Hepatocyte Growth Factor-induced Ras Activation Requires ERM Proteins Linked to Both CD44v6 and F-Actin

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In several types of cells, the activation of the receptor tyrosine kinase c-Met by its ligand hepatocyte growth factor (HGF) requires the coreceptor CD44v6. The CD44 extracellular domain is necessary for c-Met autophosphorylation, whereas the intracellular domain is required for signal transduction. We have already shown that the CD44 cytoplasmic tail recruits *e*zrin, *r*adixin and *m*oesin (ERM) proteins to the complex of CD44v6, c-Met, and HGF. We have now defined the function of the ERM proteins and the step they promote in the signaling cascade. The association of ERM proteins to the coreceptor is absolutely required to mediate the HGF-dependent activation of Ras by the guanine nucleotide exchange factor Sos. The ERM proteins need, in addition, to be linked to the actin cytoskeleton to catalyze the activation of Ras. Thus, we describe here a new function of the cytoskeleton. It is part of a "signalosome" complex that organizes the activation of Ras by Sos. So far the cytoskeleton has mainly been identified as a "responder" to signal transduction. Here, we show now that F-actin acts as an "inducer" that actively organizes the signaling cascade.

INTRODUCTION

The CD44 family of transmembrane proteins is encoded by a single highly conserved gene. The different isoforms are mainly generated by alternative splicing of 10 variant exons (designated variant exons v1 to v10) that account for sequences located in the extracellular part of the CD44 proteins. In the smallest isoform (CD44s), all variant exons have been spliced out, whereas in the variant isoforms (CD44v) several combinations of variant exons can be included (for reviews, see Naor *et al.*, 1997; Ponta *et al.*, 2003).

CD44 proteins play important roles in various cellular processes such as growth regulation, survival, differentiation, and motility. Consequently, their altered expression or dysfunction contributes to numerous pathological situations, which can give rise, in the worst cases, to metastatic spreading of tumor cells (for reviews, see Naor *et al.*, 1997; Ponta *et al.*, 2003). A CD44 variant containing exon v6 sequences has been shown to be required and is even sufficient for the metastatic spreading of a rat pancreatic tumor cell line: The metastatic spreading of these tumor cells could be inhibited by treatment of tumor-bearing animals with CD44v6-specific antibodies (Seiter *et al.*,

1993). Expression of a v6 containing CD44 isoform conferred the metastatic potential to otherwise nonmetastatic cells (Günthert *et al.*, 1991). Furthermore, CD44 variants have been detected in a variety of human tumors and their expression has been correlated with poor prognosis (for review, see Naor *et al.*, 2002).

In addition to cancer, CD44 proteins play essential roles in a variety of other physiological and pathological processes, including tissue development, neuronal axon guidance, hematopoiesis, numerous immune functions, and autoimmune diseases (for reviews, see Naor *et al.*, 1997; Ponta *et al.*, 2003).

A striking evidence for the role of CD44 variants in embryogenesis has been found during limb development in that CD44 variant-specific antibodies blocked limb outgrowth. CD44 variants are located on the apical ectodermal ridge where they act as coreceptors in trans for fibroblast growth factor receptors located on underlying mesenchymal cells (Sherman *et al.*, 1998). The coreceptor function of CD44 was dependent on modification by heparan sulfate on sequences encoded by exon v3.

Based on the work on CD44 function in limb development, we investigated whether CD44 isoforms might take part in the metastatic process also through the activation of receptor tyrosine kinases (RTK). A promising candidate seemed to be the RTK c-Met and its ligand hepatocyte growth factor or scatter factor (designated as HGF throughout the article) for several reasons. First, the activation of c-Met triggers various effects that are also related to metastasis such as migration, proliferation, or differentiation (Bardelli *et al.*, 1997). Second, c-Met is often up-regulated or amplified in human tumors such as colorectal and pancreatic cancers (Di Renzo *et al.*, 1995a,b) in which also CD44v6 is often up-regulated. Third, the introduction of c-Met into NIH3T3 cells rendered the cells metastatic (Giordano *et al.*, 1993; Jeffers *et al.*, 1996).

We have shown that the activation of c-Met and the downstream signaling to extracellular signal-regulated kinase (Erk) in response to HGF in fact requires the presence of CD44 v6-containing isoforms (Orian-Rousseau *et al.*, 2002). Antibodies against CD44v6 blocked c-Met activation in several cell lines as well as in primary cells. The physiological processes triggered by c-Met activation were also inhibited. Interestingly, one of these CD44v6-specific antibodies had been used previously to inhibit the metastatic spreading of a rat pancreatic tumor cell line (Seiter *et al.*, 1993).

In a cell line that did not express CD44 variant isoforms but the c-Met receptor, c-Met activation by HGF was restored by transfection of CD44 variant isoforms with v6 sequences (Orian-Rousseau *et al.*, 2002). The precise amino acids in the exon v6 that are required for c-Met activation had been identified by linker scan mutagenesis, being EWQ in rat and RWH in human. Peptides making up these three amino acids (the smallest being a 5mer) competed with the function of endogenous CD44v6 and blocked c-Met phosphorylation, downstream signaling, and HGF-induced invasion and scattering (Matzke *et al.*, 2005).

For the activation of c-Met, the extracellular part containing the exon v6 sequence and the transmembrane domain of CD44 were sufficient. Removal of the cytoplasmic tail of CD44, however, had an unexpected effect. Such a truncated mutant still allowed activation of c-Met, but signaling emanating from c-Met to Erk was completely blocked (Orian-Rousseau *et al.*, 2002). Activation of signaling components that directly bind to c-Met was still possible. Competition experiments by overexpressing intracellularly the cytoplasmic tail of CD44 also abrogated downstream signaling (Orian-Rousseau *et al.*, 2002), suggesting that proteins have to bind to the cytoplasmic tail to promote signaling. Candidates are *e*zrin, *r*adixin and *m*oesin (ERM) proteins, the main representative in the cells used being ezrin. This has been confirmed by the overexpression of CD44 cytoplasmic tails point-mutated in the ezrin binding site that did not interfere with signaling.

ERM proteins belong to a superfamily of proteins that link the cytoskeleton to the cell membrane (for reviews, see Arpin *et al.*, 1994; Tsukita and Yonemura, 1997). Ezrin, for example, can associate with various membrane proteins such as CD44 (Tsukita *et al.*, 1994), intercellular adhesion molecule-1,2 (Helander *et al.*, 1996), Na-H exchanger (Denker *et al.*, 2000), and type II c-AMP–dependent protein kinases (Dransfield *et al.*, 1997). These interactions are established via its N terminus, whereas its C-terminal domain binds to F-actin in vitro (Turunen *et al.*, 1994) and in vivo (Algrain *et al.*, 1993).

Here, we address two main questions. First, at which step in the Ras/mitogen-activated protein kinase (MAPK) pathway is the cytoplasmic tail of CD44v6 and the binding of ERM proteins required for signaling from the c-Met receptor? Second, is the binding of ERM proteins to F-actin necessary for signaling thus organizing the downstream signaling cascade?

MATERIALS AND METHODS

Cells

The HT29 cells (Fogh *et al.*, 1977), a gift from A. Zweibaum (Institut National de la Santé et de la Recherche Médicale, Paris, France), and the 293 cells (ATCC CRL-1573; American Type Culture Collection, Manassas, VA) were routinely grown in DMEM (Invitrogen, Karlsruhe, Germany) plus 10% fetal calf serum (FCS; PAA Laboratories, Coelbe, Germany). The BSp73AS cells (Matzku *et al.*, 1983) and its transfectants were grown in RPMI 1640 medium (PAA Laboratories) plus 10% FCS. ASv4-7 cells, ASv4-7Δcyt cells, and ASv6 cells have been described previously (Orian-Rousseau *et al.*, 2002).

Constructs

Expression vectors containing rat CD44v6 fused to full-length or truncated rat ezrin: The CD44 part was amplified by polymerase chain reaction (PCR) covering the 5'-untranslated region to the transmembrane domain from the pGKs6 vector (Sleeman *et al.*, 1997). The primers used were CGCGACCCTTT-TCCAGAGGCTACTAGATCCTTTGG TTTCATCCTGCACATCATGG and GTCTGCATTGCTGTCAACAGTAGGAGG AAGCAGACGTAACGACAGT-TGTCATCCTCCTTCCTTTGGAAAC.

Note that we have included in the forward primer an EcoRI restriction site and in the reverse primer a HindIII and an EcoRI restriction site. The PCR product was cloned into the Topo-pCRII vector (Invitrogen).

For the ezrin part, rat ezrin was amplified from a pCDNA3.1 vector containing full-length ezrin (our unpublished data) by using the following primers: CTCGGAAGCTTAGCCACCAACCAGCCAAGATGCC and CAAAGC-TTGAATT CCTACTTGCCCAGCCGGTTCATCTCGATGTCGGTGTATGGC-CTCAAAC. A vesicular stomatitis virus G (VSV-G) sequence was included in the reverse primer. The PCR product was also cloned in a Topo pCRII vector.

The Topo pCRII vector containing CD44v6 was then linearized with HindIII, and the HindIII fragment from the Topo pCRII vector containing ezrin sequences was inserted. The CD44v6–ezrin fusion was excised by EcoRI and cloned into a pCDNA3.1 vector (Invitrogen). The point mutation in ezrin at T566D (Bretscher *et al.*, 2002) in the CD44v6–ezrin fusion was introduced using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The removal of the last 34 Aas in ezrin in the CD44v6–ezrin fusion was performed by at first insertion of two MluI sites by mutagenesis followed by excision of the MluI fragment.

The fusion protein between CD44v6 and the ezrin C terminus was made out of the CD44v6 fusion with full-length rat ezrin (see above). The N-terminal part of ezrin was deleted after insertion of two MluI restriction sites by QuickChange mutagenesis. The primers used for insertion of the MluI sites at the ezrin N-terminal start site were as follows: 5'-TGCCCAAGCCAAT-CAACACGCGTGTGACCACCATGGATGC-3' and 5'-GCATCCATGGTG-GTCACACGCGTGTTGATTGGCTTGGGCA-3'.

Into the mutated CD44v6-ezrin fusion a second MluI site was introduced into the ezrin N-terminal end: 5'-CCTGACTTCGTGTTCTACACGCGTCGCCTGAGAA-TTAACAAGCG-3' and 5'-CGCTTGTTAATTCTCAGGCGACGCGTGTAGAACA-CGAAGTCAGG-3'.

An ezrin wild type and a construct, in which the sequences encoding the last 29 amino acids are deleted, both tagged with VSV-G, were kindly provided by Monique Arpin (Institut Pasteur, Paris, France). The hemagglutinin (HA)-tagged Erk2 plasmid was a gift from A. Ulrich (Martinsried, Germany), and the mitogen-activated protein kinase kinase (MEK)-glutathione *S*-transferase (GST) construct was provided by A. Cato (FZ Karlsruhe, Stutensee, Germany).

Antibodies and Other Reagents

Antibodies directed against c-Met, Erk, Shp2, and green fluorescent protein (GFP) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); antibodies against phospho-Akt, phospho-phospholipase C (PLC)γ, phospho-Erk, and phospho-MEK as well as the Akt antibodies from Cell Signaling Technology (Beverly, MA). Gab1, PLCγ, Ras, Raf, and anti-phospho-tyrosine (4G10) antibodies were purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA), and the antibody against ezrin was from Dunn Labortechnik (Asbach, Germany). Rabbit IgG was from Dianova (Hamburg, Germany), the HA antibody was from Roche Diagnostics (Mannheim, Germany), and the VSV-G antibody was from Stressgen/Biomol (Hamburg, Germany). HGF was either obtained from R&D Systems (Minneapolis, MN) or kindly provided by George Vande Woude (Van Andel Institute), and it was preactivated with 5% FCS overnight at 37°C. 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), lysophosphatidic acid (LPA), Triton X-100, latrunculin B, and cytochalasin D were from Sigma (Taufkirchen, Germany).

Immunoprecipitation and Western Blotting

Detection of c-Met, Gab1, HA-Erk, and phosphorylated signaling components has been described previously (Orian-Rousseau *et al.*, 2002). Note that throughout the article, activation was measured upon 24 h of starvation of the cells and 5 min of treatment with HGF at 37°C. This time point showed maximal activation of Met and Erk in activation kinetics (data not shown). For coimmunoprecipitation of Sos and ezrin, GFP-Sos and VSV-G ezrin constructs (Algrain *et al.*, 1993) were transfected into 293 cells by using Lipofectamine 2000 (Invitrogen). The cells were lysed in 25 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 1% NP-40, 0.5 mM MgCl$_2$, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na orthovanadate, and 1 mM aprotinine and leupeptin. Immunoprecipitation was carried out using GFP antibodies.

Activated Ras was detected in cells seeded in six-well plates by using a GST fusion protein, corresponding to the human Ras binding domain of Raf-1 (RBD; residues 1-149) bound to glutathione agarose (BIOMOL Research Laboratories) according to the manufacturer's instruction. Raf activity was measured using an in vitro kinase assay. Cell were lysed in 50 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10 mM NaF, 1 mM PMSF, 1 mM Na orthovanadate, and 1 mM aprotinine and leupeptin, and Raf was immunoprecipitated. The pellets were washed three times in lysis buffer and one time in the kinase buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 20 mM MgCl$_2$, and 1 mM ATP supplemented with

protease inhibitors). The pellets were then incubated with the kinase buffer supplemented with 10 μCi of [γ-^{32}P]ATP and GST-MEK (500 ng of purified protein) for 30 min at 30°C. After centrifugation the pellets were dissolved in SDS-sample buffer and subsequently loaded on a SDS-PAGE gel. GST-MEK was prepared from transfected *Escherichia coli* cells by affinity purification with glutathione agarose beads (Sigma) (Smith *et al.*, 1993).

Grb2 Pull-Down

Cells (10-cm plate) were induced with HGF, where indicated, lysed in the same buffer used for coimmunoprecipitation of Sos and ezrin (see above), and incubated overnight with Grb2 agarose beads (BIOMOL Research Laboratories). The beads were washed three times in lysis buffer and prepared for SDS-PAGE. Western blotting was performed with the different antibodies as indicated.

Transfection

The 293 cells were transiently transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Transfection of the BSp73AS cells was performed by electroporation using the protocol provided by Amaxa (Amaxa Biosystems, Gaithersburg, MD). In brief, 1×10^6 cells were used for each reaction. DNA (1.5 μg of HA-Erk and 2.5 μg of indicated vectors) and nucleofector solution L (200 μl) were mixed and combined with the cells. Electroporation was performed using the program T-020. Prewarmed medium without serum was then added, and the cells were seeded in a six-well plate. After 24-h incubation, the cells were starved for 24 h followed by induction with HGF. Immunoprecipitation and detection of HA-Erk has been described previously (Orian-Rousseau *et al.*, 2002).

Small interfering RNA (siRNA) sequences against human ezrin were kindly provided by S. Pust (GBF Braunschweig, Braunschweig, Germany) (sense, CCCCAAAGAUUGGCUUUCC) and Santa Cruz Biotechnology (mixture of six single-stranded RNAs; sense sequences: 1, GGAACAUCUC UUUCAAUGA; 2, CCACGUCUGAGAAUCAACA; and 3, GACUCUGUUUGCU UGUGUU) and transfected into 293 cells using Oligofectamine (Invitrogen) according to the manufacturer's protocol twice with a delay of 24 h between two transfections. For control the off-target siRNA, UUCUCCGAACGUGUCACGU (sense strand; Qiagen-Xeragon, Germantown, MD) was used.

Triton X-100 Insolubility Assay

Cells (3×10^6) were seeded in 10-cm plates and grown, starved, and induced with HGF as described previously (Orian-Rousseau *et al.*, 2002) and fixed in 10% formalin. The cells were then lysed in 25 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% Triton X-100, and 0.5 mM MgCl$_2$ together with protease and phosphatase inhibitors (see above). The lysate was centrifugated for 5 min at 1000 $\times$ g, and the supernatant was then centrifugated at 100,000 $\times$ g for 1 h. The pellet was dissolved in sample buffer and loaded on SDS gel.

Scattering Assay

HT29 cells were transfected with the indicated constructs (3 μg) by using the Amaxa electroporation device as described above. For these cells, the solution R and the program S4 were used. The scattering assay has been described previously (Orian-Rousseau *et al.*, 2002).

RESULTS

We have previously shown that the activation of c-Met and Erk in response to HGF requires the presence of CD44 v6-containing isoforms (Orian-Rousseau *et al.*, 2002). The extracellular part of CD44v6 is required for c-Met activation, whereas the cytoplasmic tail promotes signaling from c-Met to Erk. Here, we show that also other signaling pathways require the presence of the CD44 tail. We transfected the CD44v6 negative rat pancreatic adenocarcinoma cell line BSp73AS that does not respond to HGF with either CD44v4-7 or CD44v6 (both gave similar results and are referred as CD44v6). Transfection of CD44v6 into these cells leads to c-Met, Erk, Akt, and c-Jun NH$_2$-terminal kinase (JNK) activation (Figure 1A; note that a quantification of fold induction by HGF by densitrometric scanning is indicated below the respective lanes throughout Figures 1–6). Transfection of tailless CD44v6 (v6Δcyt) catalyzed HGF-induced c-Met phosphorylation as well. However, the activation of downstream signaling components was abolished (Figure 1A). These data indicate a requirement of the CD44 tail for pathways that lead to Akt and JNK activation in addition to the Ras/MAPK pathway. Transfection of the CD44v6 tail-
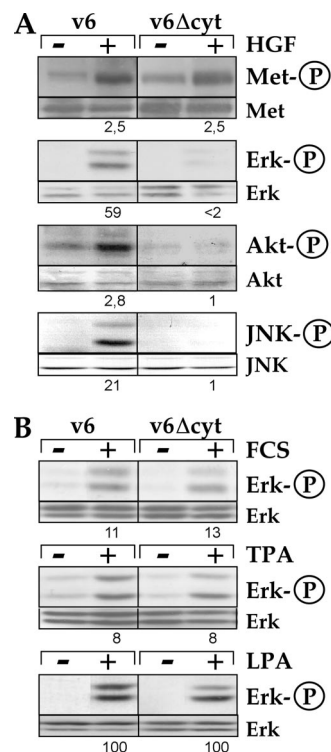

Figure 1. c-Met signaling is dependent on the CD44 cytoplasmic tail. (A) Measurement of c-Met phosphorylation as well of the downstream targets Erk, Akt, and JNK was performed in BSP73AS cells transfected with either CD44v4-7 (v6) or with CD44v4-7 truncated for the cytoplasmic tail (v6Δcyt) (Orian-Rousseau *et al.*, 2002). The numbers reflect -fold induction by HGF as determined by densitometric scanning. (B) Erk phosphorylation in the same cells as in A seeded in six-well plates and induced for 5 min by 10% FCS, 40 ng/ml TPA or 20 μM LPA.

less mutant did not affect serum-, TPA-, or LPA-dependent induction of Erk phosphorylation (Figure 1B) excluding unspecific effects on all extracellular stimuli.

To elucidate at which step in the HGF-dependent Ras/ MAPK signaling pathway the CD44 tail is required, we analyzed the activation of components downstream of c-Met. In CD44v6 transfected BSp73AS cells the activation of components that directly bind to c-Met upon HGF treatment, such as Gab1 and PLCγ were not affected by the absence of the CD44 tail (Figure 2A). However, activation of Ras, Raf, MEK, and Erk was only observed in cells transfected with wild-type CD44v6 but not with the tailless mutant (Figure 2A).

In the same cells, we measured the association of signaling components into a complex with Grb2. Grb2 is an adaptor protein that mediates signaling from c-Met to Ras via recruiting the guanine nucleotide exchange factor (GEF) Sos (Boguski and McCormick, 1993). The Grb2 complex as it is formed in cells was reconstituted on GST-Grb2 beads in vitro as shown previously (Nel *et al.*, 1995). The specificity of this reconstitution was demonstrated in that the association of c-Met, Gab1, Shp2, and Ras to GST-Grb2 was predominantly observed upon HGF induction in CD44v6-expressing cells (Figure 2B). For Gab1, a constitutive association with Grb2 has been described, however, that was strongly increased upon growth factor stimulation (Nishida *et al.*, 1999) as seen here. Sos was constitutively associated with Grb2 independently of HGF treatment (Figure 2B) in agreement
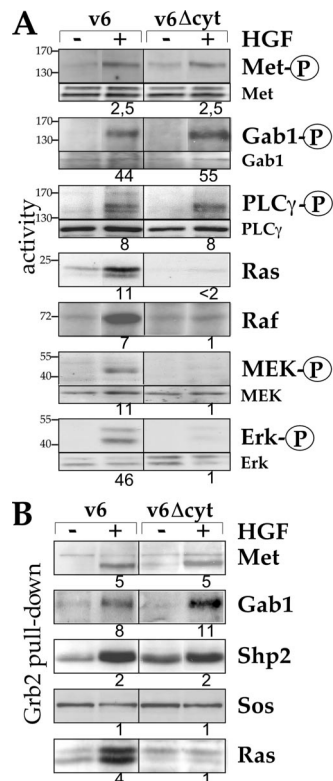
Figure 2. The CD44 cytoplasmic tail promotes activation of Ras by Sos. (A) HGF-induced phosphorylation of c-Met, Gab1, PLCγ, MEK, and Erk in ASv4-7 cells (v6) or ASv4-7Δcyt cells (v6Δcyt) (Orian-Rousseau *et al.*, 2002), respectively. Ras activity was measured by means of a pull-down by using the Ras binding domain of Raf, Raf activity was determined in an in vitro kinase assay (Huser *et al.*, 2001). The numbers at the left side of the lanes refer to molecular weight markers; the numbers below the lanes indicate -fold induction by HGF. (B) GST-Grb2 beads were used to pull-down c-Met, Gab1, Shp2, Sos, and Ras in ASv4-7 or ASv4-7Δcyt cells, respectively.

with previous reports (Egan *et al.*, 1993). Also, when using extracts from cells expressing the CD44 tailless mutant, the association of c-Met, Gab1, and Shp2 with Grb2 was increased upon HGF stimulation (Figure 2B). In striking contrast, however, there was no HGF-dependent recruitment of Ras to the complex (Figure 2B). Thus, these pull-down experiments are in complete agreement with the measurements of the activity of the proteins (Figure 2A).

Overexpression of soluble CD44 tails in the cytoplasm of 293 human kidney carcinoma cells in which c-Met activation is triggered by endogenous CD44v6 also abolished signaling from c-Met to Erk (Orian-Rousseau *et al.*, 2002), whereas CD44 tails mutated in the ERM binding site did not. These data suggested that ERM proteins are recruited to the CD44 tail to promote c-Met signaling. To further characterize the role of ERM proteins in signal transduction, we performed two types of experiments: Down-regulation of ezrin expression by siRNA in CD44v6-positive cells (the 293 cells and the HT29 cells used here contain predominantly ezrin), and transfection of a covalent CD44v6–ezrin fusion construct into CD44v6-negative BSp73AS cells.

siRNA directed against ezrin abrogated the expression of endogenous ezrin, whereas an off-target siRNA (control; ctl) did not inhibit ezrin expression (Figure 3A, shown for 293 cells; two different ezrin-specific siRNA sources were used revealing similar results). Consequently, the induction of
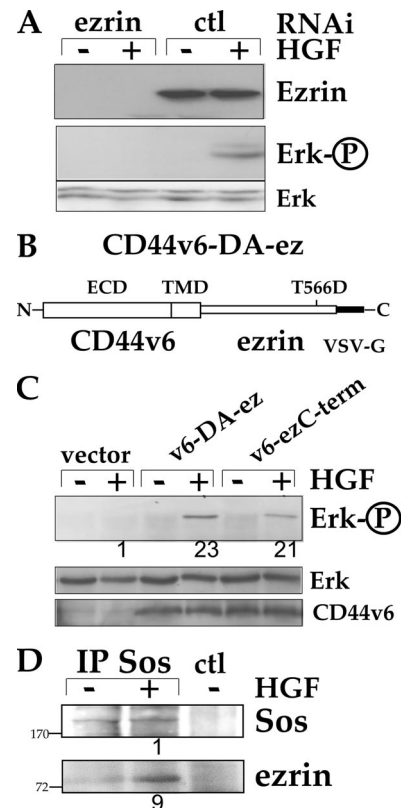
Figure 3. For c-Met signaling, the CD44 cytoplasmic tail recruits ezrin to the membrane. (A) HGF-induced Erk phosphorylation was measured in 293 cells upon down-regulation of ezrin by siRNA. ctl lane reflects treatment with off-target siRNA. (B) Scheme of a fusion construct between the CD44v6 extracellular and transmembrane domain (ECD and TMD) and a pseudophosphorylated constitutive active rat ezrin containing a T-to-D mutation. The VSV-G tag is located at the end of the ezrin part. A similar construct was produced by fusing the C terminus of ezrin (Algrain *et al.*, 1993) to CD44v6. (C) Transfection of the BSp73AS cells with the fusion constructs (scheme in B) or a control vector as indicated and HA-Erk. Detection of HGF-induced HA-Erk phosphorylation has been described previously (Orian-Rousseau *et al.*, 2002). (D) Coimmuno-precipitation of Sos and ezrin performed in 293 cells transfected with GFP-Sos and VSV-G-ezrin constructs. Precipitation was done with GFP antibodies or an isotype ctl, Western blotting was performed with VSV-G antibodies. The numbers below the various lanes indicate -fold HGF induction.
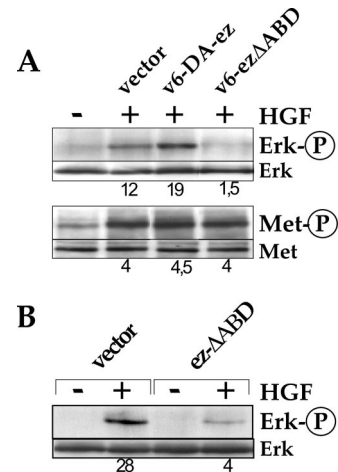
Erk phosphorylation by HGF was reduced to the basal level (Figure 3A) pointing toward a decisive role of ezrin in c-Met signaling. In the second approach, we fused pseudophosphorylated ezrin (in rat T566D, mimicking the activated state of ezrin; Gautreau *et al.*, 2000) to tailless CD44v6 (schematic representation in Figure 3B). In contrast to tailless CD44v6 that does not allow signal transduction (Orian-Rousseau *et al.*, 2002; Figure 1A), the fusion protein established HGF-induced Erk activation in the CD44v6-negative BSp73AS cells (Figure 3C). Phosphorylation of the c-Met receptor in cells transfected with the fusion protein was similar to cells transfected with CD44v6 (Figure 4C). These data prove a causal role of ezrin in HGF-dependent signal transduction and indicate that the CD44 tail serves to tether ezrin to the membrane into the proximity of c-Met. Together with the data presented in Figure 2, they suggest that the binding of ezrin to the cytoplasmic tail of CD44v6 is dispensable for the phosphorylation of the c-Met receptor and
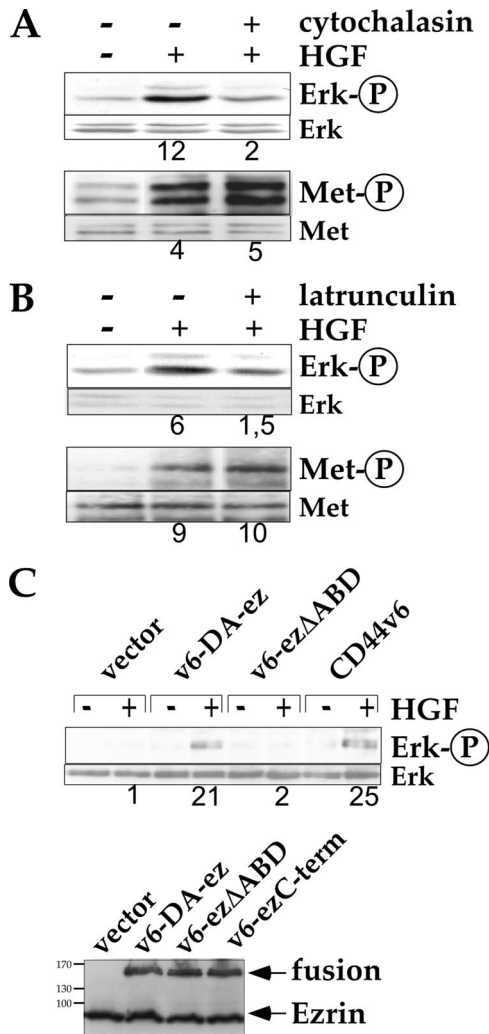
Figure 4. Requirement of the cytoskeleton for c-Met–dependent signaling. (A and B) HGF-induced Erk and c-Met phosphorylation in ASv4-7 (v6) cells pretreated with 5 μM cytochalasin D for 5 min (A) or in HT29 cells pretreated with 0.5 μg/ml latrunculin B for 5 min (B). (C) Transfection of BSp73AS cells with a control vector, the CD44v6–ezrin fusion construct (see Figure 3B), a fusion construct between CD44v6 and ezrin lacking the last 34 C-terminal amino acids (ezΔABD), or a CD44v6 full length construct (pGKs6; Sleeman *et al.*, 1997) together with HA-Erk and detection of HGF induced HA-Erk phosphorylation. The -fold induction by HGF is indicated. Expression levels of the fusion proteins were comparable.


Figure 5. Binding of ezrin to the actin cytoskeleton is a decisive step for c-Met signaling. (A and B) HGF-induced HA-Erk or c-Met, phosphorylation in 293 cells transiently transfected with HA-Erk and either an empty vector or the CD44v6 ezrin fusion constructs described in Figure 4C (A) or an ezrin construct in which the last 29 C-terminal amino acids were deleted (ezΔABD; Algrain *et al.*, 1993) (B).

the activation of receptor binding signaling components in response to HGF but is needed to activate Ras, most likely via the recruitment of Ras into a complex with Sos (or a Shp-2-regulated GEF; Kodama *et al.*, 2000). In agreement with this assumption, ezrin could be coimmunoprecipitated with Sos upon HGF stimulation in 293 cells (Figure 3D).

The highlight of ERM proteins is their ability to recruit the cytoskeleton to the cell membrane via an F-actin binding site in their C terminus (Algrain *et al.*, 1993; Tsukita *et al.*, 1994). To test whether this F-actin binding is necessary for signaling, we performed three independent experiments: 1) We constructed a fusion protein containing CD44v6 and the ezrin C terminus. 2) We measured c-Met signaling in the presence of inhibitors that prevent polymerization of F-actin, thereby abolishing the cortical organization of the actin cytoskeleton. 3) We examined c-Met activation in the presence of a CD44v6-ezrin fusion construct in which the F-actin binding domain was deleted.

Transfection of BSp73AS cells with the fusion between CD44v6 and the C terminus of ezrin allowed signaling from the c-Met receptor (Figure 3C), indicating that the F-actin binding domain is enough for signal transduction. Actin polymerization was inhibited by either cytochalasin D or latrunculin B. Cytochalasin D interferes with actin polymerization by binding to the barbed ends of actin filaments (for review, see Cooper, 1987). Latrunculin B sequesters actin monomers and thereby prevents polymerization (Spector *et al.*, 1989). Both agents severely reduced the HGF-dependent activation of Erk without affecting at all the phosphorylation of c-Met (Figure 4, A and B). The fact that c-Met phosphorylation was unaffected by both latrunculin B and cytochalasin D demonstrates the specificity of the effects of the drugs on signal transduction. The cells were still viable during these short treatments as tested by staining with trypan blue (data not shown). Finally, we made use of a CD44v6–ezrin fusion construct (Figure 3B) in which the F-actin binding domain was deleted. Transfection of this deletion construct into the CD44v6-negative BSp73AS cells did not restore HGF-dependent signaling in contrast to the fusion protein containing the F-actin binding site (Figure 4C). c-Met activation, however, was not impaired (data not shown), indicating that this construct was functional and properly expressed at the membrane. Note that similar levels of fusion proteins were expressed (Figure 4C). Thus, F-actin seems to play a decisive role in c-Met–dependent signaling but not in c-Met phosphorylation.

Based on these findings, we predicted that the fusion protein lacking the actin binding site as well as an ezrin mutant deleted in the actin binding site (Algrain *et al.*, 1993) should exert a dominant-negative effect on c-Met signaling. This was indeed the case. When the highly transfectable 293 cells, in which HGF induced c-Met signaling is dependent on endogenous CD44v6 (Orian-Rousseau *et al.*, 2002) were transiently transfected with the mutant fusion construct, Erk activation was completely abrogated (Figure 5A). The fusion protein containing the F-actin binding domain rather enhanced c-Met signaling. Note, however, that c-Met activa-
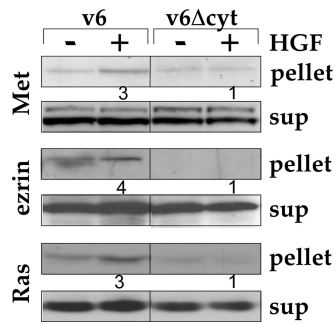
Figure 6. c-Met, ezrin, and Ras are found in complexes with F-actin only in the presence of the CD44v6 tail and HGF. Triton X-100 insolubility assay of ASv4-7 (v6) or ASv4-7Δcyt (v6Δcyt) cells pretreated with HGF where indicated and probed for c-Met, ezrin, and Ras. The numbers indicate -fold induction by HGF.
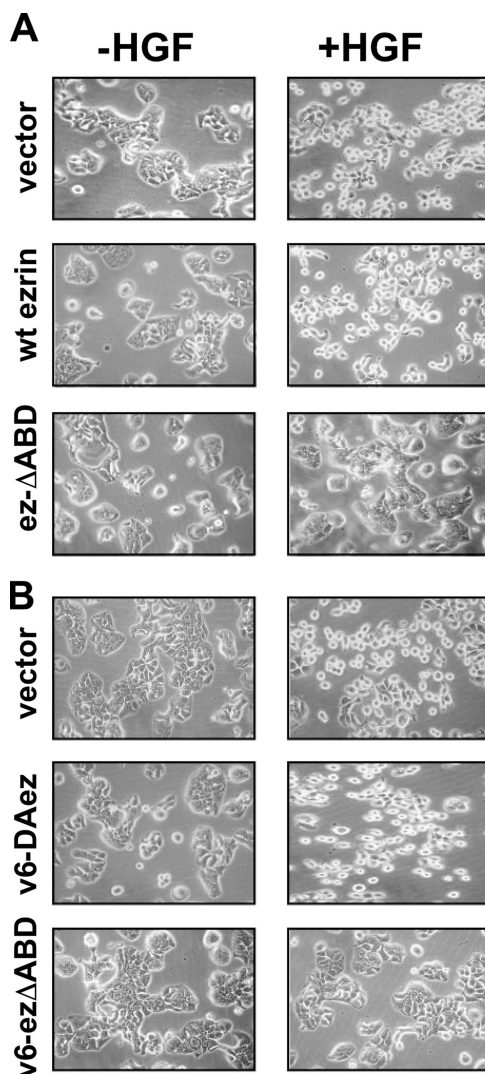
Figure 7. HGF-induced scattering requires the association of ERM proteins to the cytoskeleton. HT29 cells were transfected with a control vector, wild-type ezrin, and ezrin deleted of the 29 C-terminal amino acids, respectively (A) or with a control vector, the fusion between CD44v6 and pseudophosphorylated ezrin and the fusion between CD44v6 and ezrin deleted of the F-actin binding domain, respectively (B). HGF induced scattering was measured 48 h after HGF induction. Magnification is 40×.

tion itself was not influenced by the fusion proteins underlining the specificity of the effect on signal transduction but not on c-Met activation. Transfection of an ezrin mutant deleted in the F-actin binding domain also repressed HGF-induced Erk activation (Figure 5B).

These results demonstrate that the association of F-actin with ezrin (or other ERM proteins) through CD44v6 is required to mediate signal transfer from c-Met to the Ras pathway, shown here for Erk. Thus, the different components of the c-Met signaling cascade, including the c-Met receptor, the adaptor proteins Gab1 and Grb2, the GEF Sos and Ras together with ezrin should be in a complex linked to F-actin. Extraction of cells with Triton X-100 followed by ultracentrifugation allows separation of soluble proteins from those associated with the Triton X-100–insoluble F-actin (Musil and Goodenough, 1991). In BSp73AS cells transfected with CD44v6, the Triton X-100 insoluble fraction (pellet) indeed contained elevated amounts of c-Met, Ras, and ezrin only upon HGF induction, demonstrating the specificity of this complex. In contrast, in cells transfected with tailless CD44v6 none of these components were found in the pellet fraction, although they were detectable in the supernatant (Figure 6).

The ezrin association to the CD44v6 tail and to the F-actin cytoskeleton is required for HGF-induced biological processes (Figure 7). Untransfected human colon carcinoma HT29 cells or cells transfected with a control vector grow in clumps in the absence of HGF (Orian-Rousseau *et al.*, 2002; Figure 7). In the presence of HGF, scattering is induced. Transfection of ezrin into these cells has no effect on their behavior, neither in the presence nor in the absence of HGF. In contrast, transfection of ezrin with a truncation of the F-actin binding domain completely abrogated scattering. The same results were obtained in cells transfected either with the fusion protein between CD44v6 and an activated form of ezrin or the fusion with ezrin truncated from the F-actin binding domain. The truncated version of ezrin in the context of the fusion protein did not allow scattering. Thus, the ezrin proteins lacking the F-actin binding domain act dominant-negative on c-Met mediated signaling (Figure 5) and scattering.

DISCUSSION

Here, we describe for the first time an involvement of ERM proteins in the control of c-Met signaling and a novel function of the membrane-linked F-actin as an inducer of signaling. Whereas the phosphorylation of the c-Met receptor induced by HGF is solely dependent on the ectodomain of its coreceptor CD44v6 the cytoskeleton is required for the activation of the Ras/MAPK pathway by assembling signaling components in the vicinity of the phosphorylated c-Met receptor. The spatial organization of the cytoskeleton is mediated by binding of F-actin to ERM proteins that are anchored to the cytoplasmic tail of CD44v6. This allows the association of Ras with Sos or another GEF and consequently the activation of Ras.

These conclusions are based on several independent experiments. In cells containing CD44v6 with a cytoplasmic tail deletion, Ras was the first component in the signaling cascade that was not activated by HGF. Furthermore, in these cells Ras was not present in complexes obtained by Grb2 pull-down or cytoskeleton precipitations upon HGF stimulation (Figure 2). In contrast, the signaling components above Ras, including the c-Met receptor itself, were activated and found in the respective complexes. Therefore, we conclude that the step between Ras and Sos is the one targeted by the cytoplasmic tail of CD44v6.

The involvement of ezrin in downstream signaling was concluded from 1) experiments using the CD44v6 truncation of the cytoplasmic tail and competitions with these cytoplasmic tails (Orian-Rousseau *et al.*, 2002), 2) experiments using various CD44v6 fusion proteins containing either pseudophosphorylated ezrin or the ezrin C terminus to restore HGF-induced signaling in cells negative for CD44v6 (Figure 3), and 3) experiments using siRNA to inhibit ezrin expression (Figure 3).

The involvement of the F-actin cytoskeleton for downstream signaling was shown by means of constructs deleted in the F-actin binding domain of ezrin (Figure 4), by disturbance of the cytoskeleton integrity by drugs (Figure 4) and by the association of components of the HGF induced signaling pathway with F-actin in Triton X-100 insolubility assays (Figure 6).

In the Grb2 pull-down complexes and in the F-actin complexes in the Triton X-100 precipitations Ras could be linked to ezrin, Sos, or another GEF or a scaffold protein that binds to actin. An interesting aspect is the observation that as well Sos and Shp2 are in the complex with Grb2. It has been proposed that the activation of Ras by the c-Met receptor is predominantly mediated by a Shp2 dependent GEF and not by Sos (Schaeper *et al.*, 2000).

Also, other signaling pathways starting at the c-Met receptor, from phosphatidylinositol 3-kinase (PI3K) to Akt or the activation of JNK, depend on the function of the CD44v6 cytoplasmic tail. In principle, the activation of PI3K and of JNK could be mediated by Ras (Derijard *et al.*, 1994; Cantley, 2002), but in case of the c-Met receptor, the activation of PI3K seems to be a Ras-independent event, occurring directly at the receptor (Bardelli *et al.*, 1999). In agreement with this observation, a dominant-negative version of Ras (N17) did not interfere with HGF-mediated Akt activation (our unpublished data). The activation of JNK, however, seems to depend on Ras as a dominant-negative version of Ras prevented its phosphorylation (our unpublished data).

The physiological relevance of the involvement of the cytoskeletal organization by ERM proteins for c-Met–dependent signaling was demonstrated using dominant-negative ezrin constructs lacking the actin binding domain. These constructs interfered effectively with c-Met–dependent scattering similar to CD44v6-specific antibodies or CD44v6-specific peptides or an inhibitor of the Ras/MAPK pathway (Matzke *et al.*, 2005).

Interestingly, an involvement of ezrin in signal transduction has been demonstrated using siRNA (Khanna *et al.*, 2004). The down-regulation of ezrin led to reduced levels of MAPK and Akt activation in osteosarcoma cells. Furthermore, ezrin is overexpressed in these highly metastatic tumor cells as well as in highly metastatic rhabdomyosarcoma cells (Yu *et al.*, 2004), and ezrin inhibition reduced the metastatic capacity of these cells.

The tumor suppressor merlin, another member of the ERM protein family (Bretscher *et al.*, 2002), is a natural antagonist of the action of ERMs. Similar to ERM proteins, merlin binds to transmembrane proteins such as CD44, but its binding interrupts signaling events (Morrison *et al.*, 2001). This interference seems to occur on the step of Ras or Rac activation and seems to be the consequence of the inability of merlin to bind F-actin, because it lacks a C-terminal F-actin binding site (Morrison *et al.*, 2007).

There is ample evidence for a connection of the cytoskeleton and signaling. The majority of articles, however, deal with changes of the cytoskeleton as a consequence of signaling, e.g., by the induction of migratory processes (for recent review, see Ivetic and Ridley, 2004), the lateral movement of membrane components in the formation of the B-cell cap (de Petris and Raff, 1973), or the formation of the T-cell synapse (Penninger and Crabtree, 1999; Krawczyk and Penninger, 2001). Interestingly, a microfilament-associated large signal transduction complex containing the activated p185(neu) receptor as well as Sos and Ras was isolated from microvilli of an aggressive mammary adenocarcinoma (Li *et al.*, 1999). The connection of the cytoskeleton to this signaling complex, however, was thought to physically link mitogenic signaling to cytoskeletal remodeling (Carraway *et al.*, 1999). Recently, the ERM protein radixin was shown to be essential for the anchoring of the GABA receptor α5 subunit to the actin cytoskeleton. In this case, the ERM protein seems to mediate receptor clustering (Loebrich *et al.*, 2006).

The only direct involvement of the cortical actin cytoskeleton in signaling described so far refers to the activation of the transcription factor SRF. Here, free G-actin inhibits SRF activity by sequestering a coactivator, MAL, in the cytoplasm (Miralles *et al.*, 2003). The SRF/MAL complex addresses a subset of SRF target genes (Treisman *et al.*, 1998). Another subset requiring SRF–TCF interaction is addressed by Erk (Gille *et al.*, 1995) and is likely also dependent on the signal promoting effect of cortical actin described here.

The functional contribution of CD44 isoforms as a coreceptor seems not to be restricted to c-Met and its relative Ron. For several other receptors, e.g., of the epidermal growth factor receptor family, members of the vascular endothelial growth factor-R family, and Trk, CD44v6 isoforms seem to be also required for their activation (Matzke *et al.*, 2005; our unpublished data). Furthermore, signaling from the platelet-derived growth factor receptor, although independent of CD44, is strictly dependent on ezrin and its binding to the cytoskeleton (our unpublished data).

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