

The catalytic activity of Src is dispensable for translocation to focal adhesions but controls the turnover of these structures during cell motility

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The Src family of protein tyrosine kinases is involved in transducing signals at sites of cellular adhesion. In particular, the v-Src oncoprotein resides in cellular focal adhesions, where it induces tyrosine phosphorylation of pp125^{FAK} and focal adhesion loss during transformation. v-Src is translocated to cellular focal adhesions by an actin-dependent process. Here we have used mutant v-Src proteins that are temperature-dependent for translocation, but with secondary mutations that render them constitutively kinase-inactive or myristylation-defective, to show that neither v-Src kinase activity nor a myristyl group are required to induce association of v-Src with actin stress fibres and redistribution to sites of focal adhesions at the stress fibre termini. Moreover, switching the constitutively kinase-inactive or myristylation-defective temperature-sensitive v-Src proteins to the permissive temperature resulted in concomitant association with tyrosine-phosphorylated focal adhesion kinase (pp125^{FAK}) and redistribution of both to focal adhesions. However, both catalytic activity and myristylation-mediated membrane association are required to induce dissociation of pp125^{FAK} from v-Src, later degradation of pp125^{FAK} and focal adhesion turnover during transformation and cell motility. These observations provide strong evidence that the role of the tyrosine kinase activity of the Src family at sites of cellular focal adhesions is to regulate the turnover of these structures during cell motility.

Keywords: focal adhesions/motility/src/tyrosine kinase

Introduction

The Src family comprises a group of membrane-associated protein tyrosine kinases that are involved in transducing signals which result in altered cell growth and behaviour (reviewed in Cooper, 1989; Courtneidge, 1994). The activity of v-Src, a constitutively active variant of c-Src, induces properties associated with oncogenic transformation, including loss of normal growth control and cell rounding and detachment, a consequence of adhesion loss and disruption of the actin cytoskeleton. There is abundant evidence that v-Src exerts its oncogenic effects through its kinase activity, although it has become clear that the regulation and activity of v-Src is modulated by its interaction domains, including its myristylation signal and its Src-homology (SH) domains, SH2 and SH3 (reviewed

in Mayer and Baltimore, 1993; Pawson and Schlessinger, 1993). In particular, attachment of a myristate group to an N-terminal glycine at position 2 in the v-Src protein is required for membrane-association and transformation of CEF (Cross *et al.*, 1984; Kamps *et al.*, 1985; Catling *et al.*, 1993).

Previous studies have shown that v-Src induces changes in cell structure and morphology independently of changes in gene expression (Beug *et al.*, 1978; Frame *et al.*, 1994). Since v-Src resides in cellular focal adhesions, structures which provide the link between actin filaments and integrin-associated points of attachment with the extracellular matrix (ECM) (Burrige *et al.*, 1988; Turner and Burrige, 1991), this is most likely a consequence of tyrosine kinase activity at these sites. In particular, v-Src-induced tyrosine phosphorylation and degradation of one component of focal adhesions, the focal adhesion kinase (pp125^{FAK}) (Hanks *et al.*, 1992; Schaller *et al.*, 1992), is linked to v-Src-induced loss of focal adhesions during cell rounding and detachment as chicken embryo fibroblasts (CEF) transform (Fincham *et al.*, 1995). This, together with the observed integrin-activated c-Src/pp125^{FAK} signalling complex in NIH3T3 cells (Schlaepfer *et al.*, 1994) and the proposed requirement for association of c-Src (or Fyn) and pp125^{FAK} during pp125^{FAK}-induced migration of Chinese hamster ovary (CHO) cells (Cary *et al.*, 1996), implies a crucial role for pp125^{FAK} in the biological activity of the Src family kinases.

In order for v-Src to induce its biological effects, it must first be translocated from its site of synthesis to its sites of action in cellular focal adhesions at the cell periphery. We recently used a temperature-sensitive (*ts*) mutant of v-Src that did not locate at the cell periphery at the restrictive temperature, to show that the oncoprotein was subject to activation-dependent association with actin stress fibres and actin-mediated redistribution to the focal adhesions at the stress fibre termini (Fincham *et al.*, 1996). Furthermore, we observed that c-Src was also translocated to the cell periphery by an actin-dependent process in response to a biological stimulus (Fincham *et al.*, 1996), implying that this mode of translocation was also relevant to the endogenous Src family kinases. Although the exact nature of the actin-dependent intracellular transport of Src kinases is not known, one possibility is that, upon activation, Src is recruited into complexes which contain other focal adhesion components and that these specify focal adhesion targeting of the complex. Significantly, pp125^{FAK} contains a defined focal adhesion targeting sequence in its C-terminus (Hildebrand *et al.*, 1993) and a high-affinity binding site for Src created by pp125^{FAK} autophosphorylation (Schaller *et al.*, 1994).

These considerations led us to examine the structural determinants required for v-Src translocation to, and activity at, the cell periphery. Specifically, we have investi-

gated the role of the tyrosine kinase activity and N-terminal myristylation in both translocation and function of the v-Src oncoprotein at sites of cellular adhesion. We show that neither of these are required for association of v-Src with pp125^{FAK} and actin stress fibres or for redistribution of these complexes to focal adhesions at the cell periphery; however, kinase activity and myristylation-mediated membrane-association are both essential for focal adhesion turnover during transformation and cell motility.

Results

v-Src associated with actin and redistributed to focal adhesions; co-localization with pp125^{FAK}

Since our previous work demonstrating the actin-dependence of translocation of v-Src to focal adhesions at the cell periphery was in Swiss 3T3 cells, we first confirmed a similar mode of intracellular redistribution in chicken embryo fibroblasts (CEF), a cell type which allows us to further study the determinants of actin-dependent v-Src translocation by infecting with avian retroviruses encoding mutant v-Src proteins. Immunofluorescent staining of cells demonstrated that *ts LA29* v-Src was predominantly located in the perinuclear region of CEF at the restrictive temperature (41°C) (Figure 1A, 41°C); upon shift to the permissive temperature (35°C), v-Src was observed in discrete structures or complexes, which we could not distinguish on the basis of protein composition from focal adhesions at the cell periphery, and which we will now refer to as intracellular focal adhesions. These v-Src-containing intracellular focal adhesions redistributed with time to the cell periphery (Figure 1A, 1 and 4 h) and v-Src became associated with the cell membrane as focal adhesions were lost and the cells rounded up during the later stages of transformation (Figure 1A, 16 h). We also confirmed that the v-Src-containing focal adhesions associated with actin stress fibre termini in CEF rapidly after shift to the permissive temperature (Figure 1B), as had previously been observed in Swiss 3T3 cells (Fincham *et al.*, 1996). This, together with the cessation of translocation of v-Src to focal adhesions upon cytochalasin D treatment (not shown), and the Rho-A-induced redistribution of v-Src in quiescent cells (Fincham *et al.*, 1996), confirmed that the actin cytoskeleton is an essential component of the intracellular machinery which directs v-Src transportation to focal adhesions at the stress fibre termini.

Since pp125^{FAK} is a binding partner and substrate for the Src kinases (Guan and Shalloway, 1992; Hanks *et al.*, 1992; Schaller *et al.*, 1992; Schlaepfer *et al.*, 1994) and contains a focal adhesion targeting sequence (Hildebrand *et al.*, 1993), it is a potential mediator of Src translocation to focal adhesions. Thus, we tested whether the Src-containing intracellular focal adhesions that associated with actin soon after activation of *ts LA29* v-Src by shift to the permissive temperature also contained pp125^{FAK}. Co-staining of the cells shown in Figure 1A with anti-pp125^{FAK} demonstrated that pp125^{FAK} was predominantly located in focal adhesions (red in Figure 1C, 41°C), while v-Src was concentrated around the perinuclear region at the restrictive temperature (green in Figure 1C, 41°C). We also observed some random nuclear fluorescence after anti-pp125^{FAK}-staining, evident in some cells (Figure 1C,

upper and lower panels), but not in others (Figure 1C, middle panel); this inconsistency suggested that the nuclear staining was either non-specific or reflected the presence of a sub-nuclear focal adhesion. Upon shift to the permissive temperature, some v-Src and pp125^{FAK} rapidly co-localized (yellow in Figure 1C, 35°C), demonstrating that pp125^{FAK} was present both in intracellular actin-associated Src-containing focal adhesions (Figure 1C, 35°C 1 h) and peripheral focal adhesions (Figure 1C, 35°C 1 and 4 h). Thus, some activated *ts LA29* v-Src and pp125^{FAK} were both present in intracellular focal adhesions at early times after shift to the permissive temperature and in peripheral focal adhesions at later times.

Kinase activity and membrane-association are not required for v-Src to associate with actin and translocate to focal adhesions

The *ts LA29* v-Src mutant differs from the wild-type v-Src protein by three amino acid substitutions, one of which is responsible for both temperature-dependent tyrosine kinase activity and temperature-dependent localization to the cell periphery (Welham and Wyke, 1988). To test directly the role of v-Src kinase activity in the actin-dependent targeting of v-Src to focal adhesions, we introduced a Lys → Arg mutation at position 295 in the ATP binding site, generating a constitutively kinase-inactive, non-transforming variant of *ts LA29* v-Src. In addition, since we knew that a myristylation-defective mutant of the *ts LA29* v-Src protein was unable to transform CEF (Catling *et al.*, 1993), we addressed whether a mutation at the site of myristylation, and consequent inhibition of myristylation-mediated membrane association, interfered with the correct localization to focal adhesions. v-Src expression was similar at both the restrictive and permissive temperatures in CEF cultures infected with retrovirus encoding *ts LA29* v-Src, the constitutively kinase-inactive mutant, *ts LA29-KD-1* v-Src and the myristylation-defective derivative, *ts LA29-A2* v-Src (not shown). As expected, anti-phosphotyrosine immunoblots confirmed temperature-dependent tyrosine phosphorylation of cellular proteins in CEF expressing *ts LA29* v-Src and *ts LA29-A2* v-Src, but not in CEF expressing the constitutively kinase-inactive mutant *ts LA29-KD-1* v-Src (not shown).

In CEF expressing *ts LA29-KD-1* v-Src at the restrictive temperature, double immunofluorescence demonstrated that v-Src was located predominantly around the perinuclear region (Figure 2A, 41°C, green) and pp125^{FAK} was in focal adhesions (Figure 2A, 41°C, red). Upon shift to the permissive temperature, the constitutively kinase-inactive *ts LA29-KD* v-Src became associated with actin and intracellular focal adhesions that redistributed to the cell periphery with time (Figure 2A, 35°C). Moreover, the kinase-inactive v-Src co-localized with pp125^{FAK} in intracellular and peripheral focal adhesions, as judged by a nearly identical staining pattern in the same cells after switch to the permissive temperature (v-Src in green, pp125^{FAK} in red in Figure 2A, 35°C, 1 h). In CEF expressing *ts LA29-A2* v-Src at the restrictive temperature, v-Src was less obviously concentrated around the nucleus and was distributed more evenly throughout the cytoplasm, implying a role for the myristate group in the perinuclear retention of inactive Src molecules (Figure 2B, 41°C, green). Upon shift to the permissive temperature, myristyl-

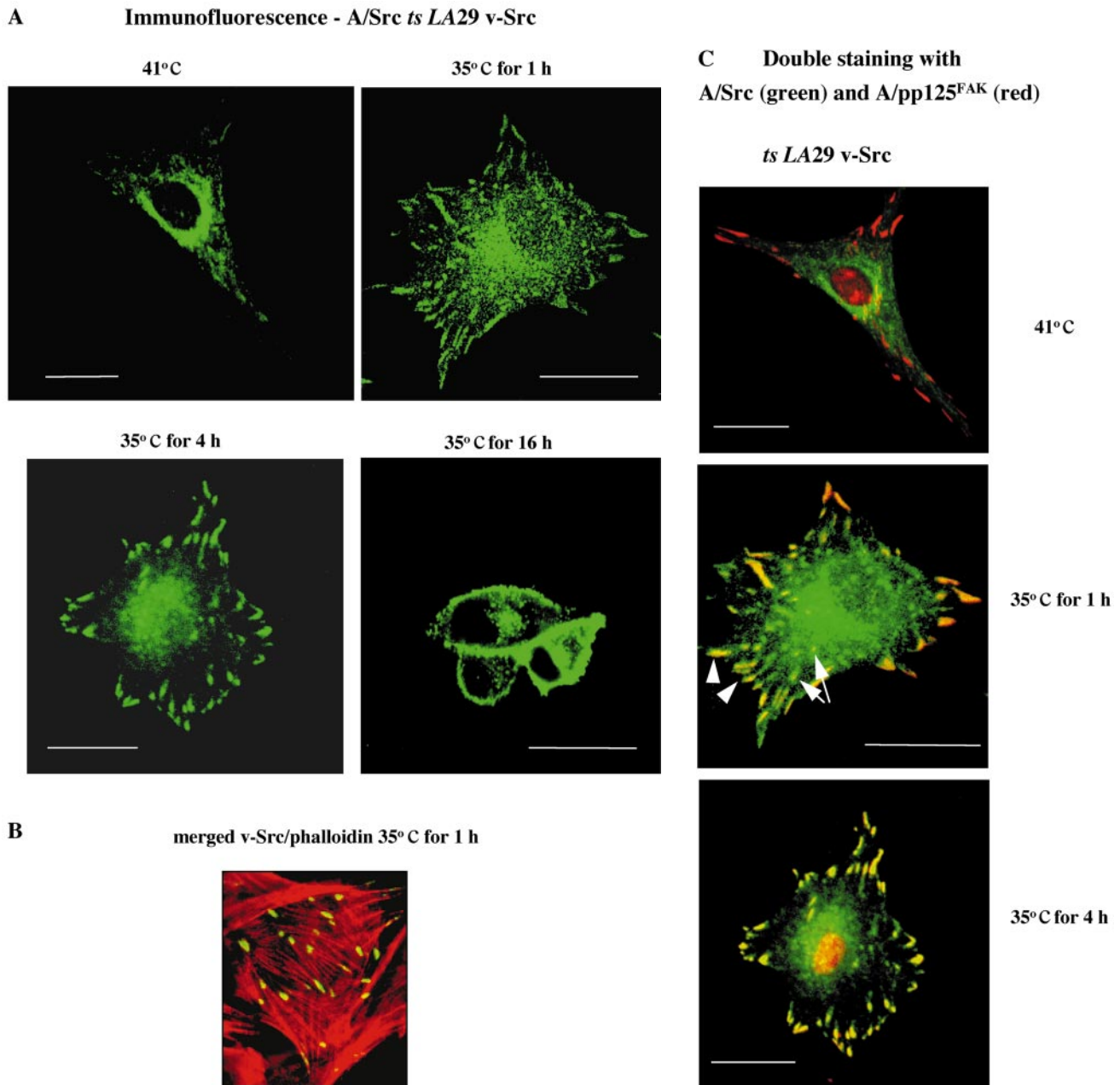


Fig. 1. Immunofluorescence confocal images of CEF stained with anti-v-Src and a second antibody coupled to FITC and phalloidin-TRITC or anti-pp125^{FAK} and a second antibody coupled to TRITC. **(A)** Cells expressing *ts LA29 v-Src* were maintained at the restrictive temperature of 41°C, or shifted to the permissive temperature of 35°C for 1, 4 or 16 h. That some of these v-Src-staining structures were intracellular and not focal adhesions on the base of the cell was confirmed by optical sectioning through the cell (not shown). Bars, 25 μm. **(B)** Merged images of cells that have been stained with anti-v-Src (detected by FITC; green) and phalloidin (stress fibres detected by TRITC; red). Co-localized actin and v-Src is visualized as yellow. In co-stained cell shown for 1 h, the arrows point to intracellular Src/pp125^{FAK}-containing complexes and arrowheads point to Src/pp125^{FAK}-containing focal adhesions at the cell periphery. **(C)** Merged images of cells shown in **(A)** stained for v-Src, were also stained with anti-pp125^{FAK} and a second antibody coupled to TRITC.

ation-defective *ts LA29-A2 v-Src* also became associated with actin filaments as focal adhesions and displayed a staining pattern which was indistinguishable from that of pp125^{FAK} (Figure 2B, 35°C, 1 h). At later times, *ts LA29-A2 v-Src* was present in focal adhesions at the cell periphery, although there also remained a relatively high level of cytoplasmic staining (Figure 2B, 35°C, 16 h). Thus, the ability of v-Src to associate with focal adhesions and actin stress fibres and to redistribute to the cell periphery does not require Src kinase activity or myristylation-mediated membrane-association.

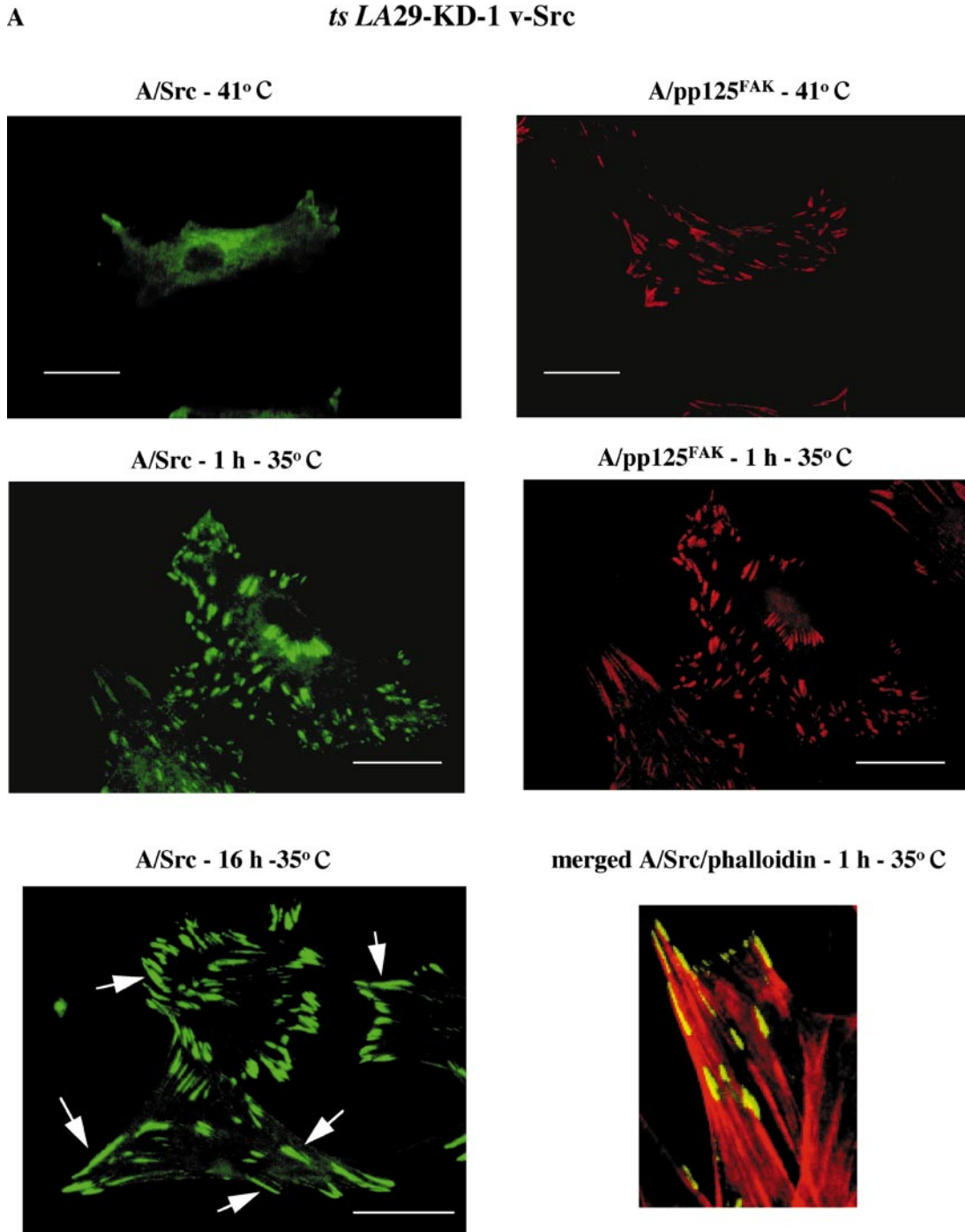
The association of v-Src with pp125^{FAK} is independent of kinase activity and membrane-association

To examine the nature of cellular proteins that associate with *ts v-Src* upon shift to the permissive temperature, and which might therefore be candidate mediators of actin association and translocation to focal adhesions, we first immunoprecipitated *ts LA29 v-Src* and probed the immunocomplexes with anti-phosphotyrosine. We identified two major species in the 120–140 kDa molecular weight range that were associated with v-Src in a tyrosine-

phosphorylated form between 0–30 minutes after activation (Figure 3A) and which were not detected in anti-Src immunoprecipitates from vector-infected cultures (Figure 3B). Upon probing anti-v-Src immunoprecipitates with anti-pp125^{FAK}, we consistently observed that pp125^{FAK} co-migrated with the lower-molecular weight tyrosine-phosphorylated species that complexed with v-Src after shift to the permissive temperature (Figure 3A, open symbol). Specific removal of the lower-molecular weight tyrosine-phosphorylated species (open symbol) by prior immunoprecipitation with anti-pp125^{FAK} was observed upon probing v-Src-immunoprecipitates with anti-phosphotyrosine (Figure 3C), confirming that this species was pp125^{FAK}. Furthermore, although we could not detect

any co-localization of pp125^{FAK} and v-Src at the restrictive temperature by immunofluorescence, some pp125^{FAK} was detected in a biochemical complex with inactive *ts LA29* v-Src (Figure 3A, lower panel, 0 h) and progressively dissociated from v-Src after shift to the permissive temperature (Figure 3A, lower panel, 1, 2 and 4 h). These data suggest that some pp125^{FAK} pre-exists in complex with inactive v-Src, becomes tyrosine-phosphorylated after activation of the v-Src tyrosine kinase, and dissociates from the complex.

If the tyrosine-phosphorylated species that associate with *ts LA29* v-Src are candidate mediators of v-Src translocation, then these should also complex in a temperature-dependent manner with the constitutively kinase-



inactive and myristylation-defective v-Src proteins that associate with actin and relocate to focal adhesions at the permissive temperature. Both kinase-inactive *ts LA29-KD-1* v-Src and myristylation-defective *ts LA29-A2* v-Src also complexed with tyrosine-phosphorylated pp125^{FAK}, rapidly after shift to the permissive temperature (Figure 3D and E, respectively, upper panels, open symbols). In

the case of *ts LA29-KD-1* v-Src, however, shift to the permissive temperature did not induce association with the slower migrating species that associated with both the catalytically active v-Src mutants, *ts LA29* and *ts LA29-A2* (Figure 3A and E, respectively, upper panels, filled symbols), implying that this v-Src-associated species became tyrosine-phosphorylated as a consequence of v-Src

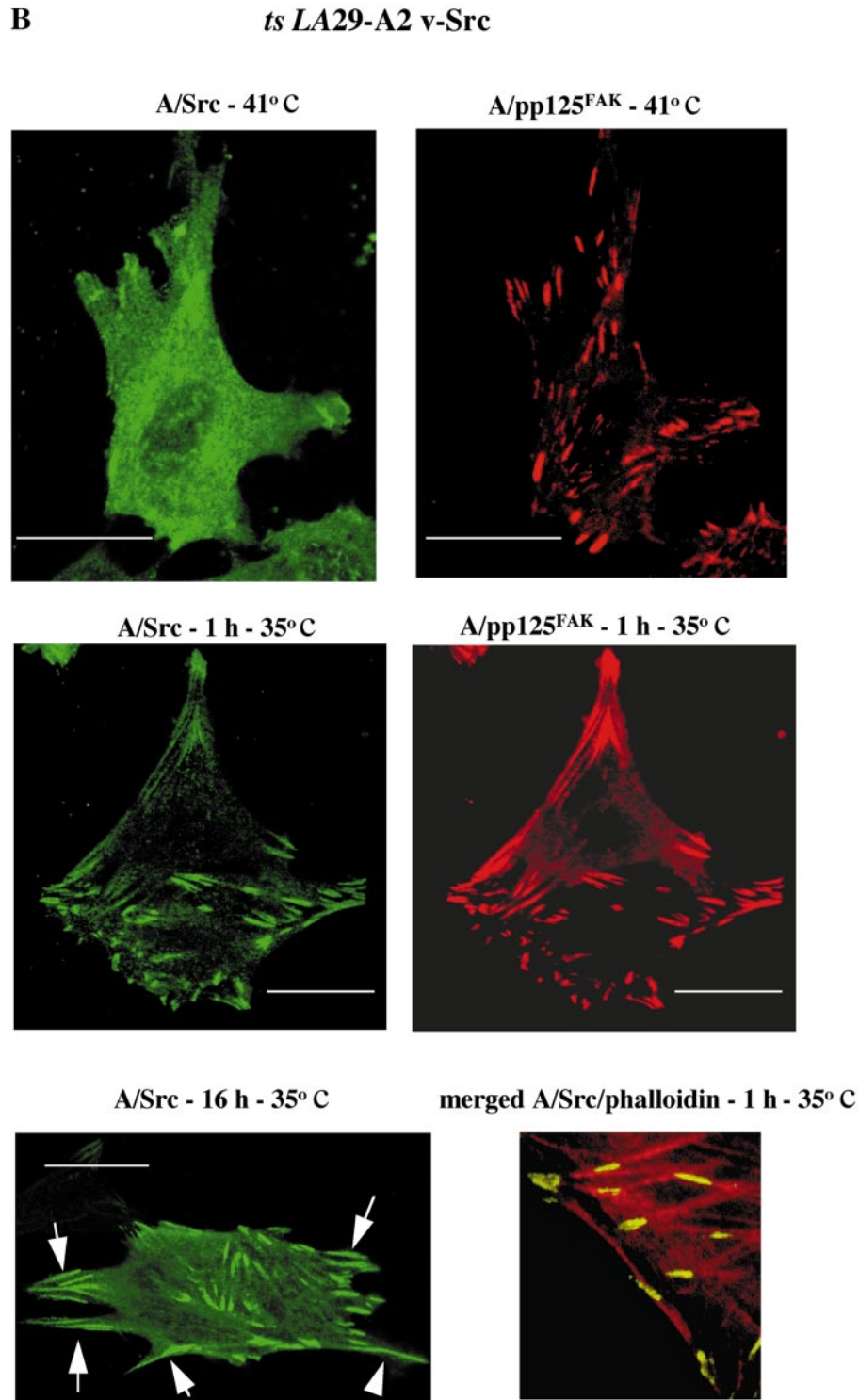


Fig. 2. Co-localization of constitutively kinase-inactive and myristylation-defective v-Src with actin and pp125^{FAK} after shift to the permissive temperature. (A) Cells expressing *ts LA29-KD-1* v-Src were maintained at the restrictive temperature of 41°C, or shifted to the permissive temperature of 35°C for 1, 4 or 16 h and stained with anti-v-Src and a second antibody coupled to FITC and phalloidin-TRITC or anti-pp125^{FAK} and a second antibody coupled to TRITC. Bars, 25 μm. (B) Cells expressing *ts LA29-A2* v-Src were treated and stained as described in (A).

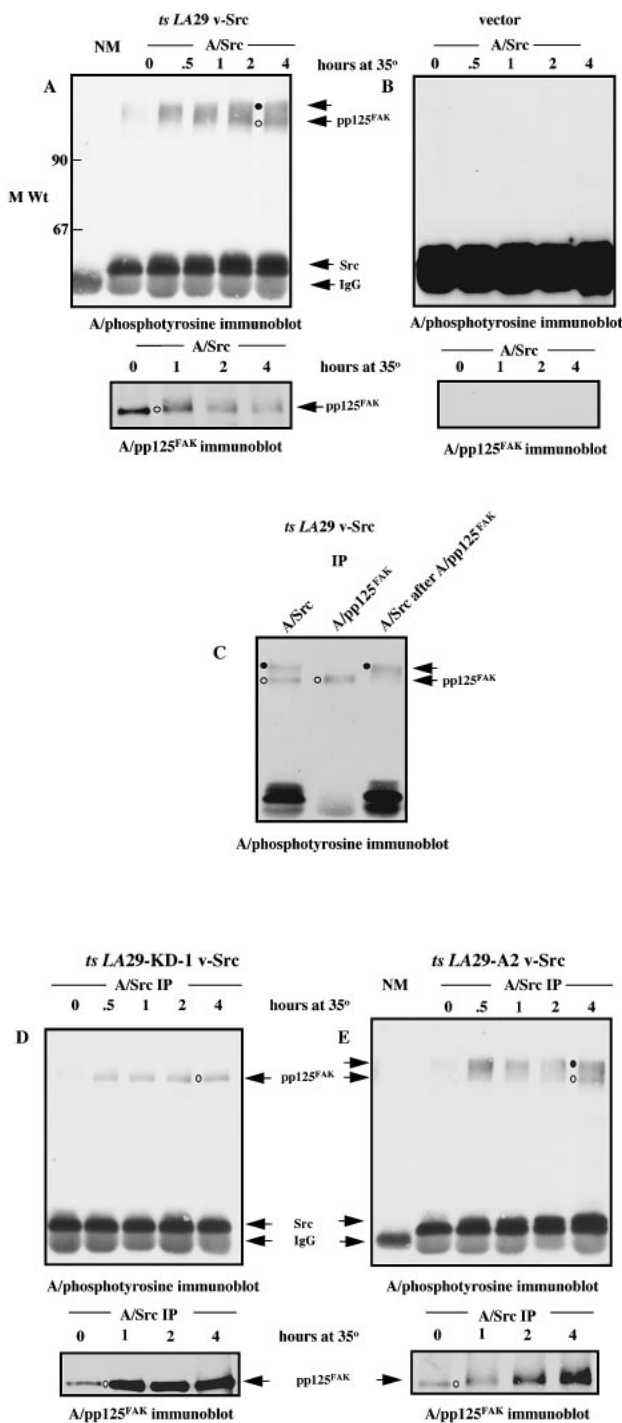


Fig. 3. Association of tyrosine-phosphorylated proteins and pp125^{FAK} with *ts v-Src*. *v-Src* was immunoprecipitated from cells expressing *ts LA29 v-Src* (A), empty vector (B), *ts LA29-KD-1 v-Src* (D) or *ts LA29-A2 v-Src* (E) which had been maintained at 41°C (0 h) or shifted to 35°C (0.5, 1, 2 or 4 h). Precipitated proteins were immunoblotted using anti-phosphotyrosine (A/phosphotyrosine, upper panels) or anti-pp125^{FAK} (A/pp125^{FAK}, lower panels) as probes. The positions of 90 and 67 kDa molecular weight markers are shown. NM is normal mouse serum which was used as control. The positions of precipitated IgG, Src, pp125^{FAK} and the faster (○) and slower (●) migrating tyrosine-phosphorylated species are indicated by arrows. (C) Lysates of *ts LA29 v-Src*-expressing CEF were immunoprecipitated with anti-*v-Src* and probed with anti-phosphotyrosine before (A/Src) and after immunodepletion with anti-pp125^{FAK} (A/pp125^{FAK}) as probes. The tyrosine-phosphorylated pp125^{FAK} removed from these lysates is also shown (A/pp125^{FAK}).

kinase activity. Moreover, complexing with this species was clearly not a prerequisite for translocation to peripheral focal adhesions or actin association of kinase-inactive *ts LA29-KD-1 v-Src* at the permissive temperature. Upon probing anti-*v-Src* immunoprecipitates with anti-pp125^{FAK}, we observed that although some pp125^{FAK} was associated with *ts LA29-KD-1 v-Src* and *ts LA29-A2 v-Src* at the restrictive temperature (Figure 3D and E, respectively, lower panel, 0 h), there was a substantial increase in the amount of pp125^{FAK} associated with these defective mutant *v-Src* proteins after shift to the permissive temperature (Figure 3D and E, respectively, lower panel, 1, 2 and 4 h). Thus, the constitutively kinase-inactive and myristylation-defective *ts v-Src* mutants undergo a temperature-induced change that stimulates further complexing with pp125^{FAK}, association with actin and translocation to focal adhesions at the cell periphery. Furthermore, we conclude that the progressive dissociation of pp125^{FAK} from the *ts LA29 v-Src* complex upon shift to the permissive temperature (Figure 3A, lower panel) requires the catalytic activity of *v-Src* and its myristylation-mediated association with the cell membrane (Figure 3A, lower panel), both properties of *v-Src* that are also required for cellular transformation and its concomitant turnover of focal adhesions.

***v-Src*-induced degradation of pp125^{FAK} requires both Src kinase activity and myristylation-mediated membrane association**

In previous work, we showed that activation of *ts LA29 v-Src* in CEF stimulated tyrosine phosphorylation of pp125^{FAK} and induced its degradation as cells rounded up and detached during transformation (Fincham *et al.*, 1995). Furthermore, pp125^{FAK} degradation was impaired in response to *v-Src* mutants that induced only weak transformation of CEF, but it preceded the onset of obvious morphological change, implying that it was not a consequence of transformation (Fincham *et al.*, 1995). Thus, we tested the ability of kinase-inactive *ts LA29-KD-1* and myristylation-defective *ts LA29-A2 v-Src* mutant proteins to induce loss of cellular pp125^{FAK} after shift to the permissive temperature. In contrast to *ts LA29 v-Src* (where pp125^{FAK} loss was evident in the experiment shown after 4 h; Figure 4), both the defective *v-Src* proteins did not induce pp125^{FAK} degradation, indicating that both the kinase activity of *v-Src*, and myristylation-mediated association with the plasma membrane, are required to induce the degradation of pp125^{FAK} that accompanies focal adhesion loss during transformation. Thus, the unregulated catalytic activity of *v-Src*, when it is correctly associated with the plasma membrane at the focal adhesion sites of stress fibre attachment, stimulates a degradative pathway that leads to focal adhesion turnover and transformation of CEF. In normal cells, the tightly regulated activity of the endogenous Src family kinases may carry out a similar function during routine aspects of cell behaviour that require controlled focal adhesion turnover, such as during cell motility.

The kinase- and myristylation-defective *v-Src* mutants induce abnormally large focal adhesions and suppress cell motility at the permissive temperature

During the course of our co-localization experiments, we noticed a difference in the size of focal adhesions in cells

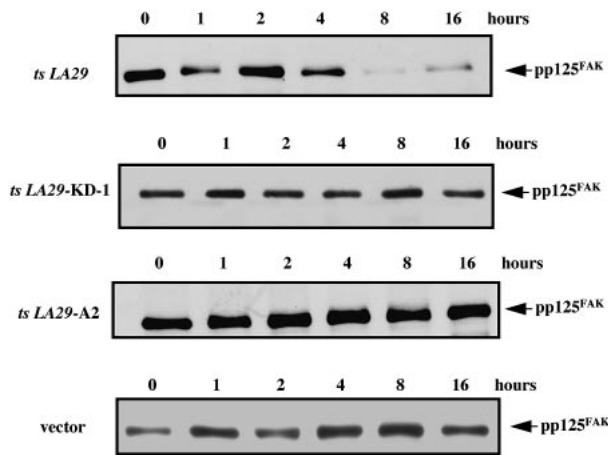


Fig. 4. Kinase-inactive and myristylation-defective v-Src mutants of *ts LA29* v-Src are impaired at inducing pp125^{FAK} degradation. Total cellular pp125^{FAK} was detected by immunoblotting lysates of CEF expressing *ts LA29*, *ts LA29-KD-1* or *ts LA29-A2* v-Src, or vector alone, which had been maintained at the restrictive temperature (0 h) or switched to the permissive temperature (1, 2, 4, 8 and 16 h). The position of pp125^{FAK} is indicated.

expressing defective v-Src mutants at the permissive temperature. In CEF expressing either the constitutively kinase-inactive *ts LA29-KD-1* v-Src or the myristylation-defective *ts LA29-A2* v-Src, the focal adhesions visualized by anti-Src staining at later times after shift to the permissive temperature (up to 16 h at 35°C, Figure 2A and B, white arrows) were consistently greater in size than those at the restrictive temperature (Figure 2A and B) or in normal CEF (not shown). Thus, the inability of kinase-inactive *ts LA29-KD-1* and myristylation-defective *ts LA29-A2* v-Src proteins to induce degradation of cellular pp125^{FAK} and focal adhesions at the permissive temperature, was associated with abnormally large Src-stained focal adhesions (Figure 2). To determine if this increase in size was specific to focal adhesions visualized by anti-Src staining, we also compared adhesions stained with anti-phosphotyrosine in CEF expressing *ts LA29* v-Src at the restrictive temperature (which have normal size focal adhesions) with CEF expressing the constitutively kinase-inactive *ts LA29-KD-1* and myristylation-defective *ts LA29-A2* v-Src mutants at the permissive temperature. Temperature-dependent control of the size of phosphotyrosine-containing adhesions at the cell periphery was again evident, with extended focal adhesions at the permissive temperature (Figure 5). Thus, in addition to impaired degradation of pp125^{FAK} and focal adhesions normally associated with v-Src-induced transformation of CEF, the presence of kinase-inactive or myristylation-defective mutant v-Src proteins in focal adhesions results in the generation of enlarged adhesion structures, implying a linked role for the Src family kinases in determining focal adhesion size and turnover.

These findings led us to examine whether the abnormally large focal adhesions were defective in turnover and impaired cell movement. CEF monolayers were wounded at the restrictive temperature (Figure 6A) and the ability of cells to migrate into the wound was monitored after 24 h at either the restrictive or the permissive temperatures (Figure 6B–H). Cells expressing *ts LA29* v-Src were able

to migrate into the wound as normal cells at 41°C (Figure 6B) or as transformed cells at 35°C (Figure 6C). However, although able to repair the wound normally at 41°C, two different cell populations expressing constitutively kinase-inactive v-Src, *ts LA29-KD-1* and *ts LA29-KD-2*, were unable to migrate into the wound at 35°C (Figure 6E and F). Expression of the myristylation-defective *ts LA29-A2* v-Src at 35°C also led to impairment of the ability of CEF to migrate into the wound (Figure 6H), although these cells were competent to migrate at 41°C (Figure 6G). Thus, the presence of kinase-inactive or myristylation-defective mutants of v-Src in focal adhesions suppressed cell motility. In order to rule out cell proliferation as a major contributing factor to wound repair in these experiments, we confirmed that the proliferation rates of CEF expressing the v-Src mutants used here were similar at the restrictive temperature and were not substantially altered upon shift to the permissive temperature (not shown).

We also observed that enforced expression of kinase-inactive v-Src was able to suppress the activity of c-Src, or its close relatives, during serum-induced cell motility of normal cells (not shown). Specifically, normal CEF that had been serum-deprived for 48 h were restrained in their ability to migrate into the wound, a restraint that was relieved by the addition of 5% serum for 24 h in normal cells but not in cells expressing kinase-inactive *ts LA29-KD-1* v-Src at the permissive temperature. Thus, in addition to increasing the size of cellular focal adhesions, kinase-inactive *ts LA29-KD-1* v-Src acts as a dominant inhibitor of serum growth factor-induced cell motility, most likely by interfering with the normal function of the endogenous cellular Src family kinases during adhesion turnover.

Discussion

Regulation of the intracellular targeting of v-Src to focal adhesions

The v-Src protein has long been recognized to reside in cellular adhesion plaques in transformed cells (Rohrschneider, 1979; Nigg *et al.*, 1982; Kreuger *et al.*, 1984; Fincham *et al.*, 1996), although it has also been found associated with the plasma membrane (Courtneidge *et al.*, 1980) and in the perinuclear region (Rohrschneider, 1979). Here we have introduced secondary mutations into the *ts LA29* v-Src protein to generate non-transforming, constitutively kinase-inactive or non-myristylated mutant derivatives, whose properties indicate that the actin-dependent re-location of v-Src from perinuclear membrane-associated sites to focal adhesions at the cell periphery is independent of both Src kinase activity and myristylation-mediated membrane association. Specifically, *ts LA29* v-Src resides in the perinuclear region of the cell at the restrictive temperature, becomes associated with intracellular actin filaments as discrete complexes that cannot be distinguished from intracellular focal adhesions after shift to the permissive temperature, and redistributes with time to peripheral focal adhesions at the stress fibre termini (Figure 1 and model in Figure 7). Furthermore, the focal adhesion component, pp125^{FAK}, co-localizes with v-Src in actin-bound focal adhesions after shift to the permissive temperature (see model in Figure 7).

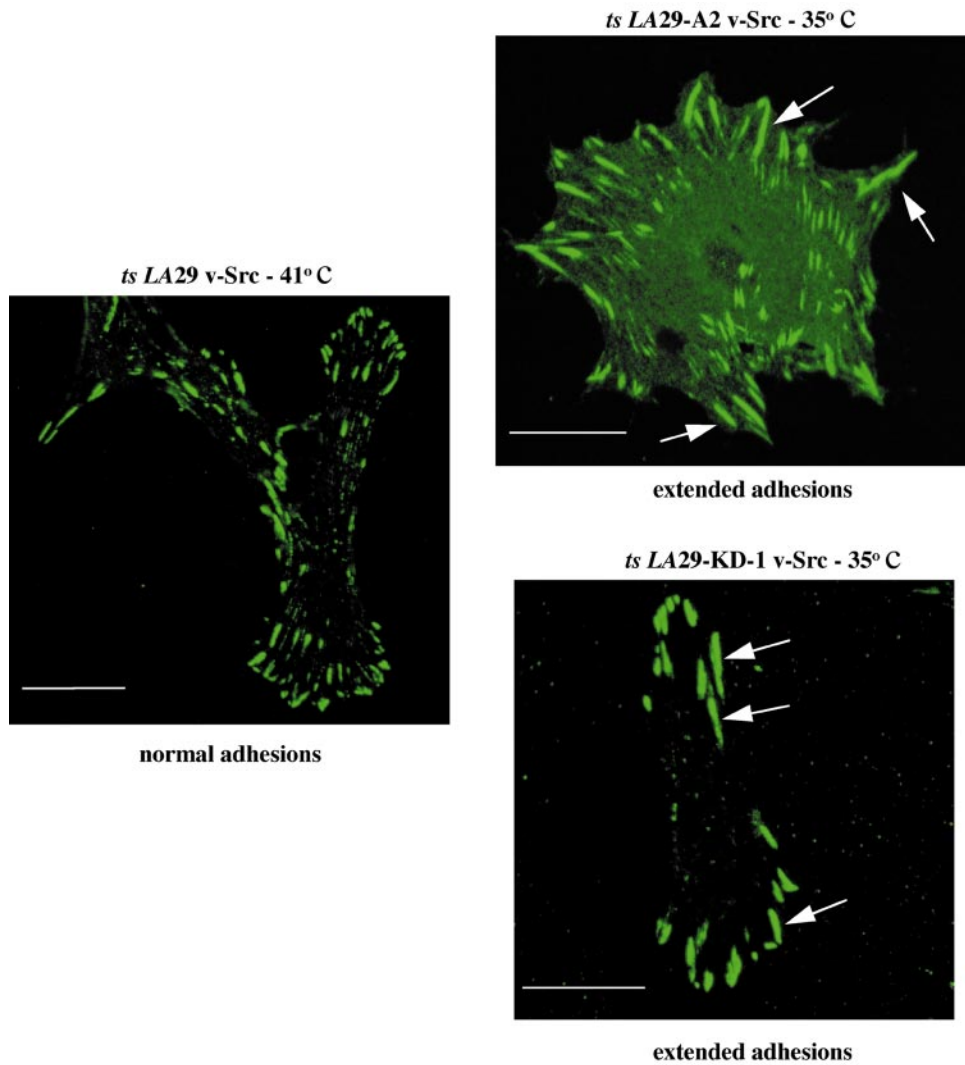


Fig. 5. Defective *ts LA29 v-Src* proteins induce large anti-phosphotyrosine-stained focal adhesions. Cells expressing *ts LA29 v-Src* at the restrictive temperature (41°C) or *ts LA29-KD-1* or *ts LA29-A2 v-Src* at the permissive temperature (35°C) were stained with anti-phosphotyrosine and a second antibody coupled to FITC. Focal adhesions were visualized by confocal microscopy. Bars, 25 μm.

Thus, v-Src, like c-Src (David-Pfeuty and Nouvian-Dooghe, 1990; Kaplan *et al.*, 1992), is most likely associated with perinuclear endosomal membranes in its inactive state at the restrictive temperature; upon shift to the permissive temperature, *ts LA29 v-Src* undergoes a kinase-independent switch which induces association with actin and translocation to focal adhesions at the cell periphery. These observations are consistent with the findings that exogenously expressed c-Src is associated with perinuclear endosomal membranes in *src -/-* fibroblasts (Kaplan *et al.*, 1994); however, c-Src which has a Tyr → Phe substitution at position 527 leading to both catalytic activation and a conformational change as a result of disruption of the intramolecular interaction between the Src SH2 domain and Tyr527 (MacAuley and Cooper, 1989), constitutively locates in focal adhesions (Kaplan *et al.*, 1994). This, together with the ability of the constitutively kinase-inactive variant of *ts LA29 v-Src* also to associate with actin and translocate to focal adhesions at the permissive temperature (Figure 2), suggests that *ts LA29 v-Src* undergoes a temperature-dependent conformational change that either induces direct association with actin,

or reveals cryptic binding sites for a protein (or proteins) which associates with v-Src and mediates actin-association and translocation to focal adhesions. pp125^{FAK} has the expected properties of a protein that could carry out this function since it contains a well-defined focal adhesion targeting sequence (Hildebrand *et al.*, 1993) and is induced to complex with the defective mutants of *ts LA29 v-Src* that also associate with actin and translocate to focal adhesions upon switch to the permissive temperature (Figure 3). However, some pp125^{FAK} is already complexed with *ts LA29 v-Src*, and its defective derivatives, at the restrictive temperature (Figure 3), although pp125^{FAK} was not significantly co-localized with v-Src in the perinuclear region of the cell as judged by immunofluorescence (Figure 2A and B). These findings imply that the level of pp125^{FAK}/v-Src complexing at the restrictive temperature was not sufficient to mediate the actin-dependent translocation of a significant proportion of v-Src to focal adhesions and that the increased complexing of pp125^{FAK} with kinase-inactive and myristylation-defective v-Src after switch to the permissive temperature (Figure 3) may be required.

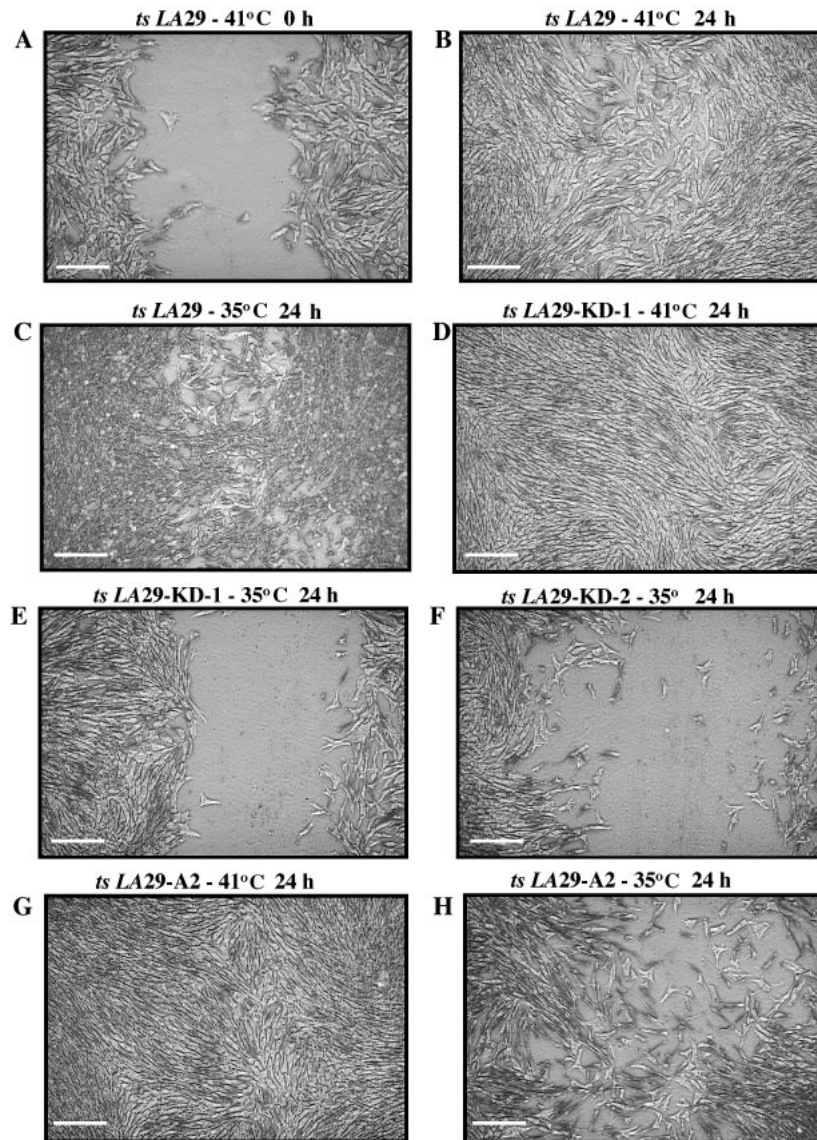


Fig. 6. Defective *ts LA29* v-Src proteins suppress cell motility at the permissive temperature. Monolayers of cells expressing *ts LA29* v-Src, *ts LA29*-KD-1 v-Src (and another population of kinase-inactive v-Src-expressing CEF, *ts LA29*-KD-2 v-Src) or *ts LA29*-A2 v-Src at the restrictive temperature (41°C) were wounded and either harvested (0 h), maintained at 41°C (24 h) or shifted to the permissive temperature (35°C) (24 h). The ability to migrate into the wound was monitored by microscopic examination of Giemsa-stained monolayers which were photographed. Bars, 100 μ m.

Regulation of focal adhesion disassembly by v-Src

During morphological transformation of CEF by *ts LA29* v-Src, cellular focal adhesions are disassembled and degraded and the organized actin cytoskeleton is lost (Kellie *et al.*, 1986; Fincham *et al.*, 1995). We previously showed that focal adhesion disassembly is linked to v-Src-induced tyrosine phosphorylation of pp125^{FAK} and degradation of pp125^{FAK}, events which are initiated before cell rounding and detachment (Fincham *et al.*, 1995). Here we show that although the v-Src kinase activity and myristylation signals are not required for the transportation of v-Src into assembled focal adhesions, both are required to induce disassembly of the v-Src/pp125^{FAK} complex (Figure 3), degradation of pp125^{FAK} (Figure 4) and adhesion disruption evident during morphological transformation (see model in Figure 7). Thus, we conclude that one consequence of unregulated Src kinase activity in focal adhesions is to stimulate turnover of these structures (Figure 7), an effect which also requires myristylation-

mediated association with the plasma membrane. One possibility is that turnover only proceeds in focal adhesion protein complexes at sites of actin stress fibre attachment to the plasma membrane, and that myristylation-mediated association of Src with the membrane may signal that the focal adhesion protein complexes are located at such a site.

In addition to impaired focal adhesion turnover and cell transformation, the focal adhesions in cells expressing kinase-inactive or myristylation-defective *ts LA29* v-Src variants at the permissive temperature were consistently greater in size than those at restrictive temperature or in uninfected CEF (Figure 5). These data are consistent with the findings of Kaplan *et al.* (1994), that expression of a truncated kinase-inactive variant of c-Src gave rise to focal adhesions which were several-fold larger in size. Thus, the catalytic activity of the Src family kinases is required to produce focal adhesions of the correct size and suggests a link between adhesion size and turnover. Of relevance here is the recent finding of Chrzanowska-

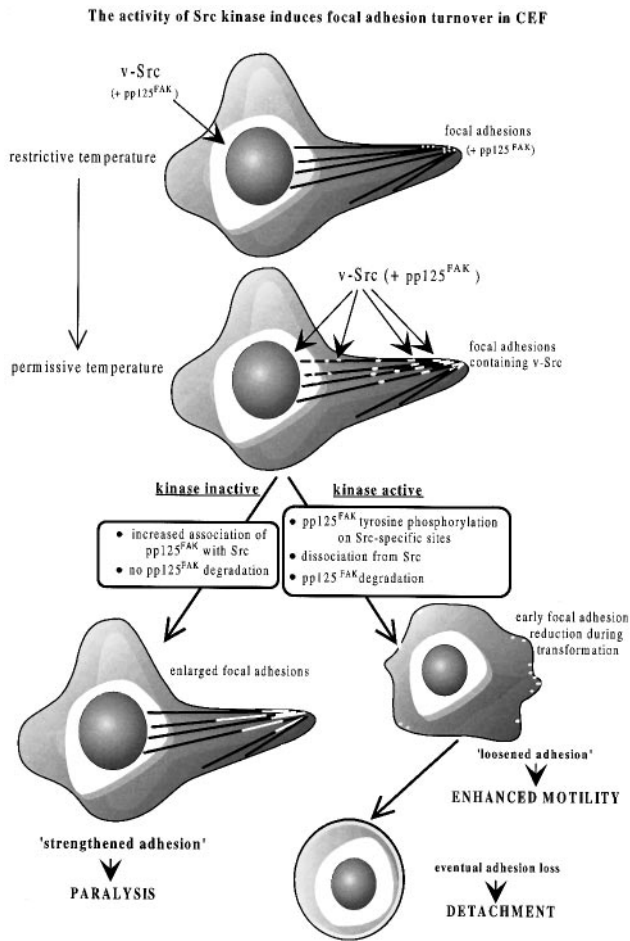


Fig. 7. Model depicting the actin-dependent re-location of v-Src from the perinuclear region of the cell to focal adhesions at the cell periphery in association with pp125^{FAK} upon shift from the restrictive to the permissive temperature. The consequences of the catalytic activity of v-Src in focal adhesions are also outlined.

Wodnicka and Burridge (1996), that focal adhesion assembly occurs as a result of tension generated by Rho-mediated actomyosin-based contractility. Thus, although the mechanism by which the defective v-Src proteins increase focal adhesion size is presently unknown, one possibility is that these mutant proteins stabilize actin stress fibres in a state of tension, leading to de-regulated focal adhesion assembly. The contractility of cells expressing kinase-active v-Src, and the non-motile cells expressing dominant-interfering mutants of v-Src, remains to be tested.

The regulation of cell motility by the Src family kinases and pp125^{FAK}

The localization of kinase-inactive and myristylation-defective mutant *ts LA29* v-Src proteins in enlarged focal adhesions led us to examine the consequences for cell behaviour. Specifically, although there is no obvious effect on cell proliferation, cell motility is suppressed in the myristylation-defective *ts* v-Src-expressing cells and almost completely abolished in the kinase-inactive *ts* v-Src-expressing cells at the permissive temperature (Figure 6). In both cases, motility of cells is unaffected by expression of these mutant v-Src proteins at the restrictive temperature, indicating that impaired motility

is most likely a consequence of their presence in enlarged cellular focal adhesions. Moreover, suppression of cell motility is associated with the impaired ability of these v-Src mutants to induce dissociation of pp125^{FAK} from the v-Src complex, degradation of pp125^{FAK}, focal adhesion turnover and transformation of CEF (see model in Figure 7). In addition, expression of kinase-inactive *ts LA29* v-Src at the permissive temperature also suppresses serum-induced motility of quiescent cells, implying that constitutively kinase-inactive v-Src acts as a dominant inhibitor of the endogenous Src family kinases in focal adhesions. These findings clearly implicate the catalytic activity of the Src family in the turnover of adhesion structures during cell motility.

Src and pp125^{FAK} functionally interact to turn over focal adhesions during transformation and cell motility

Since cell movement is controlled by a complex coordinated series of rearrangements of the actin cytoskeleton and changes in adhesion, the intracellular regulators of cytoskeletal organization and cellular adhesion strength are likely to be crucial determinants of cell motility. Specifically, contractile force at the leading edge of a migrating cell is thought to be generated by actin polymerizations that result in cell protrusions known as lamellipodia and filopodia (Lauffenburger and Horwitz, 1996; Welch *et al.*, 1997) which are, in turn, under the control of the small GTPases of the Rho family (Ridley and Hall, 1992; Ridley *et al.*, 1992). However, the strength of focal adhesions, into which polymerized actin fibres are tethered and linked to the ECM, may also be crucial in determining the rate of cell movement. For example, if cellular adhesions are too strong then the cell will adhere and not move; conversely, if adhesions are too weak then the connection to the ECM will not be strong enough to pull the cell forward. These considerations have led to the proposal that cell migration occurs at an intermediate level of cellular adhesive strength (discussed in Schwarzbauer, 1997) and predicts that cellular influences which alter adhesiveness will consequently influence the ease with which a cell can move. We propose that pp125^{FAK} and the Src family kinases act to control the rate of cell movement by influencing adhesion strength, for the reasons outlined below.

pp125^{FAK} is phosphorylated on tyrosine in response to a variety of stimuli, including adhesion to the ECM component fibronectin (Burridge *et al.*, 1992; Hanks *et al.*, 1992; Kornberg *et al.*, 1992), an effect which was interpreted as evidence for a role in focal adhesion formation during cell spreading. However, pp125^{FAK} is also extensively tyrosine-phosphorylated in response to oncogenic variants of Src (Kanner *et al.*, 1990; Guan and Shalloway, 1992; Schlaepfer *et al.*, 1994; Fincham *et al.*, 1995; this report), an effect which contrasts with cell spreading on fibronectin and is associated with focal adhesion disruption as cells round up during transformation. Our previous work, which examined cell spreading during restitution of normal morphology as *ts* v-Src-transformed cells were returned to the restrictive temperature, clearly demonstrated that tyrosine phosphorylation of pp125^{FAK} was not a prerequisite for focal adhesion formation, but suggested that pp125^{FAK} functioned more

in the turnover of adhesion structures rather than their assembly (Fincham *et al.*, 1995). Further evidence for this came from studies on cells derived from pp125^{FAK} -/- mouse embryos, which display elevated numbers of more intensely staining focal adhesions, but exhibit reduced motility as a consequence of impaired adhesion turnover (Ilic *et al.*, 1995). In addition, it has been shown that displacement of endogenous pp125^{FAK} from focal adhesions by microinjection of a competing non-functional pp125^{FAK} protein reduces cell motility (Gilmore and Romer, 1996) and a number of invasive tumour cell lines exhibit elevated expression of pp125^{FAK} (Owens *et al.*, 1995). Taken together, these observations indicate that pp125^{FAK} is involved in focal adhesion turnover, an important component of regulated cell motility, and imply that pp125^{FAK} protein may be limiting for cell invasion, perhaps by controlling motility, in some normal cells.

The role of the Src family kinases in cell motility has not been clearly resolved to date, with at least some reports suggesting that Src is not involved (Kundra *et al.*, 1994; Rodier *et al.*, 1995). However, there is now considerable evidence for a closely linked role for the Src family kinases in pp125^{FAK}-regulated cell motility, at least in some cell types. In particular, overexpression of pp125^{FAK} in CHO cells stimulates cell migration, an effect which requires the major autophosphorylation site in pp125^{FAK} (Y397) to allow complexing with c-Src or Fyn (Cary *et al.*, 1996). c-Src has also recently been implicated in hyaluronan receptor RHAMM-induced motility of mouse cells, an effect that requires Src catalytic activity (Hall *et al.*, 1996). In addition, v-Src stimulates the turnover of focal adhesions (Fincham *et al.*, 1995) and motility of rodent fibroblasts (Hall *et al.*, 1996; A.Wyke and J.A.Wyke, unpublished observations). These observations, together with our findings that dominant-interfering mutants of v-Src suppress motility of CEF, indicate that the catalytic activity of Src is generally required for cell movement.

In conclusion, while molecular control of the cytoskeletal and adhesion rearrangements which drive cell movement are incompletely understood, we have demonstrated that the catalytic activity of functional Src kinase at sites of cellular adhesion is essential for this process. Furthermore, impaired motility in cells expressing interfering Src mutants, under conditions where they are located in enlarged focal adhesions, is linked to impaired turnover of pp125^{FAK} and focal adhesions. We propose that the Src-pp125^{FAK}-linked activity induces focal adhesion turnover and consequently, loosens cellular adhesion strength, thus facilitating cell movement. Thus, the unregulated activity of v-Src stimulates a focal adhesion degradative pathway in CEF that may only be activated by normal cells in a tightly regulated fashion during cell motility.

Materials and methods

Generation of CEF cultures expressing v-Src mutants

Primary CEF cultures were grown in DMEM supplemented with 5% newborn calf serum and tryptose phosphate. Low-density cultures were transfected with replication-competent avian retroviral RAV-src constructs (5 µg per 25 cm² flask), or with vector alone, by the DOTAP method (Boehringer-Mannheim) and subcultured at the permissive temperature of 35°C until the cells were uniformly infected and expressing Src protein (judged by protein immunoblotting). The generation of

retrovirus encoding *ts LA29* v-Src or the myristylation-defective version, *ts LA29-A2*, have been described (Welham and Wyke, 1988; Catling *et al.*, 1993). Retrovirus encoding the kinase-defective variant of *ts LA29* v-Src was generated by converting the ATP-binding site at position 295 from Lys to Arg using PCR mutagenesis. The mutant sense oligonucleotide was 5'-GAG TGG CCA TAC GGA CTC TGA AGC-3'. Cell cultures infected with retrovirus encoding *ts* v-Src mutants, or with retrovirus alone, were grown either at restrictive (41°C) or permissive (35°C) temperatures and were buffered with 5% CO₂. When required, infected cultures were made quiescent by incubating in low serum-containing medium (0.2%) for 48 h at 41°C.

Protein immunoprecipitation and immunoblotting

For protein analyses, dishes of cells were washed with cold phosphate-buffered saline (PBS), drained and frozen at -70°C. For immunoprecipitation (IP) of Src, monolayers were thawed, lysed in RIPA buffer (50 mM Tris, pH 7.4, 150 mM sodium chloride, 5 mM EGTA, 0.1% SDS, 1% NP40 and 1% deoxycholate) with inhibitors [10 mM pyrophosphate, 500 µM sodium fluoride, 1 mM PMSF, 10 µg/ml aprotinin (Sigma), 100 µM sodium vanadate, 10 µg/ml leupeptin (Sigma) and 10 µg/ml benzamide (Sigma)], clarified by high-speed centrifugation at 4°C and pre-cleared with normal IgG and Protein A-Sepharose (Sigma). 750 µg cell lysate (measured by the Micro BCA Protein Assay Kit; Pierce) were immunoprecipitated with 2 µg anti-Src monoclonal antibody EC10. Immunocomplexes were collected on anti-mouse IgG-coated Protein A-Sepharose beads, washed four times with RIPA buffer, once with 0.6 M lithium chloride, eluted using high SDS-containing buffer at high temperature, and separated by 7% SDS-PAGE. v-Src and associated proteins were detected by transferring immunoprecipitated proteins to nitrocellulose and probing with either anti-phosphotyrosine (PY20; Upstate Biotechnology Inc.) at 1:1000 or with anti-pp125^{FAK} (Affiniti) at 1:500. Detection was by incubation with horseradish peroxidase-conjugated secondary antibody and visualization was by enhanced chemiluminescence (Amersham). To detect v-Src and tyrosine-phosphorylated cellular proteins, 5 µg whole-cell lysates were separated by SDS-PAGE and immunoblotted using EC10 or PY20 as described above.

Immunofluorescence

Cells were grown on glass coverslips, fixed at 4°C for 15 min with 3.7% formaldehyde, permeabilized with 0.5% Triton X-100 and incubated with 1:100 anti-Src EC10 or PY20 (1:100). (Similar results were obtained with a polyclonal antiserum raised against v-Src.) Antibody detection was by reaction with FITC-conjugated goat anti-mouse IgG (Sigma) for 45 min at room temperature. As control, cells were incubated with secondary antibody alone. Co-staining of cells was with polyclonal rabbit anti-pp125^{FAK} BC3 (Transduction Laboratories) at 1:400 and TRITC-conjugated goat anti-rabbit IgG or TRITC-conjugated phalloidin (Sigma). Cells were visualized using a confocal microscope (model MRC 600; Biorad) and images were printed on a dye sublimation printer (Kodak).

Wound-healing migration assays

The capacity of CEF expressing *ts* v-Src mutants to migrate was monitored by wound-healing assays carried out as follows. 2×10⁵ cells were seeded in 60 mm dishes at 41°C. When the cells were ~50% confluent they were switched to 35°C or maintained at 41°C as controls. After 24 h the monolayers were wounded by scoring with a sterile micropipette tip. Replicate dishes were either fixed with methanol and stained with Giemsa at the time of wounding (0 h) or maintained at 35°C for a further 24 h to enable cells to migrate into and repair the wound (24 h).

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