

Induction of p130^{cas} Signaling Complex Formation upon Integrin-Mediated Cell Adhesion: a Role for Src Family Kinases

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Integrin-mediated cell adhesion triggers intracellular signaling cascades, including tyrosine phosphorylation of intracellular proteins. Among these are the focal adhesion proteins p130^{cas} (Cas) and focal adhesion kinase (FAK). Here we identify the kinase(s) mediating integrin-induced Cas phosphorylation and characterize protein-protein interactions mediated by phosphorylated Cas. We found that expression of a constitutively active FAK in fibroblasts results in a constitutive tyrosine phosphorylation of Cas. This effect required the autophosphorylation site of FAK, which is a binding site for Src family kinases. Integrin-mediated phosphorylation of Cas was not, however, compromised in fibroblasts lacking FAK. In contrast, adhesion-induced tyrosine phosphorylation of Cas was reduced in cells lacking Src, whereas enhanced phosphorylation of Cas was observed Csk⁻ cells, in which Src kinases are activated. These results suggest that Src kinases are responsible for the integrin-mediated tyrosine phosphorylation of Cas. FAK seems not to be necessary for phosphorylation of Cas, but when autophosphorylated, FAK may recruit Src family kinases to phosphorylate Cas. Cas was found to form complexes with Src homology 2 (SH2) domain-containing signaling molecules, such as the SH2/SH3 adapter protein Crk, following integrin-induced tyrosine phosphorylation. Guanine nucleotide exchange factors C3G and Sos were found in the Cas-Crk complex upon integrin ligand binding. These observations suggest that Cas serves as a docking protein and may transduce signals to downstream signaling pathways following integrin-mediated cell adhesion.

Cell-extracellular matrix interactions profoundly influence a variety of biological processes, such as cell growth, differentiation, and migration (26, 47). Integrins, which comprise the major class of receptors used by cells to interact with the extracellular matrix proteins (26, 47), can transmit biochemical signals across the plasma membrane (30). Engagement of cell surface integrins is associated with rapid tyrosine phosphorylation of several intracellular proteins; these proteins include the focal adhesion kinase (FAK) (for references, see reference 12), paxillin (7), and tensin (5), as well as cortactin and p130^{cas} (Cas) (2, 41, 44, 62). Many of these proteins are primarily localized in focal adhesions, which are important sites of adhesion-induced signal transduction.

FAK becomes activated and autophosphorylated by integrin clustering on the cell surface (12). The mechanism for this activation is not known, but a direct *in vitro* interaction between FAK and the integrin β -subunit cytoplasmic domains has recently been described (53). Once autophosphorylated on tyrosine 397, FAK binds to Src homology 2 (SH2) domains of Src family tyrosine kinases (13, 14, 52, 63). Src then phosphorylates several other sites in FAK (8), which in turn function as binding sites for signaling molecules containing SH2 domains, such as Grb2 (55). Grb2 is constitutively associated with the Ras GDP/GTP exchange protein Sos, and therefore, the formation of a FAK-Grb2-Sos complex may lead to activation of Ras and the Raf/mitogen-activated kinase pathway (55).

In support of this notion, it has been shown that ligand binding by integrins outside the cell results in mitogen-activated protein kinase activation (11, 38, 55, 65). FAK interacts also with several other signaling molecules, including phosphatidylinositol (PI) 3'-kinase (10, 22) and C-terminal Src kinase Csk (48).

Recent studies show that paxillin is phosphorylated in a FAK-dependent manner in cells (54). Paxillin in turn has been shown to form complexes with Csk and the adapter protein Crk in an SH2-domain-dependent manner (4, 48, 54). These observations indicate that ligand binding by integrins regulates the functions of multiple docking proteins that transmit signals to downstream pathways. How these integrin-mediated tyrosine phosphorylation events and protein-protein interactions translate into the biological consequences observed upon cell-extracellular matrix interaction is largely unknown.

Cas was originally identified as a 130-kDa protein that is highly phosphorylated on tyrosine residues in cells expressing the p47^{v-crk} (v-Crk) (3, 37) and p60^{v-src} (v-Src) oncoproteins (31, 32). Cas contains an SH3 domain and a cluster of 15 possible SH2-domain-binding sites (substrate domain; Tyr-377 to Tyr-414) (49); nine of these are YD(V/T)P sequences that conform to the binding motif for the Crk SH2 domain. Another region near the C terminus of Cas contains possible binding motifs for the Src SH2 domain. Cas also has several proline-rich regions that are candidates for SH3-domain-binding sites. These structural characteristics suggest that Cas is a docking protein that can bind both SH2 and SH3 domains as well as use its own SH3 domain to engage in protein-protein interactions. Cas forms a stable complex with v-Crk and v-Src *in vivo* in a phosphorylation-dependent manner and is a major substrate for v-Src kinase activity *in vitro*. Thus, Cas may play

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a role in cellular transformation triggered by v-Crk and v-Src. Little is known, however, about the physiological function of Cas or about the pathways that promote Cas phosphorylation.

The phosphorylation of Cas and FAK occurs coordinately during cell adhesion (41, 44, 62). Moreover, these two proteins colocalize in focal adhesions, and they also bind to one another (45). Therefore, Cas is a candidate for being a substrate of FAK. In the work reported here, we seek to identify kinases that mediate tyrosine phosphorylation of Cas in response to integrin-mediated cell adhesion. Our results suggest that Src family tyrosine kinases are responsible for the integrin-initiated tyrosine phosphorylation of Cas and that FAK is not necessary for tyrosine phosphorylation of Cas but may recruit Src family kinases to phosphorylate Cas. The phosphorylated Cas was found to form complexes with a number of SH2-domain-containing signaling molecules. Thus, protein-protein interactions mediated by Cas may connect integrin signaling to downstream signaling pathways.

MATERIALS AND METHODS

Cells and cell culture. Src^{-/-}, Fyn^{-/-}, and Csk^{-/-} fibroblasts were isolated from *c-src*^{-/-}, *fyn*^{-/-}, and *csk*^{-/-} mouse embryos, respectively, and immortalized with simian virus 40 T-antigen genes (28, 58, 59). The established cell lines, as well as the corresponding wild-type cell lines established in a similar fashion, were obtained from Sheila Thomas and Philippe Soriano (Fred Hutchinson Cancer Center, Seattle, Wash.). Normal mouse (CD1 strain) embryo fibroblasts were from Hélène Baribault (La Jolla Cancer Research Foundation, La Jolla, Calif.). Low-passage-number primary FAK^{-/-} fibroblasts and the corresponding wild-type cells have been described elsewhere (27). Rat embryo fibroblasts (REF-52 cells) expressing constitutively active FAK were generated by transfecting the cells with a CD2FAK plasmid (9) together with a pSV2-Neo marker vector. Stable clones expressing the chimera CD2FAK were isolated by G418 selection. Cell lines expressing mutant forms of CD2FAK, in which either the major autophosphorylation site of FAK was mutated (Tyr-397-to-Phe [Y397F] mutation) or its kinase activity was abolished (Lys-454-to-Arg [K454R] mutation), as well as control cell lines expressing CD2 without FAK, were similarly generated. CD2, CD2FAK, CD2FAK(Y397F), and CD2FAK(K454R) constructs in expression vector pCDM8 were obtained from Alejandro Aruffo (Bristol-Myers Squibb, Seattle, Wash.) and are described elsewhere (9). REF-52 cells expressing exogenous c-Crk were generated by transfecting the cells with expression plasmid pUC-CAGGS, containing a full-length mouse c-Crk cDNA (42), together with a pSV2-Neo marker vector. Sublines stably expressing c-Crk were selected by G418 treatment and were then transfected with expression plasmid pSSRc, containing a full-length rat Cas cDNA (49), together with a hygromycin resistance marker. Cell lines expressing both Crk and Cas were selected by hygromycin treatment in the presence of G418. Quantitation of expression levels in control-transfected and Crk/Cas-transfected cells was done from anti-Crk and anti-Cas immunoblots by using an Ambis radioanalytic imaging system. COS-7 cells were used for transient transfection experiments with various FAK constructs as described below.

Cells were grown in Dulbecco modified Eagle medium (DMEM; Gibco Laboratories, Grand Island, N.Y.) supplemented with 10% fetal calf serum (Tissue Culture Biologicals, Tulare, Calif.), 50 U of penicillin per ml, and 50 µg of streptomycin (Irvine Scientific, Santa Ana, Calif.) per ml. Stably transfected cell lines were selected with 400 µg of G418 (Gibco) per ml or 400 U of hygromycin (Calbiochem, San Diego, Calif.) per ml; established cell lines were cultured in the presence of 200 µg of G418 per ml and 200 U of hygromycin per ml. Prior to adhesion experiments, cells were serum starved for 24 h in DMEM-1% fetal calf serum, detached by trypsinization, and washed with soybean trypsin inhibitor. The cells were washed twice with DMEM containing 0.5% bovine serum albumin (BSA), and cell suspensions were incubated in DMEM-0.5% BSA at 37°C for 40 min on a rotator. Cells were then plated onto dishes coated with various substrates and incubated at 37°C for 45 min; cells referred to as suspended cells were held in suspension for an additional 20 min. Dishes were coated with fibronectin (20 µg/ml; obtained from the Finnish Red Cross) or polylysine (20 µg/ml; Sigma Chemical Co., St. Louis, Mo.) overnight and blocked with 1% BSA for 1 h prior to plating of the cells.

Cell lysis and immunoprecipitation. Cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed in modified radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 1% deoxycholate, 50 mM NaF, 0.5 mM Na₃VO₄, 0.1 U of aprotinin per ml, 10 µg of leupeptin per ml, 4 µg of pepstatin A per ml), Nonidet P-40 (NP-40) buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 5 mM EDTA, 1% NP-40), phosphatase and protease inhibitors as specified above) was used for in vitro kinase assays (Fig. 2) or studies of SH3-domain-mediated interactions (Fig. 6). Immunoprecipitations and immunoblot analysis were carried out as described previously (62). Phosphotyrosine-contain-

ing proteins were visualized by incubation with horseradish peroxidase-conjugated antiphosphotyrosine antibody Py20 (Transduction Laboratories, Lexington, Ky.) followed by enhanced chemiluminescence detection (Amersham). Other antibodies used in immunoprecipitations and immunoblotting were monoclonal anti-human CD2 antibody (Pharmingen, San Diego, Calif.), monoclonal anti-FAK antibody (Transduction Laboratories), polyclonal anti-Cas antibody (Cas2 antibody [49]), monoclonal antihemagglutinin (HA) antibody (12CA5; Boehringer Mannheim, Indianapolis, Ind.), polyclonal anti-glutathione S-transferase (GST) antibody (Sigma), polyclonal anti-Csk antibody (obtained from Tony Hunter, The Salk Institute, La Jolla, Calif.), monoclonal anti-Src antibody (327; obtained from Joan Brugge, Ariad Pharmaceuticals, Cambridge, Mass.), polyclonal anti-Sos and anti-Fyn antibodies (Upstate Biotechnology, Inc., Lake Placid, N.Y.), monoclonal anti-β-tubulin antibody (Sigma), monoclonal anti-Crk antibody (Transduction Laboratories), and polyclonal anti-Crk and anti-C3G antibodies (obtained from Michiyuki Matsuda, National Institute of Health, Tokyo, Japan). Rabbit polyclonal and mouse monoclonal antibodies were detected in the immunoblot analysis by horseradish peroxidase-conjugated protein A and anti-mouse immunoglobulin G (Sigma), respectively. Immunoreactive bands were visualized by enhanced chemiluminescence.

In vitro kinase assay. CD2FAK, CD2FAK(Y397F), and CD2FAK(K454R) constructs (see above) were used to generate FAK clones tagged with the HA epitope. A 3.1-kb *PvuII-EagI* fragment containing the coding region for human FAK except for the first two amino acids (9) from each of the constructs was blunt ended and subcloned to *SmaI*-digested expression vector pJ3H (56). This vector, obtained from Jonathan Chernoff (Fox Chase Cancer Center, Philadelphia, Pa.), adds an HA tag to the amino terminus of the expressed protein. pJ3H-FAK, pJ3H-FAK(Y397F), and pJ3H-FAK(K454R) vectors were transiently expressed in COS-7 cells as described previously (56). The transfected cell populations were lysed in NP-40 lysis buffer 2 days following transfection, and the HA-tagged FAK variants were immunoprecipitated with anti-HA antibody. Cells transfected with a pJ3H vector without an insert were used as a control. Cas was immunoprecipitated from nontransfected COS cells in RIPA buffer. Immunoprecipitates were washed, combined, and incubated at 25°C for 30 min in a kinase assay buffer [piperazine-*N,N'*-bis(ethanesulfonic acid) (Pipes; pH 7.0), 10 mM MnCl₂, 1 mM dithiothreitol, 0.25 µCi of [γ-³²P]ATP (6,000 Ci/mmol; Du Pont-NEN, Boston, Mass.)]. The buffer was aspirated, and immunocomplex proteins were released by boiling in 0.5% SDS-50 mM Tris (pH 7.5)-5 mM EDTA-10 mM dithiothreitol, then diluted 10-fold in RIPA buffer, and immunoprecipitated with the anti-Cas antibody. Phosphorylated Cas was detected by autoradiography after SDS-polyacrylamide gel electrophoresis (PAGE). Immunoprecipitated Cas was similarly phosphorylated by recombinant Src (Upstate Biotechnology, Inc.). Relative amounts of FAK and Cas in each reaction were determined by immunoblot analysis with anti-FAK and anti-Cas antibodies of equal aliquots of the nonlabeled immunoprecipitates. Quantitation was performed with an Ambis radioanalytic imaging system.

Phosphorylation of an exogenous substrate, poly(Glu, Tyr) (4:1; Sigma), was carried out as described above except that the reaction mixture also contained 0.4 µg of poly(Glu, Tyr) and 5 µM unlabeled ATP. The reaction was terminated by addition of EDTA to a final concentration of 0.25 M, and the FAK-containing immunoprecipitates were removed by centrifugation. Poly(Glu, Tyr) was precipitated from the supernatant with trichloroacetic acid, and the incorporation of ³²P was monitored by liquid scintillation counting. ³²P incorporation into poly(Glu, Tyr) was normalized to the relative amounts of FAK determined by immunoblot analysis.

SH2 domain binding. In vitro association experiments were done with GST fusion proteins containing the SH2 domains of Grb2 (35), phospholipase C-γ (PLC-γ) (this construct contains the carboxy-terminal SH2 domain of PLC-γ [29]), PI 3'-kinase (this construct contains the amino-terminal SH2 domain of PI 3'-kinase [29]), Shc (51), Nck, Src, and Crk. GST-SH2 domains of Grb2, PLC-γ, PI 3'-kinase, and Shc were from Jerrold Olefsky and coworkers (University of California, San Diego, Calif.). To make GST-SrcSH2, a *XhoI-MluI* fragment of the D3 mutant of chicken c-Src (25) was blunt ended and cloned into the *SmaI* site of pGEX-1. To make GST-CrkSH2, a *SfiI-EcoRI* fragment of v-Crk was cloned by blunt-end ligation into the *SmaI* site of pGEX-1. A *StuI-BanI* fragment of Nck was cloned by blunt-end ligation to the *SmaI* site of pGEX-3X to generate GST-NckSH2. The fusion proteins were expressed in *Escherichia coli* and purified as described previously (57). The fusion protein preparations were >95% pure, as determined by SDS-PAGE and Coomassie blue staining. RIPA cell lysate (250 µg) was precleared by incubation with GST immobilized on glutathione-Sepharose beads (Pharmacia) for 30 min with rotation at 4°C. The lysates were then incubated with 5 µg of GST alone or of the GST-SH2 fusion proteins, which had been immobilized on glutathione-Sepharose beads, for 2 h with rotation at 4°C. The beads were collected and washed twice with RIPA buffer and twice with Tris-buffered saline, and the bound proteins were released by boiling in sample buffer and then subjected to SDS-PAGE and immunoblot analysis.

Blotting with GST fusion proteins. Cas immunoprecipitates were resolved by SDS-PAGE and transferred to Immobilon-P transfer membranes. Replica blots were blocked with PBS-1% BSA and then probed with 5 µg of purified GST or GST-CrkSH2 fusion protein per ml. After a 60-min incubation at room temperature with rotation, the blots were washed with PBS-1% BSA-0.1% Tween 20 and probed with a polyclonal anti-GST antiserum. Following washing, blots were

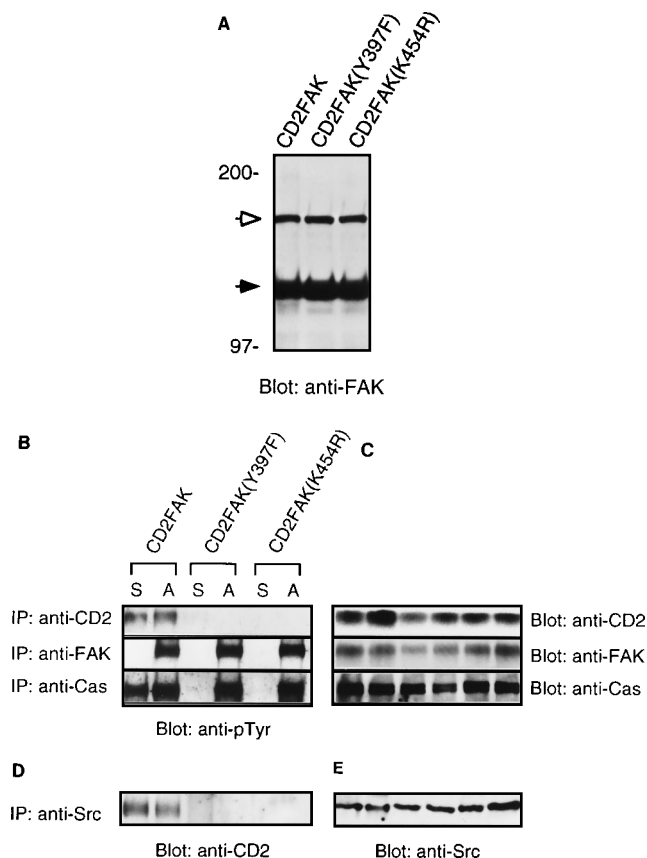


FIG. 1. Expression of wild-type and mutant forms of CD2FAK in REF-52 cells: effect on tyrosine phosphorylation of Cas. (A) RIPA cell lysates prepared from REF-52 cells stably expressing wild-type CD2FAK (CD2FAK), CD2FAK(Y397F), or CD2FAK(K454R) were subjected to immunoblot analysis with anti-FAK antibody. The molecular weight markers are indicated in kilodaltons. The open arrow indicates CD2FAK proteins, and the filled arrow indicates the endogenous FAK protein. (B to E) Lysates prepared from cells held in suspension (S) or adherent on fibronectin (A) were immunoprecipitated (IP) with anti-CD2, anti-FAK, anti-Cas, or anti-Src antibody. (B) Antiphosphotyrosine (anti-pTyr) immunoblot of the immunoprecipitates; (C) the same blot stripped and reprobed with anti-CD2, anti-FAK, and anti-Cas antibodies to assess protein loading (the band corresponding to the endogenous FAK is shown in the anti-FAK immunoprecipitates); (D) anti-CD2 immunoblot of anti-Src immunoprecipitates; (E) the same blot reprobed with anti-Src antibodies.

incubated with horseradish peroxidase-conjugated protein A and washed, and detection was performed by with enhanced chemiluminescence.

RESULTS

Tyrosine phosphorylation of Cas in cells expressing constitutively active FAK. We constructed cells expressing constitutively active FAK to study the relationship of FAK activity and tyrosine phosphorylation of Cas. Fusion of the CD2 antigen ectodomain and transmembrane domain to the amino terminus of full-length human FAK activates FAK such that it becomes autophosphorylated on tyrosine 397 and is an active tyrosine kinase, even in nonattached cells (9). REF-52 cells were transfected with expression plasmids encoding wild-type CD2FAK protein, CD2FAK lacking the major autophosphorylation site of FAK (Y397F mutation), or CD2FAK lacking tyrosine kinase activity (K454R mutation). The CD2 ectodomain alone was used as a control. Three stable sublines of each transfectant were used throughout the study, and representative experiments are shown.

Transfected cell lines immunoblotted with anti-CD2 antibodies showed expression of proteins that were of the predicted molecular weights (not shown). Immunoblotting with anti-FAK antibodies, which recognize both the endogenous rat FAK and CD2FAK proteins, indicated that the level of expression of CD2FAK was about fivefold lower than that of the endogenous FAK (Fig. 1A). CD2FAK was localized to focal contacts in immunofluorescence, presumably directed there by the C-terminal focal adhesion targeting sequence of FAK (24). The CD2 ectodomain protein expressed without a fusion to FAK localized diffusely on the cell surface (18a).

Endogenous REF-52 cell FAK was rapidly tyrosine phosphorylated in cells adhering to fibronectin but not in suspended cells (not shown). The expression of wild-type CD2FAK did not affect the tyrosine phosphorylation of the endogenous FAK, but the CD2FAK itself was highly phosphorylated in both adherent and suspended cells (Fig. 1B). The CD2FAK phosphorylation was absent in the Y397F and K454R mutants (Fig. 1B). FAK autophosphorylated on tyrosine 397 is known to form complexes with Src family tyrosine kinases in intact cells (13, 14, 52, 63). We found that wild-type CD2FAK coprecipitated with Src from the REF-52 cells (Fig. 1D). This association was not dependent on the adhesion status of the cells. The mutant forms of CD2FAK failed to associate with Src (Fig. 1D). These results indicate that wild-type CD2FAK is constitutively active and phosphorylated on tyrosine 397 in the REF-52 cells.

To study the effect of FAK on Cas tyrosine phosphorylation, Cas was immunoprecipitated from suspended and adherent cells expressing wild-type or mutant CD2FAK and subjected to antiphosphotyrosine immunoblot analysis. The results showed that Cas was tyrosine phosphorylated in both suspended and adherent CD2FAK-expressing cells. In contrast, the phosphorylation of Cas was adhesion dependent in cells expressing the mutant forms of CD2FAK and in the control REF-52 cells (Fig. 1B). These results suggest that Cas can become tyrosine phosphorylated in a FAK-dependent manner and that autophosphorylation of FAK on tyrosine 397 is an important regulatory event in Cas phosphorylation.

Tyrosine phosphorylation of Cas in vitro by FAK and Src. We next studied whether Cas could be tyrosine phosphorylated by FAK in immunoprecipitates. Wild-type FAK and Y397F and K454R mutant forms of FAK were tagged with the HA epitope, transiently expressed in COS-7 cells, and immunoprecipitated with anti-HA antibody. The immunoprecipitates were incubated together with anti-Cas immunoprecipitates in kinase assay buffer containing [γ - 32 P]ATP. To detect phosphorylated Cas, which comigrates with HA-tagged FAK, bound protein was released from the immunocomplex, and Cas was reprecipitated prior to SDS-PAGE analysis.

Incubation of Cas immunoprecipitates with HA-FAK immunoprecipitates showed that Cas was efficiently phosphorylated by wild-type FAK, whereas only background phosphorylation was seen with the kinase-deficient K454R mutant form of FAK. Our detection of a kinase activity coprecipitating with p130^{Cas} is consistent with previous observations (49). The autophosphorylation site mutant (Y397F) showed reduced kinase activity toward Cas. Compared with wild-type HA-FAK, its activity was reduced fourfold, as determined by radioanalytic quantitation of the autoradiograph (Fig. 2). These results suggest that autophosphorylation of FAK on tyrosine 397 regulates the ability of FAK to phosphorylate Cas.

It has been shown that autophosphorylation on tyrosine 397 does not significantly regulate the kinase activity of isolated FAK (8, 52). The results of our assays using poly(Glu, Tyr) as an exogenous substrate are in agreement with these findings.

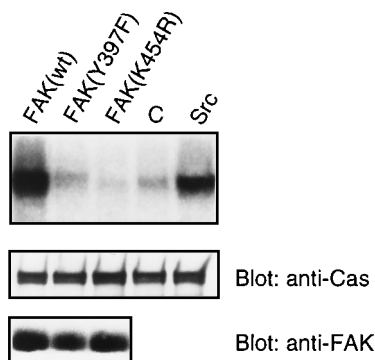


FIG. 2. Tyrosine phosphorylation of Cas *in vitro* by FAK and Src. HA-tagged wild-type FAK [FAK(wt)], FAK(Y397F), and FAK(K454R) were expressed in COS-7 cells, immunoprecipitated, and tested for the ability to phosphorylate Cas immunoprecipitated from untransfected COS-7 cells. Phosphorylated Cas was detected by autoradiography after SDS-PAGE (top). The amounts of Cas (middle) and FAK (bottom) in each reaction were determined by immunoblot analysis with anti-Cas and anti-FAK antibodies of equal aliquots of the nonlabeled immunoprecipitates. C, *in vitro* kinase assay with an anti-HA immunoprecipitate from control-transfected COS cells mixed with an anti-Cas immunoprecipitate; Src, *in vitro* kinase assay with recombinant Src mixed with an anti-Cas immunoprecipitate.

As expected, the K454R mutation essentially abolished the kinase activity of FAK (<1% of wild-type activity), whereas the Y397F mutation resulted in a modest but reproducible decrease in kinase activity, to about 60% of wild-type activity toward poly(Glu, Tyr) (not shown). Therefore, the reduced activity of the Y397F mutant toward Cas suggests that the FAK autophosphorylation site may be required to recruit and activate another tyrosine kinase, which then phosphorylates Cas (see Discussion). In support of this notion, we detected Src in the wild-type FAK but not in mutant FAK immunoprecipitates. We also found that recombinant Src efficiently phosphorylates isolated Cas (Fig. 2). That Src can phosphorylate Cas in *v*-Src immunoprecipitates has been reported (49). Together, these results indirectly suggest that Src family kinases may be responsible for Cas tyrosine phosphorylation in cells.

Integrin-mediated tyrosine phosphorylation of Cas in fibroblasts deficient in FAK, Src, Fyn, or Csk. To further study the role of FAK and Src family kinases in tyrosine phosphorylation of Cas, we took advantage of transgenic mutant (knockout) mice that do not express either FAK (27), the Src family kinase Src or Fyn (58, 59), or Csk, which is a negative regulator of Src family kinases (28). Cells isolated from these mice were used to examine tyrosine phosphorylation of Cas in response to integrin-mediated cell adhesion. Cells lacking various tyrosine kinases were compared with corresponding wild-type cells isolated in a manner similar to that used for the knockout cell lines. The results obtained with these control cell lines did not differ from those obtained with normal mouse embryo CD1 fibroblasts (not shown); the CD1 fibroblasts were used for the analyses discussed below.

CD1 fibroblasts and fibroblasts lacking FAK, Src, Fyn, or Csk were either held in suspension or plated on dishes coated with fibronectin and allowed to spread for 45 min before being harvested into RIPA buffer. Cas immunoprecipitates from the cell lysates were immunoblotted with antiphosphotyrosine and are shown in Fig. 3A. Results obtained with cells allowed to adhere on fibronectin for various time periods up to 3 h were essentially the same (not shown). Cas from FAK⁻ cells adhered on fibronectin was phosphorylated to the same degree as Cas from control cells on fibronectin, indicating that FAK is not absolutely required for integrin-mediated tyrosine phos-

phorylation of Cas in the mouse fibroblasts. In contrast, the adhesion-induced tyrosine phosphorylation of Cas was reduced in the Fyn⁻ cells and even lower in the Src⁻ cells. Moreover, increased tyrosine phosphorylation of Cas was seen in the Csk⁻ cells, in which Src family kinases are activated. Reprobing of the membranes with anti-Cas antibodies showed that a similar amount of Cas had been immunoprecipitated from each cell type (Fig. 3A). Immunoblotting experiments confirmed the absence of the appropriate tyrosine kinase from each mutant cell line (Fig. 3B). These results suggest that Cas is preferentially phosphorylated by Src and perhaps by the other Src family kinases, rather than by FAK, upon integrin-mediated cell adhesion.

Interaction of Cas *in vitro* with SH2-domain-containing signaling molecules in an adhesion-dependent manner. The tyrosine-phosphorylated sites in Cas may function as binding sites for proteins containing SH2 domains, as is the case with two other integrin-regulated proteins, FAK and paxillin (see the introduction). Cas might therefore serve as a docking protein to recruit and tether additional signaling proteins into the focal adhesion complex. To test whether SH2-domain-containing proteins would bind to Cas in an adhesion-dependent manner, binding experiments with various GST-SH2 fusion proteins were conducted. As shown in Fig. 4, the SH2 domains of several signaling proteins bound to Cas in lysates of REF-52 cells that had been plated on fibronectin for 45 min. No SH2 domain binding to Cas was seen in cell lysates prepared from suspended cells or from cells plated on polylysine. Cas does not become tyrosine phosphorylated in cells that are kept in suspension or on polylysine, which promotes cell attachment in a non-integrin-dependent manner (41, 44, 62). Among the signaling molecules found to interact with Cas in a manner dependent on integrin ligand binding were PI 3'-kinase, PLC- γ , Src, and SH2/SH3 adapter proteins Grb2, Nck, and Crk, all of

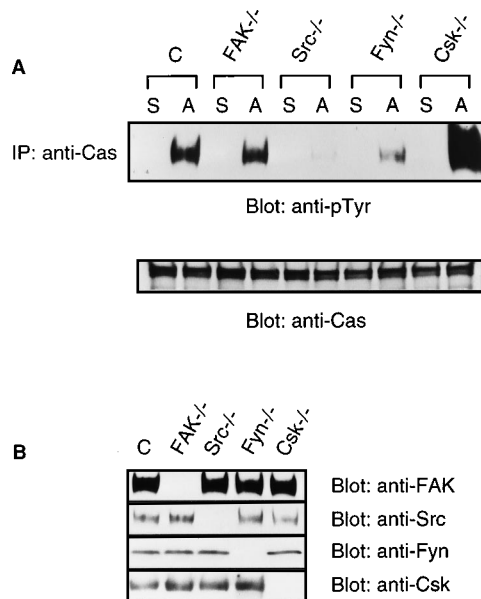
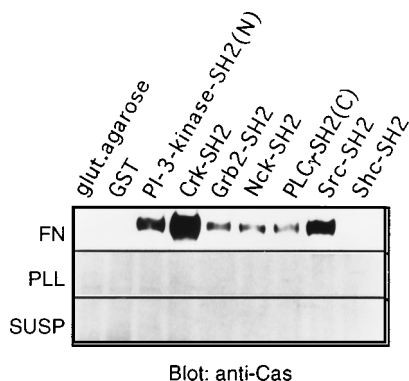


FIG. 3. Tyrosine phosphorylation of Cas in fibroblasts lacking FAK, Src, Fyn, or Csk. (A) RIPA cell lysates were prepared from suspended (S) and adherent (A) control mouse fibroblasts (C) or from fibroblasts lacking the indicated tyrosine kinases. Cas was immunoprecipitated (IP) from the lysates and analyzed by antiphosphotyrosine (anti-pTyr) immunoblotting (upper panel). Loading was assessed by immunoblotting parallel samples with anti-Cas antibodies (lower panel). Immunoblot analysis of total cell lysates confirmed the absence of the appropriate tyrosine kinase from each mutant cell line (B).



Blot: anti-Cas

FIG. 4. Adhesion-dependent interaction of Cas with multiple SH2-domain-containing molecules in binding assays. RIPA cell lysates were prepared from REF-52 cells adherent on fibronectin (FN), plated on polylysine (PLL), or held in suspension (SUSP) as described in Materials and Methods. Lysates were incubated with the indicated GST fusion proteins coupled to glutathione-agarose (glut. agarose), and precipitates were analyzed by immunoblotting with anti-Cas antibody.

which potentially couple to the Ras pathway. No interaction was detected between Cas and the SH2 domain of Shc.

Crk coprecipitates with Cas upon integrin-mediated adhesion and binds directly to Cas. Cas bound more of the Crk SH2 domain than of the other SH2 domains (Fig. 4 and data not shown); we next carried out coimmunoprecipitation experiments to determine whether Cas and Crk also associate in the cells following integrin-mediated cell adhesion. Immunoprecipitation of Crk from REF-52 cells adherent on fibronectin, but not from cells held in suspension, resulted in coimmunoprecipitation of Cas (Fig. 5A). Similarly, Cas immunoprecipitates from cells plated on fibronectin, but not from suspended cells, contained Crk (Fig. 5B). No Cas-Crk coprecipitation was observed from cells plated on polylysine. Quantitation of anti-Cas immunoblots of total cell lysates and Crk immunoprecipitates suggests that 5 to 7% of cellular Cas associates with Crk in fibronectin-adherent REF-52 cells (not shown).

To determine whether the association between Cas and Crk was direct, Cas was immunoprecipitated from suspended and fibronectin-adherent REF-52 cells and subjected to an overlay assay using the GST-CrkSH2 fusion protein as a probe. As shown in Fig. 5C, a protein of 130 kDa was detected by the GST-CrkSH2 fusion protein on the lanes of precipitates with anti-Cas antibodies from adherent but not from suspended cells. No protein was detected when control precipitates with irrelevant antibodies were probed with GST-CrkSH2 or when anti-Cas immunoprecipitates were probed with GST-ShcSH2 (not shown). The 130-kDa protein detected in the anti-Cas immunoprecipitates by GST-CrkSH2 had the same mobility as

Cas detected by reprobing of the membrane with anti-Cas antibody (Fig. 5C). These results indicate that integrin-mediated cell adhesion induces a direct, SH2-domain-dependent interaction between Cas and the SH2/SH3 adapter protein Crk.

Cas coprecipitates with Sos and C3G upon integrin-mediated cell adhesion. Crk represents an emerging class of adapter proteins consisting mostly of SH2 and SH3 domains; it can simultaneously bind to tyrosine-phosphorylated proteins via its SH2 domain and to proline-rich signaling molecules through the SH3 domains (43). Crk interacts with a number of proteins through its SH3 domain; among these proteins are Sos and C3G, which are guanine nucleotide-releasing proteins and activators of members of small GTP-binding proteins (33, 36, 60). We next studied whether these two Crk-interacting proteins could be found in Cas-Crk signaling complex induced upon integrin-mediated cell adhesion.

To facilitate the detection of Crk-Cas interaction in cells, we generated REF-52 cell lines which stably express exogenous Cas and Crk at levels that were threefold higher than the endogenous level for Cas and fourfold higher than that for Crk (Fig. 6A). Three sublines expressing exogenous Cas and Crk at similar levels were studied; representative experiments are shown.

Both Sos and C3G were detected in anti-Cas immunoprecipitates from cells adherent on fibronectin but not from suspended cells (Fig. 6B). C3G, but not Sos, was also weakly detectable in anti-Cas immunoprecipitates from adherent control-transfected cells (not shown). The amount of C3G recovered in anti-Cas immunoprecipitates thus appears higher than that of Sos, but the results may also reflect differences in the antibody detection levels. The complex formation between Sos, C3G, and Cas required the presence of Crk in the complex; Cas could not be coprecipitated with Sos and C3G from cell lysates immunodepleted of Crk (not shown). These results indicate that at least two of the proteins that are capable of binding to the SH3 domain of Crk are present in the Cas-Crk signaling complex formed upon cell adhesion. They may connect integrin ligand binding to downstream signaling pathways.

DISCUSSION

Recent work by Petch et al. (44), Nojima and coworkers (41), and us (62) has demonstrated that Cas, a focal contact protein which is heavily tyrosine phosphorylated in *v-crk*- and *v-src*-transformed cells (3, 31, 32, 37), undergoes tyrosine phosphorylation upon integrin ligand binding. The results reported herein identify Src family kinases as mediators of integrin-induced tyrosine phosphorylation of Cas. They also indicate that Cas can function as a docking protein which binds multiple SH2-domain-containing molecules upon integrin ligand binding and connects integrins to downstream signaling pathways.

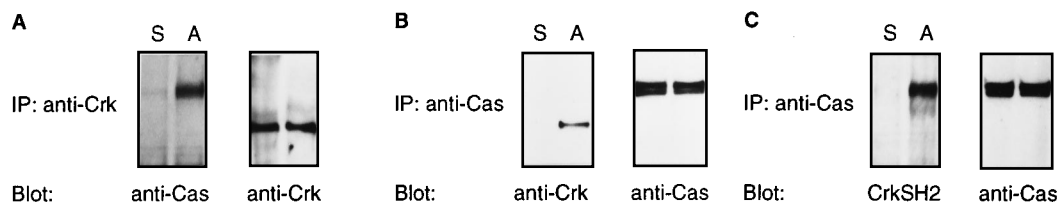


FIG. 5. Coprecipitation of Crk with Cas in extracts from adherent cells: direct binding of Crk to Cas. (A) Cell lysates from suspended (S) and adherent (A) REF-52 cells were immunoprecipitated with polyclonal antibody against Crk and analyzed by anti-Cas immunoblotting (left). The blot was reprobed with monoclonal anti-Crk to confirm equal loading (right). (B) The same lysates were immunoprecipitated with anti-Cas antibodies and analyzed by monoclonal anti-Crk immunoblotting (left). The blot was reprobed with anti-Cas (right). (C) Similarly prepared lysates were immunoprecipitated with anti-Cas antibody and blotted with GST-CrkSH2 followed by anti-GST antibody and horseradish peroxidase-conjugated protein A incubation (left). The blot was stripped and reprobed with anti-Cas antibodies (right).

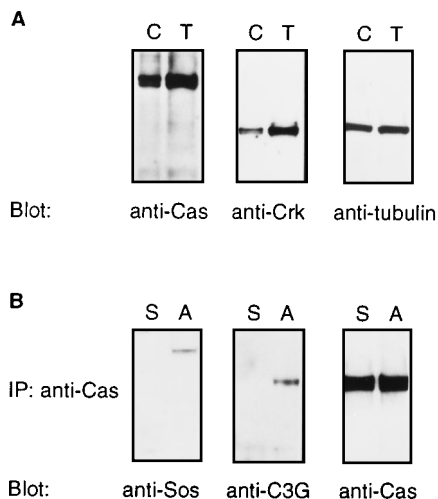


FIG. 6. Coprecipitation of Cas with Sos and C3G in extracts from adherent cells. (A) REF-52 cells were transfected with a mixture of neomycin and hygromycin resistance markers (control-transfected cell line [C]) or transfected to stably express exogenous Cas and Crk as described in Materials and Methods (Cas-Crk-transfected cell line [T]). Expression levels of Cas and Crk were determined from RIPA cell lysates by immunoblotting with the appropriate antibody. Antitubulin immunoblotting was used to confirm equal loading. (B) Cells expressing exogenous Cas and Crk were either held in suspension (S) or allowed to adhere and spread on fibronectin (A). The cells were lysed in 1% NP-40 buffer, and immunoprecipitation was carried out with anti-Cas antibodies. The immunoprecipitates were separated on SDS-PAGE and subjected to anti-Sos and anti-C3G immunoblotting. Anti-Cas immunoblotting of the precipitates was performed to ascertain equal loading.

Our results obtained with fibroblasts expressing constitutively active FAK show that the tyrosine phosphorylation of Cas can be induced in a FAK-dependent manner. This finding suggests either that Cas serves as a direct substrate for FAK or that a second protein tyrosine kinase, which is activated by FAK, phosphorylates Cas. Our results support the second possibility and suggest that Src kinases are responsible for the phosphorylation of Cas, at least upon integrin ligand binding.

We found autophosphorylation of FAK at tyrosine 397 to be crucial for the induction of Cas phosphorylation both in cells and in immunoprecipitates. However, as autophosphorylation does not significantly regulate the enzymatic activity of isolated FAK (8, 52), it is not immediately obvious how the FAK autophosphorylation might regulate the tyrosine phosphorylation of Cas. One possibility is that autophosphorylation of FAK creates binding sites for FAK substrates, and this would facilitate phosphorylation of the bound substrates. It is unlikely, however, that autophosphorylation of FAK recruits Cas, because Cas does not possess domains that are known to bind tyrosine-phosphorylated sites in other proteins. A direct interaction between FAK and Cas has recently been demonstrated; this interaction does not depend on autophosphorylation of FAK but rather is mediated by the Cas SH3 domain binding to a proline-rich region of FAK (45). Thus, a more plausible explanation for our result is that autophosphorylation of FAK is required for the binding and/or activation of a second protein tyrosine kinase, such as Src or a Src-related kinase, which then is mainly responsible for the phosphorylation of Cas. In support of this possibility, and in agreement with previous reports (13, 14, 49, 52, 63), we found that Src coprecipitates with autophosphorylated FAK and can phosphorylate Cas in vitro. Calalb and coworkers have reported that maximal kinase activity of FAK requires phosphorylation of both tyrosine 576 and tyrosine 577 (8); studies with FAK harboring mutations in

these two sites and in tyrosine 397 would be required to confirm the role of FAK in tyrosine phosphorylation of Cas under our experimental conditions.

Our experiments with cells lacking various tyrosine kinases support more directly the conclusion that Src kinases, rather than FAK, are responsible for the adhesion-induced tyrosine phosphorylation of Cas. Analysis of cells deficient of FAK demonstrated that adhesion-induced tyrosine phosphorylation of Cas has not changed in these cells compared with control cells. Tyrosine phosphorylation of Cas was greatly compromised in cells lacking Src, whereas integrin-induced Cas phosphorylation was enhanced in *Csk*⁻ cells, in which Src kinases are activated. Bockholt and Burridge (6) analyzed fibroblasts isolated from *Src*^{-/-}, *Fyn*^{-/-}, and *Yes*^{-/-} mice and identified Cas as a major protein with reduced levels of tyrosine phosphorylation, particularly in the cells lacking Src. In addition, they examined FAK phosphorylation and kinase activity and found that there were no significant differences between the knockout cells and control cells. Thus, their results also suggest that Cas is a preferential substrate for Src rather than a substrate for FAK, as FAK activity was not diminished in the *Src*⁻ cells. Because they used a monoclonal Cas antibody that recognizes a tyrosine-phosphorylated epitope (31), however, they were not able to study the possible differences in the protein levels of Cas in these cells. Our results show that the decrease in the tyrosine phosphorylation of Cas is not a result of a difference in Cas expression by the knockout cells but reflects the tyrosine phosphorylation status of Cas.

The results reported here for Cas resemble those reported for paxillin. Recent work by Schaller and Parsons shows that tyrosine phosphorylation of paxillin is FAK dependent in cells overexpressing FAK and that autophosphorylation of FAK on tyrosine 397 is required for paxillin phosphorylation (54). Similar to what we report here for tyrosine phosphorylation of Cas, tyrosine phosphorylation of paxillin has been shown to occur normally in *FAK*⁻ cells (27) but to be increased in *Csk*⁻ cells (39, 61). Similar observations have been made regarding tyrosine phosphorylation of tensin and cortactin, two other proteins which become phosphorylated upon integrin ligand binding (27, 39, 61, 62). Thus, somewhat surprisingly, FAK appears not to be required for tyrosine phosphorylation of any of the known proteins linked to integrin signaling. The *FAK*⁻ cells are valuable in the study of integrin signaling, because the roles of other signaling molecules, such as additional members of the FAK family (1, 34, 50), can be studied in isolation of FAK.

The structure of Cas suggests that it is a signal assembly protein that can bind proteins containing SH2 domains, SH3 domains, and recognition sequences for SH3 domains and can switch downstream signals on and off (49). We found that Cas interacts in vitro with multiple SH2-domain-containing proteins and that at least one of these proteins, Crk, binds to Cas in an adhesion-dependent manner in cells. Crk belongs to a family of proteins which consist almost entirely of SH2 and SH3 domains, with little intervening sequence. The Crk family presently includes the oncogenic v-Crk, two forms of c-Crk proteins, c-Crk I and c-Crk II, and a Crk-like protein, CRKL. The widely expressed c-Crk II protein, which is recognized by the antibodies used in this study, contains an amino-terminal SH2 domain followed by two SH3 domains (17). Proteins that interact with the Crk SH2 domain via phosphorylated tyrosine residues were first identified in cells transformed with *v-crk* or *v-src* and include Cas and paxillin (17). We demonstrate here that Cas-Crk interaction takes place in nontransformed cells upon integrin ligand binding; Schaller and Parsons have found that paxillin interacts with the SH2 domain of Crk in cells

overexpressing FAK, but they detected no paxillin-Crk coprecipitation in their cells (54).

Crk binds to a number of proteins through its SH3 domain; these proteins include the guanine nucleotide exchange factors Sos and C3G (33, 36, 60) and the non-receptor tyrosine kinases Abl and Arg (15, 46). We detected Sos and C3G in complexes with Cas after cells had adhered through integrins. Our finding that exogenous expression of Cas and Crk was required to detect these interactions is consistent with earlier observations showing that high levels of Crk expression are required for the formation of stable complexes between Crk and the exchange factors (15–17, 33, 60; but see also reference 36). Thus, unlike the adapter protein Grb2, Crk does not appear to be able to form constitutive signaling protein complexes and probably requires activation to form such complexes transiently (17); its binding to Cas might provide such an activating signal.

The binding of an SH3-domain-containing protein, such as Crk, to a nucleotide exchange factor can increase the catalytic activity of the factor (21). Thus, the binding of Crk to Cas upon integrin-mediated adhesion may lead to the assembly of multiprotein signaling complexes and subsequent activation of signaling pathways downstream of exchange factors Sos and C3G. The function of Sos as a Ras exchange factor has been established (19); a Cas-Crk-Sos complex may take part in Ras activation and in the mitogen-activated protein kinase activation that is seen during integrin-mediated cell adhesion (11, 38, 55, 65). Complementation experiments using yeast strains have suggested that C3G also acts as an exchange factor for a Ras protein (60); a recent report identifies Rap1 as a target for C3G (20). Rap1, a close relative of Ras, has been shown to promote Ras activity by inhibiting the GTPase activity of Ras GTPase-activating protein (18, 23). On the other hand, Rap1 may also have inhibitory effects on the Ras pathway through its binding to Raf (40). Moreover, Rap1 may also have its own signaling pathway; microinjection of Rap1 protein into 3T3 cells stimulates mitogenesis in these cells (64). Thus, the ligand binding of integrins seems to control the tyrosine phosphorylation status of a number of intracellular proteins that can function as docking proteins connecting multiple downstream signaling pathways via SH2- and SH3-domain interactions. The intracellular balance between these pathways may be responsible for the effects of the extracellular matrix on cell proliferation and differentiation.

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