The Hyaluronan Receptor RHAMM Regulates Extracellular-regulated Kinase

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We have identified two RHAMM (receptor for hyaluronan-mediated motility) isoforms that encode an alternatively spliced exon 4 (Hall, C. L., Yang, B., Yang, X., Zhang, S., Turley, M., Samuel, S., Lange, L. A., Wang, C., Curpen, G. D., Savani, R. C., Greenberg, A. H., and Turley, E. A. (1995) Cell 82, 19–26 and Wang, C., Entwistle, J., Hou, G., Li, Q., and Turley, E. A. (1996) Gene 174, 299–306). One of these, RHAMM variant 4 (RHAMMv4), is transforming when overexpressed and regulates Ras signaling (Hall et al.). Here we note using flow cytometry and confocal analysis that RHAMM isoforms encoding exon 4 occur both on the cell surface and in the cytoplasm. Epitope-tagging experiments indicate that RHAMMv4 occurs only in the cytoplasm. Several observations suggest that both cell surface RHAMM isoforms and RHAMMv4 are involved in regulating extracellular-regulated kinase (ERK) activity. Affinity-purified anti-RHAMM exon 4 antibodies block the ability of platelet-derived growth factor to activate ERK, and these reagents modify the protein tyrosine phosphorylation profile of proteins resulting from treatment with platelet-derived growth factor. A dominant negative form of RHAMMv4 inhibits mutant active Ras activation of ERK and coimmunoprecipitates with both mitogen-activated protein kinase kinase and ERK, suggesting that the intracellular RHAMMv4 acts downstream of Ras, possibly at the level of mitogen-activated protein kinase kinase-ERK interactions. Consistent with this, overexpression of RHAMMv4 constitutively activates ERK. These results identify a novel mechanism for the regulation of the Ras-ERK signaling pathway and suggest that RHAMM plays multiple roles in this regulation.

Several MAP1 kinases have been identified in mammalian cells including ERK1/2, c-Jun NH2-terminal kinase/stress-activated protein kinase, and p38/hog kinases (3–6). ERK1/2 are activated by the upstream kinases Raf and MEK (4); MEK, mitogen-activated protein kinase kinase; RHAMM, receptor for hyaluronan; R2/1, c-Jun NH2-terminal kinase/stress-activated protein kinase, and p38/hog kinases (3–6). ERK1/2 are activated by the upstream kinases Raf and MEK (4); MEK, mitogen-activated protein kinase kinase; RHAMM, receptor for hyaluronan; R2/1, c-Jun NH2-terminal kinase/stress-activated protein kinase, and p38/hog kinases (3–6). ERK activation is key to regulating proliferation and activating AP-1, that controls expression, for instance, of proteases that are necessary for tissue remodeling and that contribute to cell motility and invasion (3). Indeed, ERKs have recently been shown to be involved in the regulation of motility and chemotaxis of cells (8, 9), particularly in response to growth factors such as PDGF (10). Given these properties, it is not surprising that the Ras-ERK cascade has been linked to tumorigenesis (11–13), embryogenesis (14–16), and tissue repair (17, 18). MAP kinase cascades appear to be differentially activated by extracellular stimuli, such as growth factors, cytokines, stress, and extracellular matrix molecules (3, 7, 19–21). These localize at several subcellular sites and function in modules (4, 22–24), although the molecular basis and the functional consequences of this compartmentalization has not yet been precisely defined (8–10, 25–28).

Recently, a novel extracellular matrix binding protein, RHAMM, originally characterized for its ability to regulate cell motility (29), has been shown to control signaling through Ras. For instance, a dominant negative mutant of RHAMMv4 prevents mutant active Ras from transforming 10T1/2 fibroblasts (1). RHAMM has been reported to occur on the cell surface, intracellularly, and in the extracellular milieu (29–31). This glycoprotein is encoded by a single gene (32) that is expressed as at least five isoforms in murine 10T1/2 fibroblasts (33, 34). We report here that cell surface and intracellular RHAMM isoforms are required for the activation of ERK by PDGF and by mutant active Ras, respectively. Furthermore, overexpression of the intracellular RHAMMv4 constitutively activates ERK. These results identify novel regulatory mechanisms for the control of the ERK cascade.

EXPERIMENTAL PROCEDURES

Cell Culture—The murine fibroblast cell line 10T1/2, HA-Ras-10T1/2 CIRAS-3, RHAMMv4-transfected cells, and 10T1/2 cells expressing RHAMM antisense (U21 cells), were grown to subconfluency as described (1). Construction of Expression Plasmids—RHAMM cDNAs were amplified and assembled into the expression vector pBamHI-neo (35) using the SalI and BamHI restriction sites. All deletion and mutation constructs described in this paper utilized RHAMMv4 cDNA. RHAMM contains two identified hyaluronan binding domains (amino acids 401–411 and 423–432) (29), and these were mutated as described by Hall et al. (1). The mutant glutathione S-transferase RHAMM fusion protein was checked for its inability to bind to biotinylated hyaluronan as described (1). For glutathione S-transferase fusion protein production, all RHAMM isoforms and mutated forms were subcloned into glutathione S-transferase fusion vector pGEX-2T using EcoRI and BamHI sites as described (36). The HA-tagged RHAMM was generated by polymerase chain reaction using the 5′ primer GCTCTGAGATCTTGCATGTCGAGAAAAACATCATGATAGGAGGTATCCGTTGCTGACATTCTCAAGAGG and the 3′ primer CCCTCGAGGCGCCATGATCGGAACATCGTATGGGTAAGCCTTGGAAGGGTCAAAGTG. The HA tag is underlined. The HA tag was placed close to the C-terminus of RHAMM (610 amino acids) (33) with an XhoI site, and the resulting cDNA was subcloned into an expression vector (1). DNA
sequences were confirmed by dideoxynucleotide chain-terminal sequencing.

**RESULTS**

**Isoforms of RHAMM Encoding Exon 4 Are Expressed on the Cell Surface and in the Cytoplasm**—As shown in Fig. 1A, anti-exon 4 antibodies, which specifically recognize RHAMM glutathione S-transferase fusion proteins encoding exon 4 (data not shown), revealed the presence of this epitope on the cell surface. IgG served as a control. Cell surface display of RHAMM was affected by culture confluence so that, at even 70% confluency, cell surface RHAMM expression was lower. These results indicate that RHAMM isoforms encoding exon 4 can be expressed at the cell surface depending upon cell culture conditions and are consistent with multiple reports of a cell surface location for RHAMM in subconfluent cultures (29, 39–41). Confocal analysis using the same antibody also indicated the existence of RHAMM isoforms intracellularly (Fig. 1, B and C). HA or myc epitope-tagged RHAMMv4 was not detected by flow cytometry analysis using anti-epitope tag antibodies (data not shown), but confocal analysis revealed the presence of tagged RHAMM isoform intracellularly, particularly in cell processes (shown in Fig. 1C for HA epitope). These results suggest that RHAMMv4 is not located at the cell surface in detectable levels, but rather is concentrated intracellularly in cell processes, a distribution we previously noted for RHAMM expressed in Ras-transformed cells (29). These results are consistent with other reports showing a cytoplasmic localization of RHAMM in transformed cells (31) and with an absence of a signal peptide in the RHAMMv4 cDNA (33).

**Cell Surface RHAMM Is Required for Activation of ERK by PDGF**—10T1/2 cell lines that express varying levels of RHAMM isoforms encoding exon 4 (Fig. 2A) exhibited variations in the activation state of ERK, a kinase that acts downstream of Ras. Thus, cell lines expressing the highest levels of RHAMM isoforms encoding exon 4 exhibited the highest levels of ERK activity (i.e. Ras-transformed and RHAMMv4-transfected cells) (Fig. 2B). These results were consistent with our previous report suggesting that RHAMM regulates Ras signaling (1). Interestingly, cell lines shown in Fig. 2 also differed in their ability to further activate ERK in response to PDGF (Fig. 2C). Thus, cells that make little cell surface RHAMM (U21 cells) due to expression of RHAMM antisense, showed a poor ability to respond to PDGF (Fig. 2C), even though the PDGF receptor was expressed in these cells (data not shown). In contrast, RHAMMv4- or Ras-transformed cells that expressed high levels of RHAMM isoforms encoding exon 4 (Fig. 2A) responded to PDGF with a 18–20-fold increase in ERK activity (Fig. 2C). The 10T1/2 parent cell line, which expressed less cell surface RHAMM encoding exon 4 than either Ras- or RHAMMv4-transfected cells but more than U21 cells (Fig. 2A), exhibited an intermediate response (8-fold stimulation) to PDGF (Fig. 2C). To more directly assess the role of cell surface RHAMM in PDGF-mediated ERK activation, we added anti-RHAMM antibodies to the culture media before the addition of PDGF. Anti-RHAMM antibodies inhibited ERK stimulation by 50% (Fig. 2C), except in U21 cells, which expressed little RHAMM (1) and where the level of ERK activity was not blocked by anti-RHAMM antibodies. Stimulation of ERK activity by PDGF was also blocked by the addition of anti-RHAMM antibodies in NIH3T3 fibroblasts, indicating that this effect of RHAMM was not specific to one cell background (data not shown).

To begin to address the mechanisms by which cell surface
RHAMM may regulate PDGF-mediated activation of ERK, we assessed whether or not RHAMM was required for protein tyrosine phosphorylation by the PDGF receptor, which is an immediate response resulting from PDGF-PDGF receptor interactions and which is required for ERK activation. As shown in Fig. 2D, cells that made little RHAMM due to expression of RHAMM antisense (U21 cells) showed a strongly reduced ability to phosphorylate p200, p54, p42/44 (ERKs, data not shown), and p36 proteins, which were strongly phosphorylated in cells that overexpress RHAMM (compare U21 cells to RHAMMv4-transfected cells, Fig. 2D). Anti-RHAMM antibodies had no effect on the profile of proteins phosphorylated in U21 cells in response to PDGF, consistent with the low levels of RHAMM expressed by these cells. In contrast, RHAMM antibodies strongly inhibited the protein tyrosine phosphorylation of p200, p54, p44/42, and p36 in RHAMMv4-transfected cells. These RHAMM antibody-treated cells therefore resembled U21 cells in their ability to respond to PDGF by phosphorylating proteins on tyrosine. The identity of the proteins has not yet been established, but the p42/44 proteins were ERK1/2 by immunological criteria (data not shown). These results suggest that cell surface RHAMM plays an important role in regulating ERK activity by regulating the ability of the PDGF receptor to acutely effect phosphorylation of proteins.

**RHAMMv4 Is Required for Activation of ERK by Mutant Active Ras—** We next assessed whether RHAMMv4, which is cytoplasmic, was required for activation of ERK by a cytoplasmic activator of this kinase cascade, mutant active Ras. U21 (RHAMM antisense-transfected) cells that expressed little RHAMMv4 (Fig. 2A) were transiently transfected with mutant active Ras, and the effect on ERK activation was assayed (Fig. 3A). Although both the parental cells and U21 cells were equally efficiently transfected with Ras (see Hall et al. (1) and data not shown), Ras activated ERK to a lesser extent in U21 than in parental 10T1/2 cells. Ras-transformed cells were next stably transfected with a dominant negative mutation of...
RHAMMv4, and the effect on ERK activity toward myelin basic protein of two independently selected clones was assessed (Fig. 3B). The dominant negative mutation of RHAMM strongly suppressed ERK activity (Fig. 3B), indicating that intracellular RHAMMv4 and Ras cooperated to activate ERK. To begin to assess the mechanism(s) by which RHAMMv4 regulates activation of ERK through Ras, we conducted transient transfections of 10T1/2 cells with mutant active Raf, mutant active MEK, and a dominant negative mutant of RHAMMv4 (1) and assessed focus formation, which requires ERK activation by these regulators (3) to rapidly screen for the step on the Ras ERK pathway that RHAMMv4 might be regulating. RHAMMv4 was required for both Raf-CAAX and mutant active MEK-mediated transformation of 10T1/2 cells (control foci number/dish, 21: Raf-CAAX and mutant RHAMMv4, 3.2, active MEK and mutant RHAMMv4, 4.1). Next, the ability of RHAMM antibodies to coimmunoprecipitate components of MAP kinase cascades was assessed (Fig. 4). As noted in Fig. 4, A and B, anti-RHAMM antibodies coimmunoprecipitated both ERK1 and MEK in RHAMMv4-transfected cells. Further, ERK antibodies coimmunoprecipitated an HA epitope-tagged RHAMMv4 transfected into parental 10T1/2 cells, which normally express little of this isoform (Fig. 2A), confirming that ERK interacts with the cytoplasmic RHAMMv4. Dominant mutant HA-tagged RHAMMv4 did not coimmunoprecipitate with ERK or MEK (data not shown). These data relate the ability of RHAMMv4 to regulate ERK activity to its ability to coassociate with this kinase. We were unable to observe coimmunoprecipitation of RHAMM with either ERK2, Ras, or Raf (data not shown). Furthermore, an association of RHAMMv4 with other MAP kinases including c-Jun NH2-terminal kinase and p38 kinases was not detected (Fig. 4D). Overall, these results are consistent with an effect of RHAMMv4 at the level of MEK-ERK interactions.

RHAMMv4 Overexpression Activates ERK—Consistent with its increased activity toward myelin basic protein (Fig. 2B),
phosphorylated forms of ERK were also uniquely observed in Ras- or RHAMMv4-transfected cells (Fig. 5A). Furthermore, activated ERK was uniquely observed in the nucleus of mutant active Ras- and RHAMMv4-transfected cells (Fig. 5B).

**DISCUSSION**

Our results indicate that RHAMM isoforms encoding exon 4, which occur at the cell surface and in the cytoplasm, are both required for activation of the ERK by growth factors such as the PDGF as well as by cytoplasmic regulators such as mutant active Ras. A role for cell surface RHAMM in controlling signaling is consistent with a number of previous studies that have shown a variety of RHAMM antibodies (39–41, 43) and peptides mimicking RHAMM sequence (42) to block cell motility. The ability of cell surface RHAMM to regulate protein tyrosine phosphorylation effected by PDGF is consistent with our previous demonstration that RHAMM controls protein tyrosine phosphorylation cascades during cell motility (44). The molecular mechanisms by which RHAMM modifies protein tyrosine phosphorylation effected by the PDGF receptor are not yet clear but an effect on Src, which is recruited to the PDGF receptor after autophosphorylation (45) and which is regulated by RHAMM (34) is possible. Known RHAMM isoforms do not encode a transmembrane domain (33, 48), and this protein may therefore associate with the cell surface by linkage to a glycosylphosphatidylinositol tail (49) or as a peripheral protein (50). Both of such classes of cell surface molecules appear to act as co-receptors, modifying the signaling ability of a transmembrane protein. For instance, glycosylphosphatidylinositol-linked proteins contribute to the signaling capability of the T cell receptor complex (51), transforming growth factor β receptor complexes (52), and integrins (53).
many of which regulate MAP kinase activity. The ability of RHAMM to modify the signaling of a receptor tyrosine kinase is reminiscent of the recent demonstration that another cell surface hyaluronan binding protein, CD44, regulates protein tyrosine phosphorylation by the Her2/Neu receptor (46). Indeed the effects of CD44 (46) and of RHAMM noted here are consistent with increasing evidence pointing to the presence of accessory cell surface proteins that modify protein tyrosine phosphorylation cascades initiated by receptor and non-receptor protein-tyrosine kinases and that are required for cell functions such as motility (i.e. Ref. 47).

The cytoplasmic location of RHAMMv4 and the ability of a RHAMMv4 dominant mutant to block mutant active Ras activation of ERK also predicts a role for this isoform in regulating ERK. The data presented here are consistent with an action of RHAMMv4 dominant mutant to block mutant active Ras activation of ERK, but whether these interactions occur directly or via other proteins remains to be demonstrated. It is interesting that even though Ras has been shown to co-distribute with RHAMM in the cell surface and in the cytoplasm and to perform a function at both of these locations is similar to the family of ectoenzymes (56), which include glycosyltransferases (57, 58), and TM4 proteins, which regulate cell motility (47). Cell surface glycosyltransferases have been shown to be involved in cell adhesion and in transmitting signals while they perform synthetic functions intracellularly (59).

In summary, we show that the hyaluronan binding protein RHAMM may function at the cell surface level as a co-stimulatory requirement for PDGF activation of ERK. Our studies also suggest a role for intracellular RHAMM in mutant Ras regulation of ERK, possibly as a result of an interaction of RHAMMv4 with MEK/ERK complexes. These results identify a novel regulation of a MAP kinase cascade that is key to controlling cell proliferation and motility.

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