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Integrin-mediated RON Growth Factor Receptor Phosphorylation Requires Tyrosine Kinase Activity of Both the Receptor and c-Src*

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Cooperation between integrins and growth factor receptors plays an important role in the regulation of cell growth, differentiation, and survival. The function of growth factor receptor tyrosine kinases (RTKs) can be regulated by cell adhesion to extracellular matrix (ECM) even in the absence of ligand. We investigated the pathway involved in integrin-mediated RTK activation, using RON, the receptor for macrophage-stimulating protein. Adhesion of RON-expressing epithelial cells to ECM caused phosphorylation of RON, which depended on the kinase activity of both RON itself and c-Src. This conclusion is based on these observations: 1) ECM-induced RON phosphorylation was inhibited in cells expressing kinase-inactive c-Src; 2) active c-Src could phosphorylate immunoprecipitated RON from ECM-stimulated cells but not from unstimulated cells; and 3) ECM did not cause RON phosphorylation in cells expressing kinase-dead RON, nor could active c-Src phosphorylate RON immunoprecipitated from these cells. The data fit a pathway in which ECM-induced integrin aggregation causes both c-Src activation and RON oligomerization followed by RON kinase-dependent autophosphorylation; this results in RON becoming a target for activated c-Src, which phosphorylates additional tyrosines on RON. Integrin-induced epidermal growth factor receptor (EGFR) phosphorylation also depended on both EGFR and c-Src kinase activities. This sequence appears to be a general pathway for integrin-dependent growth factor RTK activation.

Growth, differentiation, and survival of anchorage-dependent cells are regulated through signals generated by adhesion to ECM[1] and by soluble growth factors (1–5). Cell-matrix interaction is mediated by integrins, transmembrane noncovalently linked heterodimeric receptors consisting of α and β subunits (6). Integrin engagement by ECM can modulate growth factor signaling pathways, increasing the activity of growth factor RTKs (7, 8) and their downstream intracellular mediators (9, 10). Integrin-based effects on growth factor receptors include enhancement of cell migration (11, 12), survival (13), and proliferation (14–17).

As to the basis for their collaboration, integrins and growth factor receptors may form macromolecular complexes on the cell membrane (7, 8, 13, 16–18). In that case, adhesion-induced aggregation of integrins might trigger co-aggregation (5) and autophosphorylation of growth factor RTKs (13). Integrin-induced epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) RTK phosphorylation depends on the kinase activity of the receptor (7, 13). Recent data suggest that integrin association with RTKs might also protect the latter against the activity of phosphatases (17, 19) and/or ensure the correct subcellular juxtaposition of cytoplasmic tails of dimerized growth factor receptors (17). Despite the cited progress in this area of research, the molecular mechanisms underlying growth factor receptor activation by integrins remain to be defined.

RON is an RTK that mediates the biological effects of macrophage-stimulating protein (MSP) (20, 21). MSP was discovered as a serum factor that regulates the motility of macrophages (22). Recent investigations have shown that the RON receptor is expressed in various cell types including epithelial cells (23) and that MSP-mediated effects on epithelial cells are integrin-dependent (23, 24). In the present work, we investigated the pathway involved in integrin-mediated activation of RON.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis of the RON Receptor—A kinase-dead K1114M RON mutant was generated using the GeneEditor (Promega, Madison, WI) mutagenesis kit with oligonucleotide GTGCCATCATGTCACTAAG.

Cells and Transfections—RE7 (20) (MDCK RON-expressing cells), MDCK, and HEK 293 cells (ATCC, Manassas, VA) were grown in DMEM with 10% FCS. HaCat cells (donated by Dr. N. Fusenig, Heidelberg, Germany) were grown in KSF medium with supplements.

For transient transfection of the HEK 293 cell line, cells were grown to 70–80% confluence on 15-cm dishes and transfected with 20 μg of RON cDNA or empty vector pCI-neo (Promega, Madison, WI) using Superfect reagent (Qiagen, Santa Clarita, CA). For co-transfection, 10 μg of RON cDNA plus 10 μg of empty vector (MOCK), FAK Y397F, or dominant-negative (dn) c-Src (K295M/Y527F) mutant DNAs were used.

For transient transfection of MDCK cells, 15 μg of empty vector or dn c-Src were co-transfected together with 5 μg of MACS4 plasmid (Miltenyi Biotec, Auburn, CA) using Superfect reagent. After 36 h, successfully transfected cells were selected using a MACS4 selection kit (Miltenyi Biotec).

Cell Stimulation, Lysis, Immunoprecipitation, and Western Blotting—Cells were starved overnight in medium without serum and then collected from dishes and stimulated with 5 nM MSP (Toyobo, Japan) or 50 μg/ml EGF (Life Technologies, Inc.) for 30 min in suspension or on

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[1] The abbreviations used are: ECM, extracellular matrix; dn, dominant-negative; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; EGFR, EGF receptor; FAK, focal adhesion kinase; FCS, fetal calf serum; MBP, myelin basic protein; MDCK, Madin-Darby canine kidney cells; MSP, macrophage-stimulating protein; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PDGFR, platelet-derived growth factor receptor; RTK, receptor tyrosine kinase; PAGE, polyacrylamide gel electrophoresis; RON, "recepteur d'origine Nantais"; RONkd, kinase-dead RON; RONwt, wild type RON.

noncoated or poly-lysine-coated dishes. For stimulation by ECM (mouse collagen type IV, human collagen type I, or human fibronectin), cells were plated on ECM-coated dishes in the presence or absence of 5 nM MSP or 50 μg/ml EGF for 30 min. After stimulation, cells were lysed in lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 100 mM NaF, 1% Triton X-100, 10 μg/ml leupeptin, 10 units/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride). Insoluble material was removed by centrifugation, and RON receptor was immunoprecipitated from supernatants using mouse monoclonal anti-RON antibodies (clone ID2). RON tyrosine phosphorylation was detected by Western blotting using anti-phosphotyrosine antibodies (anti-PY, clone 4G10, Upstate Biotechnology, Inc., Lake Placid, NY). Rabbit anti-RON antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used for detection of RON on the membranes.

RON Kinase Assay in Vitro—RON was immunoprecipitated from cell lysates by RON antibodies. Immunoprecipitates were washed twice in HNTG buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol) and twice in kinase buffer (20 mM HEPES, pH 7.4, 10% glycerol, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 150 mM NaCl). To initiate kinase reactions, 15 μCi of [γ^{32}P]ATP (3000 Ci/mmol, 10 μCi/ml) was added, and immunoprecipitates were incubated for 30 min at room temperature in 15 μl total volume. The exogeneous substrate, myelin basic protein (MBP), was added to the kinase reaction mixture at a concentration of 0.5 μg/reaction tube. Reactions were stopped with 5 μl of 4× sample buffer. Phosphorylated RON or its substrate, MBP, were visualized after SDS-PAGE by autoradiography.

Phosphorylation of RON by c-Src in Vitro—Pure constitutively active c-Src enzyme (3 units/sample; Upstate Biotechnology, Inc.) was incubated with immunoprecipitated RON and 15 μCi of [γ^{32}P]ATP (3000 Ci/mmol, 10 μCi/ml) in kinase buffer for 30 min at room temperature, and incorporation of ^{32}P into RON was detected by autoradiography after SDS-PAGE.

Assay of Cell Accumulation in Tissue Culture—RE7 cells (MDCK cells stably expressing the RON receptor, 1×10^4 cells/well) were plated in triplicate into 96-well tissue culture plates, uncoated (control) or coated with mouse collagen type IV, and incubated in the presence or absence of 1 nM MSP in DMEM without FCS. Cell numbers were measured after 48 h by adding 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) into culture wells and measuring A_{570} 2 h later. The number of cells was determined from an MTT calibration curve for this cell line.

RESULTS AND DISCUSSION

In the present work, we investigated the effect of integrin-dependent adhesion on the functional activity of RON and the molecular mechanisms mediating this effect. Plating of RON-expressing HEK 293 epithelial cells on a collagen-coated substrate induced ligand-independent RON tyrosine phosphorylation (Fig. 1*A*, *lane 3*) and kinase activity (Fig. 1B, *lane 3*). The Addition of MSP to collagen-adherent cells caused a higher level of RON phosphorylation and kinase activity than either MSP or collagen alone (Fig. 1, *A* and *B*). The time courses of RON phosphorylation induced by these different stimuli were comparable, with a plateau at about 30 min (data not shown). RGD-containing peptide or anti-β_1-integrin antibodies blocked ECM-induced RON activation (data not shown), indicating that the effect of ECM (collagen types I and IV, fibronectin) on RON phosphorylation and activation is mediated by integrins. Similar results were obtained with MDCK cells stably expressing transfected RON and with the human HaCat keratinocyte cell line that expresses endogenous RON (data not shown). In addition to the effect on RON phosphorylation and kinase activity, the combination of MSP and ECM maximized epithelial cell numbers, measured after 48 h in culture (Table I). Effects of ECM, alone or with growth factor, on receptor phosphorylation or downstream mediators have been described for Met (25), EGF (7, 8, 13–16), PDGF (7, 8, 16), fibroblast growth factor (8), VEGFR-2 (17), and insulin (16) receptors in various cell types, indicating that cell-ECM interactions frequently regulate growth factor-RTK responses.

In thinking about a molecular mechanism mediating the ECM effect on RON, we considered two possibilities, which are not mutually exclusive. 1) Inasmuch as RON is associated with β_1 integrin (24), ECM-induced integrin aggregation could result in RON oligomerization, transphosphorylation, and increased RON kinase activity; and (2) tyrosine kinases activated by integrins might phosphorylate and activate RON.

Two tyrosine-specific cytoplasmic kinases, focal adhesion kinase (FAK) and c-Src, can be activated in response to cell-ECM

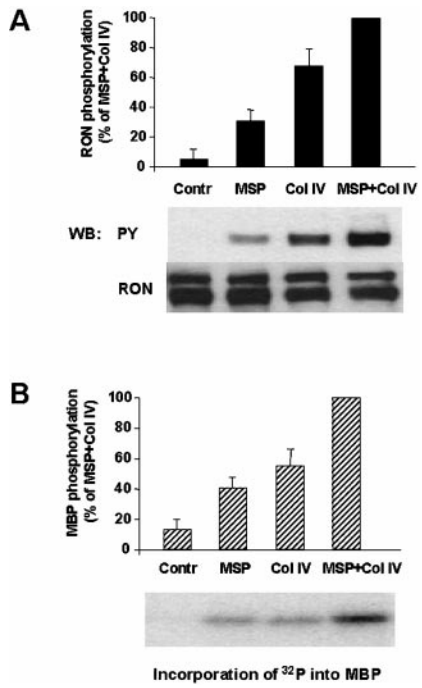

FIG. 1. **Stimulus-induced tyrosine phosphorylation and kinase activity of RON transiently expressed in HEK 293 cells.** Cells in serum-free medium were stimulated in suspension with 5 nM MSP or plated on mouse collagen IV (*Col IV*)-coated dishes in the presence of MSP or medium alone for 30 min. Cells were then lysed, and RON was immunoprecipitated from the lysates for detection of tyrosine phosphorylation or kinase activity. *A*, RON tyrosine phosphorylation was detected by Western blotting with anti-phosphotyrosine (*WB: PY*) antibodies. The level of RON phosphorylation was calculated by densitometry of the phosphorylated RON bands on the blots and expressed as a percent of the RON phosphorylation level induced by the combination of MSP and collagen IV. Bar graph data are means ± S.E. of three independent experiments. The *upper blot* shows results of a representative experiment demonstrating the levels of RON phosphorylation. The *lower blot*, developed with anti-RON, is a control for the amount of RON in precipitates. The two bands represent immature (*upper band*) and mature RON (*lower band*). *B*, RON kinase activity was assayed *in vitro* using [^{32}P]ATP and MBP as substrates. The level of RON kinase activity was calculated by densitometry of the phosphorylated MBP bands on the autoradiographs and expressed as a percent of the MBP phosphorylation induced by the combination of MSP and collagen IV. Bar graph data are means ± S.E. of three independent experiments. The autoradiograph shows results of one experiment demonstrating incorporation of ^{32}P into MBP.

TABLE I
Effect of collagen type IV on MSP-induced accumulation of RE7 (MDCK RON-expressing) cells

Cells (10^4/well) were distributed in triplicate in 96-well plates, uncoated or coated with mouse collagen IV, and incubated with or without 1 nM MSP in DMEM without FCS. Cell numbers were measured 48 h later by adding MTT to culture wells and measuring A_{570} 2 h later. The number of cells was determined from a standard calibration curve. The means ± S.E. for three independent experiments are shown in the table.

	Cell no. × 10^{-4}
Uncoated wells	1.5 ± 0.2
Uncoated wells + 1 nM MSP	3.2 ± 0.3
Collagen IV-coated wells	3.8 ± 0.4
Collagen IV-coated wells + 1 nM MSP	5.6 ± 0.5

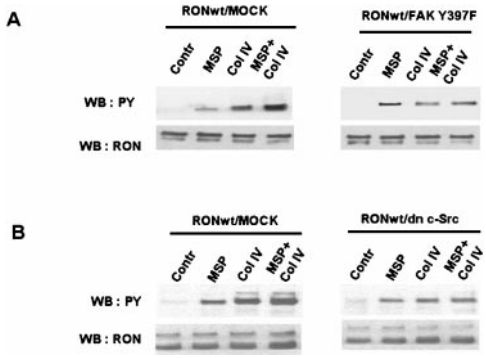

FIG. 2. **FAK major autophosphorylation site Y397F mutant (*A*) or dn c-Src (*B*) inhibits collagen IV-induced RON tyrosine phosphorylation in HEK 293 cells.** HEK 293 cells transiently co-transfected with RONwt plus FAK Y397F mutant (*A*) or with RONwt plus dn c-Src (*B*) were stimulated with MSP, collagen IV, or both, as described in the legend to Fig. 1. After stimulation, RON was immunoprecipitated from cell lysates, and tyrosine phosphorylation was determined by Western blotting with anti-PY antibodies (*WB: PY*) (*A* and *B, upper panels*). The *lower panels* represent re-blotting with anti-RON antibodies to estimate the amount of RON in precipitates. MOCK: empty vector.

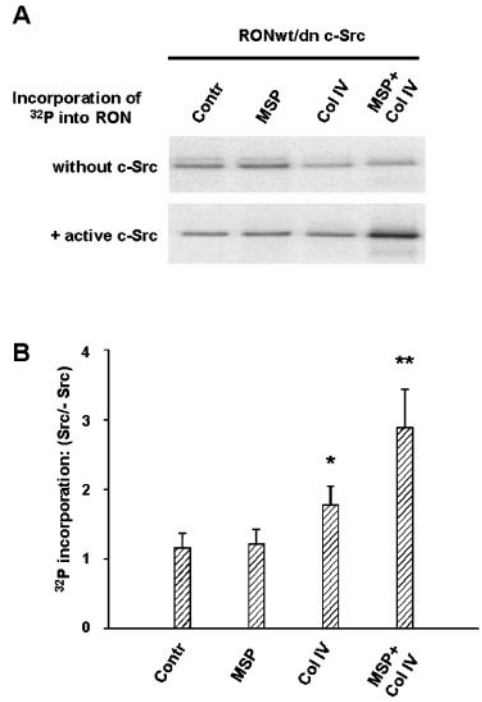

FIG. 3. **C-Src kinase phosphorylates RON from ECM-stimulated cells but not from unstimulated or MSP-stimulated cells.** *A*, HEK 293 cells transiently co-transfected with RONwt and dn c-Src were stimulated with MSP, collagen IV (*Col IV*), or both, as described in the legend to Fig. 1. After stimulation, RON was immunoprecipitated from cell lysates and tested *in vitro* as a substrate for purified active c-Src enzyme. RON phosphorylation was determined by incubation of immunoprecipitated RON with [^{32}P]ATP alone (*upper panel*) or in the presence of active c-Src (3 units/sample) (*lower panel*). ^{32}P incorporation was detected by SDS-PAGE and autoradiography. *B*, c-Src-induced RON phosphorylation was calculated by densitometry of the phosphorylated RON bands and expressed as a ratio of ^{32}P incorporation into RON in the presence and absence of c-Src. Data are means ± S.E. for five independent experiments. *p* values are shown *above* the *bars*: *, $p < 0.05$, differences between the cells stimulated with collagen or collagen plus MSP (*MSP + Col IV*) *versus* nonstimulated control (*Contr*) or MSP-stimulated cells; **, $p < 0.05$, difference between the cells stimulated with collagen + MSP *versus* collagen-stimulated cells.

adhesion (26). Cell adhesion to ECM induces integrin aggregation and activation of FAK, which is associated with β integrin. The initial step in FAK activation is transphosphorylation of

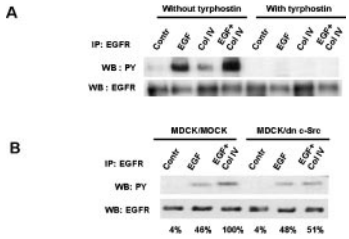

FIG. 4. **Collagen-induced EFGR tyrosine phosphorylation depends on EFGR (*A*) and c-Src (*B*) kinase activities.** MDCK cells expressing endogenous EGFR were stimulated in suspension with 50 ng/ml EGF or plated on mouse collagen IV (*Col IV*)-coated dishes in the presence of EGF or in medium alone (control (*Contr*)) for 30 min. For inhibition of EGFR activity, 250 nM tyrphostin AG1478 was added at the beginning of cell stimulation. For detection of c-Src effect on EGFR phosphorylation, MDCK cells were co-transfected with empty vector (*MOCK*) and MACS4 selection vector or with dn c-Src cDNA and MACS4 vector; after selection, positively transfected cells were used. After stimulation, EGFR was immunoprecipitated (*IP: EGFR*) from cell lysates and used for the detection of tyrosine phosphorylation by Western blotting with anti-PY (*WB: PY*) antibodies. Re-probing of the membrane with anti-EGFR antibodies provided an estimate of the amount of EGFR in precipitates. The *numbers* at the *bottom* of the figure are densitometric data for phosphorylated EFGR bands expressed as a percent of EGFR phosphorylation levels induced by the combination of EGF and collagen IV.

Tyr^{397}, after which c-Src can bind phosphorylated Tyr^{397} via its SH2 domain (27). This interaction between FAK and c-Src increases c-Src kinase activity. Activation of FAK and c-Src is a key point in integrin-mediated signal transduction (26).

To study the role of FAK in integrin-mediated RON phosphorylation, we co-expressed transiently wild type RON with the major autophosphorylation site (Y397F) FAK mutant in HEK 293 cells. In cells expressing the Y397F FAK mutant, the level of collagen-induced RON phosphorylation was about one-half that of cells transfected with vector alone (Fig. 2*A*). The fact that the FAK Y397F mutant decreased ECM-induced RON phosphorylation suggested the involvement of c-Src, a downstream kinase, for which FAK-phosphorylated Tyr^{397} is a binding site (27).

To test for a role of c-Src in ECM-induced RON phosphorylation, we transfected HEK 293 cells with a dn kinase-inactive c-Src construct. Collagen-induced RON phosphorylation (Fig. 2*B*) in these cells was reduced comparable with the Y397F FAK mutant (Fig. 2*A*). These data were consistent with the postulated ECM-integrin-FAK-c-Src pathway, suggesting that RON is phosphorylated by activated c-Src.

We therefore determined the capacity of c-Src to phosphorylate RON *in vitro* by adding active purified c-Src enzyme to RON immunoprecipitated from HEK 293 cells. These cells were also transfected with the kinase-inactive c-Src construct, in addition to RON, to prevent possible *in vivo* c-Src activity. RON was immunoprecipitated from cells that were either unstimulated or stimulated with MSP or collagen. Active c-Src *in vitro* phosphorylated RON from collagen-stimulated cells but failed to phosphorylate RON from unstimulated or MSP-stimulated cells (Fig. 3). These results indicate that c-Src can phosphorylate RON but that sites become available for phosphorylation by c-Src only on RON from cells stimulated with ECM. In contrast to our findings, it has been reported that c-Src can phosphorylate nonstimulated or ligand-stimulated growth factor receptors (28–30); these published results are with cultured adherent cells, the integrins of which might be engaged by fibronectin derived from cells or serum.

Fig. 3 also shows that although MSP alone does not make RON a target for phosphorylation by c-Src, it enhances the response to collagen. This apparent synergy between MSP and collagen in making RON a target for c-Src might be the result

of increased RON kinase activity (Fig. 1*B, last bar*).

How does stimulation by ECM make RON a target for c-Src? A clue was provided by the fact that expression of either Y397F FAK or kinase-inactive c-Src only partially decreased ECM-induced RON phosphorylation (Figs. 2 and 3), suggesting that a kinase distinct from FAK and c-Src is involved in ECM-induced RON phosphorylation. We considered the possibility that RON itself might play that role. To test whether RON kinase activity was required for ECM-mediated RON phosphorylation, we expressed a kinase-dead RON (RONkd) construct in HEK 293 cells. Stimulation of RONkd-293 cells with MSP or collagen did not cause RON phosphorylation (data not shown). Moreover, active purified c-Src *in vitro* did not phosphorylate RONkd immunoprecipitated from collagen- or MSP-stimulated cells (data not shown), which is additional evidence that non-phosphorylated RON is not a substrate for c-Src. Thus, RON kinase activity is essential for ECM-mediated effects on RON. This finding correlates with observations that PDGF (7) and EGF (13) receptor kinase activity is necessary for ECM-induced receptor phosphorylation.

Our results suggest that ECM-induced RON phosporylation and activation occur in two steps. Step 1: because RON is associated with β_1 integrin (24), ECM-induced integrin aggregation can lead to RON oligomerization and transphosphorylation, which causes an increase in RON kinase activity above the basal level that initiates the autophosphorylation. Step 2: RON molecules phosphorylated in step 1 are a target for c-Src activated via the ECM-integrin-FAK pathway, which results in the phosphorylation of additional tyrosines on RON. Data consistent with step 1 are the requirement for RON kinase activity for the ECM effect (no ECM effect on cells with RONkd) and ECM-induced RON phosphorylation above unstimulated levels in cells with step 2 blocked by kinase-inactive c-Src (Fig. 3). Data consistent with step 2 are prevention of the additional increment of ECM-induced phosphorylation in cells with kinase-inactive c-Src (Fig. 3), and phosphorylation *in vitro* by active c-Src of RON from ECM-adherent cells but not from unstimulated cells.

We also found that adhesion to ECM by epithelial cells expressing endogenous EGFR caused ligand-independent EGFR tyrosine phosphorylation as well as increased EGF-dependent tyrosine phosphorylation (Fig. 4*A*). In agreement with our data for RON, ECM-mediated phosphorylation of the EGFR requires kinase activity of both the EGFR itself and c-Src. Inhibition of EGFR kinase activity by tyrphostin blocked both EGF- and ECM-induced EGFR phosphorylation (Fig. 4*A*). In contrast, inhibition of endogeneous c-Src kinase activity by overexpression of kinase-inactive c-Src had no effect on EGF-induced phosphorylation, but it prevented the collagen-mediated increment (Fig. 4*B*). Thus, it appears that ECM-induced EGFR phosphorylation occurs via the pathway outlined above for RON, where EGFR catalytic activity and autophosphorylation are essential for c-Src to phosphorylate additional tyrosines on EGFR. Potentiation by c-Src of the mitogenic and tumorigenic capacity of EGFR is mediated by phosphorylation of additional tyrosines in EGFR by c-Src, when c-Src interacts with phosphorylated EGFR (29). The fact that receptor phosphorylation induced by ECM-dependent adhesion occurs via similar pathways for both RON and EGFR, which belong to different growth factor receptor kinase families, suggests that this is a common pathway that integrins may use for regulation of growth factor RTK activity.

The nature of this regulation is a subject for further investigation. The fact that c-Src can phosphorylate RON from cells stimulated by ECM, but not by MSP (Fig. 3*B*), is evidence that the pattern of RON tyrosine phosphorylation induced by ECM is distinct from that induced by MSP. There are 14 tyrosines in the cytoplasmic domain of RON. The similarity among the columns of Fig. 1, *A* and *B*, suggests that incremental increases in phosphorylation of some of these tyrosines may correlate with increments in RON kinase activity. It is also possible that ECM or MSP stimulation results in phosphorylation of different tyrosines, which could be unique docking sites for particular downstream mediators. This could result in different cellular responses, depending on whether RON was stimulated by ECM or MSP. An example is the fact that epithelial cells cultured in serum-free medium in collagen-coated dishes become apoptotic, despite stimulation of RON by ECM adherence; and yet stimulation of RON in the presence of MSP in these cultures prevents the apoptosis (31). Our next step in this investigation will therefore be to determine whether stimulation by ECM and MSP results in phosphorylation of different tyrosines in the RON cytoplasmic domain.

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