Brief Report

Hyaluronic Acid (HA) Binding to CD44 Activates Rac1 and Induces Lamellipodia Outgrowth✪

Snezhana Oliferenko,* Irina Kaverina,‡ J. Victor Small,‡ and Lukas A. Huber*

*Research Institute of Molecular Pathology (IMP), A-1030 Vienna, Austria; and ‡Institute of Molecular Biology, Austrian Academy of Sciences, A-5020 Salzburg, Austria

Abstract. Both cell adhesion protein CD44 and its main ligand hyaluronic acid (HA) are thought to be involved in several processes ultimately requiring cytoskeleton rearrangements. Here, we show that the small guanine nucleotide (GTP)-binding protein, Rac1, can be activated upon HA binding to CD44. When applied locally to a passive cell edge, HA promoted the formation of lamellipodial protrusions in the direction of the stimulus. This process was inhibited by the prior injec- tion of cells with dominant-negative N17Rac recombi- nant protein or by pretreatment of cells with mono- clonal anti-CD44 antibodies, interfering with HA binding, implying the direct involvement of CD44 in signaling to Rac1.

Key words: extracellular matrix • cell guidance • Rac1 • cytoskeleton • mammary epithelial cells

Introduction

A widely distributed cell adhesion protein, CD44, serves as the major cell surface receptor for hyaluronic acid (HA)[1]. HA is a main carbohydrate component of the ex- tracellular matrix with a simple, repeated disaccharide lin- ear copolymer structure. It is mostly found in skin, joints, and eyes, but is also present in other organs and tissues. HA expression is tightly regulated; it is elevated in a vari- ety of inflammatory conditions (Gerdin and Hallgren, 1997). Transient overexpression of HA also takes place in granulation tissue during the wound healing process (Chen and Abatangelo, 1999). The biological roles attrib- uted to HA range from a purely structural function in the extracellular matrix to regulation of cell motility and adhe- sion (Itano et al., 1999; Delpech et al., 1997) and receptor- mediated alterations in gene expression (Fujii et al., 1999). Both CD44 and HA are thought to be involved in a variety of physiological processes, such as tumor formation and metastasis (Günthert, 1996), wound repair (Kaya et al.,

1997), the inflammatory immune response (Günthert, 1999), lymphocyte homing and adhesion (Siegelman et al., 1999), and embryonic development (Ponta and Herr- lich, 1998). All these processes require concordant rear- rangements of the actin cytoskeleton being the basis of various cell adhesion and migration events. Yet, there is almost no information available regarding possible intra- cellular signals transmitted upon CD44 binding to HA. Similarly, the exact roles of CD44 in vivo remain to be de- termined. Here, we show that in mouse mammary epithe- lial cells (EpH4), CD44 interaction with HA triggers the local signaling cascade, resulting in the direct activation of small GTP-binding protein Rac1, actin cytoskeleton rear- rangements, and reorientation of cells in the direction of the provided cue.

Materials and Methods

Cells, Antibodies, Reagents, and Constructs

Media, tissue culture reagents, and FCS were purchased from GibcoBRL and Boehringer Mannheim Corp. EpH4 cells, a spontaneously immortal- ized mouse mammary epithelial cell line (Fialka et al., 1996), were cul- tured at 37°C in 5% CO_2 and 98% humidity in Eagle's medium, supple- mented with 10 mM Hepes/KOH, pH 7.3, 50 IU/ml penicillin, 50 mg/ml streptomycin, and 5% FCS.

Anti-CD44 antibody, KM114, blocking hyaluronate recognition by

---

✪The online version of this article contains supplemental material.

Address correspondence to Dr. Lukas A. Huber, IMP, Research Insti- tute of Molecular Pathology, Dr. Bohr-Gasse 7, A-1030 Vienna, Austria. Tel.: (+43)-1-79730-885. Fax: (+43)-1-7987-153. E-mail: huber@nt.imp. univie.ac.at

[1]*Abbreviations used in this paper:* EpH4, mouse mammary epithelial cell line; HA, hyaluronic acid.

CD44, and anti-CD44 antibody, IM7.8.1, were purchased from RDI. Anti-Rac1 antibodies were purchased from Transduction Laboratories. Oligosaccharide of HA (H-9649) was purchased from Sigma Chemical Co., and high molecular weight HA, labeled with FITC, was a kind gift from Dr. C. Isacke and coworkers (Imperial College, London, UK). Laminin (20–200 µg/ml) and fibronectin (12–300 µg/ml) were from Boehringer Mannheim. Lipofectamine PLUS™ Reagent was purchased from Gibco-BRL. Rhodamine–phalloidin was from Molecular Probes (R-415). Recombinant N17Rac and N17Cdc42 were kindly provided by Dr. K. Rottner (Austrian Academy of Sciences, Salzburg, Austria; using a construct originally provided by Dr. A. Hall, University College, London, UK), dialyzed into 50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM MgCl$_2$, and 1 mM DTT. Concentrations of 1–2 mg/ml were used for injections. GST-CRIB construct was kindly donated by Dr. R. Cerione (Cornell University, Ithaca, NY). Human zyxin and paxillin in pEGFP-N1 vector were kindly supplied by Dr. J. Wehland and coworkers (GBF, Braunschweig, Germany). NH$_2$-terminal EGFP human β-actin, under the control of CMV promoter, cloned in pcDNA3 vector was kindly provided by Dr. M. Way (EMBL, Heidelberg, Germany).

Microinjections and Local Applications

Microinjections were performed with sterile Femtotips (Eppendorf, 5242 952.008) held in a Leitz micromanipulator with a pressure supply from an Eppendorf Microinjector 5242. Cells were injected with continuous outflow mode from the needle under a constant pressure of 20–40 hPa. Local applications of HA were performed essentially as described in Kaverina et al. (1999), under a constant pressure of 25–50 hPa.

Videomicroscopy

Cells were observed in an open chamber at 37°C on an inverted microscope (Axiovert 135TV, Zeiss) equipped for epifluorescence and phase-contrast microscopy. Injections were performed at 40× (NA 1.3 Plan Neofluar), and videomicroscopy with a 100× (NA 1.4 Plan-Apochromat) with or without 1.6× optovar intermediate magnification. Data were acquired with a back-illuminated cooled CCD camera from Princeton Research Instruments driven by IPLabs software (Visitron Systems). The microscope was additionally equipped with shutters driven through a homemade interface to allow separate recordings of video sequences in phase-contrast and fluorescent channels. Time between frames was 18 s. The video sequences were analyzed and processed on a Macintosh Power PC using IPLabs and Adobe Photoshop 5.2.1 software.

Online Supplemental Material

The online version of this article includes videos that accompany the figures presented here. Videos are supplied in the QuickTime format and are available at http://www.jcb.org.cgi/content/full/148/6/1159/DC1.
    Video 1. Depicts Fig. 1 a.
    Video 2. Depicts Fig. 1 c.
    Video 3. Depicts Fig. 2 a.
    Video 4. Depicts Fig. 2 b.
    Video 5. Depicts Fig. 2 b, inset.
    Video 6. Depicts Fig. 2 c.
    Video 7. Depicts Fig. 3 a.
    Video 8 and the Supplemental Figure. Microinjection of the dominant-negative N17Cdc42 recombinant protein does not block HA/CD44-induced lamellipodial formation. EGFP-β-actin–expressing cell was microinjected into N17Cdc42 recombinant protein and, subsequently, treated with HA. Note the formation of new lamellipodia in response to HA.

Results

EpH4 cells derive from a spontaneously immortalized mouse mammary epithelial cell line and display a fully polarized epithelial cell phenotype when grown to high density on semipermeable filter supports or in a three-dimensional collagen matrix (Fialka et al., 1996). However, when sparsely seeded, EpH4 cells often display a fan-shaped and motile fibroblastoid phenotype. Fibroblastoid cells typically exhibit protruding, mobile lamellipodial regions, interspersed by mainly concave, inactive cell edges, delim-

ited by peripheral actin filament bundles (Small, 1988). EpH4 cells express large amounts of CD44, consisting of standard and several variant isoforms (data not shown). In many cell types, CD44 exhibits a nonrandom distribution in the plasma membrane. In polarized epithelial cells, CD44 localization is restricted to the basolateral membrane domain, whereas in sparsely seeded, unpolarized EpH4 cells, CD44 is more broadly distributed, but clearly concentrated in the trailing regions and in the cell body (Oliferenko et al., 1999).

When sparsely seeded, EpH4 cells were treated with soluble oligosaccharides of HA. The majority of cells appeared depolarized, often with multiple lamellipodia around their entire circumference. This phenomenon was followed in more detail in single cells by phase-contrast time-lapse microscopy. By these means, we could confirm the fast outgrowth of new lamellipodia from cell edges previously exhibiting an inactive actin cytoskeleton, including trailing regions (Fig. 1 a, see also supplemental video). We quantified the percentage of cells exhibiting the polarized fibroblastoid phenotype in control and HA-treated cells. Almost three quarters (69%) of sparsely seeded EpH4 cells in control cultures exhibited unidirectional movement and a polarized morphology. Already 15 min after addition of HA, only 28% EpH4 cells remained polarized, whereas the rest exhibited multiple lamellipodia. However, the proportion of polarized cells in the presence of HA was comparable to control (68%) upon pretreatment of cells with anti-CD44 antibody, KM114 (Fig. 1 b). This mAb efficiently inhibits binding of HA to CD44 (Miyake et al., 1990). Inhibition of HA-induced lamellipodial protrusion by KM114 implied the direct involvement of CD44 in mediating the HA effect. To evaluate changes in the actin cytoskeleton in real time, we repeated HA treatment on EpH4 cells transiently expressing EGFP-β-actin. We observed the reorganization of the actin cytoskeleton, new lamellipodial outgrowth, characterized by a band of actin fluorescence, active ruffling of the leading edges, and the appearance of very bright EGFP-actin containing spots, or microruffles, on the cell surface (Fig. 1 c, see also supplemental video).

The immediate effect of HA treatment on the cell shape led us to investigate whether the observed changes could be induced locally. This was tested by the local application of HA through a microinjector needle to selected regions of cells. When HA was applied to inactive cell edges we could indeed observe a spatially restricted outgrowth of lamellipodia underneath the micropipette (Fig. 2 a, see also supplemental video). Pretreatment of cells with KM114 antibody effectively prevented these rearrangements, in a way similar to after the general application of HA (data not shown). HA-induced lamellipodia harbored small focal complexes that subsequently elongated and grew into mature focal adhesions, as revealed by experiments with cells transiently transfected with EGFP fusion constructs of the focal adhesion proteins, zyxin (Fig. 2 b, see also supplemental video) and paxillin (data not shown). Identical results were obtained with high molecular weight HA (Fig. 2 c, see also supplemental video). The local applications of the matrix proteins, laminin and fibronectin, under identical conditions did not produce lamellipodia outgrowth (data not shown).
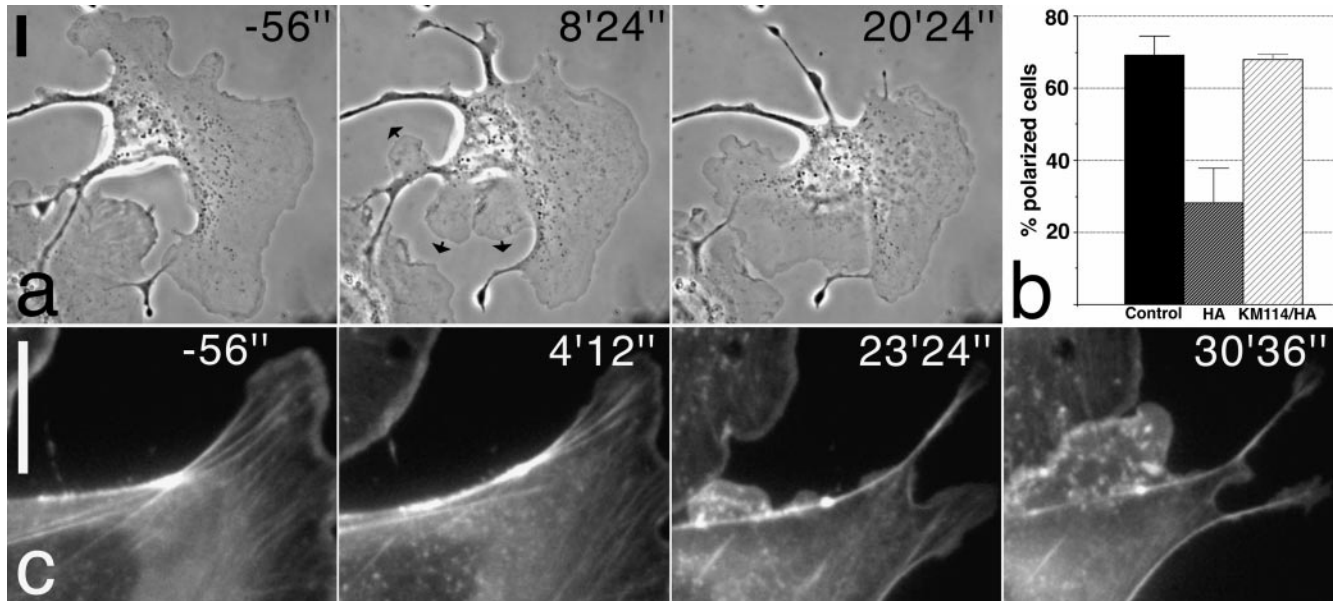
Figure 1. HA treatment promotes lamellipodial outgrowth. a, Oligosaccharide of HA (1 μg/ml) was applied to sparsely grown EpH4 cells. Note lamellipodial outgrowth from previously inactive cell edges. Numbers refer to the time, in minutes and seconds, in the time-lapse sequence. Arrows show the area of outgrowth. b, Histogram of the number of polarized cells in control, HA-treated (HA), and HA-treated cells preincubated with anti-CD44 HA-blocking antibodies, KM114 (KM114/HA). Mean ± SD values are shown ($n = 200$ cells, three experiments). c, Time-lapse sequence of EGFP-β-actin expressing EpH4 cells treated with HA. Note growth of lamellipodia from inactive cell edge and appearance of multiple microruffles. Videos depicting a and c are available at http://www.jcb.org.cgi/content/full/148/6/1159/DC1. Bar, 10 μm.

HA-induced cytoskeleton rearrangements were highly reminiscent of lamellipodial protrusion and ruffling caused by the activated GTPase Rac1 (Ridley et al., 1992), a member of the Rho family of small GTPases (Tapon and Hall, 1997). Rac1 is thought to be activated by various stimuli, including growth factors (Peppelenbosch et al., 1995; Ridley et al., 1995; Wennstrom et al., 1994; Nobes et al., 1995). To analyze whether Rac1 is indeed involved in HA-induced lamellipodial outgrowth, we analyzed the effect of HA on cells injected with the mutant form of Rac1, N17Rac, which acts as a dominant inhibitor of Rac1 activity. When cells were microinjected with N17Rac recombinant protein before HA treatment, the formation of lamellipodia was efficiently blocked (Fig. 3 a, see also supplemental video). Analysis of the effect of N17Rac was performed in ten independent experiments; the HA-induced lamellipodia outgrowth was inhibited in all cells observed. Likewise, de novo–formed HA-induced lamellipodia were retracted when cells were injected with N17Rac after HA treatment (data not shown). Injection of dominant-negative N17Cdc42 did not prevent HA-induced lamellipodia outgrowth, arguing for a direct effect on Rac, rather than via Cdc42 (data not shown; see supplementary figure and video available at http://www.jcb.org.cgi/content/full/148/6/1159/DC1).

To determine whether HA/CD44-induced lamellipodial outgrowth could be attributed to the direct activation of Rac1 instead of an activation of its downstream targets, we performed an affinity pull-down assay, allowing us to directly measure the amount of the active, GTP-bound form of Rac1 (Bagrodia et al., 1998). The CRIB domain of the downstream Rac1 effector, PAK, specifically binds the GTP-bound form of Rac1, which allows a quantification of actual amounts of active Rac1 in cell lysates. The affinity matrix, representing GST-fusion of CRIB domain coupled to the glutathione–Sepharose beads, was incubated with cell lysates derived from either control cells or cells treated with HA for various times. As shown in Fig. 3 b, the amount of GTP-bound Rac1 was considerably increased already after two minutes of HA treatment, and continued to a plateau after 15 min. However, the total amount of Rac1 did not change upon HA treatment (Fig. 3 b). Taken together, these results argue for the direct activation of Rac1 upon HA/CD44 engagement.

Discussion

Application of soluble HA to EpH4 cells in culture promoted activation of Rac1 and rapid outgrowth of lamellipodia from previously inactive cell edges. This effect was dependent on the ability of CD44 to bind HA. More interestingly, when HA was applied locally, a spatially restricted outgrowth of lamellipodia was observed precisely in the region of HA application. This result means that activation of Rac1 probably takes place directly in the vicinity of the plasma membrane. Rac1 activation appears to be independent on Cdc42, since microinjection of dominant-negative Cdc42N17 protein does not block HA-induced lamellipodia outgrowth. HA-induced lamellipodia also contained mature focal adhesions. This strongly implies that, in this case, not only is Rac1 activated, but also that Rho remains active.

The precise details of Rac1 activation upon HA/CD44 engagement remain to be elucidated. However, it is tempting to speculate that one of the Rac1 accessory proteins could participate in complex formation with CD44. One
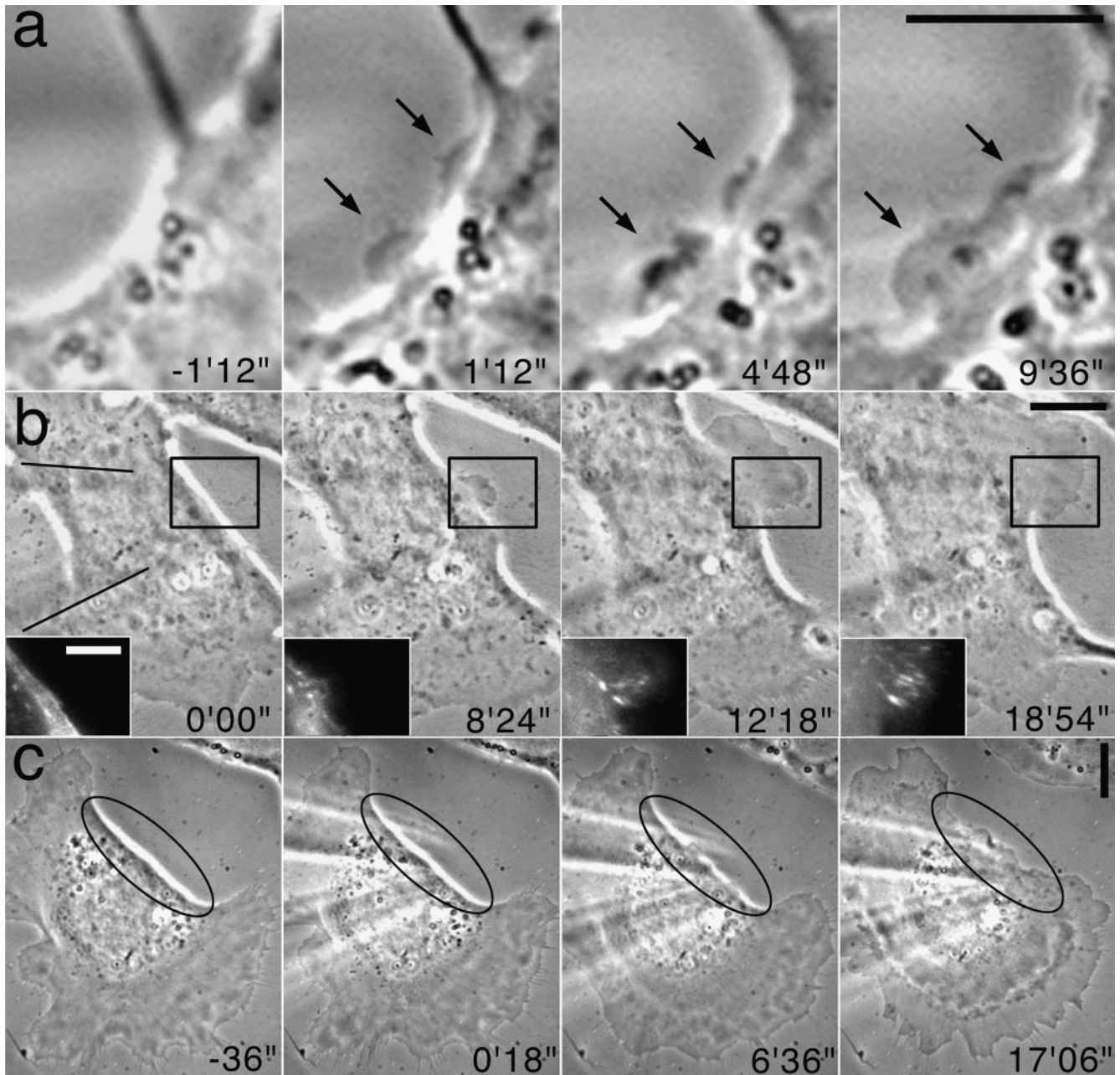
Figure 2. Lamellipodial outgrowth is spatially restricted and confined to the region of local HA application. When oligosaccharide HA (40 μg/ml) was supplied through the microinjection needle to the defined region of the cell, we could observe rapid lamellipodia formation exclusively underneath the micropipette (a). The lamellipodia being formed exhibited normal focal contact formation and growth, as shown for the EGFP-zyxin–expressing cell (b). High molecular weight HA (40 μg/ml) was equally potent in inducing local lamellipodial outgrowth (c). Videos depicting A–C and the inset in B are available at http://www.jcb.org.cgi/content/full/148/6/1159/DC1. Bars: (a–c) 10 μm, (b, inset) 5 μm.

of the logical candidates would be the guanine nucleotide exchange factor, Tiam-1, which was shown to specifically activate Rac1, but not Cdc42 (Sander et al., 1999). Recently, while this paper was under review procedure, the biochemical evidence of the direct CD44/Tiam-1 interaction was reported (Bourguignon et al., 2000). This result might provide the potential straightforward link between CD44/HA binding and Rac1 activation. CD44 was also found in a complex with Rho-GDP dissociation inhibi-

tor (RhoGDI; Olofsson, 1999) and proteins of the ERM (ezrin-radixin-moesin) family in fibroblasts (Hirao et al., 1996). The interaction of ERM proteins with RhoGDI was shown to activate Rho subfamily members (Hirao et al., 1996; Takahashi et al., 1997). It might also be possible that HA binding to CD44 would trigger the conformational change in this complex, inactivating RhoGDI as a result, and ultimately contributing to the activation of Rac1. Yet, CD44/ERM interaction appears to be rather
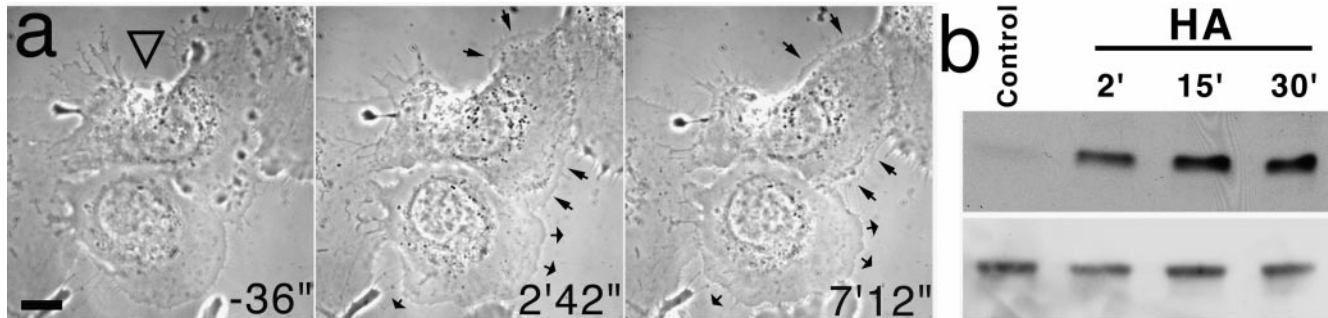
Figure 3. Rac1 is required for HA/CD44-induced lamellipodial formation and is activated upon HA treatment. a, Lamellipodial outgrowth upon HA treatment could be inhibited by microinjection of dominant-negative N17Rac before HA treatment. Note that only the upper cell was injected with N17Rac, as depicted with empty arrowhead; the bottom cell exhibited the typical protrusive activity in response to HA. A representative anti-Rac1–probed immunoblot of affinity-precipitated Rac-GTP from control and HA-treated cells is shown in b, upper panel, together with a Western blot of total cellular Rac1 (lower panel). Rac-GTP amounts increased upon HA treatment, indicating the activation of Rac1. A video depicting a is available at http://www.jcb.org.cgi/content/full/148/6/1159/DC1. Bar, 10 μm.

cell-type restricted. So far, we have no indication that they interact with each other in EpH4 mammary epithelial cells (Oliferenko et al., 1999).

Interestingly, when the dominant active mutant of Rac1 was overexpressed in human glioblastoma cells (U251MG), CD44 was shown to redistribute to lamellipodia where it subsequently was cleaved and shed from the surface (Okamoto et al., 1999). A possible mechanism for downregulating CD44-mediating signaling can be suggested by these experiments.

The observation we describe here would suggest the possibility of the direct involvement of CD44 and HA in cell guidance. We would like to propose that HA may serve as an external stimulus influencing cell orientation, with CD44 mediating signaling to the actin cytoskeleton.

References

Bagrodia, S., S.J. Taylor, K.A. Jordon, L. Van Aelst, and R.A. Cerione. 1998. A novel regulator of p21-activated kinases. *J. Biol. Chem.* 273:23633–23636.

Bourguignon, L.Y., H. Zhu, L. Shao, and Y.W. Chen. 2000. CD44 interaction with Tiam1 promotes Rac1 signaling and hyaluronic acid-mediated breast tumor cell migration. *J. Biol. Chem.* 275:1829–1838.

Chen, W.Y., and G. Abatangelo. 1999. Functions of hyaluronan in wound repair. *Wound Repair Regen.* 7:79–89.

Delpech, B., N. Girard, P. Bertrand, M.N. Courel, C. Chauzy, and A. Delpech. 1997. Hyaluronan: fundamental principles and applications in cancer. *J. Intern. Med.* 242:41–48.

Fialka, I., H. Schwarz, E. Reichmann, M. Oft, M. Busslinger, and H. Beug. 1996. The estrogen-dependent c-JunER protein causes a reversible loss of mammary epithelial cell polarity involving a destabilization of adherens junctions. *J. Cell Biol.* 132:1115–1132.

Fujii, K., Y. Tanaka, S. Hubscher, K. Saito, T. Ota, and S. Eto. 1999. Cross-linking of CD44 on rheumatoid synovial cells up-regulates VCAM-1. *J. Immunol.* 162:2391–2398.

Gerdin, B., and R. Hallgren. 1997. Dynamic role of hyaluronan in connective tissue activation and inflammation. *J. Intern. Med.* 242:49–55.

Günthert, U. 1996. CD44 in malignant disorders. *Curr. Top. Microbiol. Immunol.* 213:271–285.

Günthert, U. 1999. Importance of CD44 variant isoforms in mouse models for inflammatory bowel disease. *Curr. Top. Microbiol. Immunol.* 246:307–312.

Hirao, M., N. Sato, T. Kondo, S. Yonemura, M. Monden, T. Sasaki, Y. Takai, and S. Tsukita. 1996. Regulation mechanism of ERM (ezrin/radixin/moesin) protein/plasma membrane association: possible involvement of phosphatidylinositol turnover and Rho-dependent signaling pathway. *J. Cell Biol.* 135:37–51.

Itano, N., T. Sawai, O. Miyaishi, and K. Kimata. 1999. Relationship between hyaluronan production and metastatic potential of mouse mammary carcinoma cells. *Cancer Res.* 59:2499–2504.

Kaverina, I., O. Krylyshkina, and J.V. Small. 1999. Microtubule targeting of substrate contacts promotes their relaxation and dissociation. *J. Cell Biol.* 146:1033–1044.

Kaya, G., I. Rodriguez, J.L. Jorcano, P. Vassalli, and I. Stamenkovic. 1997. Selective suppression of CD44 in keratinocytes of mice bearing an antisense CD44 transgene driven by a tissue-specific promoter disrupts hyaluronate metabolism in the skin and impairs keratinocyte proliferation. *Genes Dev.* 11:996–1007.

Miyake, K., C.B. Underhill, J. Lesley, and P.W. Kincade. 1990. Hyaluronate can function as a cell adhesion molecule and CD44 participates in hyaluronate recognition. *J. Exp. Med.* 172:69–75.

Nobes, C.D., P. Hawkins, L. Stephens, and A. Hall. 1995. Activation of the small GTP-binding proteins rho and rac by growth factor receptors. *J. Cell Sci.* 108:225–233.

Okamoto, I., Y. Kawano, M. Matsumoto, M. Suga, K. Kaibuchi, M. Ando, and H. Saya. 1999. Regulated CD44 cleavage under the control of protein kinase C, calcium influx, and the Rho family of small G proteins. *J. Biol. Chem.* 274:25525–25534.

Oliferenko, S., K. Paiha, T. Harder, V. Gerke, C. Schwärzler, H. Schwarz, H. Beug, U. Günthert, and L.A. Huber. 1999. Analysis of CD44-containing lipid rafts: recruitment of annexin II and stabilization by the actin cytoskeleton. *J. Cell Biol.* 146:843–854.

Olofsson, B. 1999. Rho guanine dissociation inhibitors: pivotal molecules in cellular signaling. *Cell Signal.* 11:545–554.

Peppelenbosch, M.P., R.G. Qiu, A.M. de Vries-Smits, L.G. Tertoolen, S.W. de Laat, F. McCormick, A. Hall, M.H. Symons, and J.L. Bos. 1995. Rac mediates growth factor-induced arachidonic acid release. *Cell.* 81:849–856.

Ponta, H., and P. Herrlich. 1998. The CD44 protein family: roles in embryogenesis and tumor progression. *Front. Biosci.* 3:650–656.

Ridley, A.J., H.F. Paterson, C.L. Johnston, D. Diekmann, and A. Hall. 1992. The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell.* 70:401–410.

Ridley, A.J., P.M. Comoglio, and A. Hall. 1995. Regulation of scatter factor/hepatocyte growth factor responses by Ras, Rac, and Rho in MDCK cells. *Mol. Cell. Biol.* 15:1110–1122.

Sander, E.E., J.P. ten Klooster, S. van Delft, R.A. van der Kammen, and J.G. Collard. 1999. Rac downregulates Rho activity: reciprocal balance between

both GTPases determines cellular morphology and migratory behavior. *J. Cell Biol.* 147:1009–1022.

Siegelman, M.H., H.C. DeGrendele, and P. Estess. 1999. Activation and interaction of CD44 and hyaluronan in immunological systems. *J. Leukoc. Biol.* 66:315–321.

Small, J.V. 1988. The actin cytoskeleton. *Electron Microsc. Rev.* 1:155–174.

Takahashi, K., T. Sasaki, A. Mammoto, K. Takaishi, T. Kameyama, S. Tsukita, and Y. Takai. 1997. Direct interaction of the Rho GDP dissociation inhibitor with ezrin/radixin/moesin initiates the activation of the Rho small G protein. *J. Biol. Chem.* 272:23371–23375.

Tapon, N., and A. Hall. 1997. Rho, Rac and Cdc42 GTPases regulate the organization of the actin cytoskeleton. *Curr. Opin. Cell Biol.* 9:86–92.

Wennstrom, S., P. Hawkins, F. Cooke, K. Hara, K. Yonezawa, M. Kasuga, T. Jackson, L. Claesson-Welsh, and L. Stephens. 1994. Activation of phosphoinositide 3-kinase is required for PDGF-stimulated membrane ruffling. *Curr. Biol.* 4:385–393.