v-Src SH3-enhanced Interaction with Focal Adhesion Kinase at β_1 Integrin-containing Invadopodia Promotes Cell Invasion*

Received for publication, December 23, 2001,
and in revised form, January 30, 2002
Published, JBC Papers in Press, February 11, 2002,
DOI 10.1074/jbc.C100760200

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In viral Src (v-Src)-transformed cells, focal adhesion kinase (FAK) associates with v-Src by combined v-Src SH2 and gain-of-function v-Src SH3 domain binding to FAK. Here we assess the significance of the Arg-95 to Trp gain-of-function mutation in the v-Src SH3 domain through comparisons of Src−/− fibroblasts transformed with either Prague C v-Src or a point mutant (v-Src-RT) containing a normal (Arg-95) SH3 domain. Both v-Src isoforms exhibited equivalent kinase activity, enhanced Src−/− cell motility, and stimulated cell growth in both low serum and soft agar. The stability of a v-Src·FAK signaling complex and FAK phosphorylation at Tyr-861 and Tyr-925 were reduced in v-Src-RT- compared with v-Src-transformed cells. v-Src but not v-Src-RT promoted Src−/− cell invasion through a reconstituted Matrigel basement membrane barrier and v-Src co-localized with FAK and β_1 integrin at invadopodia. In contrast, v-Src-RT exhibited a partial perinuclear and focal contact distribution in Src−/− cells. Adenovirus-mediated FAK overexpression promoted v-Src-RT recruitment to invadopodia, the formation of a v-Src-RT·FAK signaling complex, and reversed the v-Src-RT invasion deficit. Adenovirus-mediated inhibition of FAK blocked v-Src-stimulated cell invasion. These studies establish that gain-of-function v-Src SH3 targeting interactions with FAK at β_1 integrin-containing invadopodia act to stabilize a v-Src·FAK signaling complex promoting cell invasion.

c-Src is a modular protein-tyrosine kinase (PTK)[1] consisting of a unique N-terminal segment, a Src homology (SH) 3 domain, a SH2 domain, and a kinase domain. Highly transforming strains of Rous sarcoma virus contain v-Src isoforms with C-terminal truncations (1) and activating mutations within the kinase or SH3 domains (2, 3). Importantly, v-Src SH3 domain substitutions (Trp at Arg-95 and Ile at Thr-96) introduced into normal c-Src, converted c-Src into a transforming protein (4).

Crystal structure analyses revealed that the v-Src SH3 domain mutations were within the RT loop and were near the surface ligand binding groove (5). Substitution of murine c-Src RT loop residues (Trp at Arg-97 and Ile at Thr-98) did not disrupt the binding of normal c-Src SH3 targets such as p130Cas (6). Instead, RT loop residue changes at Trp-97 promoted the binding of proteins such as connexin 43 and FAK to the c-Src SH3 domain (6, 7). In FAK, the v-Src SH3 domain binding sites were mapped to three proline-rich motifs conforming to a P*XXPXXϕ* consensus where *ϕ* is a hydrophobic residue (6). This extended P*XXPXXϕ* motif differs from c-Src class I or class II SH3 binding motifs (5) and is conserved in other v-Src SH3 domain-binding proteins (7, 8). Since the v-Src SH3 domain binds to additional targets compared with the c-Src SH3 domain, the RT loop substitutions can be considered gain-of-function mutations.

FAK is a non-receptor PTK that associates with transmembrane integrins to promote matrix-initiated signaling. In normal cells, a transient c-Src·FAK PTK signaling complex is formed by c-Src SH2 binding to FAK in an integrin-stimulated manner (9, 10). This PTK complex is linked to increased cell motility, cell cycle progression, and cell survival signals (for reviews, see Refs. 11–14). FAK was first identified as a v-Src substrate (15), and combined v-Src SH2 and SH3 binding to FAK stabilizes an integrin-independent signaling complex (6, 9, 16). Within this complex, v-Src promotes elevated FAK kinase activity, the SH2-mediated binding of Grb2 to phosphorylated FAK Tyr-925, and the enhanced tyrosine phosphorylation of FAK-associated proteins such as p130Cas and paxillin (9). Although p130Cas-null cells are refractory to transformation by activated Src (17), the role of the FAK in a v-Src signaling complex remains undefined. Here we show that a v-Src SH3 domain-stabilized signaling complex with FAK is localized to β_1 integrin-containing invadopodia and promotes cell invasion.

EXPERIMENTAL PROCEDURES

Cells, DNA Constructs, and Retroviruses—Large T immortalized Src−/− fibroblasts were maintained as described (10). Mutagenesis was used to change the codon for Trp-95 to Arg-95 within v-Src Prague C to create v-Src-RT and this construct was subcloned into pRetroOff (CLONTECH, Palo Alto, CA). 293 Phoenix-Eco packaging cells (G. Nolan, Stanford) were transfected, and the retrovirus-containing media was collected after 72 h. Src−/− cells were infected for 24 h and selected for growth in 3 μg/ml puromycin. Pooled populations of cells were used in all assays.

Antibodies, Immunoprecipitation, and Blotting—Anti-phosphotyrosine (Tyr(p), 4G10) monoclonal antibody (mAb), avian-specific mAb to v-Src (EC10), and anti-paxillin mAb were from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-hemagglutinin (HA) epitope tag mAb (16B12) was from Covance Research (Berkeley, CA). Anti-c-Src mAb (2–17), anti-HA epitope tag mAb (12CA5), and affinity-purified polyclonal antibodies to the N- and C-terminal domains of FAK were used (18). Polyclonal antibodies to c-Src (Src-2) were from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal phosphospecific antibodies to FAK (Tyr(p)-397, Tyr(p)-861, and Tyr(p)-925) were from BIOSOURCE

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[1] The abbreviations used are: PTK, protein-tyrosine kinase; FAK, focal adhesion kinase; FN, fibronectin; FRNK, FAK-related non-kinase; HA, hemagglutinin; IP, immunoprecipitation; IVK, *in vitro* kinase; m.o.i., multiplicity of infection; mAb, monoclonal antibody; SH, Src homology; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine B isothiocyanate; AMCA, 7-amino-4-methylcoumarin-3-acetic acid; CT, C-terminal.

International (Hopkinton, MA). Immunoprecipitations (IPs), blotting, and sequential polyvinylidene fluoride membrane (Millipore, Bedford, MA) reprobing were performed as described (18).

Cell Growth and Soft Agar Assays—1×10^4 serum-starved cells were plated onto fibronectin (FN)-coated (5 μg/ml) in media containing 0.5% or 10% serum. Every 24 h cells were counted. For measurement of anchorage-independent growth, 4×10^4 cells suspended in 0.3% agar were seeded onto a solidified base of growth medium containing 0.6% agar and overlaid with 1 ml of growth medium. Ordinary one-way analysis of variance the Tukey-Kramer multiple comparisons t test were used to determine significance.

Migration and Invasion Assays—For haptotaxis assays, the membrane underside of Millicell (12-mm diameter with 8-μm pores) chambers (Millipore) was coated with 10 μg/ml FN (Sigma). For invasion assays, diluted (30 μg in 100 μl of H_2O) growth factor-reduced Matrigel (BD PharMingen) was used as described (19).

In Vitro Kinase (IVK) Assays—Assays were performed on immunoisolated proteins at 32 °C for 15 min as described (19).

Immunolocalization—Cells plated onto FN-coated slides in the presence of serum for 2 h were incubated with a mixture of 5 μg/ml anti-β_1 integrin 9EG7 rat mAb (BD PharMingen) and 280 μg/ml ChromPure donkey IgG (Jackson ImmunoResearch, West Grove, PA) for 1 h; fixed in 3.8% paraformaldehyde, PBS; and permeabilized in cold acetone. Slides were blocked (Vector Laboratories, San Francisco, CA) and incubated overnight at 4 °C with 10 μg/ml mouse anti-v-Src (EC10) 4 μg/ml rabbit anti-FAK and 140 μg/ml donkey IgG. After washing in PBS, slides were incubated (40 min) with FITC-conjugated donkey anti-mouse, TRITC-conjugated donkey anti-rat, and biotinylated anti-rabbit. Cells were washed in PBS, incubated (15 min) with 1 μg/ml AMCA-avidin D conjugate (Vector Laboratories), washed in PBS, mounted, and visualized using a Zeiss Axiophot epifluorescence microscope. Staining of invadopodia on the Millicell membrane lower surface was performed as described previously (20).

Adenovirus Production and Infection—β-Galactosidase- (LacZ) and FRNK-expressing adenoviruses were used as described (19). HA-tagged murine FAK was subcloned into pShuttle-CMV to prepare recombinant E1-deleted adenovirus (Stratagene). Src−/− cells were infected at a matched multiplicity of infection (m.o.i.) between 30 and 100 plaque-forming units/cell and analyzed after 2 days.

RESULTS AND DISCUSSION

Comparisons of v-Src Isoforms with Either Trp-95 or Arg-95 Residues in the SH3 Domain—To test the biological significance of gain-of-function v-Src SH3 binding interactions, comparisons were made between Src−/− fibroblasts stably reconstituted with murine c-Src, Prague C v-Src with a Trp-95-containing SH3 domain, and Prague C v-Src-RT with a normal c-Src-like Arg-95-containing SH3 domain (Fig. 1*A*). Pooled Src−/− cell populations equally expressed either c-Src, v-Src, or v-Src-RT (Fig. 1*A*). Analyses of Src-associated IVK activity from serum-starved cells revealed that both v-Src and v-Src-RT possessed higher activity compared with normal c-Src (Fig. 1*B*).

v-Src and v-Src-RT Transform Src−/− Cells—Cell proliferation analyses were performed. When plated onto FN in 0.5% serum, Src−/− cells did not proliferate and c-Src-reconstituted cells underwent one round of cell division within 6 days, whereas v-Src and v-Src-RT readily proliferated and formed foci after 6 days (Fig. 1*C*). Src−/− and c-Src-reconstituted cells showed increased growth in 10% serum-containing media (Fig. 1*D*). However, v-Src- and v-Src-RT-expressing cells exhibited a similar growth rate in 10% serum that was not significantly different ($p > 0.05$) from cell proliferation in 0.5% serum (Fig. 1, *C* and *D*). Comparisons between v-Src- and v-Src-RT-reconstituted Src−/− cells revealed no significant growth differences ($p > 0.1$). Further, both v-Src and v-Src-RT promoted the anchorage-independent growth of Src−/− cells in soft agar (Fig. 1*E*). No differences in either the size or number of soft agar colonies were observed (data not shown). Together these results show that v-Src and v-Src-RT promote equivalent serum- and anchorage-independent Src−/− cell growth.

v-Src-RT Promotes Src−/− Cell Motility but Not Matrigel Invasion—Since Src−/− cells exhibit integrin-stimulated cell spreading and motility defects (21), FN haptotaxis motility

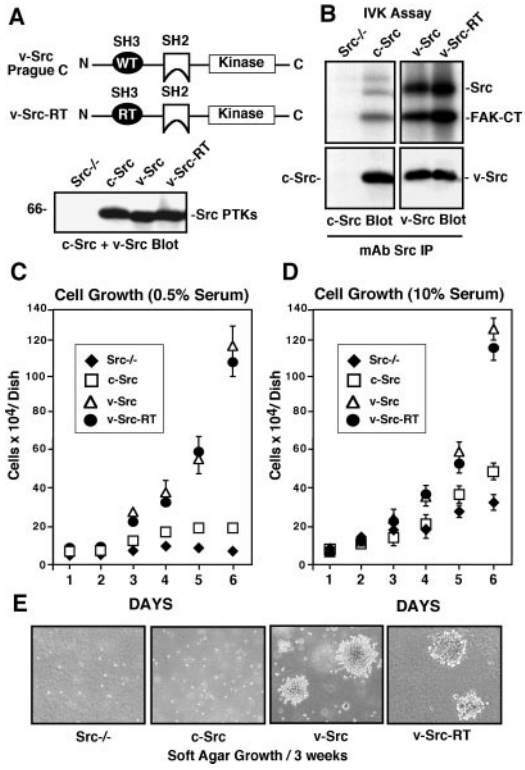

FIG. 1. **Prague C v-Src and v-Src-RT exhibit elevated *in vitro* kinase activity and promote Src−/− cell growth in low serum and soft agar.** *A*, schematic of Prague C v-Src and v-Src-RT. Src−/− fibroblasts were stably reconstituted with normal c-Src, v-Src, or v-Src-RT, and expression was verified by pan-Src blotting. *B*, Src IPs were analyzed for associated IVK activity using the GST·FAK-CT substrate (*top panels*). c-Src or v-Src present in the IVK IPs was detected by blotting (*lower panels*). *C* and *D*, growth of the indicated cells in 0.5% serum (*C*) or in 10% serum (*D*). Mean values ± S.D. are from two independent experiments. *Black diamonds*, Src−/−; *open squares*, Src−/− (+c-Src); *open triangles*, Src−/− (+v-Src); and *filled circles*, Src−/− (+v-Src-RT). *E*, anchorage-independent cell growth in soft agar. Images show typical fields at 60×.

assays were performed with the reconstituted Src−/− cells. Normal c-Src expression enhanced cell motility 3-fold compared with Src−/− cells (Fig. 2*A*). Both v-Src and v-Src-RT equally enhanced FN-stimulated Src−/− cell motility but at levels less than c-Src (Fig. 1*D*). This lower level of v-Src-stimulated haptotaxis is consistent with studies showing that v-Src phosphorylation of integrins inhibited *in vitro* cell migration compared with normal cells (22). Since a fully transformed cell phenotype encompasses changes in the growth, motility, and invasive property of cells, the reconstituted Src−/− cells were analyzed for the acquisition of an invasive phenotype. c-Src-reconstituted cells did not penetrate through a Matrigel basement membrane barrier (Fig. 2*B*). Src−/− cells transformed by v-Src but not v-Src-RT possessed invasive activity in 24-h assays (Fig. 2*B*). Although v-Src-RT was able to penetrate the Matrigel barrier at low numbers after 48 h (data not shown), our results support the conclusion that gain-of-function v-Src SH3 domain binding interactions promote enhanced cell invasion activity that is independent of changes in cell growth.

Reduced FAK Activation and Phosphorylation in Both Suspended and Adherent v-Src-RT-transformed Src−/− Cells—To determine whether the stability of the v-Src·FAK signaling complex was altered in v-Src-RT-expressing cells, FAK IP analyses were performed (Fig. 2*C*). In lysates from suspended cells, FAK exhibited high phosphorylation levels and strong association with v-Src compared with reduced autophosphorylation and weak association with v-Src-RT, respectively (Fig. 2*C*). To

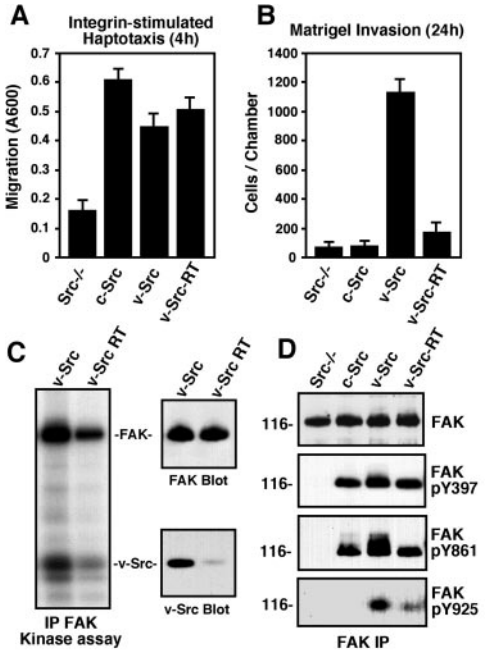

FIG. 2. **v-Src-RT promotes Src−/− cell motility but not Matrigel invasion.** *A*, haptotaxis motility assays (10 μg/ml FN) were performed. Mean values ± S.D. are from two independent experiments. *B*, cells were evaluated for invasion activity through a reconstituted Matrigel basement membrane barrier. Mean values ± S.D. are from two independent experiments. *C*, FAK IPs from cells held in suspension for 45 min were analyzed for associated IVK activity and visualized by autoradiography (*left panel*). The amount of FAK or associated v-Src present in the FAK IPs was determined by blotting. *D*, FAK IPs from serum-starved cells were sequentially analyzed by blotting with antibodies to FAK or phosphospecific FAK antibodies to Tyr-397 (*pY397*), Tyr-861, (*pY861*), and Tyr-925 (*pY925*).

analyze whether the Arg-95 reversion mutation in v-Src-RT was associated with reduced FAK phosphorylation in adherent cells, FAK IPs were analyzed with phosphospecific FAK antibodies (Fig. 2*D*). Consistent with previous studies (10), FAK phosphorylation at Tyr-397 is reduced in Src−/− cells, and this is rescued by c-Src re-expression (Fig. 2*D*). v-Src promoted increased FAK phosphorylation at Tyr-861 and Tyr-925 but not at FAK Tyr-397 compared with c-Src-expressing cells. FAK phosphorylation at Tyr-861 and Tyr-925 were reduced in v-Src-RT- compared with v-Src-transformed Src−/− cells (Fig. 2*D*), and these results are consistent with the reduced association of v-Src-RT with FAK (Fig. 2*C*).

Localization of v-Src, FAK, and β_1 Integrin to Invadopodia—To evaluate whether differences in v-Src-stimulated cell invasion and FAK phosphorylation were associated with either altered cell morphology or v-Src-RT localization, Src−/− cells expressing v-Src or v-Src-RT were triple stained for v-Src, FAK, and β_1 integrin (Fig. 3). When plated onto FN-coated slides, v-Src-expressing cells formed fine cell extensions that were enriched with v-Src (Fig. 3*A*, *arrowheads*). Staining for FAK revealed a co-localization with v-Src at cell extensions (Fig. 3*B*) and with v-Src in ventral focal contact sites (data not shown). v-Src-RT-expressing cells formed reduced numbers of long cell extensions and instead formed short pointed projections around the cell periphery (Fig. 3*A*). v-Src-RT and FAK co-localized at pointed cell projections (Fig. 3*B*); however, v-Src-RT also exhibited strong perinuclear staining that was not detected in v-Src-expressing cells (Fig. 3*A*). When plated onto FN, c-Src-reconstituted Src−/− cells exhibited a morphology similar to v-Src-RT cells, and c-Src had a cellular distribution similar to v-Src-RT (data not shown).

Previous studies have shown that Rous sarcoma virus-transformed chicken fibroblasts and human melanoma cells form β_1 integrin-enriched peripheral and ventral cell extensions termed invadopodia (23, 24). Strong co-localization of β_1 integrin with v-Src at both perimeter and ventral invadopodia sites were observed in v-Src-transformed Src−/− cells (Fig. 3*C*). β_1 integrin staining of v-Src-RT cells showed a strong perimeter distribution with minimal staining in the central or ventral region of cells (Fig. 3*C*). v-Src-RT was co-localized with β_1 integrin only in a subset of cell perimeter pointed projections. Whereas SH3 do-

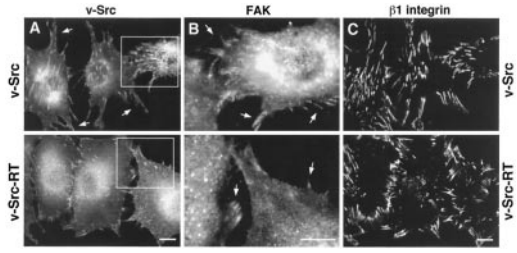

FIG. 3. **Co-localization of FAK, v-Src, and β_1 integrin at invadopodia.** Src−/− cells expressing v-Src or v-Src-RT were plated onto FN-coated slides and evaluated for FAK, v-Src, and β_1 integrin indirect immunofluorescence. *A*, FITC staining shows v-Src localization to cell extensions (*arrowheads*) and ventral focal contact site within cells. v-Src-RT exhibits perinuclear and perimeter focal contact distribution. *B*, the *boxed region* in *panel A* shows AMCA staining of FAK localized to cell extensions resembling invadopodia (*arrowheads*) within both v-Src and v-Src-RT cells. *C*, TRITC staining shows β_1 integrin co-distribution to invadopodia and ventral focal contact regions in v-Src-expressing cells. TRITC β_1 integrin staining at perimeter focal contact sites in v-Src-RT-expressing cells. The *scale bar* is 10 μm. No FITC v-Src staining was detected using control Src−/− cells.

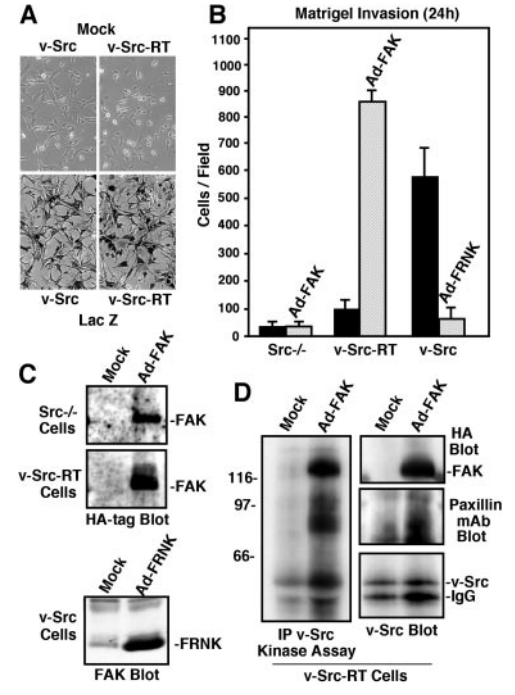

FIG. 4. **Adenoviral modulation of FAK expression or activity controls v-Src-stimulated cell invasion.** *A*, Src−/− cells expressing v-Src or v-Src-RT were infected (m.o.i. = 30) with a recombinant LacZ-expressing adenovirus. β-Galactosidase activity was measured in mock- or LacZ-infected cells. *B*, cells were infected (m.o.i. = 100) with adenoviruses expressing HA-FAK or FRNK for 24 h, switched to serum-free media for 16 h, and then analyzed in Matrigel invasion assays. Mean values ± S.D. are from two independent experiments. *C*, HA tag or FAK C-terminal antibody blotting of lysates from cells mock-treated or infected as described in *panel B*. *D*, v-Src-associated *in vitro* kinase activity was evaluated from adherent and serum-starved mock- or FAK adenovirus-infected v-Src-RT cells (*left panel*). The associated proteins in the v-Src IPs were detected by either v-Src, paxillin, or HA tag blotting. *Ad*, adenoviral.

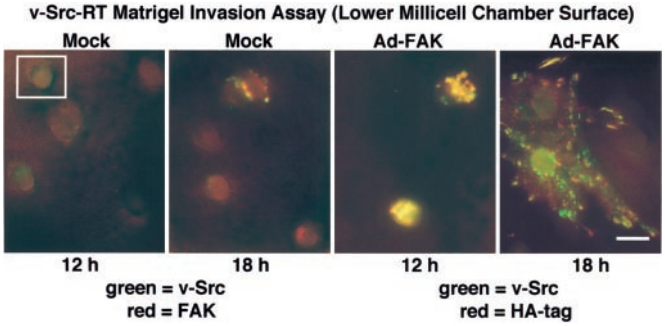

FIG. 5. **FAK overexpression promotes cell invasion and the recruitment of v-Src-RT to invadopodia.** Indirect immunofluorescence analysis of invadopodia projections through Matrigel emerging on the lower Millicell surface after 12 or 18 h. Analyses of either mock- or adenoviral FAK-infected v-Src-RT cells. Merged images show v-Src staining (*green*) and either endogenous FAK or HA-FAK staining (*red*). A representative Millicell membrane pore is *boxed*, and the *scale bar* is 8 μm. *Ad*, adenoviral.

main integrity is important for targeting both c-Src and v-Src to focal contact sites (25, 26), our studies suggest that specific gain-of-function v-Src SH3 interactions with targets such as FAK may enhance the formation of invadopodia.

Modulation of FAK Expression or Activity Alters v-Src-stimulated Cell Invasion—To assess the importance of FAK in v-Src-stimulated cell invasion and invadopodia formation, recombinant adenovirus infection was used to overexpress HA epitope-tagged FAK in either Src−/− or v-Src-RT-transformed cells (Fig. 4). Use of a recombinant adenovirus expressing β-galactosidase (LacZ) showed >90% infectivity of v-Src- and v-Src-RT-transformed Src−/− cells (Fig. 4*A*). FAK overexpression in Src−/− cells did not promote Matrigel invasion, whereas equivalent FAK overexpression in v-Src-RT-transformed Src−/− cells promoted a 9-fold increase in Matrigel invasion (Fig. 4, *B* and *C*). This level of FAK-enhanced v-Src-RT cell invasion exceeded that of v-Src-transformed Src−/− cells (Fig. 4*C*). When expressed in v-Src-RT cells, HA-FAK was phosphorylated at Tyr-397, Tyr-861, and Tyr-925 as opposed to being weakly phosphorylated only at Tyr-397 when expressed in Src−/− cells (data not shown). To determine the mechanism of increased HA-FAK phosphorylation, v-Src-RT-associated *in vitro* kinase assays were performed (Fig. 4*D*). Compared with noninfected cells, HA-FAK overexpression facilitated the formation of an active signaling complex with v-Src-RT and also promoted the co-immunoprecipitation of the integrin-associated protein paxillin with v-Src-RT (Fig. 4*D*).

Since FAK overexpression promoted increased v-Src-RT but not control Src−/− cell invasion through Matrigel, indirect immunofluorescence staining of Millicell membranes was used to evaluate whether FAK functioned to recruit v-Src-RT to invadopodia during Matrigel invasion (Fig. 5). In the absence of FAK overexpression, no v-Src-RT cell protrusions were present at 12 h, and after 18 h, partial co-localization of v-Src and endogenous FAK were detected at the low numbers of emerging invadopodia (Fig. 5). In v-Src-RT cells infected with HA-FAK adenovirus, strong co-localization of v-Src-RT and HA-FAK were detected in invadopodia projections at 12 h (Fig. 5). After 18 h, the adenovirus-infected v-Src-RT cells had fully emerged from the Millicell pores, and at this point, v-Src-RT and HA-FAK were not significantly co-localized. These results suggest that FAK activation and connections to integrins potentially through the binding of integrin-associated proteins such as paxillin (Fig. 4*D*) are important in stabilizing the localization of a v-Src-RT signaling complex targeted to invadopodia.

To support the importance of FAK in mediating v-Src-stimulated cell invasion, the FAK C-terminal domain termed FRNK, which functions as a specific inhibitor of FAK activity (11–13), was expressed in v-Src-transformed Src−/− cells by recombinant adenovirus infection (Fig. 4, *B* and *C*). FRNK potently blocked v-Src-stimulated cell invasion (Fig. 4*B*), and FRNK inhibited v-Src-stimulated endogenous FAK tyrosine phosphorylation (data not shown).

In summary, our results show that the introduction of a gain-of-function v-Src SH3 domain point mutation results in the activation of an invasion-promoting signaling pathway in Src−/− cells. This cell invasion activity was independent of changes in either v-Src-stimulated haptotaxis motility or v-Src-stimulated cell growth in low serum and soft agar. Instead this cell invasion activity was connected to the SH3-mediated stabilization of a v-Src·FAK signaling complex, increased FAK tyrosine phosphorylation, and the co-localization of v-Src with FAK and β_1 integrin at invadopodia cell projections. Our results support the hypothesis that FAK acts to promote invadopodia formation and to increase cell invasion through the recruitment of v-Src into an activated signaling complex. Since FAK functions to promote normal cytotrophoblast-mediated invasion of the uterus during placental formation (20) and there is a strong correlation between the acquisition of an invasive phenotype and FAK overexpression in human tumor cells (19, 27), future studies using v-Src-transformed cells may yield important insights into the molecular mechanisms and signals through which FAK promotes cell invasion.

Acknowledgments—We thank Philippe Soriano for the Src−/− cells and Amanda Moore for administrative assistance.

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