Activated Src Oncogene Phosphorylates R-Ras and Suppresses Intigrin Activity

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One of the prominent effects of the Src kinase is to reduce cell adhesion. The small GTPase, R-Ras, affects cell adhesion by maintaining integrin activity, and the ability of R-Ras to do so can be regulated by phosphorylation of a tyrosine residue located in its effector domain by an Eph receptor kinase (Zou, J. X., Wang, B., Kalo, M. S., Zisch, A. H., Pasquale, E. B., and Ruoslahti, E. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 13813–13818). Here we show that Src regulates cell adhesion through R-Ras and integrins. Reduced substrate attachment of 293T cells transfected with the cDNA for an activated form of Src (v-Src) was accompanied by phosphorylation of endogenous R-Ras. v-Src also phosphorylated R-Ras in vitro. An activated form of Src similar to one that has been found in human cancers, Src527, shared with v-Src the ability to phosphorylate R-Ras. Stronger R-Ras phosphorylation was seen in Madin-Darby canine kidney cells cells transformed with temperature-sensitive v-Src at the permissive temperature than at the non-permissive temperature, and R-Ras and Src co-immunoprecipitated at the permissive temperature. Mutation analysis showed that the Src phosphorylation site in R-Ras was tyrosine 66, the position critical to the ability of R-Ras to support integrin activity. Finally, activated R-Ras in which tyrosine 66 is mutent to phenylalanine rendered cells partially resistant to the effects of Src on cell adhesion. Regulation of cell adhesion by Src through R-Ras may be at least partially responsible for the reduced adhesion and the resulting increased invasiveness of Src-transformed cells.

Reduced adhesiveness is one of the hallmarks of malignantly transformed cells, as exemplified by the rounding up of transformed cells in culture. Transformed cells elaborate less fibronectin matrix than their normal counterpart cells. These properties are thought to be important in facilitating the ability of malignant cells to leave their original site and invade other tissues (1, 2). Cell adhesion and extracellular matrix assembly are largely controlled by integrins, a family of heterodimeric membrane proteins that mediate cell-extracellular matrix interactions (3).

Cells control the ability of their integrins to bind to extracellular ligands. Thus, certain cells can convert from non-adherent to highly adherent cells through integrin activation. This happens when platelets are activated and aggregate to initiate blood clotting and when leukocytes adhere to the vascular endothelium at inflammatory sites (4, 5). The degree of activation of integrins in adherent cells can also vary (5).

Src and the oncogenic Ras proteins are among the oncoproteins that reduce cell adhesion and fibronectin matrix formation (1, 2), presumably by affecting integrin activity. A mechanism whereby H-Ras interferes with integrin activity through the ERK MAPK pathway has been proposed recently (6).

R-Ras, a small intracellular GTPase that is related to H-Ras and K-Ras, but that is only weakly oncogenic (7–9), can control integrin function (10). The ability of R-Ras to control integrin activity can be regulated by phosphorylation. An Eph receptor, EphB2, phosphorylates a tyrosine residue in the effector domain of R-Ras and, as the phosphorylated R-Ras no longer supports integrin activity, cell-extracellular matrix adhesion is lost upon activation of EphB2 (11). Another Eph receptor, EphA2, has also been reported to down-regulate integrin-mediated adhesion (12).

Based on the sequence of the kinase domain, the substrate specificity of the Eph receptor kinases is predicted to be similar to that of non-receptor tyrosine kinases such as Src (13); and Src is associated with integrin complexes in focal adhesions (14, 15). In this paper, we examine the possibility that Src might also regulate integrin activity by phosphorylating the effector domain tyrosine in R-Ras. We show that v-Src and an activated mutant Src protein both phosphorylate the critical tyrosine residue in R-Ras in vitro and in cells and that Src can be co-immunoprecipitated with R-Ras from cell extracts. A variant R-Ras that cannot be phosphorylated in the effector domain renders cells partially resistant to the cell adhesion-reducing effects of activated Src. These results indicate that the R-Ras pathway is, at least in part, responsible for the reduced adhesiveness of Src-transformed cells. This may be a contributory mechanism in tumor invasion.

MATERIALS AND METHODS

Cell Lines and Plasmids—The ts-Src MDCK cell line expressing a temperature-sensitive mutant of v-Src was provided by Dr. Steven Frisch at this institute. The kinase-inactive v-Src plasmid, pcDNA3-v-srcK295R, was generated by mutating a lysine at position 295 to an arginine in the v-Src expression plasmid, pcDNA3-v-src, by using a QuikChange Site-Directed Mutagenesis Kit (Stratagene). Construction of Myc-tagged forms of wild-type R-Ras and the mutants R-RasY66F and R-Ras66YE, and the plasmids for the expression of GST-R-Ras and GST-H-Ras have also been described (11). The plasmid

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for the expression of constitutively activated chicken c-Src, pCIS2+c-Src-Y527F (16, 17) was a gift from Dr. Christine Holt.

Abbreviations—A monoclonal anti-Myc antibody and a polyclonal anti-R-Ras antibody used for immunoblotting were from Santa Cruz Biotechnology. A polyclonal anti-Myc antibody used for immunoprecipitation was from Upstate Biotechnology, and an anti-v-Src antibody (clone 327) used for immunoblotting was from Oncogene Research Products. An anti-v-Src antibody (clone GD11) used for immunoprecipitation was obtained from Upstate Biotechnology.

An anti-R-Ras antiserum was prepared by immunizing a rabbit with bacterially produced GST-R-Ras protein. Specific antibodies were purified from the antiserum on a GST-R-Ras affinity column. The antibody was then absorbed on a GST-H-Ras affinity column to eliminate any possible cross-reactivity with H-Ras. The antibody immobiloblated a single protein band of the appropriate size in 293T cells transfected with R-Ras, but not in cells transfected with H-Ras, Rap-1, or Rap.

Cell Transfections—Transient transfections were performed with 293T cells. Cells were transfected in 10-cm plates at 60% confluency with 5–10 μg of each plasmid DNA by using Superfect Transfection Reagent (Qiagen). The total amount of transfected DNA was kept constant by addition of pcDNA3. To examine cell morphology, the transfected cells were marked by co-transfection with 1 μg of the enhanced green fluorescent protein vector pEGFP (CLONTECH).

Immunoprecipitation and Immunoblot Analysis—Cell extracts were prepared in lysis buffer (50 mM Hepes, pH 7.6, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 2 mM EDTA, 2 mM EGTA, 150 mM NaCl, 50 mM NaF, 20 mM sodium vanadate, and 10% glycerol (v/v)). Protease inhibitor phenylmethylsulfonyl fluoride and the protease inhibitor mixture (Sigma) were added to the lysis buffer prior to the use. For immunoprecipitation, cell lysates were first incubated with antibodies at 4 °C for 1.5 h and then incubated with GammaBind-Sepharose beads (Amersham Biosciences, Inc.) or Protein A-Sepharose beads (Amersham Pharmacia Biotech) for another 1.5 h at 4 °C. Immunoprecipitates were boiled in SDS-containing sample buffer, separated by SDS-PAGE, and transferred to Immobilon membranes (Millipore). An anti-phosphotyrosine antibody conjugated to horseradish peroxidase (PY-20H; Transduction Laboratories) was used for immunoblotting at 1:2000 dilution. An anti-Myc antibody (9E10) and anti-R-Ras antibody, both from Santa Cruz Biotechnology were used at a dilution 1:1000, followed by a secondary goat-anti-mouse IgG antibody conjugated to horseradish peroxidase. An anti-v-Src antibody (Oncogene) was used at 10 μg/ml, followed by protein A conjugated to horseradish peroxidase (Sigma). Immunoblots were developed by enhanced chemiluminescence (Amersham Biosciences, Inc.).

In Vitro Kinase Assays—v-Src immunoprecipitates were split into equal amounts and washed with a kinase buffer consisting of 50 mM Hepes, pH 7.6, 20 mM MnCl₂, 20 mM MgCl₂, 0.25 mM Na₃VO₄, 10 μg/ml leupeptin, 5 μg/ml aprotinin, and 2.5 mM phenylmethylsulfonyl fluoride. Kinase reactions were carried out for 15 min at room temperature in kinase buffer containing 1 μCi of [γ-³²P]ATP. The kinase reaction was stopped by adding SDS sample buffer. The samples were boiled for 5 min and subjected to SDS-PAGE, then transferred to Immobilon membranes (Millipore). Anti-phosphotyrosine antibody (clone 4G10) and anti-R-Ras antibody were used at a dilution 1:2000, followed by a secondary goat-anti-mouse IgG antibody conjugated to horseradish peroxidase (Sigma). Immunoblots were developed by enhanced chemiluminescence (Amersham Biosciences, Inc.).

RESULTS

Phosphorylation of R-Ras by v-Src—The ability of v-Src to phosphorylate R-Ras was studied in 293T cells transfected with v-Src. Green fluorescent protein (EGFP) vector was co-transfected to identify the cells that had received the vector. The transfected cells showed morphological changes typical of Src transformation, leading to the spreading and growth into higher saturation density (not shown). A kinase-inactive v-Src and the vector alone caused no changes. Immunoprecipitation of R-Ras from these cells showed that R-Ras was phosphorylated in the cells transfected with v-Src, but not in cells transfected with kinase-inactive v-Src, v-SrcK295R (Fig. 1). Incubation of a full-length R-Ras GST fusion protein with v-Src immunoprecipi-
rylation at Tyr-66. We used the activated form R-Ras38V to prepare this mutant because the activated R-Ras more efficiently increases the adhesion of poorly adherent cells than wild-type R-Ras (10). Cell attachment assays showed that expression of v-Src or c-Src527 decreased the attachment of 293 cells to fibronectin by 60–70% (Fig. 7). As the transfection efficiency in the 293 cells was about 70% (not shown), this level of reduction indicates that the transfected cells have become essentially non-adhesive. The ability of v-Src to decrease cell adhesion was partially suppressed by the co-expression of the R-Ras38VY66F mutant, but not by R-Ras38V (Fig. 7). Similar results were obtained with c-Src527, and by plating the cells on type I collagen instead of fibronectin (data not shown). These results suggest that activated Src inhibits cell adhesion through phosphorylation of R-Ras, which suppresses the ability of R-Ras to support integrin activity.

**DISCUSSION**

We show here that activatedSrc can phosphorylate a tyrosine residue in R-Ras that is located in the effector domain of R-Ras. The phosphorylation of this effector domain residue affects the ability of R-Ras to regulate integrin activation. As a consequence, integrin-mediated cell adhesion is impaired. This mechanism may, at least in part, explain the reduced cell

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adhesion that is characteristic of Src-transformed cells.

Earlier results have shown that the activity of endogenous R-Ras is required for cells to maintain their ability to attach to extracellular matrix substrates through integrins (10). Furthermore, the ability of the activated EphB2 receptor to decrease cell adhesion is mediated by the phosphorylation of R-Ras (11). EphA2 has a similar effect on cell adhesion (12), indicating that this Eph receptor may also act through R-Ras. The present results indicate that v-Src and constitutively activated c-Src reduce cell adhesion at least in part by the same mechanism as the Eph receptors.

Cell transformation by v-Src causes a pleiotropic change in cellular properties, including loss of normal growth control, reduced adhesion and cell rounding. The mechanism underlying this v-Src-induced transformation is still obscure. Previous studies have shown that the ability of v-Src to induce changes in cell structure and morphology is independent of changes in gene expression (18, 19) and, therefore, most likely a direct result of the tyrosine kinase activity of Src. Many of the known v-Src targets are associated with the cellular cytoskeletal network and focal adhesions (14, 15, 20). Our results showing that oncogenic Src also phosphorylates R-Ras add a new and potentially important substrate to the list of proteins phosphorylated by Src. The binding of Src to R-Ras is another novel aspect of our findings.

The R-Ras variant resistant to Src phosphorylation only partially reversed the effects of oncogenic Src on cell adhesion, suggesting the existence of additional pathways whereby Src can affect cell adhesion. H-Ras, which like Src interferes with integrin activity, regulates integrins through the ERK MAPK pathway (6). As Src is capable of activating ERK, this pathway could contribute to the effects of Src transformation on cell adhesion.

c-Src has been found to be activated in colon cancers metastatic to the liver (21, 22). A truncating mutation in human c-Src at codon 531 (corresponding to 528 in chicken c-Src) has been found in 12% of cases of advanced human colon cancer (23). This mutation activates c-Src, conferring transforming, tumorgenic and metastasis-promoting activity to Src. We tested another similarly activated c-Src mutant, c-Src527 (23, 24), and found that it phosphorylated R-Ras as effectively as v-Src. As reduced cell adhesion can promote tumor invasion and metastasis (2), the Src-R-Ras pathway we describe here may contribute to the oncogenic properties of Src. However, as activation of the α5β3 integrin has been shown to promote rather than to inhibit metastasis (25), the effects of the Src-integrin pathway on tumor progression may be complex. Although our results do not address that question, regulating cell adhesion through R-Ras phosphorylation may also be part of the normal function of c-Src.

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