certain conditions such as *ras* transformation or PKC activation, the re-entry of HA into the cell. It must also be considered that some HA may not be extruded but retained within the cytoplasm, where it is therefore available within a non-endosomal compartment to interact with key HA binding proteins.

We note here that the rapid uptake of fluorochrome-tagged

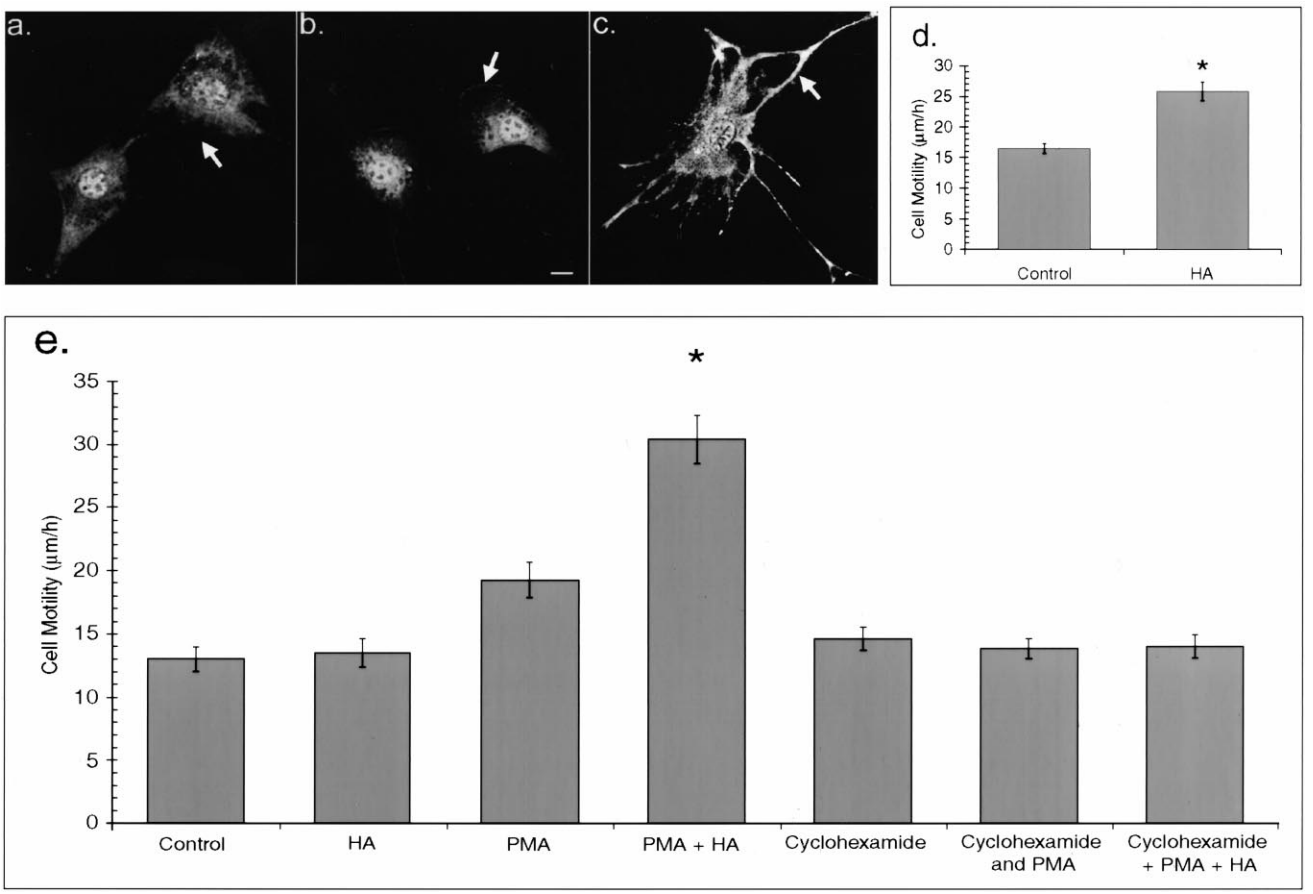

Fig. 3. a: Confocal analysis of 10T1/2 fibroblasts in an optical section midway through the cell. TR-HA is diffusely present in the the nucleus and the cytoplasm and is reduced in the perinuclear area. b: 10T1/2 fibroblasts treated with 100 nM PMA for 45 min prior to TR-HA addition show TR-HA localized at the edge of cell processes and in the perinuclear area although nuclear staining remains unchanged. Bar = 25 μm. c: 10T1/2 fibroblasts treated with 100 nM PMA for 4 h prior to TR-HA addition show enhanced uptake of TR-HA into cell processes. As well, an altered cytoplasmic TR-HA distribution is observed which is not seen in *ras*-transformed cells. d: Addition of HA to *ras*-transformed 10T1/2 cells significantly enhances their motility (also previously reported [33], $P < 0.05$, Student's *t*-test). e: Motility of parental 10T1/2 cells is not significantly promoted by HA (10 ng/ml) or PMA alone (100 nM). However, phorbol ester combined with the addition of HA now significantly enhances motility ($P < 0.001$, Student's *t*-test). These effects of phorbol ester and HA are abolished by cycloheximide. The bars indicate S.E.M. of 100 cells. The asterisk indicates significance. Arrows indicate cell processes.

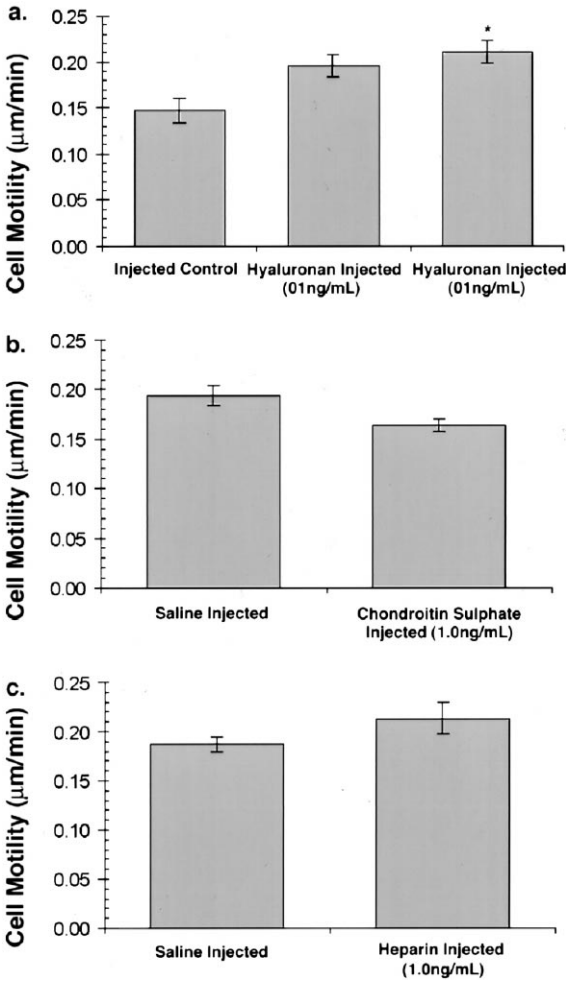

Fig. 4. a: Microinjection of HA (0.1 ng/ml or 1 ng/ml based on cell volume calculation) into 10T1/2 fibroblasts significantly promotes random motility, while microinjection of (b) chondroitin sulfate (1 ng/ml, $P < 0.0001$) or (c) heparin (1 ng/ml, $P < 0.0001$) does not promote random motility of 10T1/2 fibroblasts. The bars indicate S.E.M. of 100 cells (Student's *t*-test).

HA into *ras*-transformed cells appears to be independent of the traditional receptor-mediated endocytic pathway since uptake is acute, does not appear to be blocked by CD44 antibodies (at least in *ras*-transformed cells), and results in the accumulation within cell processes, the nucleus and the perinuclear area [19]. The localization of HA in the nucleus requires an interaction with proteins since exposing cells to peptides that mimic HA binding motifs of RHAMM blocks this targeting and results in accumulation of HA only within cell processes. HA binding motifs [35] are found in RHAMM, cdc37 and p68 and these proteins therefore represent potential candidates involved in the uptake of HA. Our results also suggest targeting to cell processes involves a mechanism distinct from nuclear targeting.

The ability of HA to be directed into 10T1/2 cell processes after acute phorbol ester treatments suggest an involvement of protein phosphorylation by protein kinase C in this HA update. This protein serine/threonine kinase has previously been implicated in cell motility [46] and its kinase activity has also previously been linked to release of HA from the cell [47], which may involve analogous mechanisms to uptake noted here.

A multifaceted role of intracellular HA is suggested by its localization in the nucleus, previous reports of HA binding to nuclear proteins [24,25], and the demonstration that a hyaluronan binding protein interacts with RNA splicing machinery [8]. Our results provide the first preliminary evidence of the ability of exogenously added HA to accumulate within multiple subcellular compartments, and to directly affect cell motility. Further analysis is required to determine the precise role(s) of HA within the cell, to define the mechanisms involved in this uptake and accumulation process, and to determine the signaling processes that intracellular HA might regulate.

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