v-Src SH3-enhanced interaction with focal adhesion kinase at β1 integrin-containing invadopodia promotes cell invasion

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SUMMARY

In viral Src (v-Src) transformed cells, focal adhesion kinase (FAK) associates in a stable signaling complex with v-Src that is mediated 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. Notably, 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 to v-Src-transformed cells. Significantly, 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 β1 integrin at invadopodia. In contrast, v-Src-RT exhibited a partial peri-nuclear and focal contact distribution in Src-/- cells. Adenoviral-mediated FAK overexpression promoted the recruitment of v-Src-RT to invadopodia, facilitated the formation of a v-Src-RT-FAK signaling complex, and reversed the v-Src-RT invasion deficit. Adenoviral-mediated dominant-negative inhibition of FAK blocked v-Src-stimulated cell invasion. These studies establish that gain-of-function v-Src SH3 targeting interactions with FAK at β1 integrin-containing invadopodia act to stabilize a v-Src-FAK signaling complex promoting cell invasion.
c-Src is a modular protein-tyrosine kinase (PTK) 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 were found to contain v-Src isoforms with C-terminal truncations (1) and activating mutations within the kinase or SH3 domains of v-Src (2, 3). Importantly, when v-Src SH3 domain substitutions (Trp at Arg-95 and Ile at Thr-96) were introduced into normal c-Src, these changes converted c-Src into a transforming protein (4).

Crystal structure analyses revealed that the v-Src SH3 domain mutations were within the SH3 domain 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) singly or in combination 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 additional target 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 PXXPXXφ consensus where φ is a hydrophobic or proline residue (6). This extended PXXPXXφ 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 to 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 (reviewed in 11-14). However, FAK was first identified as a v-Src substrate (15) and combined v-Src SH2 and SH3 binding to FAK stabilize 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 β1 integrin-containing invadopodia and functions to promote cell invasion.
EXPERIMENTAL PROCEDURES

**Cells, DNA constructs, and retroviruses**—Large T immortalized Src-/- fibroblasts were maintained in DMEM containing 10% fetal calf serum as described (10). Quikchange mutagenesis (Stratagene, La Jolla, CA) was used to change the codon for W-95 to R-95 within v-Src Prague C using the sense primer 5’-

TACGACTACGAGTCGCGAACTGAAACGGACTTGTC-3’.

A NruI site (underlined) was used for v-Src-RT screening, the full-length construct was verified by sequencing, and subcloned into pRetroOff (Clontech, Palo Alto, CA). The murine c-Src cDNA was used as described (6). 293 Phoenix-Eco packaging cells (from G. Nolan, Stanford) were transfected and the retrovirus-containing media was collected after 72 h as described (18). Src-/- cells were infected for 24 h in media containing 5 µg/ml polybrene and selected for growth in 3 µg/ml puromycin. Pooled populations of cells were used in all assays.

**Antibodies, immunoprecipitation, and blotting**—Anti-phosphotyrosine (P.Tyr, 4G10) monoclonal antibody (mAb), avian-specific mAb to v-Src (EC10), and anti-paxillin mAb were from UBI (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 as described (18). Polyclonal antibodies to MMP-2 (C19) and c-Src (Src-2) were from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal phospho-specific antibodies to FAK (pY397, pY861, and pY925) were from BioSource International (Hopkinton, MA). Cells were lysed in a buffer containing 1% Triton X-100, 1% sodium deoxycholate and 0.1% SDS and antibody immunoprecipitations (IP) were performed as described (18). Blotting and sequential polyvinylidene fluoride (PVDF) membrane (Millipore Bedford, MA) reprobing were performed as described (18).

**Cell growth and soft agar assays**—1x10^4 serum starved cells were plated onto fibronectin (FN) coated (5 µg/ml) 60 mm dishes in media containing 0.5% or 10% serum. Every 24 h, cells were collected after trypsin treatment and counted. For measurement of anchorage-independent growth, 4x10^4 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. Colonies were scored after 3 weeks. Values were determined in triplicate. Ordinary one-way ANOVA was used to
determine significance within data groups. The Turkey-Kramer multiple comparisons \( t \) test was used to determine significance between groups.

**Migration and invasion assays** - For haptotaxis assays, the membrane underside of MilliCell (12 mm diameter with 8 \( \mu \)m pores) chambers (Millipore) was coated with 10 \( \mu \)g/ml FN (Sigma, St. Louis, MO) for 2 h at RT. For invasion assays, diluted (30 \( \mu \)g in 100 \( \mu \)l H\( _2 \)O) growth factor-reduced Matrigel (BD Biosciences) was added to the membrane topside, allowed to polymerize for 1 h at 37\( ^\circ \)C, air-dried for 16 h, and the Matrigel barrier was reconstituted with 100 \( \mu \)l DMEM for 2 h at 37\( ^\circ \)C. Serum starved cells (1 \( \times \)10\(^5 \) cells in 0.3 ml) in migration media (DMEM with 0.5% BSA) were added to the top-side chamber and the Millicell unit was placed into 24-well dishes containing 0.4 ml migration media for motility or with DMEM with 10% serum for invasion assays, respectively. After 4 h (haptotaxis) or 24 h (invasion) at 37\( ^\circ \)C, cells on the lower membrane surface were enumerated as described (19). Mean values were obtained from three chambers for each experimental point per assay.

**In vitro kinase (IVK) assays** - Assays were performed on immuno-isolated proteins at 32\( ^\circ \)C for 15 min as described (19). Briefly, \( (\gamma^{32}P) \)ATP was added in the presence (Src IVK) or absence (FAK IVK) of a Src substrate (GST-FAK-CT), labeled proteins were visualized by autoradiography, and the equal recovery of the immuno-isolated kinase was verified by blotting as described (18).

**Immunolocalization**- Cells plated onto FN coated slides in the presence of serum for 2 h, were washed in cold PBS, incubated with a mixture of 5 \( \mu \)g/ml anti-\( \beta \)1 integrin 9EG7 rat mAb (BD Biosciences, San Diego, CA) and 280 \( \mu \)g/ml ChromPure donkey IgG (Jackson ImmunoResearch, West Grove, PA) for 1 h, washed in cold PBS, fixed in 3.8% paraformaldehyde/PBS, and permeabilized in cold acetone. Slides were blocked (Vector Labs, San Francisco, CA) and incubated overnight at 4\( ^\circ \)C with 10 \( \mu \)g/ml mouse anti-v-Src (EC10, UBI), 2 \( \mu \)g/ml rabbit anti-FAK (A-17, Santa Cruz), 2 \( \mu \)g/ml rabbit anti-FAK (C-20, Santa Cruz) and 140 \( \mu \)g/ml donkey IgG. After washing in PBS, the slides were incubated (40 min) with FITC-conjugated donkey anti-mouse, TRITC-conjugated donkey anti-rat, and biotinylated antirabbit. Cells were washed in PBS, incubated (15 min) with 1 \( \mu \)g/ml AMCA-avidin D conjugate (Vector), washed in PBS, mounted in Vectashield (Vector), and images visualized using a Zeiss Axiophot epifluorescence microscope. Staining of invadopodia on the Millicell membrane lower surface was performed as described (20) using rabbit polyclonal anti-HA-tag antibodies (HA.11,
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). All viruses were propagated in 293 cells, clonally isolated, and titered using agar plaque assays as described (19). Src−/− cells were infected at a matched multiplicity of infection (MOI) between 30 and 100 plaque forming units/cell and analyzed for protein expression or invasion activity 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. 1A). Pooled Src-/- cell populations equally expressed either c-Src, v-Src, or v-Src RT (Fig. 1A). Analyses of Src-associated in vitro kinase (IVK) activity from serum-starved cells revealed that both v-Src and v-Src RT possessed higher activity compared to normal c-Src (Fig. 1B) and repeated assays did not reveal significant differences between v-Src and v-Src-RT autophosphorylation or IVK activity toward GST-FAK-CT (Fig. 1B) or acid-denatured enolase as substrates (data not shown).

v-Src and v-Src-RT Transform Src-/- cells - Cell proliferation analyses were performed to ascertain whether v-Src or v-Src-RT expression result in Src-/- fibroblast transformation (Figs. 1C and D). When plated onto FN in 0.5% serum, Src-/- cells did not proliferate, 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. 1C). Src-/- and c-Src reconstituted cells showed increased growth in 10% serum-containing media (Fig. 1D). 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 (Figs. 1C 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. 1E). 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 assays were performed with the reconstituted Src-/- cells. Normal c-Src expression enhanced cell motility 3-fold compared to Src-/- cells (Fig. 2A). Both v-Src and v-Src-RT equally enhanced FN-stimulated Src-/- cell motility, but at levels less than c-Src (Fig. 1D). Compared to c-Src-reconstituted cells, 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 to
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. Whereas c-Src readily promoted Src-/- motility, c-Src-reconstituted cells did not penetrate through a Matrigel basement membrane barrier (Fig. 2B). Notably, Src-/- cells transformed by v-Src but not v-Src-RT possessed invasive activity in 24 h assays (Fig. 2B). Although v-Src-RT are 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 immunoprecipitation (IP) analyses were performed (Fig. 2C). In lysates from suspended cells, FAK exhibited high phosphorylation levels and strong association with v-Src compared to reduced autophosphorylation and weak association with v-Src-RT, respectively (Fig. 2C). To 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 phospho-specific FAK antibodies (Fig. 2D). 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. 2D). v-Src promoted increased FAK phosphorylation at Tyr-861 and Tyr-925 but not at FAK Tyr-397 compared to c-Src-expressing cells. FAK phosphorylation at Tyr-861 and Tyr-925 were reduced in v-Src-RT compared to v-Src-transformed Src-/- cells (Fig. 2D) and these results are consistent with the reduced association of v-Src-RT with FAK (Fig. 2C). Together, these results support the conclusion that gain-of-function mutations in the v-Src SH3 domain stabilize v-Src-FAK binding and promote enhanced v-Src-mediated FAK phosphorylation.

Localization of v-Src, FAK, and β1 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 β1 integrin (Fig. 3). When plated onto FN-coated slides, v-Src-expressing cells formed fine cell extensions that were enriched with v-Src (Fig. 3A, arrowheads). Staining for FAK revealed a co-localization with v-Src at cell extensions (Fig. 3B)
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. 3A). v-Src-RT and FAK co-localized at pointed cell projections (Fig. 3B), however, v-Src-RT also exhibited strong peri-nuclear staining that was not detected in v-Src-expressing cells (Fig. 3A). When plated onto FN, c-Src reconstituted Src/-/- cells exhibited a similar morphology as v-Src-RT cells and c-Src had a similar cellular distribution as v-Src-RT (data not shown). These results support the hypothesis that gain-of-function v-Src SH3 interactions both enhance the formation of cell extensions and stabilize v-Src localization to these sites.

Previous studies have shown that Rous Sarcoma virus transformed chicken fibroblasts and human melanoma cells form β1 integrin-enriched peripheral and ventral cell extensions termed invadopodia (23, 24). Strong co-localization of β1 integrin with v-Src at both perimeter and ventral invadopodia sites were observed in v-Src transformed Src/-/- cells (Fig. 3C and D). β1 integrin staining of v-Src-RT cells showed a strong perimeter distribution with minimal staining in the central or ventral region of cells (Fig. 3C). v-Src-RT was co-localized with β1 integrin only in a subset of cell perimeter pointed projections (Fig. 3D). Whereas SH3 domain 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 (Lac Z) showed >90% infectivity of v-Src and v-Src-RT transformed Src/-/- cells (Fig. 4A). Adenovirus-mediated 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. 4B and C). This level of FAK-enhanced v-Src-RT cell invasion exceeded that of v-Src-transformed Src/-/- cells (Fig. 4C). 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 potential mechanism of increased HA-FAK phosphorylation, v-Src-RT-
associated in vitro kinase assays were performed (Fig. 4D). Compared to non-infected 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. 4D).

Since FAK overexpression promoted increased numbers of v-Src-RT but not control Src-/- cell invasion through Matrigel after 24 h as visualized by crystal violet staining of invasive cells on the lower Millicell membrane surface (Fig. 5A), indirect immunofluorescence staining of Millicell membranes was used to evaluate whether FAK functioned to recruit v-Src-RT to invadopodia during Matrigel invasion (Fig. 5B). 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. 5B). 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 through the Matrigel barrier located within pores of the Millicell membrane at 12 h (Fig. 5B). After 18 h, the adenoviral-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 in the absence of a Matrigel matrix cue. These results suggest that FAK activation and connections to integrins potentially through the binding of integrin-associated proteins such as paxillin (Fig. 4D) 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, that functions as a specific dominant-negative inhibitor of FAK activity (11-13), was expressed in v-Src-transformed Src-/- cells by recombinant adenovirus infection (Fig. 4B and C). FRNK potently blocked v-Src-stimulated cell invasion (Fig. 4B) and FRNK inhibited v-Src-stimulated endogenous FAK tyrosine phosphorylation (data not shown). Together, these results support the conclusion that v-Src co-localization with FAK at invadopodia and signals generated by the v-Src-FAK signaling complex function to promote cell invasion.

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. Notably, 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 β1 integrin at invadopodia cell projections. By using adenovirus to either overexpress FAK or a dominant-negative inhibitor of FAK function, 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 also containing integrin-associated proteins such as paxillin. 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.
**FIGURE LEGENDS**

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 overview of Prague C v-Src and v-Src-RT with a single amino acid change at Trp-95 to Arg-95 in the SH3 domain of v-Src-RT. Src-/- fibroblasts were stably reconstituted with normal c-Src, v-Src, or v-Src-RT and equal expression in pooled cell populations was verified by pan-Src blotting of whole cell lysates. B, Src IPs from serum-starved cells were analyzed for associated IVK activity using the GST-FAK-CT substrate and visualized by autoradiography (top panels). The amount of 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) was evaluated by plating 1x10^4 cells onto FN-coated dishes. Mean values +/- SD are from two independent experiments. Src-/- (black diamonds), Src-/- [+c-Src] (open squares), Src-/- [+v-Src] (open triangles), and Src-/- [+v-Src-RT] (filled circles). E, anchorage-independent cell growth in soft agar was evaluated by plating the indicated cells in 0.3% agar with 10% serum. Images show typical fields at 60X.

Fig. 2. **v-Src-RT promotes Src-/- cell motility but not Matrigel invasion. FAK tyrosine phosphorylation and v-Src association are elevated in v-Src compared to v-Src-RT-transformed Src-/- cells.** A, Millicell chamber (10 µg/ml FN) haptotaxis motility assays were performed with the indicated cells. Mean values +/- SD are from two independent experiments. B, the indicated cells were evaluated for invasion activity through a reconstituted Matrigel basement membrane barrier. Mean values +/- SD are from two independent experiments. C, FAK IPs from the indicated 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 the indicated serum-starved cells were sequentially analyzed by blotting with antibodies to FAK or phospho-specific FAK antibodies to Tyr-397 (pY397), Tyr-861, (pY861), and Tyr-925 (pY925).

Fig. 3. **Co-localization of FAK, v-Src, and β1 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 β1-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 peri-
nuclear and perimeter focal contact distribution. B, the boxed region in panel A was enlarged 2-fold and 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 β1 integrin co-distribution to invadopodia and ventral focal contact regions in v-Src-expressing cells. TRITC β1 integrin staining at perimeter focal contact sites in v-Src-RT-expressing cells. D, merged images of v-Src or v-Src-RT (green) with β1 integrin (red) staining from panels A and C. 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 (MOI=30) with a recombinant Lac Z-expressing adenovirus. β-galactosidase activity was measured in mock or Lac Z-infected cells using X-gal as a substrate. B, the indicated cells were infected (MOI = 100) with recombinant 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 +/- SD are from two independent experiments. C, HA-tag or FAK C-term antibody blotting of lysates from the indicated cells mock-treated or infected with HA-FAK or FRNK adenoviruses as described in (B). D, v-Src-associated in vitro kinase activity was evaluated from adherent and serum-starved mock or FAK adenoviral-infected v-Src-RT cells and visualized by autoradiography (left panel). The associated proteins in the v-Src IPs were detected by either v-Src, paxillin, or HA-tag blotting.

Fig. 5. FAK overexpression promotes cell invasion and the recruitment of v-Src-RT to invadopodia. A, representative images (60X) of the lower porous membrane surface from invasion assays with the indicated cells as performed in Fig. 4. Crystal violet-stained cells that invaded through the Matrigel barrier can be distinguished from the 8 µm membrane pores. B, indirect immunofluorescence analysis of invadopodia projections through Matrigel emerging on the lower Millicell surface after 12 h 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.
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Abbreviations
FAK, focal adhesion kinase; FN, fibronectin; FRNK, FAK-related non-kinase; HA, hemagglutinin; IP, immunoprecipitation; IVK, in vitro kinase; MOI, multiplicity of infection; MMP, matrix metalloproteinase; mAb, monoclonal antibody; P.Tyr, phosphotyrosine; PAGE, poly-acrylamide gel electrophoresis; PTK, protein-tyrosine kinase; SD, standard deviation; SH, Src homology; WCL, whole cell lysate.
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**A**

![Diagram of Src and v-Src proteins with SH3 and SH2 domains and kinase activity]

**B**

**IVK Assay**

- Src
- v-Src
- v-Src-RT

- FAK-CT

**C**

**Cell Growth (0.5% Serum)**

- Src-/-
- c-Src
- v-Src
- v-Src-RT

**D**

**Cell Growth (10% Serum)**

- Src-/-
- c-Src
- v-Src
- v-Src-RT

**E**

**Soft Agar Growth / 3 weeks**

- Src-/-
- c-Src
- v-Src
- v-Src-RT

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**Fig. 1A-E**
Fig. 2A-D
Fig. 3

Beta integrin / Src

Beta integrin

FAK

v-Src

v-Src-RT

v-Src
Fig. 4A-D
Fig. 5A and B
v-Src SH3-enhanced interaction with focal adhesion kinase at β1 integrin-containing invadopodia promotes cell invasion
Christof R. Hauck, Datsun A. Hsia, Dusko Ilic and David D. Schlaepfer

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