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# Interaction between focal adhesion kinase and Crk-associated tyrosine kinase substrate p130$^{Cas}$

**(Fyn/integrins/signal transduction/Src homology domains/two-hybrid screen)**

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**ABSTRACT** **The focal adhesion kinase (FAK) has been implicated in integrin-mediated signaling events and in the mechanism of cell transformation by the v-Src and v-Crk oncoproteins. To gain further insight into FAK signaling pathways, we used a two-hybrid screen to identify proteins that interact with mouse FAK. The screen identified two proteins that interact with FAK via their Src homology 3 (SH3) domains: a v-Crk-associated tyrosine kinase substrate (Cas), p130$^{Cas}$, and a still uncharacterized protein, FIPSH3-2, which contains an SH3 domain closely related to that of p130$^{Cas}$. These SH3 domains bind to the same proline-rich region of FAK (APPKPSR) encompassing residues 711–717. The mouse p130$^{Cas}$ amino acid sequence was deduced from cDNA clones, revealing an overall high degree of similarity to the recently reported rat sequence. Coimmunoprecipitation experiments confirmed that p130$^{Cas}$ and FAK are associated in mouse fibroblasts. The stable interaction between p130$^{Cas}$ and FAK emerges as a likely key element in integrin-mediated signal transduction and further represents a direct molecular link between the v-Src and v-Crk oncoproteins. The Src family kinase Fyn, whose Src homology 2 (SH2) domain binds to the major FAK autophosphorylation site (tyrosine 397), was also identified in the two-hybrid screen.**

Focal adhesion kinase (FAK) is a widely-expressed nonreceptor protein tyrosine kinase found in the focal adhesion plaques of cultured cells (1, 2). Elevation of the phosphotyrosine content of FAK is a rapid response to integrin-mediated cell adhesion (2–6) and correlates with increased kinase activity (3, 7, 8). Activation of FAK appears to involve an interplay with members of the Src family of protein tyrosine kinases. FAK phosphotyrosine content and kinase activity are elevated several-fold in v-Src-transformed fibroblasts (3), where FAK is readily detected in Src immunoprecipitates (9–11). In nontransformed cells, associations between FAK and either c-Src (11) or the Src-family kinase Fyn (9) have been reported. The major FAK autophosphorylation site is tyrosine 397 (10), which is a high-affinity binding site for the Src homology 2 (SH2) domain (9, 10, 12). This interaction leads to further modification of FAK by Src, including phosphorylation of catalytic-domain tyrosines 576 and 577, which elevates FAK kinase activity toward exogenous substrates (8), and phosphorylation of tyrosine 925, which is a binding site for the Grb2 SH2 domain (11). In addition to integrin ligands and oncogenic forms of Src, several other agents stimulate increases in the phosphotyrosine content of FAK, including mitogenic neuropeptides (13–15), lysophosphatitic acid (16–18), platelet-derived growth factor (19), activated Rho (20), and v-Crk (21). Thus, FAK is a point of convergence in the actions of a variety of factors known to influence cell morphology, locomotion, growth, and differentiation. However, the precise cellular responses to FAK activation remain poorly understood.

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To further elucidate FAK signaling pathways, we employed a yeast two-hybrid screen to identify proteins that interact with mouse FAK. Such proteins are likely candidates as either upstream regulators of FAK kinase activity or downstream effectors, including substrates. Here, we report the identification of the v-Crk-associated tyrosine kinase substrate (Cas), p130$^{Cas}$, as a FAK-interacting protein.§ The interaction between FAK and p130$^{Cas}$ is a likely key step in integrin-mediated signal transduction. The FAK–p130$^{Cas}$ complex further represents a direct molecular link between the v-Src and v-Crk oncoproteins. Fyn was also identified from the screen interacting with FAK through the SH2 domain.

## MATERIALS AND METHODS

**Materials.** All plasmids, libraries, and yeast strains used in the two-hybrid screen were generously provided by Stan Hollenberg (Vollum Institute). The polyclonal antisera (330 and 331) raised against mouse FAK have been described (2, 8). FAK polyclonal antibodies C-20 and C-903 were from Santa Cruz Biotechnology. Monoclonal antibody against p130$^{Cas}$ was from Transduction Laboratories (Lexington, KY). BALB/c 3T3 mouse fibroblasts (passage 12 or less) were maintained as described (8).

**Two-Hybrid Screen.** The two-hybrid screen was carried out essentially as described by Vojtek *et al.* (22) and Hollenberg *et al.* (23). The bait plasmid, pBTM116-FAKNX, was pBTM116-ADE2 (carries *TRP1* gene as selectable marker) expressing a carboxyl-terminal truncation of mouse FAK (FAKNX; residues 1–748) in frame with the LexA DNA-binding domain. The L40 yeast strain, which carries *HIS3* and *lacZ* reporter genes, was transformed first with pBTM116-FAKNX and subsequently with a 10.5-day mouse embryo cDNA library in pVP16 (pVP16 carries the *LEU2* gene as a selectable marker). His$^+$ colonies were selected after 2–4 days of growth on plates lacking histidine and then individually assayed for β-galactosidase activity by filter assay. A total of 300 His$^+$/lacZ$^+$ colonies were grown for 2 days in medium containing tryptophan but lacking leucine to allow segregation of the pBTM116-FAKNX plasmid. Leu$^+$/Trp$^-$ isolates were then tested both for the ability to grow on plates lacking histidine and for β-galactosidase activity, thereby allowing elimination of library plasmids that permitted bait-independent transactivation of reporter genes. Leu$^+$/Trp$^-$/His$^-$/lacZ$^-$ isolates were then mated to the AMR70 yeast strain containing either a LexA–lamin-encoding plasmid, to test for nonspecific interactions, or the original LexA-FAKNX bait. Diploid colonies were again assayed for reporter-gene activation, and library cDNAs that

Abbreviations: Cas, Crk-associated tyrosine kinase substrate; FAK, focal adhesion kinase; FAKNX, carboxyl-terminal truncation of mouse FAK; FIP, FAK-interacting polypeptide; SH2, Src homology 2; SH3, Src homology 3.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. U28151).

specifically required coexpression of LexA-FAKNX for reporter-gene activation were shuttled into *Escherichia coli* and sequenced.

**Isolation and Sequencing of Full-Length Mouse p130$^{Cas}$.** cDNA clones encompassing the entire coding sequence of p130$^{Cas}$ were obtained by screening two phage libraries: a λgt10 8.5-day mouse embryo library (gift from B. L. M. Hogan; Vanderbilt University) and a λgt11 11.5-day mouse embryo library (Clontech). Nucleotide sequences were determined from both strands from several overlapping clones to obtain the composite sequence.

**Site-Directed Mutagenesis.** FAKNX prolines 712 and 715 were changed to alanines by site-directed mutagenesis using the Muta-Gene Phagemid *in vitro* mutagenesis kit (Bio-Rad) and oligonucleotide 5′-GAAGCAGCACCCAAGGCCAG-CAG-3′. The mutations were confirmed by DNA sequencing. Mutation of FAK tyrosine 397 to phenylalanine was described (8).

**Coimmunoprecipitation of FAK and p130$^{Cas}$.** Subconfluent cultures of BALB/c 3T3 mouse fibroblasts were lysed in no salt NP40 buffer (1% Nonidet P-40/50 mM Tris·HCl, pH 7.4/50 mM NaF/1% aprotinin/0.1 mM $Na_3VO_4$), 0.5% SDS RIPA buffer (50 mM Tris·HCl, pH 7.4/150 mM NaCl/5 mM EDTA/1% Nonidet P-40/1% sodium deoxycholate/0.5% SDS/50 mM NaF/1% aprotinin/0.1 mM $Na_3VO_4$), or 0.05% SDS RIPA buffer [0.5% SDS RIPA buffer containing reduced amounts of sodium deoxycholate (0.5%) and SDS (0.05%)]. Immunoprecipitations were carried out by using various polyclonal antibodies against FAK (C-20, C-903, or 330), essentially as described (2, 14).

For immunoblotting, proteins in the immunoprecipitates were fractionated by SDS/7% PAGE and transferred to Immobilon [polyvinylidene fluoride (PVDF)] membrane (Millipore). To detect p130$^{Cas}$, blots were probed with 0.25 μg of anti-p130$^{Cas}$ monoclonal antibody per ml, followed by 10 μg of rabbit anti-mouse IgG per ml and detection with $^{125}$I-labeled protein A, essentially as described (2). Identical blots were probed with anti-FAK polyclonal antibody 331 (2).

## RESULTS

**Two-Hybrid Screen Identifies p130$^{Cas}$ as a FAK-Interacting Protein: Isolation of cDNAs Encoding Full-Length Mouse p130$^{Cas}$.** Full-length FAK was found unsuitable as a "bait" for the two-hybrid screen as it promoted "prey"-independent activation of reporter genes. This problem was alleviated, however, when a truncated version of mouse FAK (FAKNX; Fig. 1) was tested (data not shown). FAKNX was expressed as a fusion protein with the LexA DNA-binding domain in the reporter strain L40 and used to test for interactions with a library of mouse embryo cDNA-encoded polypeptides fused to the transcriptional activation domain of VP16. From about 20 × 10$^6$ independent library transformants, 40 FAK-interacting polypeptide (FIP) cDNAs that promoted FAK-dependent transactivation of two independent reporter genes (*HIS3* and *lacZ*) were ultimately isolated and sequenced (see *Materials and Methods*).

Fourteen of the FIP cDNAs (7 distinct clones) encoded an SH3 domain identical to that from rat p130$^{Cas}$ (Fig. 2*A*). The relatively short SH3-flanking regions of these FIPs indicated that the SH3 domain is mediating the interactions with FAK. p130$^{Cas}$ is a recently described (24) protein implicated in cell transformation by both v-Crk and v-Src, as its phosphotyrosine content is greatly elevated in cells transformed by either of these oncoproteins (21, 24). In addition to an amino-terminal SH3 domain, a notable feature of p130$^{Cas}$ is a series of nine YDXP motifs which, upon tyrosine phosphorylation, could potentially serve as binding sites for the Crk SH2 domain. Two forms (short and long) of rat p130$^{Cas}$ which apparently arise from alternative splicing near the 5′ end of the cDNA, immediately upstream of the SH3 domain, have been reported (24). Of the seven mouse p130$^{Cas}$ FIPs, five represent the short form described for rat p130$^{Cas}$ (designated Cas$_a$ in Fig. 2), while the other two encode a unique amino-terminal region (Cas$_b$) that differs from the rat long form (Fig. 2*B*). We isolated and sequenced cDNAs encoding the full-length mouse p130$^{Cas}$ proteins and determined that they are ≈97% identical to the reported rat sequence (short form) over the entire 874 amino acids.

**A Second SH3 Domain, Closely Related to That of p130$^{Cas}$, Also Interacts with FAK.** Two additional FIP cDNAs (representing the same clone) encode a second SH3 domain (designated FIPSH3-2) that is closely related to the SH3 domain of p130$^{Cas}$ (Fig. 2*B*). Aside from the SH3 domain, however, FIPSH3-2 does not apparently resemble p130$^{Cas}$.

Another cDNA obtained from the screen encoded the Fyn SH3 domain, together with the adjacent SH2 domain, but in this case it appears to be the SH2 domain that mediates the interaction with FAK (see below). No other cDNAs encoding SH3 domains were obtained from the two-hybrid screen, indicating that the FAKNX interactions with the p130$^{Cas}$ and FIPSH3-2 SH3 domains are quite specific.

**Identification of the SH3 Binding Site on FAK.** FAKNX contains one proline-rich region, APPKPSR, encompassing residues 711–717 (Fig. 1), which closely matches the consensus (XPPXPXR) for class II SH3 ligands (25). To determine if this region is responsible for the interactions with the SH3 domains from p130$^{Cas}$ and FIPSH3-2, prolines 712 and 715 were mutated to alanines, and tested for interaction with the SH3 domains by using the two-hybrid system. As shown in Fig. 3 *A–D*, the mutations resulted in loss of *HIS3* reporter gene transactivation when coexpressed with either p130$^{Cas}$ or FIPSH3-2 SH3 domain–VP16 fusions. Thus the aa 711–717 proline-rich region of FAK is required for the interaction with these SH3 domains, and it is highly likely that the interactions are direct.

**p130$^{Cas}$ Associates with FAK in Mammalian Cells.** The identification of p130$^{Cas}$ as a FAK-interacting protein in the two-hybrid system indicated that these two proteins may also interact in mammalian cells. To obtain evidence for this, coimmunoprecipitation experiments were carried out with lysates from BALB/c 3T3 mouse fibroblasts. Immunoprecipitates were formed by using three different polyclonal antibodies against FAK (C-903, C-20, or 330), and the presence of p130$^{Cas}$ in the complex was determined by immunoblot anal-

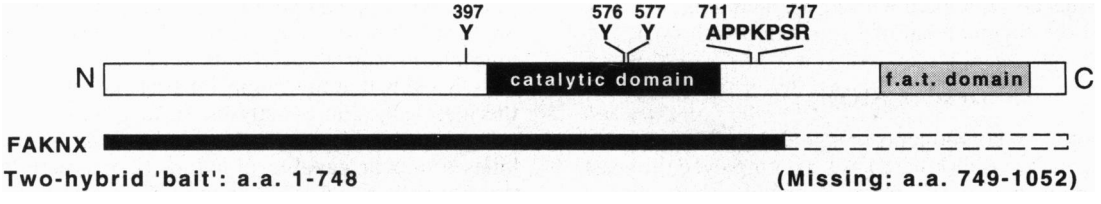


FIG. 1. Two-hybrid bait. The region of mouse FAK protein (FAKNX) used in the two-hybrid screen is indicated by the bar under diagram of full length FAK. Positions of the kinase catalytic domain, three major tyrosine phosphorylation sites (397, 576, and 577), the Src homology 3 (SH3) binding site (APPKPSR; see text), and the focal adhesion targeting (f.a.t.) domain are shown.

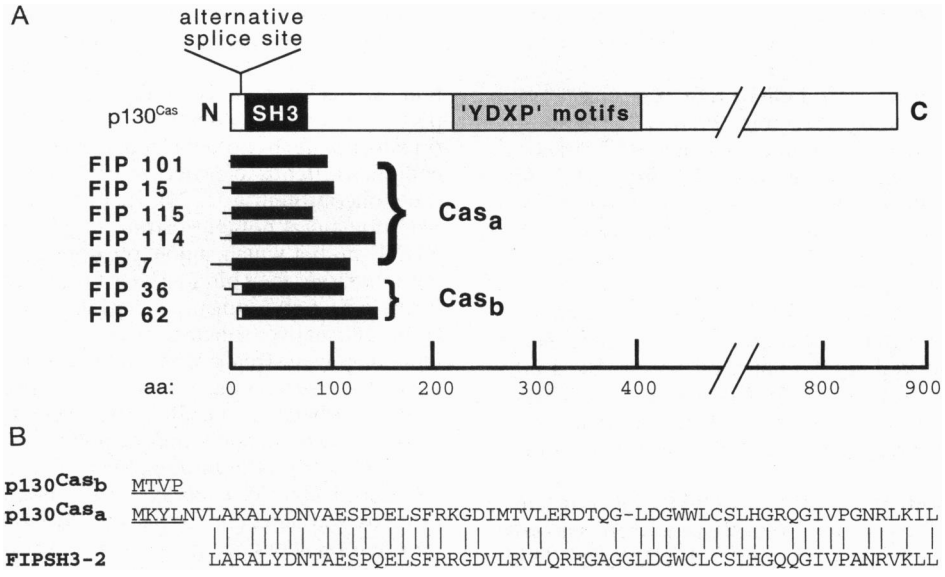


FIG. 2. SH3 domains identified in the two-hybrid screen. (*A*) Alignment of p130$^{Cas}$ FIPs with respect to their positions along the full-length protein. Bars represent coding sequences and thin lines represent 5′ untranslated regions. FIP 36 and FIP 62 contain a distinct sequence upstream of the SH3 domain which gives rise to an alternative amino terminus (open regions of bars). (*B*) Sequence alignment of SH3 domains of mouse p130$^{Cas}$ and FIPSH3-2. Also shown are the alternative amino-terminal sequences of the mouse p130$^{Cas}$ clones (Cas$_a$ and Cas$_b$).

ysis using a monoclonal antibody against p130$^{Cas}$. As shown in Fig. 4, p130$^{Cas}$ was clearly detected in the immunoprecipitates prepared with each FAK antibody. The amount of coprecipitating p130$^{Cas}$ was greatest under milder lysis conditions (no salt NP40 buffer or 0.05% SDS RIPA buffer) and greatly reduced when lysis was in the higher stringency 0.5% SDS RIPA buffer. p130$^{Cas}$ was not detected in immunoprecipitates formed when using normal rabbit IgG. These results indicate that FAK and p130$^{Cas}$ can associate in mammalian cells.

**Fyn Also Interacts with FAK Through Its SH2 Domain.** Among the other FIPs isolated, most notable were five cDNAs (two distinct clones) that encoded regions of Fyn (nonthymic form). The Fyn FIPs overlapped in the region that encodes the SH2 domain, with one clone also encoding the entire SH3 domain. This suggested that the LexA–FAKNX fusion was enzymatically active such that FAK tyrosine 397 (the autophosphorylation site) was becoming phosphorylated to provide a docking site for the Fyn SH2 domain. This was confirmed by coexpressing a mutation of LexA–FAKNX (F397) in which tyrosine 397 is changed to phenylalanine in the two-hybrid system along with either of the two VP16–Fyn FIP fusions and observing failure of the yeast to grow on plates lacking histidine (Fig. 3 *E* and *F*). The failure of the Fyn FIP containing the SH3 domain to interact with the F397 mutation of FAKNX is a further indication of the binding specificity of FAKNX for the SH3 domains of p130$^{Cas}$ and FIPSH3-2.

No other SH2 domains, including those from other Src-family kinases, were obtained in the two-hybrid screen. This probably reflects their poor representation in the 10.5-day mouse embryo library used in the screen. We have tested a variety of SH2 domains from other mouse Src-family kinases for their ability to bind FAKNX in the two-hybrid system. All SH2 domains tested interacted with FAK, including Src, Yes, Fgr, Lck, and the thymic form of Fyn (data not shown).

## DISCUSSION

To identify potential components of signal transduction pathways involving the tyrosine kinase FAK, we employed the yeast two-hybrid screen to identify proteins encoded by a mouse embryo cDNA library that interact with mouse FAK. Among the proteins that emerged from the screen were the v-Crk-associated tyrosine kinase substrate p130$^{Cas}$ and a second protein (given the preliminary designation FIPSH3-2) that contains an SH3 domain closely related to that of p130$^{Cas}$. In the two-hybrid system, p130$^{Cas}$ and FIPSH3-2 appear to interact with FAK via their SH3 domains binding to a proline-rich domain (APPKPSR) encompassing FAK residues 711–717. Our coimmunoprecipitation experiments with lysates from mouse BALB/c 3T3 fibroblasts confirm that FAK and p130$^{Cas}$ are also found associated in mammalian cells.

Since FAK is a tyrosine kinase and p130$^{Cas}$ is known as a tyrosine kinase substrate, the observation of a physical association between these two proteins engenders speculation that FAK may contribute to the tyrosine phosphorylation of p130$^{Cas}$ *in vivo*. Indeed, much indirect evidence has already been reported in support of this idea. For example, a monoclonal antibody that recognizes tyrosine-phosphorylated p130$^{Cas}$ prominently stains focal adhesions (26), placing p130$^{Cas}$ and FAK in the same cellular location. In addition, elevation of the phosphotyrosine content of p130$^{Cas}$ occurs under many of the same circumstances known to activate FAK, including transformation by v-Src and v-Crk (21, 24), plating cells onto fibronectin (26), and exposure of cells to bombesin, vasopressin, or endothelin (13), lysophosphatitic acid (18), or platelet-derived growth factor (19). It will be of interest to determine if any of these cellular stimuli also promote the association between FAK and p130$^{Cas}$.

Consistent with previous reports (24), we have observed a tyrosine kinase activity in immune complexes formed when using antibody against p130$^{Cas}$. When FAK and p130$^{Cas}$ immunoprecipitates are mixed prior to the kinase assay, a significant increase in the level of p130$^{Cas}$ phosphorylation is observed (unpublished results). This result is consistent with the idea that p130$^{Cas}$ serves as a substrate for FAK. However, since FAK appears to function in concert with associated Src-family kinases, it is premature to conclude that tyrosine phosphorylation of p130$^{Cas}$ is mediated solely by FAK. It is equally likely that Src-family kinases phosphorylate p130$^{Cas}$ in the focal adhesion complexes. In fact, we have observed (by using two-hybrid and *in vitro* binding assays) that Src-family kinases appear capable of interacting directly with p130$^{Cas}$ through either their SH2 or their SH3 domains (unpublished observations). Evidence that Src can phosphorylate p130$^{Cas}$ has been reported from assays of v-Src immunoprecipitates (24), and we have observed that baculovirus-expressed c-Src

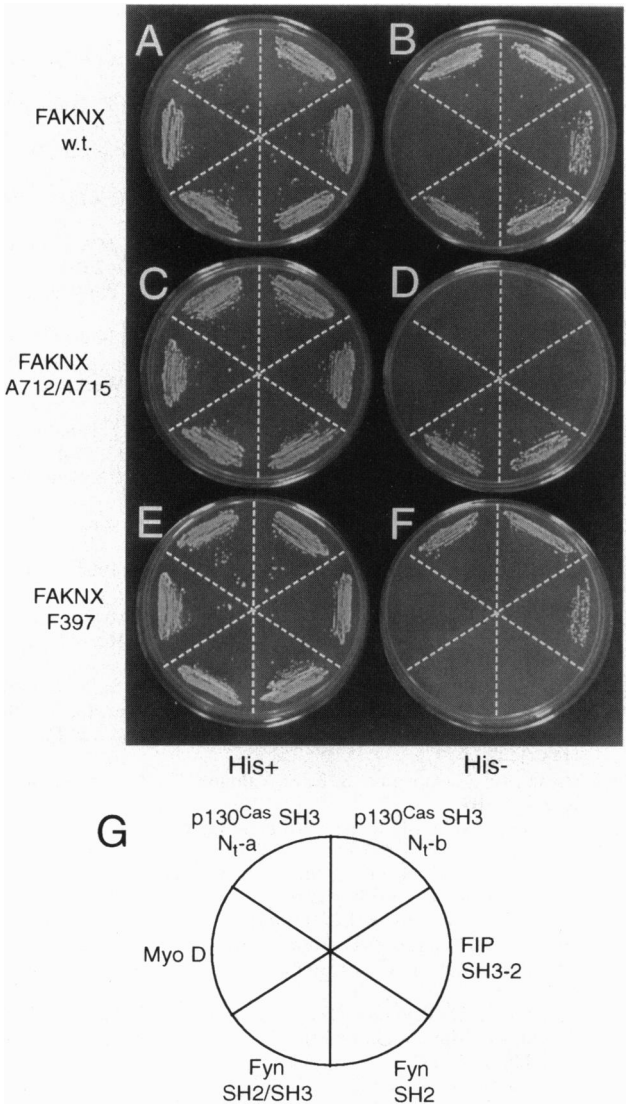


FIG. 3. Analysis of FAK residues required for two-hybrid interactions. AMR70 yeast cells expressing LexA–FAKNX$^{w.t.}$ (*A* and *B*), LexA–FAKNX$^{A712/A715}$ (*C* and *D*), or LexA–FAK$^{F397}$ (*E* and *F*) were mated to strain L40 expressing various VP16–FIP hybrids, as indicated in *G*. p130$^{Cas}$ FIPs tested included the two alternative amino-terminal sequences, $N_t$-a and $N_t$-b. Both Fyn FIPs were tested, one containing essentially just the SH2 domain, and the other containing both the SH3 and SH2 domains. MyoD served as a negative control. Diploid growth in the absence of histidine (*B*, *D*, and *F*) indicates a two-hybrid interaction driving expression of *HIS3*.

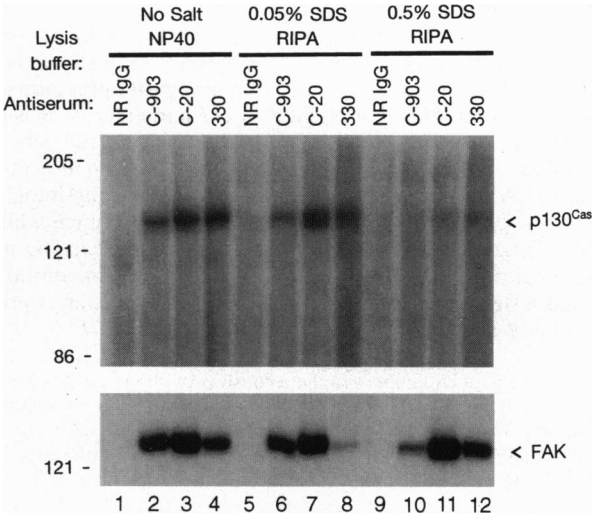


FIG. 4. Association of FAK and p130$^{Cas}$ in mammalian cells. Immunoprecipitates were formed in lysates from BALB/c 3T3 mouse fibroblasts by using one of three different polyclonal antibodies against FAK (C-903, C-20, or 330) or control IgG from normal rabbit serum (NR IgG) and analyzed by immunoblotting for the presence of either p130$^{Cas}$ (*Upper*) or FAK (*Lower*). Cells were lysed with one of three different buffers of varying stringency (see *Materials and Methods*): no salt NP40 buffer (lanes 1–4), 0.05% SDS RIPA buffer (lanes 6–8), and 0.5% SDS RIPA buffer (lanes 9–12). The positions of molecular mass standards (in kDa) are shown on the left.

efficiently phosphorylates p130$^{Cas}$ *in vitro* (unpublished data). Determining the relative contributions of FAK and Src-family kinases to the *in vivo* phosphorylation of p130$^{Cas}$ will require extensive further investigation.

Tyrosine phosphorylation of p130$^{Cas}$ is likely to be a key step in integrin-activated signaling. Tyrosine-phosphorylated p130$^{Cas}$ may serve primarily as a docking protein to recruit additional signaling proteins containing SH2 domains into the focal adhesion complex. One such protein may be c-Crk. In turn, c-Crk could promote Ras activation through an SH3-mediated association with guanine nucleotide-exchange proteins C3G and SOS (27–29). This would represent an adhesion-induced route to Ras activation distinct from the proposed FAK–Grb2 pathway (11). Another potential p130$^{Cas}$-interacting SH2 protein is the actin-binding protein tensin. The recruitment of both FAK and tensin to sites of integrin clustering has been suggested as an early step in the cascade of events involved in integrin signaling (30). Recruitment of tensin to the complex could serve to anchor newly assembled actin filaments, providing the final step in the transmembrane linkage between the extracellular matrix and the actin cytoskeleton. Indeed, work from our lab (X. Zhang and S.K.H., unpublished data) has shown that the tensin SH2 domain binds tyrosine-phosphorylated p130$^{Cas}$ but not FAK.

Our results also emphasize common features involved in the mechanisms of cell transformation by v-Src and v-Crk. Previous studies have shown that morphological transformation by both oncoproteins is strongly correlated with elevation of the phosphotyrosine content of a common set of proteins predominantly localized in focal adhesions, including FAK (21) and p130$^{Cas}$ (21, 24). Furthermore transformation by v-Crk is enhanced by overexpression of c-Src (31). The interaction between FAK and p130$^{Cas}$ provides a direct molecular link between v-Src and v-Crk. In v-Src-transformed cells, enhanced phosphorylation of p130$^{Cas}$ could in part be mediated by FAK, whose kinase activity appears elevated after phosphorylation by v-Src (8); however, direct phosphorylation of p130$^{Cas}$ by v-Src is also likely to be involved. In v-Crk-transformed cells, increased tyrosine phosphorylation of p130$^{Cas}$ could be due primarily to the SH2-mediated interaction with v-Crk, which would protect p130$^{Cas}$ from the action of tyrosine phosphatases (32). In either case, the constitutive tyrosine phosphorylation of p130$^{Cas}$ is viewed as a key event in morphological transformation. Paxillin, another potential FAK substrate (33) and v-Crk-interacting protein (34), could similarly be involved.

FAK interaction with other SH3-containing proteins may contribute to signaling responses. For example, another proline-rich region of FAK, corresponding to mouse residues 875–880 (not included in our FAKNX two-hybrid bait), has recently been suggested to interact with the SH3 domain of the p85 subunit of 1-phosphatidylinositol 3-kinase in thrombin-stimulated platelets (35).

A third protein identified in the two-hybrid screen is the Src-family tyrosine kinase, Fyn, which interacts with FAK through its SH2 domain binding to the major FAK autophosphorylation site, tyrosine 397. Our identification of Fyn as a

FIP is a further indication that Fyn acts as an upstream activator of FAK's signaling function. The Fyn–FAK interaction characterized in the two-hybrid system is consistent with previous observations demonstrating coimmunoprecipitation of these two proteins from lysates of chicken embryo cells (9) and the recognition of FAK tyrosine 397 as the site of interaction with the Src SH2 domain (9, 10, 12). An indication that the FAK–Fyn interaction may have primary physiological relevance comes from a study of *Fyn* knockout mice which, unlike *Src* and *Yes* knockouts, are impaired in learning and long-term potentiation (36). Specifically in the *Fyn* mutants, there is a significant reduction in the phosphotyrosine content and kinase activity of FAK in forebrain tissue (37).

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