Soluble Hyaluronan Receptor RHAMM Induces Mitotic Arrest by Suppressing Cdc2 and Cyclin B1 Expression

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Summary

The hyaluronan (HA) receptor RHAMM is an important regulator of cell growth. Overexpression of RHAMM is transforming and is required for H-*ras* transformation. The molecular mechanism underlying growth control by RHAMM and other extracellular matrix receptors remains largely unknown. We report that soluble RHAMM induces G_2/M arrest by suppressing the expression of Cdc2/Cyclin B1, a protein kinase complex essential for mitosis. Downregulation of RHAMM by use of dominant negative mutants or antisense mRNA also decreases Cdc2 protein levels. Suppression of Cdc2 occurs as a result of an increased rate of cdc2 mRNA degradation. Moreover, tumor cells treated with soluble RHAMM are unable to form lung metastases. Thus, we show that mitosis is directly linked to RHAMM through control of Cdc2 and Cyclin B1 expression. Failure to sustain levels of Cdc2 and Cyclin B1 proteins leads to cell cycle arrest.

Hyaluronan (HA)[1], a glycosaminoglycan (1), is one of the matrix molecules that regulates cell locomotion via its interaction with a unique 58–70-kD cell surface receptor for HA-mediated motility, RHAMM (2). RHAMM is constitutively overexpressed in H-*ras*–transformed fibroblasts (2), terminally differentiated multiple myelomas (3), and breast carcinoma cells. It is transiently expressed in activated T cells (4) and in bovine aortic smooth muscle cells after injury (5), coincident with cell locomotion. Polyclonal and monoclonal antibodies to RHAMM that block HA binding to RHAMM, or peptides corresponding to its HA-binding domains, completely inhibit HA-induced cell locomotion (2–6). Moreover, interaction of HA with RHAMM promotes focal adhesion turnover and transient phosphorylation of focal adhesion kinase that leads to cell locomotion (7).

In contrast to other extracellular matrix receptors, overexpression of RHAMM by transfection into nonsenescing fibroblasts results in elevated motility, anchorage-independent growth, and transformation to a fully metastatic fibrosarcoma (8). Furthermore, mutation of the HA-binding domains in RHAMM not only destroys its ability to transform nonsenescing fibroblast, but the overexpression of this dominant negative mutant also prevents mutant H-*ras* transformation, indicating that HA:RHAMM signaling is on an essential growth regulatory pathway and downstream of *ras* (8). In this report, we examined the role of RHAMM in controlling cell growth by interfering with its function either using soluble recombinant glutathione-S-transferase (GST)–RHAMM fusion protein (9), or by suppressing its function with either a dominant negative mutant (8) or antisense RHAMM (8). Our results suggest that RHAMM signaling controls the synthesis of Cdc2 and Cyclin B1 proteins and thus entry into mitosis.

[1]*Abbreviations used in this paper:* GST, glutathione-S-transferase; HA, hyaluronan; KBG, Kentucky Bluegrass pollen; MEF, mouse embryonic fibroblast; RHAMM, receptor for hyaluronan-mediated motility.

Materials and Methods

Cell Lines. The CIRAS-3 (C3) cell line is derived from a 10T1/2 fibroblast cell line transfected with the H-*ras* and *neo*^{R} genes as described previously (10). Mouse embryonic fibroblasts (MEFs) from 15-d-old embryos were kindly provided by Dr. Junying Yuan (Harvard Medical School, Boston, MA). Three dominant negative C3 clones (MR-C3-4D, -5B, and -5C) were derived from C3 cells stably transfected with the RHAMM cDNAs mutated in their HA-binding domains as described elsewhere (8). Cell lines OR1 and OR2 were derived from 10T1/2 after stable transfection of antisense RHAMM cDNA as described previously (8). The cells were maintained in DMEM supplemented with 10% FCS. Murine mammary carcinoma cell line FT210, which contains a temperature-sensitive mutation in Cdc2 (11), was cultured as described previously (12).

Preparation of GST Fusion Proteins. RHAMM cDNA (2) was PCR-cloned into pGEX-2T plasmid as per the method described

previously (9). Plasmids containing GST–Kentucky Bluegrass pollen (KBG) and GST were obtained from Dr. Shyam Mohapatra (University of Manitoba). GST fusion proteins were purified from the cell lysates by affinity chromatography using glutathione agarose and eluted in 50 mM Trsi-Cl, pH 8.0, containing 5 mM glutathione as described elsewhere (9).

Cell Cycle Analysis. Cells were harvested, fixed with 70% ethanol, treated with 2N HCl containing 0.5% Triton X–100 at room temperature for 30 min, and then neutralized by treatment with 0.1 M $Na_2B_4O_7$, pH 8.5. Total DNA content was detected by propidium iodide (PI) staining. The stained cells were analyzed by flow cytometry using a cell sorter (Epics model 753) and PARA1 cell cycle analysis software (both from Coulter Electronics, Inc., Hialeah, FL).

In Vitro Growth Assay. Cell density assay was carried out by seeding 5×10^4 cells in 60-mm dishes in DMEM supplemented with 10% FCS. At each point, cells were harvested by trypsinization and viable cell concentrations were determined using trypan blue exclusion.

Western Analysis. Cells were lysed in buffer containing 50 mM β-glycerophosphate, pH 7.3, 1% NP-40, 10 mM NaF, 1% aprotinin, and 1 mM sodium vanadate (12). The lysates were centrifuged at 30,000 *g* for 20 min at 4°C. Protein concentration was determined by protein assay kit (Bio-Rad Laboratories, Richmond, CA). Lysates were loaded at equal concentrations and resolved on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes in a Tris-Cl buffer containing 25 mM Tris, 192 mM glycine, and 20% (vol/vol) methanol, pH 8.3, at 100 V for 1 h at 4°C. Filters were incubated with either a 1:5,000 dilution of polyclonal rabbit antiserum raised against the COOH-terminal peptide (LDNQIKKM) of human Cdc2 protein (12), or 1 μg/ml of polyclonal rabbit antiserum raised against the COOH-terminal domain (287-298) of human Cdk2 (Upstate Biotechnology, Inc., Lake Placid, NY), or 1 μg/ml of monoclonal anti–human Cyclin B1 (Upstate Biotechnology, Inc.) and developed with horseradish peroxidase–conjugated goat antibody to rabbit or mouse IgG (Sigma Chemical Co., St. Louis, MO). Blotting was visualized by the enhanced chemiluminescence Western blotting system (Amersham Corp., Arlington Heights, IL) following the manufacturer's instructions.

Northern Analysis. Total cellular RNA was extracted by a rapid RNA isolation method using Trizol reagent (GIBCO BRL, Gaithersburg, MD) as per the manufacturer's instructions (13). For half-life measurements, transcription was inhibited after addition of 20 μg/ml actinomycin D for 2 h. RNA was isolated at different times and 20 μg of total cellular RNA was electrophoresed through 1% formamide-agarose gels and blotted onto Nytran membranes. The blots were prehybridized and probed with random primer ^{32}P-labeled murine cdc2 cDNA (provided by Paul Nurse, ICRF Cell Cycle Control Laboratory, London, UK) as described previously (6). RNA loading was determined with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. Autoradiography was analyzed by Phosphorimager SF (Molecular Dynamics, Sunnyvale, CA) using storage phosphorimaging. Nuclear run-on assay was performed as described (14) using ^{32}P-labeled RNA to probe filter-bound cdc2 and GAPDH single strand cDNA inserts. Quantification of transcription was determined by scanning phosphorimages using Multiquant software program (Molecular Dynamics).

Tumorigenicity and Metastasis Assay. Cells were cultured in presence of serum, GST protein or GST-RHAMM for 48 h. For assessing tumorigenicity, 3×10^5 viable cells were injected subcutaneously into the right hind leg and maintained for 6–8 wk measuring tumor size with calipers (10). Lungs and other organs

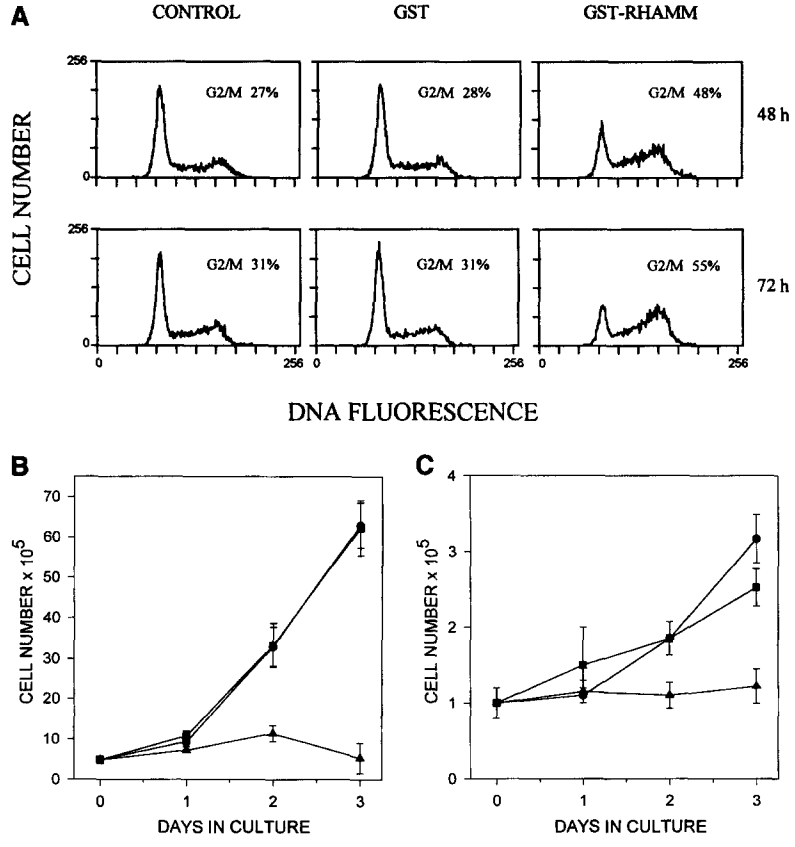

Figure 1. Soluble GST-RHAMM fusion protein inhibits cell growth by arresting cells at G_2/M. (*A*) Flow cytometric analysis of C3 fibrosarcoma cells in which relative DNA content of PI-treated cells is plotted against cell number. C3 cells were cultured in the absence of serum for 40 h and then incubated in 5 μg/ml of GST-RHAMM or GST in 10% serum. After 48 and 72 h, cells were harvested, fixed, and stained with PI. Growth curves of triplicate cultures of untreated (●), 5 μg/ml GST (■) and 5 μg/ml GST-RHAMM (▲) treated C3 cells (*B*) and primary MEFs (*C*). C3 cells were cultured as in (*A*). MEFs were cultured in 3% serum for 24 h before RHAMM treatment. Live cells were counted at daily intervals and the mean values of the triplicates with standard deviation are plotted versus time.

were visually surveyed for spontaneous metastasises at the termination of the assay. For experimental metastasis assays, 4×10^5 cells were injected into the tail vein. The mice were maintained for 16–18 d, euthanized, and the lungs stained by Bouins' solution (picric acid, formaldehyde, and acetic acid [15:5:1]), and metastatic foci were counted under a dissecting microscope (10).

Results and Discussion

Soluble RHAMM Inhibits Cell Cycle Progression. H-*ras*–transformed C3 fibrosarcoma cells (10) were treated with GST-RHAMM and cell cycle progression was monitored by flow cytometry. As shown in Fig. 1 *A*, in the presence of GST-RHAMM for 48–72 h, 48–55% of the cells accumulated in G_2/M, whereas in the presence or absence of the control GST protein, C3 cells cycled normally. No change in the cell cycle profile or in cell growth (Fig. 1 *B*) was seen during 24 h of RHAMM treatment. However, RHAMM treatment completely ablated the growth of C3 cells for 48–72 h (Fig. 1 *B*). A similar pattern of growth inhibition was obtained with primary MEFs (Fig. 1 *C*). Thus, soluble RHAMM arrests cells at G_2/M without affecting their progression through S-phase. Although the mechanism by which soluble RHAMM suppresses cell cycle progression is not known, it likely interferes with receptor function because anti-RHAMM antibody treatment of cells also results in growth arrest (data not shown). Similarly, HA-induced cell motility is blocked by GST-RHAMM fusion protein and antibody (Entwhistle, J., B. Yang, C. Hall, S. Mohapatra, A.H. Greenberg, and E.A. Turley, manuscript in preparation). These results suggest that the RHAMM fusion protein blocks receptor function, possibly by binding to its ligand HA.

Soluble RHAMM Suppresses Expression of Cdc2 and Cyclin B1 Proteins. Since progression through G_2 and initiation of mitosis requires Cdc2 kinase (15–17) and RHAMM-treated cells arrest at G_2/M, we examined the expression of Cdc2 protein by Western blotting. As shown in Fig. 2 *A*

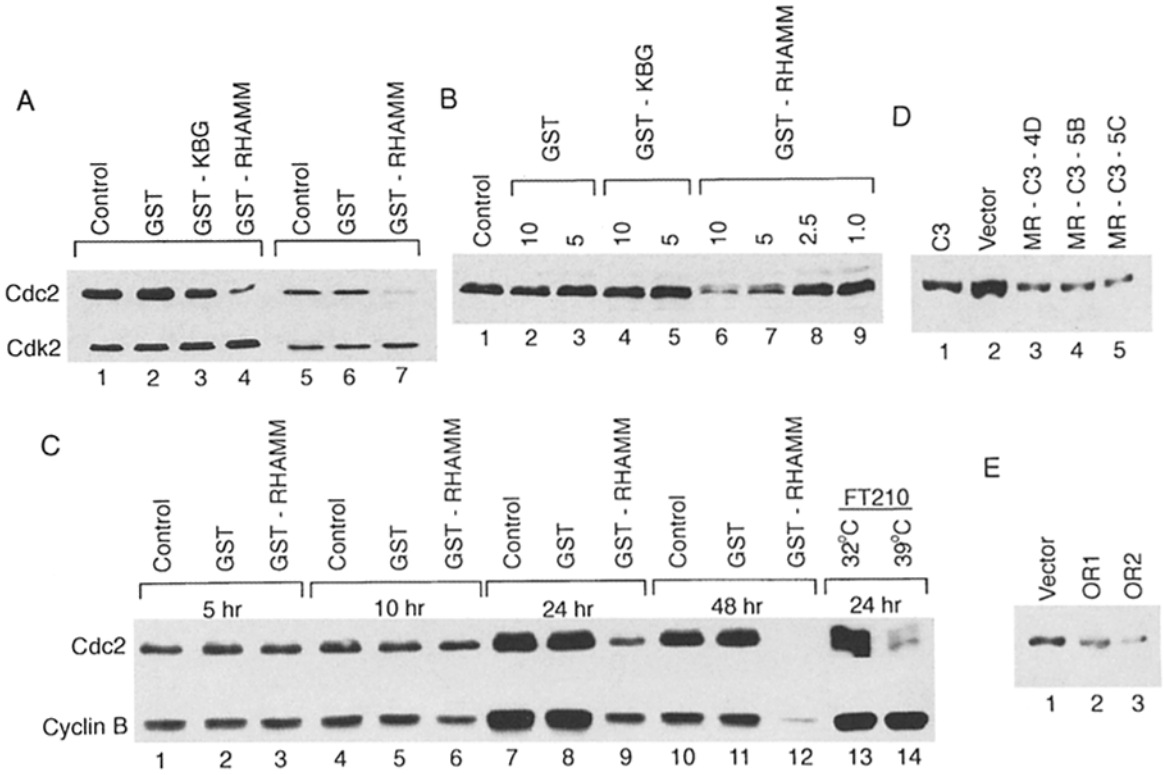

Figure 2. RHAMM treatment suppresses Cdc2 and Cyclin B1 proteins. (*A*) RHAMM suppression of Cdc2 protein levels. C3 fibrosarcoma cells (lanes *1–4*) and primary MEFs (lanes *5–7*) were cultured as in Fig. 1 and then incubated with 10 μg/ml of either GST (lanes *2* and *6*), GST linked to an unrelated protein (GST-KBG) (lane *3*), or GST-RHAMM (lanes *4* and *7*). After 30 h, cells were lysed and Cdc2 and Cdk2 proteins were determined by Western blotting (12). (*B*) Dose-dependent inhibition of Cdc2 protein expression in C3 cells by GST-RHAMM. Cells were cultured as in *A* with the indicated dose of proteins (μg/ml). Lane *1*, untreated; lanes *2* and *3*, GST; lanes *4* and *5*, GST-KBG; lanes *6–9*, GST-RHAMM treated. (*C*) Expression of Cdc2 and Cyclin B1 proteins in GST-RHAMM–treated C3 cells and temperature-sensitive FT210 cells. C3 cells were cultured as in *A* for 5, 10, 24, and 48 h in 10 μg/ml GST-RHAMM before lysis. Lanes *1*, *4*, *7*, and *10*, untreated; lanes *2*, *5*, *8*, and *11*, GST treated, and lanes *3*, *6*, *9*, and *12*, GST-RHAMM treated. FT210 cells were cultured for 24 h at the permissive temperature of 32°C (lane *13*) or the restrictive temperature of 39°C (lane *14*). (*D*) Cdc2 protein expression in cells overexpressing a dominant negative RHAMM mutated in its HA-binding domains. Cells were cultured overnight in the presence of 0.2 μg nocodazole which arrested >85% cells at G_2/M; they were then harvested, washed three times in PBS, and lysed. Cdc2 protein was determined by Western blotting in parental C3 cells (lane 1), empty vector–transfected C3 cells (lane *2*), and three C3 clones expressing mutant RHAMM (*MR-C3-4D*, *-5B*, and *-5C*; lanes *3–5*). (*E*) Cdc2 expression in 10T1/2 clones OR1 and OR2 which over express antisense RHAMM and have 50 and 10% of control RHAMM protein levels, respectively. Cells were cultured in nocodazole and analyzed as in *D*. Lane *1* is a vector-transfected 10T1/2; lane *2*, clone OR1; and lane *3*, clone OR2.

untreated cells or cells treated with GST, or GST linked to the unrelated KBG protein, expressed high levels of Cdc2. However, after RHAMM treatment, Cdc2 protein levels drastically decreased in both in C3 cells and primary MEFs, whereas Cdk2 protein levels remained unaffected (Fig. 2 *A*). The suppression of Cdc2 protein was dependent on the dose (Fig. 2 *B*) and the length of RHAMM treatment (Fig. 2 *C*). No significant reduction in Cdc2 protein was seen before 24 h of RHAMM treatment. Similar results were obtained when 10T1/2, Rat-1, and HeLa cells were treated with RHAMM (data not shown). Taken together, these data strongly suggest that soluble RHAMM arrests cells at G_2/M by suppressing Cdc2 expression.

We next analyzed levels of Cyclin A and B proteins that associate with Cdc2 during entry into G_2 and mitosis, respectively. In RHAMM-treated cells, the level of Cyclin B1 dropped concomitantly with the Cdc2 protein 24 h after GST-RHAMM treatment (Fig. 2 *C*) whereas the level of Cyclin A remained unchanged (data not shown). In cycling cells, the level of Cyclin B rises in S phase, peaks at G_2/M, and drops drastically at the metaphse/anaphase transition (18), but the level of Cdc2 normally remains constant throughout the cell cycle because of its relatively long half-life (15). The possibility that decreased levels of Cdc2 protein may lead to premature degradation of Cyclin B1 before anaphase was ruled out by the observation that suppression of the temperature-sensitive Cdc2 in FT210 cells (11) at restrictive temperature did not alter Cyclin B1 expression (Fig. 2 *C*). These results suggest that treatment with soluble RHAMM leads to suppression of Cdc2 as well as its mitotic partner Cyclin B1.

To establish whether RHAMM signaling is essential for progression through G_2/M, we examined Cdc2 protein levels in several C3 fibrosarcoma cell clones that express RHAMM mutated in the HA-binding domains that acts as a dominant suppressor (8). These C3 clones, MR-C3-4D, -5B, and -5C, resemble nontransformed fibroblasts in their reduced growth rate and are nontumorigenic despite expressing high levels of H-*ras* (8). Cdc2 protein levels were examined in cells arrested at G_2/M by nocodazole and, as shown in Fig. 2 *D*, the three clones bearing the dominant suppressor mutant of RHAMM expressed four- to sixfold less Cdc2 compared with C3 parental or vector-transfected control cells. Similarly, 10T1/2 fibroblasts that expressed low levels of RHAMM as a result of transfection and constitutive expression of antisense RHAMM cDNA and are

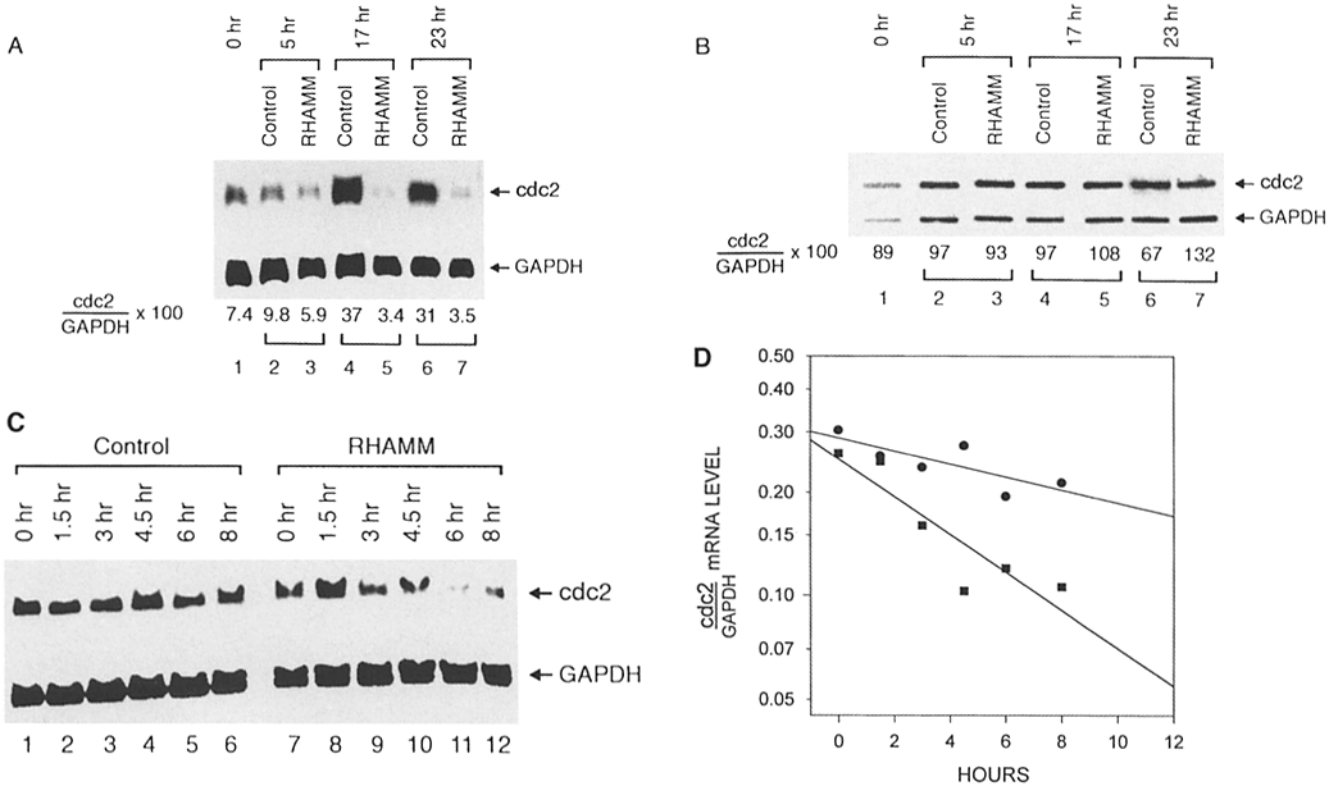

Figure 3. RHAMM treatment increases cdc2 mRNA degradation without altering transcription. (*A*) Expression of cdc2 mRNA of GST-RHAMM–treated C3 cells as determined by Northern blotting. Lanes *1*, *2*, *4*, and *6* are untreated; lanes *3*, *5*, and 7 are RHAMM treated (10 μg/ml) for the indicated times. Cdc2/GAPDH ratios × 100 are indicated below. (*B*) Nuclear run-on assays. Nuclei from C3 cells were isolated after 5, 17, and 23 h of treatment with 10 μg/ml GST-RHAMM. ^{32}P-labeled RNA isolated from control (lanes *1*, *2*, *4*, and *6*) and GST-RHAMM–treated cells (lanes *3*, *5*, and 7) were used to probe nitrocellulose containing linearized cdc2 and GAPDH DNA. Cdc2/GAPDH ratios × 100 are indicated below. (*C*) Cdc2 message stability in the presence of GST-RHAMM after actinomycin D (20 μg/ml) treatment. RNA was isolated from different samples at the indicated time after 2 h of actinomycin D treatment and analyzed by Northern blotting (12). Lanes *1–6*, untreated; lanes *7–12*, GST-RHAMM treated. (*D*) Determination of cdc2 half-life of C3 cells in presence of GST-RHAMM, 2 h after addition of 20 μg/ml actinomycin D. Cdc2 half-life in untreated cells (●) is 15 h and after RHAMM treatment (■) is 4.5–5 h. One of three experiments with similar results is shown.

Table 1. *Suppression of C3 Fibrosarcoma Growth and Metastasis by Soluble RHAMM Receptor*

Treatment	Tumorigenicity*		Metastasis‡	
	Tumor latency (days ± SE)	Tumor size (day 20) (mm^2 ± SE)	Spontaneous (frequency)	Experimental (lung tumors ± SE)
Control	7 ± 1	112 ± 5	8/8	>300
GST	5 ± 0.5	136 ± 17	8/8	>300
GST-RHAMM	22 ± 1	6 ± 6	0/8	10 ± 6

*After 48-h incubation in GST-RHAMM (10 μg/ml), 3×10^5 viable tumor cells were injected subcutaneously into C3H/HeN mice. After day 20, the tumor growth rate was equivalent to control cells.
‡Spontaneous metastases were assessed in mice 8 wk after receiving subcutaneous tumor. Experimental lung metastases were examined 21 d after intravenous injection of 4×10^5 viable cells.

resistant to H-*ras* transformation (8), also have reduced Cdc2 protein levels (Fig. 2 *E*). Taken together, these results indicate that HA:RHAMM–mediated signaling is necessary for maintenance of Cdc2 protein levels.

Soluble RHAMM Increases cdc2 mRNA Degradation Rate. In cycling cells, Cdc2 protein levels are maintained at a nearly constant steady-state level, however cdc2 mRNA synthesis is initiated during each cell cycle beginning at the G_1/S transition reaching a maximum at G_2 (19, 20). As shown in Fig. 3 *A*, an increase in cdc2 mRNA levels was seen in control C3 cells after serum stimulation. However, 5 h after treatment with RHAMM in the presence of serum, cdc2 mRNA levels of C3 cells decreased, and by 17–24 h of treatment, a greater than 10-fold reduction was seen. Nuclear run-on assays performed with C3 nuclei prepared from cells cultured in the presence or absence of RHAMM revealed no change in transcription of cdc2 mRNA that could account for the reduced levels (Fig. 3 *B*). To explore the alternative possibility that RHAMM alters cdc2 mRNA degradation rates, we next determined the mRNA half-life by examining RNA levels over time after treatment of C3 cells with actinomycin D. In the presence of RHAMM, the cdc2 mRNA half-life was reduced to 4.5–5 h from the normal 15 h (Fig. 3, *C* and *D*). These results indicate that soluble RHAMM suppressed Cdc2 protein and RNA expression by increasing the degradation rate of cdc2 mRNA.

Soluble RHAMM Inhibits Tumorigenicity and Metastasis. We have previously shown that HA:RHAMM signaling is essential for H-*ras* transformation (8). In the present study we wanted to determine if suppression of signaling induced by soluble RHAMM reduced the tumorigenicity and metastatic ability of C3 fibrosarcomas (10). Mice receiving 3×10^5 viable cells subcutaneously after treatment with RHAMM for 48 h in vitro developed subcutaneous tumors that were smaller and appeared later than controls, and no spontaneous metastases were observed in these mice (Table 1). Moreover, after intravenous injection, RHAMM-treated cells formed very few lung nodules (10/lung) compared with both control groups (>300/lung). These results suggest that soluble RHAMM can suppress receptor signaling and tumorigenicity similar to altering receptor function (8). It is also possible that tumor cells that were treated with soluble RHAMM failed either to migrate through extracellular matrix or to interact with HA at a distant site, and thus inhibited metastasis. A similar approach has been used for CD44, another HA receptor, in which infusion of soluble CD44H-Ig fusion protein inhibited metastasis (21, 22).

Our study indicates that RHAMM signaling may coordinately regulate the synthesis of Cdc2 and the detachment required for cells to enter mitosis. The synthesis of HA, which is the RHAMM ligand, has been correlated to many cellular functions including cell proliferation and cell division (1). In particular, HA synthesis and synthase activity increase during mitosis when cells round and are loosely adherent, possibly because HA is required for detachment of cells from the supporting matrix (23). Synergistic interactions between growth factors and integrin-mediated signal transduction involving sustained tyrosine phosphorylation regulate cell proliferation and cell adhesion during G_1 to S transition (24–27) and Cyclin A expression has been shown to be a target for adhesion-dependent signals (28). In contrast, HA:RHAMM interactions that involve rapid but transient tyrosine phosphorylation and promote deadhesion (7) may be required specifically during G_2/M transition where increased HA synthesis occurs (23). Thus, Cdc2 seems to be a target of RHAMM-mediated signaling events at mitosis. Whether Cyclin B1 is directly suppressed by RHAMM or occurs secondary to suppression of Cdc2 remains to be elucidated.

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