The focal adhesion kinase (FAK) and Crk-associated substrate, \( p130^{\text{Cas}} \) (Cas), have been implicated in diverse signaling pathways including those mediated by integrins, G-protein-coupled receptors, tyrosine kinase receptors, and the v-src and v-erbB oncogenes. The recent identification of a direct interaction between FAK and Cas prompted the examination of potential regulation of FAK-Cas complexes by factors that result in concomitant increase in their phosphotyrosine content, namely cell adhesion and transformation by Src. Both processes increased FAK phosphotyrosine content, namely cell adhesion and transformation by Src. Both conditions resulted in elevated FAK-Cas complex levels in nonionic detergent-insoluble fractions, indicating increased association with the cytoskeleton. For activated Src, this effect requires an active Src catalytic domain but not its Src homology 2 (SH2) or Src homology 3 (SH3) domains. FAK kinase domain tyrosines 576 and 577 are also required, suggesting that direct phosphorylation of these sites by Src may influence the solubility and/or stability of the complex. FAK-Cas association was only observed in the context of Cas binding to at least one of two distinct proline-rich sites on FAK. These findings firmly establish a direct interaction between FAK and Cas and demonstrate that Src can influence the subcellular localization of the complex by a tyrosine phosphorylation-dependent mechanism.

Focal adhesion kinase (FAK) is a widely expressed nonreceptor protein-tyrosine kinase found in the focal adhesion plaques of cultured cells (1, 2). FAK’s phosphotyrosine content is elevated in response to distinct cellular stimuli including integrin-mediated cell adhesion (2–6), mitogenic neuropeptides (7–9), lysophosphatidic acid (10–12), platelet-derived growth factor (13), and oncogenic transformation by v-Src (14, 16). Cloning of Cas cDNAs revealed a protein consisting of a single amino-terminal Src homology 3 (SH3) domain, and multiple putative Src homology 2 (SH2) and SH3 domain binding sites (17, 18), suggesting a function as a docking protein for recruiting proteins involved in protein-tyrosine kinase-mediated signaling pathways. For example, Cas association with v-Crk and c-Crk SH2 domains (18–20) may lead to MAP kinase activation by Ras or Rap1-dependent pathways (21–24). Recent studies revealed that Cas interacts directly with FAK via its SH3 domain (17, 25), supporting a role for this protein in FAK-mediated signaling. In addition, Cas localizes to focal adhesions (25, 26), and elevation of its phosphotyrosine content occurs under circumstances known to activate FAK, including oncogenic transformation (14, 16), cell adhesion (25–28), and treatment with mitogens such as bombesin (29), lysophosphatidic acid (11), or platelet-derived growth factor (30). Finally, overexpression of activated FAK enhances tyrosine phosphorylation of Cas (20).

Increasing evidence indicates that FAK-mediated signaling involves a close interplay with Src family protein-tyrosine kinases. Members of this family bind to FAK’s autophosphorylation site, tyrosine 397 (31), via their SH2 domains (17, 31–33). This interaction results in further modification of FAK by Src family protein-tyrosine kinases, including phosphorylation of catalytic domain tyrosines 576 and 577, which elevates FAK’s kinase activity toward exogenous substrates (34), and phosphorylation of tyrosine 925, which is a binding site for the Grb2 SH2 domain (35, 36). Reduced tyrosine phosphorylation of FAK has been reported in cells isolated from Src- (37) and Fyn- (38) deficient mice. Src family kinases also interact directly with Cas by both SH2- and SH3-dependent mechanisms (39–43), and phosphorylation of Cas is likely to be mediated at least in part by Src as evidenced by in vitro data (18) and analysis of Src-deficient mice (20).

To gain insight into the mechanisms that govern signaling mediated by FAK and Cas, we examined potential regulation of FAK-Cas complex formation and subcellular distribution by factors known to result in a concomitant increase in their phosphotyrosine content, namely cell adhesion and transformation by Src. Both processes increased FAK-Cas complex levels in nonionic detergent-insoluble cellular fractions, suggesting increased association with the cytoskeleton. In the case of activated Src, increased association of the FAK-Cas complex with this fraction requires an active Src catalytic domain but not its SH2 or SH3 domains. This effect was observed in the context of Cas binding to each of two distinct proline-rich sites on FAK. Moreover, Src’s ability to enhance the FAK-Cas com-
plex required FAK tyrosines 576 and 577, suggesting a role for direct phosphorylation of these sites by Src.

**EXPERIMENTAL PROCEDURES**

**Cells and Cell Culture**—The source and maintenance of COS-7 cells, mouse Balb/c3T3 fibroblasts, rat 3Y1 fibroblasts, and the Src-transformed 3Y1 cell line PS3Y1 have been described previously (34). The NIH 3T3 fibroblast line and the Src-transformed NIH 3T3 fibroblast lines, F3Rc11, were both obtained from T. Hunter (Salk Institute) and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

**Cell Lysis and Coimmunoprecipitation Analysis**—Cell lysis of adherent cells was carried out as described previously (2, 8). Detached cells were prepared by trypsinization followed by two washes in phosphate-buffered saline with 0.5% bovine serum albumin. Cell lysis was performed in the following buffers as indicated: low salt Nonidet P-40 buffer (50 mM Tris-Cl, pH 7.4, 1% Nonidet P-40, 10% glycerol), Nonidet P-40 buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.05% SDS-RIPA buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.05% SDS, 0.5% sodium deoxycholate), 0.1% SDS-RIPA buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.1% SDS, 1% sodium deoxycholate), or 0.5% SDS-RIPA buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.5% SDS, 1% sodium deoxycholate). All lysis buffers were supplemented with 50 mM NaF, 1% Nonidet P-40, and 0.1 mM Na3VO4. In some experiments the insoluble material from low salt Nonidet P-40 lysates was solubilized in 0.1% SDS-RIPA buffer and homogenized by passing several times through a 26-gauge needle. Protein concentration of lysates was determined by the bicinchoninic acid assay (Pierce), and samples were standardized as stated in the figure legends.

**Antibodies, Immunoprecipitation, and Immunoblotting**—Immunoprecipitations were carried out using anti-FAK C-20 polyclonal antibody (Upstate Biotechnology, Inc., Lake Placid, NY) at 1 mg/ml, or anti-Cas monoclonal antibody at approximately 0.8 mg/ml (Santa Cruz Biotechnology) when specified. Immunoblotting was performed (2) using anti-Cas monoclonal antibodies (Santa Cruz Biotechnology) at 1 μg/ml, anti-FAK C-19 polyclonal antibodies (Santa Cruz Biotechnology) at 1 μg/ml, anti-FAK 331 polyclonal antibody (Upstate Biotechnology, Inc., Lake Placid, NY) at 1 μg/ml and detected by rabbit anti-mouse IgG (Cappel) and anti-P-Polyclonal antibody (1C, Swiss Immuno AG). Proteins detected by immunoblotting were quantified by PhosphorImager analysis (PhosphorImager and ImageQuant software for volume integration; Molecular Dynamics).

**Triton X-100 Fractionation**—Cell fractionation was carried out according to the methods of Kaplan et al. (44). Briefly, subconfluent normally growing cells were chilled on ice for 15 min and then washed twice with cold phosphate-buffered saline at 4°C. Cells were lysed at 0°C in 0.1% Triton X-100, 10% glycerol, or 0.1% Triton X-100, 10% glycerol, and 2% aprotinin, and 0.1 mM Na3VO4. In some experiments the insoluble material from low salt Nonidet P-40 lysates was solubilized in 0.1% SDS-RIPA buffer and homogenized by passing several times through a 26-gauge needle. Protein concentration of lysates was determined by the bicinchoninic acid assay (Pierce), and samples were standardized as stated in the figure legends.

**RESULTS**

**Coimmunoprecipitation of Cas with FAK Is Differentially Regulated by Cell Adhesion in Nonidet P-40 Buffer Lysates versus RIPA Buffer Lysates**—To determine if interactions between FAK and Cas are regulated by cell adhesion, the ability of Cas to co-precipitate with FAK immune complexes was compared in lysates of adherent versus detached Balb/c3T3 fibroblasts. The relative stability and solubility of the complexes was determined by employing five different buffers of increasing stringency based on concentration of NaCl, SDS, and sodium deoxycholate: low salt Nonidet P-40 buffer, Nonidet P-40 buffer, 0.05% SDS-RIPA buffer, 0.1% SDS-RIPA buffer, or 0.5% SDS-RIPA (see “Experimental Procedures” for complete buffer compositions). Cas was readily detected in FAK immune complexes formed in lysates of both adherent and detached cells under each lysis condition tested with the exception 0.5% SDS-RIPA lysis, which failed to recover FAK-Cas complexes from either adherent or detached cells (Fig. 1 and data not shown).

The effect of cell adhesion on Cas coprecipitation with FAK

2 S. Reddy, T. R. Polte, and S. K. Hanks, unpublished results.

3 Minor levels of Cas were detected in some immune complexes formed with control IgG. These background levels were highest for Nonidet P-40 buffer lysates (and CSK buffers; see below) of adherent cells and were taken into account when determining the significance of coprecipitating Cas.

**Plasmids and Mutagenesis**—pBluescript-II (SK+) (Stratagene) and pReCMV (Invitrogen) plasmids containing hemagglutinin (HA) epitope-tagged FAK plasmids have been described previously (34). Site-directed mutagenesis was carried out on pBluescript-II (SK+)-HA-tagged FAK plasmids using the Mutagen phagemid in vitro mutagenesis kit (Stratagene Inc.). Protocols for mutagenesis were carried out using the protocols of the manufacturer. Tyrosine to phenylalanine mutations at positions 397, 407, 576, and 577 have been described previously (34). An additional tyrosine to phenylalanine mutation was made at position 861 using the following DNA oligomer: 5'-CACATCCTTTACGCCTTGTG-3'. FAK mutants containing proline to alanine mutations were made using the following oligomers: P712A/P715A (mPR-1), 5'-GAAAGCACAGCCCAAGGCGACACGAG-3'; and P873A/P876A (mPR-2), 5'-CTCCAGCTTCAGGCTGCGGCT-3'. Mutagenized fragments were sequenced to confirm the mutations and to ensure integrity of the remaining sequence prior to subsequent subcloning into the pReCMV eukaryotic expression vector or pBTM116 yeast expression vector.
FIG. 1. Coimmunoprecipitation of FAK and Cas from adherent versus detached Balb/c3T3 fibroblasts. Balb/c3T3 were lysed either directly on plates as normally growing cell cultures (Att) or after trypsinization (Det) in each of the following buffers (see “Experimental Procedures”): low salt Nonidet P-40, Nonidet P-40, 0.05% SDS-RIPA, 0.1% SDS-RIPA, and 0.5% SDS-RIPA. Immunoprecipitates were formed from equal amounts of protein from each lysate with C-20 anti-FAK polyclonal antibody (α-FAK) or control IgG from normal rabbit serum (IgG) and analyzed by immunoblotting for the presence of either Cas (upper bands) or FAK (lower bands) in the immune complex. The positions of the FAK and Cas bands are marked on the right.

varied with the buffer used to lyse the cells. Equal or slightly more Cas coprecipitated from detached cells as compared with adherent cells when lysates were prepared with low salt Nonidet P-40 and Nonidet P-40 buffers (Fig. 1, compare lanes 2 with lane 4 and lane 6 with lane 8). In contrast, significantly less Cas coprecipitated with FAK from detached cells as compared with adherent cells when lysates were prepared with 0.05–0.1% SDS RIPA buffers (Fig. 1, compare lane 10 with lane 12 and lane 14 with lane 16).

These findings suggest the presence of distinct subsets of FAK-Cas complexes, differing with respect to solubility and/or stability and differentially regulated by cell adhesion. Less coprecipitating Cas was observed in Nonidet P-40-lysates compared with low salt Nonidet P-40 lysates (Fig. 1, compare lanes 2 and 4 with lanes 6 and 8), suggesting that nonionic detergent-soluble FAK-Cas complexes are sensitive to salt concentrations, possibly reflecting a weak interaction. In contrast, more coprecipitating Cas was detected in 0.05–0.1% SDS-RIPA lysates compared with Nonidet P-40 buffer lysates (Fig. 1, compare lane 6 with lanes 10 and 14) despite the fact that each buffer contains equal concentrations of NaCl. This indicates that RIPA buffers solubilize a different subpopulation of complexes in which FAK-Cas interactions are more stable. Further supporting the presence of distinct FAK-Cas complex subpopulations, different electrophoretic mobility characteristics were observed for the coprecipitating Cas under different lysis conditions. In low salt Nonidet P-40 buffer, FAK-associated Cas comprised a major band migrating near 125 kDa and a minor band near 120 kDa (Fig. 1, lanes 2, 4, 6, and 8). In contrast, the two bands were of nearly equal intensity in RIPA lysates (Fig. 1, lanes 10, 12, 14, and 16). It is not known what changes in Cas give rise to these different migrating species; however, it is unlikely that tyrosine phosphorylation is a major contributing factor, since only trace amounts of phosphotyrosine were detected in Cas immunoprecipitates formed under these conditions (Fig. 3, and see “Discussion”).

To determine if elevated levels of FAK-Cas complexes in RIPA lysates of Src-transformed cells are dependent on cell adhesion, coimmunoprecipitation of FAK and Cas was assayed in lysates of detached transformed cells. Detachment of PS3YI cells by trypsinization decreased the coprecipitating Cas 2–3-fold (Fig. 2C, compare lanes 3 and 4). Nevertheless, residual coprecipitating Cas was still greater than that found in normal adherent or detached 3YI fibroblasts (Fig. 2C, compare lanes 1 and 2 with lane 4).
transformation leads to redistribution of FAK-Cas complexes to a less soluble fraction rather than elevated overall complex formation. However, these experiments do not distinguish between recruitment of pre-existing complexes to insoluble cellular fractions and changes in de novo Nonidet P-40-soluble versus Nonidet P-40-insoluble FAK-Cas complex formation.

Fractionation in Cytoskeleton Stabilization Buffer Reveals Differences in Subcellular Distribution of FAK, Cas, and FAK-Cas Complexes in 3YI versus PS3YI Fibroblasts—Lysis buffers that act to stabilize cytoskeletal structures commonly have been used to fractionate the less soluble cytoskeleton-associated proteins from noncytoskeletal proteins. Such a system was adopted to further study the effect of Src transformation on the subcellular distribution of FAK, Cas, and FAK-Cas complexes. Adherent 3YI and PS3YI cells were lysed for 30 s, 2 min, and 10 min in CSK (see “Experimental Procedures”) without scraping (soluble fraction). Insoluble fractions remaining on the plates were solubilized in 0.1% SDS-RIPA buffer followed by scraping and homogenization (insoluble fraction) and used for comparison with the soluble fraction. Soluble and insoluble fractions were first assayed for total FAK and Cas content by direct immunoblot analysis. As judged by the ratio of soluble to insoluble protein at each timepoint, FAK and Cas displayed roughly equal solubility characteristics in normal 3YI fibroblasts (Fig. 3A, lanes 1–6). In PS3YI cells (Fig. 3A, lanes 7–12), a strikingly higher percentage of FAK is found in the insoluble fractions (4–8-fold increase) when compared with the nontransformed cells at each timepoint. On the other hand, Cas solubility was not appreciably affected by Src transformation (Fig. 3A). To determine if tyrosine-phosphorylated forms of FAK or Cas are preferentially retained in either the soluble or insoluble fractions, each protein was separately immunoprecipitated and analyzed by anti-phosphotyrosine immunoblotting. In PS3YI cells, the stoichiometry of FAK tyrosine phosphorylation was 2–4-fold higher in insoluble fractions as compared with soluble fractions at each timepoint (Fig. 3B, lanes 2–7). Thus, increased tyrosine phosphorylation of FAK in Src-transformed cells correlates with its decreased solubility in CSK. Tyrosine-phosphorylated Cas was found almost exclusively in the CSK-soluble fractions of Src-transformed cells at each time point (Fig. 3C, lanes 9–14). Despite low levels of tyrosine-phosphorylated Cas in the insoluble fractions, even at 30 s, longer lysis times yielded increased levels of tyrosine-phosphorylated Cas in the soluble fractions. This paradox could indicate that phosphorylated Cas is refractory to solubilization with 0.1% SDS-RIPA buffer and thus not detected in the insoluble fractions. In fact, increased insolubility of hyperphosphorylated Cas has been observed in 3YI cells transformed with v-Crk (18). Nevertheless, even harsher solubilization procedures such as boiling in SDS-lysis buffer did not release significant amounts of hyperphosphorylated Cas from insoluble fractions (data not shown). An alternative explanation is that Cas becomes phosphorylated during lysis in CSK buffer.

To analyze the distribution of FAK-Cas complexes in CSK soluble and insoluble fractions, each fraction was assayed for Cas coprecipitating with FAK. A clear elevation in the ratio of Cas associated with FAK in insoluble versus soluble fractions was observed at 30-s and 2 min time points in Src-transformed cells as compared with the parental line (Fig. 4). This supports the idea that FAK-Cas complexes shift to a cytoskeleton-associated cell fraction upon transformation by Src. Immunoprecipitation of Cas was low in the insoluble fractions taken at 10 min (Fig. 4), consistent with our inability to detect coprecipitating Cas in insoluble fractions of low salt Nonidet P-40 buffer lysates (Fig. 2B, lane 10).

Increased Coimmunoprecipitation of FAK and Cas in RIPA Buffer Requires a Functional Src Catalytic Domain—To gain insight into the mechanism leading to elevation of FAK-Cas complex formation in Src-transformed cells, a coexpression system was employed to assay the functional domain requirements of Src. COS-7 cells were transiently transfected with plasmids expressing either activated mouse neuronal c-Src (Y527F, tyrosine to phenylalanine mutation at residue 535;
Regulation of FAK-Cas Complex

Fig. 3. Distribution of FAK and Cas in CSK-fractionated normal and Src-transformed fibroblasts. As detailed under “Experimental Procedures,” subconfluent normally growing cultures of normal (3YI) and Src-transformed (PS3YT) rat fibroblasts were lysed for the indicated times in CSK to obtain the soluble fraction (Sol.). The CSK-insoluble material was solubilized in 0.1% SDS-RIPA buffer to obtain the insoluble fraction (Insol.). A, FAK and Cas in each fraction were assayed by immunoblotting using anti-FAK c-903 polyclonal antibodies (upper bands) and anti-Cas monoclonal antibodies (lower bands), respectively. Samples were standardized with respect to total protein solubilized at each time point in the CSK and RIPA fractions combined. B, immune complexes were formed using rabbit anti-FAK c-20 polyclonal antibodies (for controls, lanes 1 and 8, IgG from normal rabbit serum was used with 0.1% SDS-RIPA lysates of normally growing cells) and analyzed by immunoblotting for the presence of FAK (lower bands) and phosphotyrosine (upper bands). C, immune complexes were formed using a rabbit anti-Cas monoclonal antibody (for controls, lanes 1 and 8, IgG from normal rabbit serum was used with 0.1% SDS-RIPA lysates of normally growing cells) and analyzed by immunoblotting for the presence of Cas (lower bands) and phosphotyrosine (upper bands). The positions of the FAK and Cas bands are marked on the right.

Fig. 4. Distribution of FAK-Cas complexes in Triton X-100-fractionated normal and Src-transformed fibroblasts. As described in the legend to Fig. 3, subconfluent normally growing cultures of normal (3YI) and Src-transformed (PS3YT) rat fibroblasts were fractionated into CSK-soluble and -insoluble fractions after lysis for the indicated times in CSK buffer. Immune complexes were formed using rabbit anti-FAK c-20 polyclonal antibodies (c-FAK) or control IgG from normal rabbit serum (IgG) and analyzed by immunoblotting for the presence of either Cas (upper bands) or FAK (lower bands). The positions of the FAK and Cas bands are marked on the right.

equivalent to residue 527 of non-neuronal c-Src) or activated neuronal c-Src harboring deleterious point mutations in either the SH2 domain (D101N, aspartate 101 to asparagine), SH3 domain (R183K, arginine 183 to lysine), and/or kinase domain (K303R, lysine 303 to arginine). To assay for FAK and Cas association specifically in transfected cells, an influenza virus HA-tagged FAK expression construct was cotransfected with the Src expression plasmids, and immune complexes were formed in 0.1% SDS-RIPA lysates using monoclonal antibody 12CA5, which recognizes the HA epitope. Consistent with results from Src-transformed fibroblasts, activated Src expression in COS cells increased the amount of coprecipitating Cas in HA-FAK immune complexes by 3–4-fold (Fig. 5, compare lanes 3 and 4). Mutations in either the SH3 or SH2 domain,
previously shown to reduce affinity for peptide ligands (48–50) had no effect on the ability of activated Src to enhance Cas coprecipitation with HA-FAK (Fig. 5, lanes 5 and 6). However, inactivation of the Src catalytic domain by mutating the ATP binding site (lysine 303 to arginine; equivalent to residue 295 of non-neuronal c-Src) eliminated the enhanced Cas coprecipitation (Fig. 5, lanes 7–9). Likewise, expression of a C-terminal truncated form of Src, which lacks the kinase domain altogether (Nt-Src), did not enhance Cas coprecipitation (Fig. 5, lanes 10–12).

Although Src requires an active catalytic domain to enhance FAK-Cas coimmunoprecipitation, it is clear from the decreased electrophoretic mobility of FAK (Fig. 5, lanes 7–12) as well as from immunoblotting with anti-phosphotyrosine antibodies (data not shown) that FAK's phosphotyrosine content is moderately elevated in cells overexpressing either catalytically inactivated Src or Nt-Src, which lacks the catalytic domain. These results are reminiscent of the studies by Kaplan et al. (51), who reported that expression of a similar Src variant increased tyrosine phosphorylation of FAK when expressed in Src-deficient fibroblasts. Elevation of FAK's phosphotyrosine content by Nt-Src, as measured by immunoblotting with anti-phosphotyrosine antibodies, is approximately one-half that induced by full-length activated Src (data not shown). Thus, only a subset of FAK tyrosine residues with increased phosphorylation in cells expressing full-length activated Src appear to be targets for increased phosphorylation by Nt-Src. Phosphorylation of FAK induced by either catalytically inactive Src or Nt-Src appears to be at least partially dependent on an intact SH2 domain as demonstrated by abrogation of induced phosphorylation in the SH2 domain mutants (Fig. 5, lanes 9 and 12, and data not shown). The SH2 domain may serve to protect FAK phosphotyrosine residues against the action of phosphatases, or it could function to activate other protein-tyrosine kinases and/or target FAK for phosphorylation.

Identification of a Second Cas Binding Site on FAK—To determine if Src-enhanced FAK-Cas complex formation is dependent on the interaction of the Cas SH3 domain with proline-rich sites on FAK, HA-tagged FAK mutants were coexpressed with activated Src in COS-7 cells, lysed in RIPA buffer, and assayed for coimmunoprecipitation of FAK and Cas. First, a proline-rich site (APPKPSR) in FAK encompassing residues 711–717 (PR-1), previously identified as a Cas binding site by two-hybrid analysis (17), was mutated by changing prolines 712 and 715 to alanines (mPR-1). As shown in Fig. 6 (lanes 2–5), coimmunoprecipitation of Cas requires an intact PR-1. However, residual co-precipitating Cas is clearly detected in the PR-1 mutant (Fig. 6, lane 4), and co-expression of activated Src with this mutant elevated the levels of coprecipitating Cas (Fig. 6, lane 5). A second proline-rich site (PPKKPPR) in FAK encompassing residues 872–878 (PR-2) closely resembles PR-1 and thus could serve as a second site for interaction with the SH3 domain of Cas. To test this, HA-tagged FAK variants containing proline to alanine mutations at positions 873 and 876 (mPR-2) and at positions 712, 715, 873, and 876 (mPR-1/mPR-2) were assayed. As shown in Fig. 6 (lanes 6–9), mutation of PR-2 alone had only a minor effect on the amount of co-precipitating Cas either in the absence or presence of activated Src. However, combining mutations at PR-1 with PR-2 eliminated co-precipitating Cas, whether in the presence or absence of activated Src.

The interaction of the Cas SH3 domain with PR-2 was confirmed using the two-hybrid system. A LexA fusion protein consisting of amino acids 841–895 of mouse FAK (LexA-FAKKPP) and encompassing PR-2, but not PR-1, was shown to interact specifically with VP16 fusion proteins consisting of the SH3 domains of Cas and a closely related family member Efs/Sin (52, 53), resulting in growth in the absence of histidine (Fig. 7, A and B). As expected, mutation of PR-2 prolines 873 and 876 eliminated the interaction with the SH3 domains of both Cas and Efs/Sin (Fig. 7, C and D). The interaction appears to be specific, since VP16-SH3 domain hybrids derived from Src or Fyn did not permit growth in the absence of histidine (Fig. 7).

Requirement for Tyrosine Residues 576 and 577 of FAK for Src-enhanced FAK-Cas Complex Formation—As described above, Src requires an active catalytic domain to induce enhanced coimmunoprecipitation of FAK and Cas in 0.1% SDS-RIPA lysates. To test if phosphorylation of FAK at specific tyrosines is required for Src-enhanced FAK-Cas association,
hybrids, derived from Cas, Efs/Sin, Src, or Fyn as indicated in the absence of histidine indicate two-hybrid interaction driving expression of versus cytoskeleton, activated forms of Src, including v-Src, localize to and stabilization of FAK.

FAK.

demonstrate that enhanced association of FAK-Cas complexes with a nonionic detergent-insoluble cellular fraction is a common property of adherent versus detached and Src-transformed versus normal cells, suggesting an increased association with the cytoskeleton. In the case of activated Src, elevated levels of the FAK-Cas complexes in this fraction require an active Src catalytic domain but not its SH2 or SH3 domains. Moreover, FAK tyrosines 576 and 577 are critical for enhanced FAK-Cas complex levels, suggesting that increased kinase activity in response to phosphorylation of tyrosines 576 and 577 was not required for enhanced association with Cas.

DISCUSSION

Our results demonstrate that enhanced association of FAK-Cas complexes with a nonionic detergent-insoluble cellular fraction is a common property of adherent versus detached and Src-transformed versus normal cells, suggesting an increased association with the cytoskeleton. In the case of activated Src, elevated levels of the FAK-Cas complexes in this fraction require an active Src catalytic domain but not its SH2 or SH3 domains. Moreover, FAK tyrosines 576 and 577 are critical for enhanced FAK-Cas complex levels, suggesting that direct phosphorylation of these sites by Src stabilizes FAK-Cas interactions and/or interactions with other cytoskeletal component.

All FAK-Cas interactions, whether induced by activated Src or not, require at least one of two distinct proline-rich sites on FAK, implying that activated Src does not lead to association of Cas with FAK by mechanisms, direct or indirect, that are independent of the interaction of the Cas SH3 domain with FAK.

In support of a role for Src in pathways leading to activation and stabilization of FAK-Cas signaling complexes with the cytoskeleton, activated forms of Src, including v-Src, localize to cytoskeletal fractions and focal adhesions, whereas nonactivated Src localizes predominantly to perinuclear membranes (51, 55–58). Activation and redistribution of c-Src to focal adhesions occurs during integrin-mediated cell adhesion (44, 59), which parallels localization of FAK (1, 2) and Cas (26, 28, 40) to these same structures. Finally, Src can interact directly with both FAK and Cas by distinct mechanisms (31, 39–43), and both FAK and Cas can be directly phosphorylated by Src (18, 34). Thus, FAK, Cas, and Src appear to be part of a distinct signaling complex, which becomes stably associated with focal adhesion structures upon FAK and Src activation.

The mechanisms that regulate interaction of FAK, Cas, and Src with focal adhesions remain unclear. For FAK, focal adhesion targeting sequences (FAT domain) lie near the C terminus (60, 61) and are sufficient for localization of heterologous proteins to focal adhesions (61). Although paxillin and talin can bind to sequences that overlap the FAT domain, localization to focal adhesions can occur in the absence of their binding to FAK (61, 62). Focal adhesion targeting sequences of Cas have not been mapped, but interaction with FAK via its SH3 domain may contribute. For Src, myristoylation and SH3 domain accessibility are key elements in Src’s localization to focal adhesions, while kinase activity is not needed (51). Based on these findings, there appears to be no obvious role for tyrosine phosphorylation in regulating the association of the FAK-Cas/Src complex with focal adhesions. However, our finding that tyrosine-phosphorylated FAK preferentially distributes to cytoskeletal fractions of Src-transformed cells, an event accompanied by increased association with Cas, supports a role for tyrosine phosphorylation in the assembly of this complex in focal adhesions. That Src kinase activity and FAK phosphoacceptor tyrosines 576 and 577 are required for this effect further supports this notion. Maximum kinase activity of FAK requires phosphorylation of tyrosines 576 and 577 (34). Nevertheless, it is unlikely that increased kinase activity of FAK directly contributes to enhanced interaction with Cas and/or distribution of the complex to cytoskeletal fractions, since complexes composed of catalytically inactive FAK demonstrate solubility/stability characteristics similar to wild-type complexes. The possibility remains that increased kinase activity of endogenous FAK could effect cellular changes, or phosphorylation of transfected FAK and/or Cas, that alter their subcellular distribution and/or ability to interact.

How then does activation of Src and/or FAK enhance the
association of FAK-Cas complexes with the cytoskeleton? One possibility is that tyrosine phosphorylation serves to stabilize pre-existing interactions or to promote new interactions by creating SH2 binding sites or altering protein conformation. For example, phosphorylation of FAK tyrosines 576 and 577 could lead to conformational changes that allow enhanced interaction with Cas, perhaps via interaction with the catalytic domain, which stabilizes pre-existing interactions. It is also possible that phosphorylation of these tyrosines promotes association with other proteins that indirectly stabilize FAK association with Cas or other cytoskeletal components. There is likely to be a close interdependence among proteins that contribute to cytoskeletal structure and proteins involved in classical signaling cascades, as evidenced by the fact that normal activation of FAK as well as tyrosine phosphorylation of other focal adhesion components including Cas is abrogated by disruption of actin filaments by cytochalasin D (4, 63).

The interaction of Src family SH2 domains with FAK's autophosphorylation site (Tyr397) and Cas is well established (31–34, 39–43). Recently, Src's SH2 domain was demonstrated to bind to a proline-rich site on Cas, spanning residues 733–741, near the C terminus (43). Also, we recently pulled the SH2 domains of both Src and Fyn from a two-hybrid screen using a Cas polypeptide as bait.2 This raises the possibility that Src could stabilize the FAK-Cas interaction by forming a bridge between FAK and Cas, interacting simultaneously with each protein via its SH2 and SH3 domains, respectively. Our data argues against this hypothesis, however, since the Src SH3 and SH2 domains are not required for enhanced complex formation. Moreover, FAK tyrosine 397 is not required for increased FAK-Cas complex formation in RIPA-soluble fractions.

Tyrosine phosphorylation of Cas does not appear to contribute to increased association of the FAK-Cas complex with the cytoskeleton or increased affinity for FAK, since nearly all detectable hyperphosphorylated Cas is extracted in nonionic detergent-soluble fractions. One possible explanation for this finding is that Cas is more accessible to phosphorylation by Src when not tightly associated with the cytoskeleton. On the other hand, it is possible that association between tyrosine-phosphorylated Cas and FAK is inherently unstable in nonionic detergents, such that Cas would be effectively stripped away from FAK, which is tightly associated with the cytoskeleton. This would explain why FAK-Cas complexes could not be detected in cytoskeletal fractions after extended exposure to nonionic detergents.

The identification of FAK-Cas complexes in the absence of cell adhesion suggests that pre-existing FAK-Cas complexes may serve to nucleate higher order signaling complexes upon activation, tyrosine phosphorylation, and recruitment of signaling and/or structural proteins, implicating this complex in an early step of the signaling cascade. Disruption of the normal tyrosine phosphorylation status of such complexes by oncocogenic transformation could serve as a mechanism for promoting inappropriate assembly of an active signaling complex. Consistent with this idea, Src transformation results in increased association of Cas with FAK in RIPA buffers even in detached cells, suggesting increased affinity of FAK-Cas interactions or maintenance of a less soluble, higher order signaling complex found normally under adherent conditions.

Several lines of evidence suggest that FAK-Cas complexes exist in two discrete forms: one tightly associated with cytoskeletal structures, presumably focal adhesions, and a second that exists independent of focal adhesion formation and thus is only loosely associated with or excluded from the cytoskeleton. First, coimmunoprecipitation of Cas with FAK under different lysis conditions displays differential regulation by cell adhesion and Src transformation. Second, qualitative differences in co-precipitating Cas are observed under each condition. In normal fibroblasts, FAK-associated Cas consists predominantly of a single band migrating near 125 kDa when assayed in Nonidet P-40 buffer, whereas two bands of roughly equal intensity migrating at 125 and 120 kDa are coprecipitated in RIPA lysates. In Src-transformed fibroblasts, FAK-associated Cas migrates as a 125-kDa band and a slower migrating smear when assayed in Nonidet P-40 buffer, whereas a single band migrating at roughly 125 kDa coprecipitates in RIPA lysates.

This supports the notion that FAK-Cas complexes from different cellular fractions consist of differentially modified proteins. It is likely that the smear detected by anti-Cas antibodies in FAK immune complexes formed in Nonidet P-40 lysates represents tyrosine-phosphorylated Cas, based on its co-migration with phosphotyrosine detected in Cas immunoprecipitates. The modifications giving rise to the Cas species migrating at 120 and 125 kDa are not known, but they probably do not reflect gross differences in phosphotyrosine content, since we detected only trace amounts of phosphotyrosine in either band isolated from untransformed cells. Furthermore, Sakai et al. (18) have observed similar Cas bands in rat 3Y1 fibroblasts and demonstrated that their electrophoretic mobility characteristics were not affected by protein phosphatases.

Our results demonstrate that Cas associates in vivo with a second proline-rich site (PR-2) distinct from the site initially identified by two-hybrid analysis (PR-1). Despite the fact that PR-2 mediates binding to only a minor fraction of the total coprecipitating Cas, complex formation mediated by this site is regulated by activated Src in a manner similar to that mediated by PR-1. Less efficient binding of Cas to PR-2 could be due to competition by other proteins for this region. The sequence between 869 and 879 contains three overlapping PXPF motifs, and several other proteins have been shown to bind to this region including the Cas family members Efs/Sin (17, 52, 53) and human enhancer of filamentation 1 (HEF1) (64), the p85 subunit of PI3 kinase (65), and the recently described GTPase regulator associated with FAK (Graf) (66).

In summary, FAK-Cas complex levels are elevated in cytoskeletal fractions, most likely focal adhesions, under conditions that elevate the phosphotyrosine content of both proteins, reinforcing the concept that Cas is involved in FAK signaling. Moreover, the observation that FAK-Cas complexes are regulated by mechanisms involving Src kinase activity and FAK tyrosine phosphorylation adds to accumulating evidence for a close interplay among FAK, Cas, and Src in transducing signals initiated by diverse stimuli. This study provides groundwork for future studies to assess the significance of FAK-Cas complex formation and subcellular localization in regulating FAK and Cas signaling functions.

Acknowledgments—We thank Samyukta Reddy for excellent technical assistance, Xiaoe Zhang for help with the two-hybrid experiments, and Seth Grant for providing the neuronal e-Src cDNA.

REFERENCES
1. Schaller, M. D., Borgman, C. A., Cobb, B. S., Vines, R. B., Reynolds, A. B., and Parsons, J. T. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 5192–5196
2. Hanks, S. K., Calalb, M. B., Harper, M. C., and Patel, S. K. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 8487–8491
3. Guan, J. L., and Shaloway, D. D. (1992) Nature 358, 690–692
4. Burridge, K., and Krieg, T. (1992) J. Biol. Chem. 267, 19031–19034
5. Kornberg, L., Earp, H. S., Parsons, J. T., Schaller, M., and Juliano, R. L. (1992) J. Biol. Chem. 267, 23439–23442
6. Roeckel, D., and Krieg, T. (1994) Exp. Cell Res. 211, 42–48
7. Zachary, I., Sinnett-Smith, J., and Rozengurt, E. (1992) J. Biol. Chem. 267, 19031–19034
8. Polte, T. R., Naftilan, A. J., and Hanks, S. K. (1994) J. Cell. Biochem. 55, 106–119
9. Lee-Lundberg, L. M. F., Song, X.-H., and Mathis, S. A. (1994) J. Biol. Chem. 269, 24328–24334
