Integrin-mediated growth factor receptor activation

Integrin-mediated RON growth factor receptor phosphorylation requires tyrosine kinase activity of both the receptor and c-Src

Alla Danilkovitch-Miagkova*, Debora Angeloni, Alison Skeel, Shannon Donley, Michael Lerman and Edward J. Leonard

Laboratory of Immunobiology, NCI-Frederick Cancer Research and Development Center, Frederick, Maryland 21702

*-corresponding author: Dr. A. Danilkovitch-Miagkova
Tel. (301) 846-1560
Fax (301) 846-6145
E-mail: danilkovitch@mail.ncifcrf.gov

Running title: Integrin-mediated growth factor receptor activation
Summary.

Cooperation between integrins and growth factor receptors plays an important role in regulation of cell growth, differentiation and survival. The function of growth factor receptor tyrosine kinases (RTKs) can be positively regulated by integrin-dependent 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 (MSP). Adhesion of RON-expressing epithelial cells to ECM caused phosphorylation of RON, which depended on kinase activity of both RON itself and c-Src. This conclusion is based on the following 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 unstimulated cells. [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.
Introduction.

Growth, differentiation and survival of anchorage-dependent cells are regulated through signals generated by adhesion to ECM and by soluble growth factors (1-5). Cell-matrix interaction is mediated by integrins, transmembrane non-covalently 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 collaboration between integrins and growth factor receptors, they 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 latter against the activity of phosphatases (17;19) and/or insure 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.
Here, we investigated the pathway involved in integrin-mediated RTK activation using RON, the receptor for Macrophage Stimulating Protein (MSP) (20). RON is a RTK that mediates biological effects of MSP (20;21). MSP was discovered as a serum factor that regulates motility of macrophages (22). Recent investigations have shown that the RON receptor is expressed in various cell types including epithelial cells (23), and MSP-mediated effects on epithelial cells are integrin-dependent (23;24). We found that plating epithelial cells on ECM induced ligand-independent RON phosphorylation and kinase activation. Addition of ligand to ECM-adherent cells caused an additional increment of phosphorylation and kinase activity. ECM mediated RON phosphorylation required kinase activity of both RON itself and c-Src. 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. We also observed that integrin-induced EGFR phosphorylation likewise depended on both EGFR and c-Src kinase activities. Thus, the defined two-step sequence may represent a general pathway for integrin-dependent growth factor RTK activation.
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 mutagenesis oligonucleotide GTGCCATCATGTCACTAAG.

Cells and transfections. RE7 (20) (MDCK RON expressing cells), and MDCK, HEK 293, cells (ATCC, Rockville, Maryland) 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 RON cDNA or empty vector pCL-neo (Promega, Madison, WI) using Superfect reagent (Quiagen, Santa Clarita, CA). For co-transfection 10 µg of RON cDNA plus 10 µg of empty vector (MOCK) or FAK Y397F or dn c-Src (K295M/Y527F) mutant DNAs were used.

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

Cell stimulation, lysis, immunoprecipitation and western blotting. Cells were starved overnight in medium without serum, then collected from dishes and
stimulated with 5 nM MSP (Toyobo, Japan) or 50 µg/ml EGF (Gibco, BRL, Gaithersburg, MD) for 30 min. in suspension or on non-coated 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 (50mM HEPES, pH 7.4, 150mM NaCl, 10% glycerol, 1 mM EDTA, 1mM sodium orthovanadate, 10mM sodium pyrophosphate, 100mM NaF, 1% Triton X-100, 10 µg/ml leupeptin, 10U/ml aprotinin, 1 mM PMSF). 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, UBI, Lake Placid, NY). Rabbit anti-RON antibodies (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 (IPs) were washed twice in HNTG buffer (50mM HEPES, pH7.4, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol) and twice in kinase buffer (20mM HEPES, pH7.4, 10% glycerol, 10 mM MgCl2, 10mM MnCl2, 150mM NaCl). To initiate kinase reactions 15 µCi of γ32P-ATP (3000Ci/mmoll, 10 µCi/ml) was added, and IPs were incubated 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
Integrin-mediated growth factor receptor activation

0.5µg/reaction tube. Reactions were stopped with 5 µl 4x 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 3U/sample (UBI, Lake Placid, NY) was incubated with immunoprecipitated RON and 15 µCi of γ32P-ATP (3000Ci/mmol, 10 µCi/ml) in kinase buffer for 30 min. at room temperature, and incorporation of 32P into RON was detected by radioautography after SDS-PAGE.

**Assay of cell accumulation in tissue culture.** RE7 cells (MDCK cells stably expressing the RON receptor, 1x10^4 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 number was measured after 48 hr by addition of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) into culture wells and measuring A₅₇₀ 2 hrs 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 functional activity of RON, and 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. 1A, lane 3) and kinase activity (Fig. 1B, lane 3). Addition of MSP to collagen-adherent cells caused a higher level of RON phosphorylation and kinase activity than either MSP or collagen alone (Fig. 1A and B). The time course of RON phosphorylation induced by these different stimuli was comparable, with a plateau at about 30 min (data not shown). RDG-containing peptide or anti-β1-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 number, measured after 48 hrs in culture (Table 1). 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), FGF (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 β1 integrin (24), ECM-induced integrin aggregation could result in RON oligomerization, transphosphorylation and increased RON kinase activity; [2] cytoplasmic 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 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 Y397, after which c-Src can bind phosphorylated Y397 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 together 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 ½ that of cells transfected with vector alone (Fig.2A). The fact that the FAK Y397F mutant decreased ECM-induced RON phosphorylation suggested involvement of c-Src, a downstream kinase, for which FAK phosphorylated Y397 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 dominant negative kinase-inactive c-Src (dn c-
Src) construct. Collagen-induced RON phosphorylation (Fig. 2B) in these cells was reduced, comparable to the results with the Y397F FAK mutant (Fig. 2A). These data were consistent with the postulated ECM-integrin-FAK-c-Src pathway, and suggested that RON was 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. In addition to RON, these cells were also transfected with the kinase-inactive c-Src construct 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 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 non-stimulated 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 due to increased RON kinase activity (Fig. 1B, last bar).
Integrin-mediated growth factor receptor activation

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). This suggested 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 if RON kinase activity was required for ECM-mediated RON phosphorylation, we expressed a kinase dead RON (RON kd) 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 that was 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 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 phosphorylation and activation occurs in two steps. Step 1: because RON is associated with β1 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 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 kdRON), 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. 4A). Like 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. 4A). In contrast, inhibition of endogeneous c-Src kinase activity by overexpression of kinase-inactive c-Src had no effect on EGF-induced phosphorylation, but prevented the collagen-mediated increment (Fig. 4B). 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 in the columns of Figures 1A and 1B 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 by the presence of MSP in these cultures prevents the apoptosis (31). Our next step in this investigation will therefore be to determine if stimulation by ECM and MSP results in phosphorylation of different tyrosines in the RON cytoplasmic domain.
Acknowledgements

We thank Dr. N. Fusenig (Heidelberg, Germany) for his HaCat keratinocyte cell line, Dr. D. Schlaepfer (Scripps Research Institute, La Jolla, CA) for mouse FAK mutant cDNAs, Dr. P. Schwarzberg (NIH, Bethesda, MD) for the chicken c-Src mutant cDNAs, Dr. R. Breathnach (INSERM U211, Nantes Cedex 01, France) for the human RON cDNA, and Dr. F.A. Montero-Julian (Immunotech, Nantes Cedex 01, France) for mouse monoclonal anti-RON antibodies, Dr. B. Zbar (NIH-FCRDC, Frederick, MD), Dr. M. Andreazzoli (NIH, Bethesda, MD) and Dr. S. Makarov (UNC, Chapel Hill, NC) for useful discussions, and Dr. A. Miagkov (Johns Hopkins University, Baltimore, MD) for his help in preparing figures.
Integrin-mediated growth factor receptor activation

References

1. Porter, J.C. and Hogg, N. (1998) Trends Cell Biol. 8, 390-396

2. Juliano, R. (1996) BioEssays 18, 911-917

3. Schwartz, M.A. (1997) J Cell Biol. 139, 575-578

4. Schwartz, M.A. and Baron, V. (1999) Curr. Opin. Cell Biol. 11, 197-202

5. Giancotti, F.G. and Ruoslahti, E. (1999) Science 285, 1028-1032

6. Hynes, R.O. (1992) Cell 69, 11-25

7. Sundberg, C. and Rubin, K. (1996) J. Cell Biol. 132, 741-752

8. Miyamoto, S., Teramoto, H., Gutkind, J.S., and Yamada, K.M. (1996) J. Cell Biol. 135, 1633-1642

9. Renshaw, M.W., Ren, X.D., and Schwartz, M.A. (1997) EMBO J 16, 5592-5599

10. Aplin, A.E. and Juliano, R.L. (1999) J Cell Sci. 112, 695-706

11. Woodard, A.S., Garcia-Cardena, G., Leong, M., Madri, J.A., Sessa, W.C., and Languino, L.R. (1998) J. Cell Sci. 111, 469-478

12. Li, J., Lin, M.L., Wiepz, G.J., Guadarrama, A.G., and Bertics, P.J. (1999) J Biol. Chem. 274, 11209-11219
13. Moro, L., Venturino, M., Bozzo, C., Silengo, L., Altruda, F., Beguinot, L., Tarone, G., and Defilippi, P. (1998) *EMBO J* **17**, 6622-6632

14. Cybulsky, A.V., McTavish, A.J., and Cyr, M.D. (1994) *J Clin. Invest* **94**, 68-78

15. Jones, P.L., Crack, J., and Rabinovitch, M. (1997) *J Cell Biol.* **139**, 279-293

16. Schneller, M., Vuori, K., and Ruoslahti, E. (1997) *EMBO J.* **16**, 5600-5607

17. Soldi, R., Mitola, S., Strasly, M., Defilippi, P., Tarone, G., and Bussolino, F. (1999) *EMBO J* **18**, 882-892

18. Plopper, G.E., McNamee, H.P., Dikem, L.E., Bojanowski, K., and Inberg, D.E. (1995) *Mol. Biol. Cell* **6**, 1349-1365

19. DeMali, K.A., Balciunaite, E., and Kazlauskas, A. (1999) *J Biol. Chem.* **274**, 19551-19558

20. Wang, M.-H., Ronsin, C., Gesnel, M.-C., Coupey, L., Skeel, A., Leonard, E.J., and Breathnach, R. (1994) *Science* **266**, 117-119

21. Gaudino, G., Follenzi, A., Naldini, L., Collesi, C., Santoro, M., Gallo, K.A., Godowski, P.J., and Comoglio, P.M. (1994) *EMBO J.* **13**, 3524-3532

22. Leonard, E.J. and Skeel, A. (1976) *Exp. Cell Res.* **102**, 434-438

23. Wang, M.-H., Iwama, A., Dlugosz, A.A., Sun, Y., Skeel, A., Yuspa, S.H., Suda, T., and Leonard, E.J. (1996) *Exp. Cell Res.* **226**, 39-46
Integrin-mediated growth factor receptor activation

24. Danilkovitch, A., Skeel, A., and Leonard, E.J. (1999) *Exp.Cell Res.* **248**, 575-582

25. Wang, R., Kobayashi, R., and Bishop, J.M. (1996) *Proc.Natl.Acad.Sci.U.S.A* **93**, 8425-8430

26. Hanks, S.K. and Polte, T.R. (1997) *BioEssays* **19**, 137

27. Eide, B.L., Turck, C.W., and Escobedo, J.A. (1995) *Mol.Cell Biol.* **15**, 2819-2827

28. Peterson, J.E., Kulik, G., Jelinek, T., Reuter, C.W., Shannon, J.A., and Weber, M.J. (1996) *J Biol.Chem.* **271**, 31562-31571

29. Tice, D.A., Biscardi, J.S., Nickles, A.L., and Parsons, S.J. (1999) *Proc.Natl.Acad.Sci.U.S.A* **96**, 1415-1420

30. Biscardi, J.S., Maa, M.C., Tice, D.A., Cox, M.E., Leu, T.H., and Parsons, S.J. (1999) *J Biol.Chem.* **274**, 8335-8343

31. Danilkovitch, A., Donley, S.M., Skeel, A., and Leonard, E.J. (2000) *Mol. Cell. Biol.* **20**, in press
Footnotes.

The abbreviations used are: dn, dominant-negative; ECM, extracellular matrix; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FAK, focal adhesion kinase; kd, kinase dead; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; MSP, macrophage stimulating protein; PDGFR, platelet-derived growth factor receptor; RTK, receptor tyrosine kinase; PAGE, polyacrylamide gel electrophoresis.
Legends to figures.

**Figure 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 in 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 (PY) antibodies. The level of RON phosphorylation was calculated by densitometry of the phosphorylated RON bands on the blots and expressed as a % of the RON phosphorylation level induced by the combination of MSP and Collagen IV. Bar graph data are means±SE of three independent experiments. The blot shows results of a representative experiment demonstrating the levels of RON phosphorylation (WB: PY). 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 myelin basic protein (MBP) as a substrate. The level of RON kinase activity was calculated by densitometry of the phosphorylated MBP bands on the radioautographs and expressed as a % of the MBP phosphorylation induced by the
combination of MSP and Collagen IV. Bar graph data are means±SE of three independent experiments. The radioautograph shows results of one experiment demonstrating incorporation of $^{32}$P into MBP.

Figure 2. FAK major autophosphorylation site Y397F mutant (A) or dominant negative (dn) c-Src (B) inhibits collagen IV-induced RON tyrosine phosphorylation in HEK 293 cells.

HEK 293 cells transiently co-transfected with wt RON plus FAK autophosphorylation site Y397F mutant or with wt RON plus dn kinase-inactive c-Src were stimulated with MSP, collagen IV or both as described in legend to Fig.1. After stimulation RON was immunoprecipitated from cell lysates and tyrosine phosphorylation was determined by western blotting with anti-PY antibodies (A and B upper panels). The lower panels represent re-blotting with anti-RON antibodies to estimate the amount of RON in precipitates.

Figure 3. C-Src kinase phosphorylates RON from ECM-stimulated cells, but not from unstimulated or MSP-stimulated cells.

HEK 293 cells transiently co-transfected with wt RON and dn c-Src were stimulated with MSP, collagen IV or both as described in 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 (3U/sample) (lower panel). $^{32}$P
incorporation was detected by SDS-PAGE and radioautography. C-Src-induced RON phosphorylation was calculated by densitometry of the phosphorylated RON bands and expressed as a ratio of $^{32}\text{P}$ incorporation into RON in the presence and absence of c-Src. Data are means±SE for five independent experiments. $P$ values are shown above the bars: $^*P<0.05$ showing differences between the cells stimulated with collagen or collagen+MSP versus non-stimulated (Contr.) or MSP-stimulated cells; $^{**}P<0.05$ showing difference between the cells stimulated with collagen+MSP versus collagen-stimulated cells.

**Figure 4.** Collagen-induced EFGFR tyrosine phosphorylation depends on EFGFR (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 medium alone for 30 min. For inhibition of EGFR activity 250 nM of 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, and after selection positively transfected cells were used. After stimulation EGFR was immunoprecipitated from cell lysates and used for detection of tyrosine phosphorylation by western blotting with anti-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 densitometry
data for phosphorylated EFGR bands (WB:PY), expressed as a % of EGFR phosphorylation levels induced by the combination of EGF and Collagen IV.
Table 1. Effect of collagen type IV on MSP-induced accumulation of RE7 (MDCK RON expressing) cells.

| Condition                          | Cell number x 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         |

Cells (10^4/well) were distributed in triplicate in 96-well plates, uncoated or coated with mouse collagen IV, and incubated with or without 1nM MSP in DMEM without FCS. Cell number was measured 48 hr later by addition of MTT to culture wells and measuring $A_{570}$ 2 hrs later. The number of cells was determined from a standard calibration curve. The mean±SE for three independent experiments is shown in the Table.
A

RON phosphorylation (% of MSP+Col IV)

|       | Contr | MSP | Col IV | MSP+Col IV |
|-------|-------|-----|--------|------------|
| RON   |       |     |        |            |

WB: PY

RON

B

MBP phosphorylation (% of MSP+Col IV)

|       | Contr | MSP | Col IV | MSP+Col IV |
|-------|-------|-----|--------|------------|
| Inc 32P into MBP |       |     |        |            |
