

Identification of Sequences Required for the Efficient Localization of the Focal Adhesion Kinase, pp125^{FAK}, to Cellular Focal Adhesions

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Abstract. The integrin family of heterodimeric cell surface receptors play critical roles in multiple biological processes by mediating cellular adhesion to the extracellular matrix (ECM). Adhesion triggers intracellular signaling cascades, including tyrosine phosphorylation and elevation of $[Ca^{2+}]_i$. The Focal Adhesion Kinase (FAK or pp125^{FAK}), a protein tyrosine kinase that colocalizes with integrins in cellular focal adhesions, is a prime candidate for a mediator of integrin signaling events. Here we report an analysis of the domain structure of FAK in which we have

identified a contiguous stretch of 159 amino acids within the COOH terminus essential for correct subcellular localization. When placed in the context of an unrelated cytosolic protein, this Focal Adhesion Targeting (FAT) sequence functions to efficiently mediate the focal adhesion localization of this fusion protein. Furthermore, this analysis suggests that pp125^{FAK} cannot be activated oncogenically by mutation. This result could be explained if pp125^{FAK} either exhibits a narrow substrate specificity or is diametrically opposed by cellular phosphatases or other cellular processes.

ADHESION of cells to the extracellular matrix (ECM)¹ is a critical step in biological processes such as development and differentiation, metastasis, and normal cell growth (Hynes, 1992). The integrin family of cell surface receptors play a prominent role in mediating many of these cellular processes. The integrins are heterodimers consisting of one α and β subunit (Albelda and Buck, 1990; Hynes, 1992). To date, 15 different α chains and 8 different β chains have been identified. The complexity of receptor-ligand interactions is augmented by the fact that an individual integrin receptor may bind more than one adhesive ligand and a particular cell may express a large repertoire of integrins. The integrins are prominent in focal adhesions, subcellular structures formed at regions of close contact between the cell and its underlying substratum. In addition to binding specific ECM components through their extracellular domain, the cytoplasmic tail of the $\beta 1$ integrin binds to at least two defined elements of the cytoskeleton, talin, and α -actin (Horwitz et al., 1986; Tapley et al., 1989; Otey et al., 1990). These two cytoskeletal proteins associate with other proteins present in focal adhesions including vinculin, paxillin, zyxin, and actin (Turner and Burridge, 1991; Burridge et al., 1988). Through these interactions, integrins serve to link the ECM to the cytoskeleton. The architecture

of a focal adhesion is complex and its assembly/disassembly appears to be dependent upon an extensive array of protein-protein interactions.

Increasing evidence indicates that integrin-ligand interactions play a much greater role in biological signaling than simply serving as mediators of ECM-cytoskeleton linkage. Engagement of integrins with defined ligands induce several intracellular signals, including increase in pH_i , elevated $[Ca^{2+}]_i$ levels (Jaconi, 1991; Schwartz et al., 1983; Richter et al., 1990; Pelletier et al., 1992), tyrosine phosphorylation of cellular proteins (Guan et al., 1991; Kornberg et al., 1991, 1992; Golden et al., 1990), and activation of gene expression (Werb et al., 1989). The mechanisms by which integrins initiate signal cascades are unknown, but probably employ intermediary signaling proteins since integrins themselves contain no identified intrinsic enzymatic activity. The increase in phosphotyrosine (pTyr)-containing proteins triggered by ECM-integrin interactions suggests the involvement of integrin-regulated protein tyrosine kinases in these potential signaling events. Recently, we identified a 125-kD cytoplasmic protein tyrosine kinase that localizes to focal adhesions (pp125^{FAK}, Focal Adhesion Kinase, or FAK) (Schaller et al., 1992). The FAK protein is structurally distinct from other identified cytoplasmic tyrosine kinases. The catalytic domain is flanked by large NH₂- and COOH-terminal domains of unknown function. Neither domain exhibits significant sequence similarity to other identified proteins. In addition, FAK contains no SH2 or SH3 domains, motifs which mediate protein-protein interactions and are present in most other cytoplasmic protein tyrosine kinases (Pawsons and Gish, 1992). The sequence of avian, mouse,

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1. *Abbreviations used in this paper:* CE, chicken embryo; dl, deletion; ECM, extracellular matrix; FAK, Focal Adhesion Kinase; FAT, focal adhesion targeting; pTyr, phosphotyrosine.

and human FAK has been determined by cDNA cloning and is highly conserved across species (Schaller et al., 1992; Hanks et al., 1992; Andre and Becker-Andre, 1993). FAK is expressed in most cell lines and tissues examined (Hanks et al., 1992; Andre and Becker-Andre, 1993; Turner et al., 1993; Schaller, M. D., unpublished observations). In some cells the COOH-terminal domain of pp125^{FAK} is expressed autonomously as a 41-kD protein (called *FRNK-FAK-Related-Non-Kinase*) (Schaller et al., 1993).

Evidence from several laboratories has shown that engagement of integrin with ECM ligands or cross-linking of surface integrins with antibodies leads to increased phosphorylation of pp125^{FAK} (Guan and Shalloway 1992; Kornberg et al., 1992; Hanks et al., 1992; Schaller et al., 1993; Burrige et al., 1992) and a concomitant increase in pp125^{FAK} kinase activity in vitro (Guan and Shalloway, 1992). These data, in conjunction with the localization of pp125^{FAK} to focal adhesions, supports the hypothesis that pp125^{FAK} may be directly involved in the transduction of signals transmitted through cell surface integrins.

To understand the regulation of pp125^{FAK} and its potential role in integrin directed signaling, we have initiated an analysis of the structural domains of the FAK protein. Using a series of FAK deletion variants, we have identified a sequence element(s) necessary for the localization of FAK to cellular focal adhesions. This sequence (termed the FAT sequence, for *F*ocal *A*dhesion *T*argeting sequence), resides within a sequence of 159 amino acids embedded in the COOH-terminal domain of pp125^{FAK}. Addition of 199 residues derived from the FAK COOH terminus, which contains the FAT sequence, to a cytosolic form of pp60^{src} directed its localization to focal adhesions. Thus, the FAT sequence appears to be both obligatory for directing FAK to focal adhesions and sufficient to function independently as a focal adhesion localization domain. The role of pp125^{FAK} in mediating cell signaling from the ECM is discussed.

Materials and Methods

Cells and Viruses

Primary chicken embryo (CE) cells were maintained and transfected with retroviral vectors as described (Reynolds et al., 1989). Replication-competent RCASA (BH) and RCAS B (BH) retroviral vectors (Hughes et al., 1987) were used for the expression of FAK and Src derivatives.

Molecular Biology

The full length *FAK* cDNA was cloned into pBluescript (Stratagene, La Jolla, CA) and this plasmid (pBSFAK) (Schaller et al., 1992) was used for the generation of *FAK* variants. Deletion (dl) mutations are designated by the amino acid residues deleted. Base pair designation corresponds to the nucleotide sequences of the *FAK* cDNA which contains 5' untranslated sequences (Schaller et al., 1992). The mutation dl31-144 was generated by deletion of sequences between *Sma*I (bp 137) and *Dra*I (bp 479). To construct dl51-377, pBSFAK was digested with *Bst*XI (bp 200) and the resulting 3' extension removed with T4 DNA polymerase. This blunt end was ligated to DNA digested with *Msc*I (bp 1177), thus deleting nucleotides 197-1176. Mutation dl686-1011 was created by digesting pBSFAK with *Pst*I (which cleaves at nucleotides 2105 and 3083) followed by religation of the *Pst*I termini. Mutation dl721-857 was made by digesting pBSFAK with *Bst*EII (which cleaves at bps 2208 and 2619) followed by rejoining of the *Bst*EII termini. To generate dl853-963, pBSFAK was digested with *Apa*I (position 2608) and the 3' extension removed by treatment with T4 DNA polymerase. This blunt end was ligated to a blunt end formed by digesting pBSFAK with *Nhe*I (position 2941) followed by incubation with T4 DNA polymerase to fill in the 5' extension. The resulting deletion removes nucleotides

2605-2936. The mutation dl965-1012 was created by digesting pBSFAK with *Nhe*I (bp 2941) and *Eco*NI (bp 3083), followed by incubation with T4 DNA polymerase and T4 DNA ligase, to modify the termini and ligate the blunt ends. The resulting mutation deletes nucleotides 2944-3081. In the process of generating dl965-1012, a new codon was created, such that a glutamic acid to alanine substitution occurred at position 965.

The point mutation pmK454R (Lys⁴⁵⁴ to Arg) was generated by oligonucleotide-directed mutagenesis. pBSFAK was used to transform CJ236 *E. coli* (Biorad Labs., Hercules, CA), a strain which allows for uracil incorporation into DNA. Mutagenic primers were annealed to the single-stranded template isolated from CJ236 and second strand synthesis was carried out in vitro using T4 DNA polymerase. In vitro synthesized DNA was transformed into an *E. coli* strain which selects against the DNA strand containing uracil (MV1190, Biorad Labs.). Clones were isolated and analyzed by dideoxy DNA sequencing (Un. States Biochem., Cleveland, OH).

The plasmid constructs encoding the fusion proteins 2ASrcFAT and 2ASrcFATdl were generated as follows. Using PCR, the cDNA encoding a variant of pp60^{src} containing a glycine to alanine substitution at residue 2 was amplified and an *Apa*I restriction site created at nucleotide position 1551. The amplified cDNA was ligated in frame to the *Apa*I site in the *FAK* cDNA at nucleotide position 2608. The resulting clone encodes a fusion protein consisting of the first 517 residues of pp60^{src} fused to residues 853-1052 of pp125^{FAK}. A similar approach was used to generate the 2ASrcFATdl construct, however, the amplified *src* cDNA was ligated in frame to the *Apa*I site of the *FAK* cDNA encoding the pp125^{FAK} mutant dl965-1012 containing the epitope tag at the COOH terminus (see below). Two other variants of pp60^{src} were used. The first contains an amber mutation at amino acid residue 518 (termed Src518Am). Expression of Src518Am results in cellular transformation (Reynolds et al., 1989). The second variant contains the 518 amber mutation and the glycine to alanine substitution at amino acid residue 2 (termed 2ASrc518Am). The 2A mutation abrogates the transforming potential of activated Src variants (Reynolds et al., 1989). cDNAs encoding FAK, the FAK mutations, and Src-FAK fusion proteins were cloned into the retroviral vector RCAS A (BH). cDNAs encoding pp60^{src}, Src518Am, and 2ASrc518Am were cloned into RCAS B (BH).

Epitope-tagged pp125^{FAK} was generated using the pBluescript derivative ptag as previously described (Schaller et al., 1993). Similarly, epitope-tagged FAK variants were created by subcloning into ptag containing the full length *FAK* cDNA. The epitope tag consists of the final 11 COOH-terminal residues of SV40 large T antigen (KPPTPPPEPET). In the tagged constructs, the epitope replaces the final 13 COOH-terminal residues of FAK variants expressed. The 11 residue epitope tag is specifically recognized by the mAb KT3 (MacArthur and Walter, 1984).

Protein Expression and Analysis

Cells were lysed in modified radioimmunoprecipitation assay buffer (Kanner et al., 1989), protein concentration determined by BCA protein assay (Pierce, Rockford, IL), and the lysates evaluated directly or used for immunoprecipitation as previously described (Schaller et al., 1992). Total cell lysates or immune complexes were subjected to SDS-PAGE (Laemmli, 1970) and immunoblotted for the detection of FAK and FAK variants, Src and Src variants, and pTyr. The FAK-specific polyvalent serum BC3 has been described previously (Schaller et al., 1992). The FAK-specific polyvalent serum BC1 was raised against a bacterially expressed trpE-FAK fusion protein (Koerner et al., 1991) containing residues 311-701 of FAK. Immunoprecipitation and Western blot detection of Src and Src derivatives was performed using the mouse mAb EC10 (Parsons et al., 1984). Western blot detection of tyrosine phosphorylated proteins was carried out as described previously using affinity-purified rabbit antibodies specific for pTyr (Kanner et al., 1990). For in vitro kinase assays, BC3 immune complexes or EC10 immune complexes were incubated for 15 min at room temperature in FAK kinase buffer (10 mM Pipes [pH 7.2], 3 mM MnCl₂) or Src kinase buffer (20 mM Pipes [pH 7.2], 10 mM MnCl₂) plus 10 μ Ci [γ -³²P]ATP (Dupont/New England Nuclear, 6,000 Ci mmole⁻¹) (Schaller et al., 1992; Bryant and Parsons, 1984) and the complexes visualized by SDS-PAGE and autoradiography.

Immunofluorescence

Immunofluorescence experiments were carried out as previously described (Reynolds et al., 1989; Wu et al., 1991). FAK variants and Src variants were detected with mAbs KT3 or EC10, respectively, affinity purified goat anti-mouse secondary antibody, and FITC-conjugated donkey anti-goat im-

munoglobulin. Talin was detected with a talin-specific rabbit serum and a Texas red-conjugated donkey anti-rabbit immunoglobulin.

Results

Expression of pp125^{FAK} Variants

A mutational analysis of pp125^{FAK} was initiated to identify regions of the protein that mediate various aspects of its function, including subcellular localization and regulation of its enzymatic activity. Epitope-“tagged” FAK variants bearing either deletions or a Lys to Arg point mutation within the ATP-binding site of the catalytic domain (Knighton et al., 1991; Hanks et al., 1988) were generated as described in Materials and Methods (Fig. 1). Cells overexpressing either pp125^{FAK} or FAK variants retained a normal cellular morphology (data not shown). To assess the level of FAK protein expression and the extent of *in vivo* tyrosine phosphorylation, transfected cells were lysed and FAK proteins immunoprecipitated using the FAK-specific polyvalent serum BC3. Analysis of immune complexes by Western blot analysis using BC3 (Fig. 2 *a*) or anti-pTyr antibody (Fig. 2 *b*) confirmed both the expression and tyrosine phosphorylation of polypeptides of the correct predicted molecular weight. Expression of endogenous pp125^{FAK} was readily detected by BC3 Western blot upon longer exposure of the autoradiographs (data not shown). Interestingly, the expression of dl51-377 (*a*, lane 8) resulted in the appearance of two BC3 immunoreactive proteins of ~83 and 87 kD. We speculate that the additional polypeptide may be generated by alternative translational initiation or proteolytic cleavage of NH₂-terminal sequences. Also evident is the attenuated expression of dl31-144, which was reproducibly diminished relative to the expression of other variants (*a*, lane 7; see also Figs. 3 and 4). The altered expression of this variant did not appear to reflect incomplete infection of the cells, as these cells were resistant to superinfection by a transformation competent virus of the same subgroup (data not shown).

This suggests the possibility that deletion of NH₂-terminal sequences may either increase the rate of mutant protein degradation or inhibit the rate of protein synthesis. Finally, BC3 immunoprecipitation and Western blotting of extracts from cells transfected with dl686-1011 yielded little detectable signal (*a*, lane 12). However, since this mutation deletes the majority of the sequences used to generate the BC3 antisera, extracts were immunoprecipitated and immunoblotted with a second polyvalent sera, BC1, which is directed against the FAK kinase domain. As shown in Fig. 2, BC1 recognized a polypeptide of correct predicted molecular weight that was phosphorylated on tyrosine (*a* and *b*, lanes 15 and 17).

Anti-pTyr Western blotting of immune complexes prepared in parallel, indicated that each of the deletion variants was phosphorylated on tyrosine *in vivo*, and the pTyr signal was proportional to the level of protein expression. As expected, the predicted kinase deficient variant pmK454R showed significantly reduced levels of tyrosine phosphorylation (*b*, lane 13). The low level of tyrosine phosphorylated FAK protein observed in BC3 immune complexes from cells expressing pmK454R was similar to that present in immune complexes from mock- or vector-transfected cells, and was consistent with the presence of endogenous pp125^{FAK} in the immune complex. This observation implies that the majority of tyrosine phosphorylation of FAK *in vivo* is primarily the consequence of autophosphorylation and not transphosphorylation by other cellular protein tyrosine kinases.

To assess the *in vivo* kinase activity of the pp125^{FAK} mutants, total cell lysates from transfected cells were analyzed for pTyr-containing proteins by anti-pTyr Western blotting (Fig. 3). In each case, overexpression of pp125^{FAK} or individual FAK variants did not result in a significant increase in tyrosine phosphorylation of cellular proteins, indicating that none of the deletions deregulate FAK catalytic activity and lead to unscheduled tyrosine phosphorylation of cellular proteins.

The *in vitro* kinase activity of individual FAK variants was

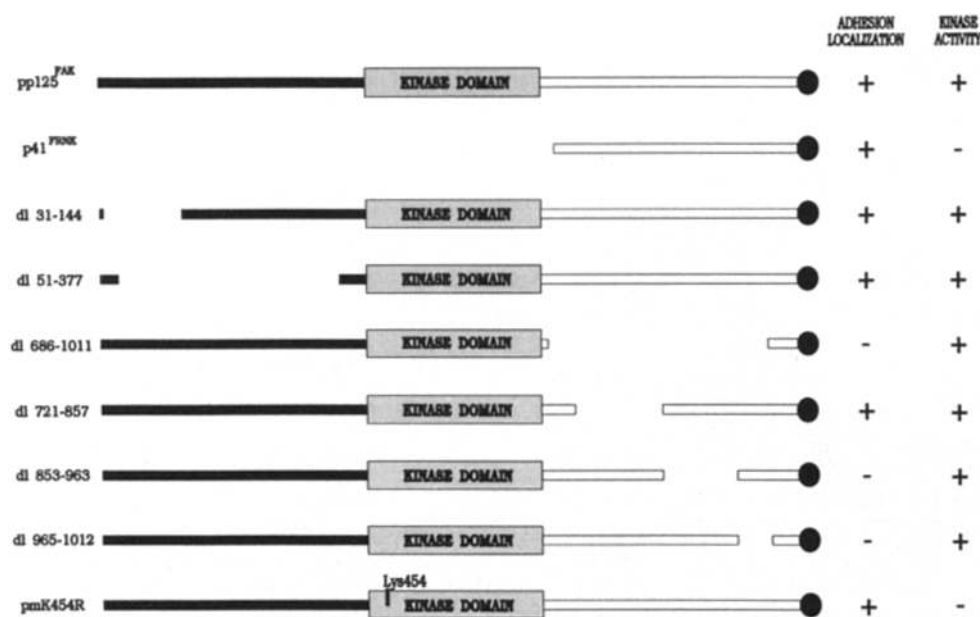


Figure 1. Diagrammatic representation of pp125^{FAK}, p41^{FRNK}, and pp125^{FAK} variants. The NH₂-terminal domain is represented by the black region, the kinase domain by the shaded region, and the COOH-terminal domain by the white region. Filled circles indicate the presence and position of the epitope tag. Internal deletions were generated using specific restriction enzymes and modifying enzymes as described in Materials and Methods. The subcellular localization and enzymatic activity of each variant is indicated at the right (+ indicating focal adhesion localization or enzymatic activity as measured by *in vitro* kinase assay, respectively).

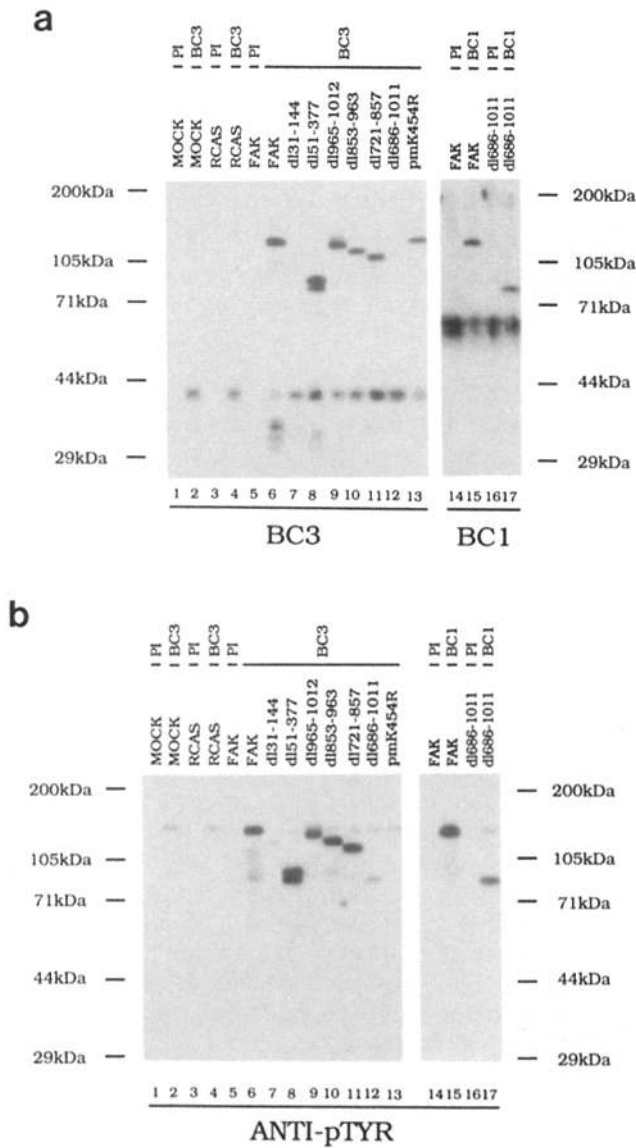


Figure 2. Expression and tyrosine phosphorylation of pp125^{FAK} and pp125^{FAK} variants. The cDNAs encoding FAK or FAK variants were cloned into the RCAS A (BH) retroviral vector and expressed in CE cells. Cell lysates (500 μ g) from control cells or cells transfected with the various constructs were evaluated by immunoprecipitation using polyvalent serum BC3 (*a* and *b*, lanes 2, 4, and 6-13) or BC1 (*a* and *b*, lanes 15 and 17). Immunoprecipitates were divided in half and analyzed by Western blotting using BC3 (*a*, lanes 1-13), BC1 (*a*, lanes 14-17), or anti-pTyr (*b*). Control immunoprecipitations were carried out in parallel using preimmune serum (PI, *a* and *b*, lanes 1, 3, 5, 14, and 16). Retroviral constructs used are listed across the top, where *Mock*, mock transfected; *RCAS*, empty vector; and *FAK*, wild-type pp125^{FAK}.

measured using an *in vitro* immune complex kinase assay (Fig. 4). BC3 immune complexes containing individual deletion variants exhibited greatly elevated autophosphorylation activity in comparison to the activity of BC3 immune complexes from control cells. This elevated level of kinase activity detected *in vitro* correlated with the level of protein expression. The point mutant pmK454R showed only a background level of activity (Fig. 4, lane 13), comparable to that observed in control immune complexes containing en-

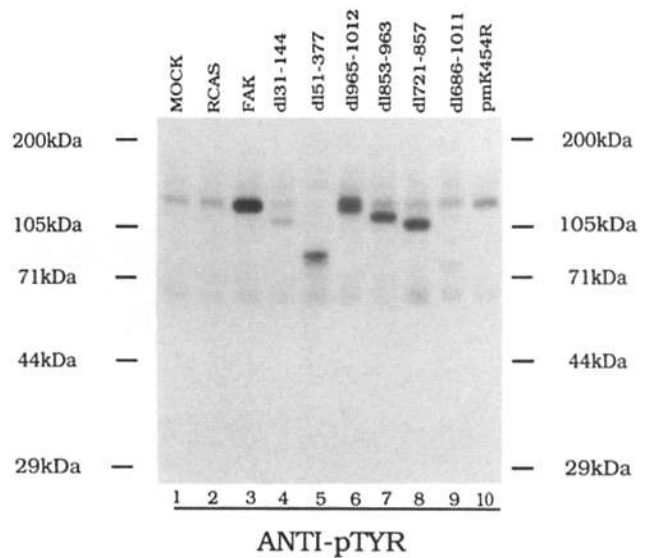


Figure 3. Phosphorylation state of cellular proteins. Cell lysates (50 μ g) from control cells or cells expressing the various *FAK* cDNAs were subjected to SDS-PAGE and the samples analyzed by Western blotting with anti-pTyr.

dogenous pp125^{FAK}, confirming that this mutant is enzymatically inactive.

Subcellular Localization of pp125^{FAK} Mutants

Our previous results indicate that both FAK and FRNK localize to focal adhesions (Schaller et al., 1993), suggesting that the sequences responsible for localization reside within the COOH-terminal one-third of pp125^{FAK}. To more thoroughly identify the FAK sequences required for targeting FAK to focal adhesions, the subcellular localization of individual FAK variants was assessed (Fig. 5). To distinguish between endogenous and exogenously expressed FAK, cells

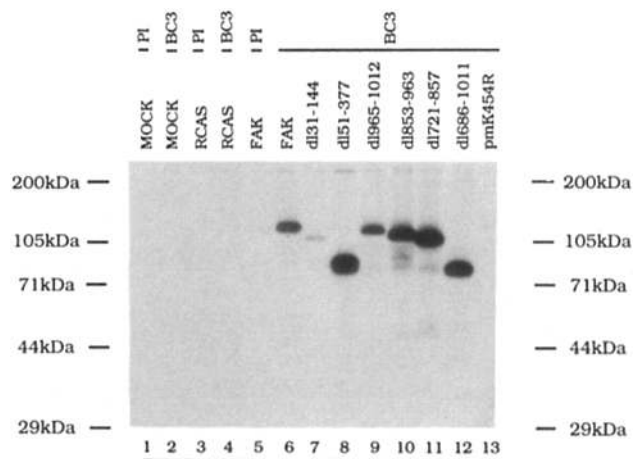


Figure 4. *In vitro* kinase activity of pp125^{FAK} variants. BC3 immune complexes (lanes 2, 4, and 6-13) from control cells and cells expressing the various pp125^{FAK} retroviral constructs were incubated in kinase buffer and [γ -³²P]ATP, subjected to SDS-PAGE, and visualized by autoradiography. Control immune complexes using preimmune serum were performed in parallel (lanes 1, 3, and 5).

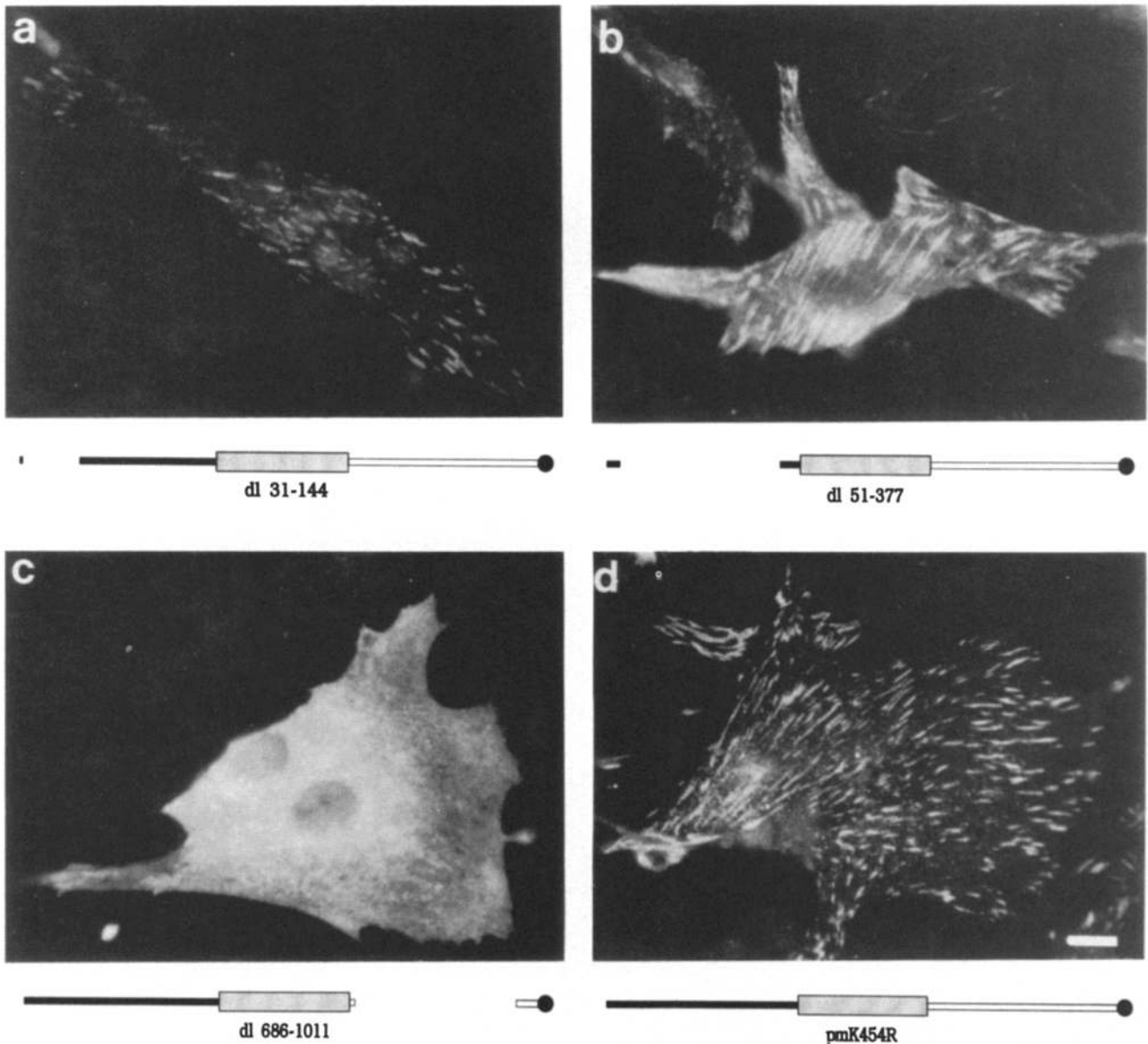


Figure 5. Immunostaining of NH₂-terminal, COOH-terminal, and kinase defective FAK variants. CE cells expressing epitope-tagged variants of dl31-144 (a), dl51-377 (b), dl686-1011 (c), and pmK454R (d) were grown overnight on glass coverslips, fixed, and immunostained with mAb KT3 as described in Materials and Methods. Bar, 3.5 μ m.

expressing epitope-tagged variants of FAK were immunostained with mAb KT3. Cells expressing epitope-tagged variants dl31-144 (Fig. 5 a), dl51-377 (Fig. 5 b), or pmK454R (Fig. 5 d) exhibited characteristic focal adhesion staining upon incubation with mAb KT3. In contrast, little or no staining of focal adhesion structures was observed in cells expressing the COOH-terminal deletion variant dl686-1011 (Fig. 5 c). These data not only support earlier findings which indicate that the FAT sequence is contained in the COOH-terminal domain of FAK, but also imply that the NH₂ terminus is not required for focal adhesion localization. Furthermore, the presence of kinase deficient FAK in focal adhesions indicates that kinase activity is not a necessary prerequisite for translocation of FAK to focal adhesions.

To further define the sequences necessary to mediate the localization to focal adhesions, three additional COOH-

terminal deletion variants of FAK were analyzed (Fig. 6). Cells expressing epitope-tagged dl721-857, when stained with mAb KT3, exhibited extensive focal adhesion structures (Fig. 6 a). In contrast, mAb KT3 immunostaining of cells expressing either epitope-tagged dl853-963 or dl965-1012 revealed a punctate cytoplasmic fluorescence (Fig. 6, c and e, respectively). Costaining of these cells with an antibody directed to the focal adhesion protein talin revealed a distinctive focal adhesion staining pattern, confirming that these cells retain intact adhesion structures (Fig. 6, d and f). The immunostaining experiments clearly indicate that the FAT sequence resides within or overlaps the 159 residues between amino acid positions 853 and 1012.

Focal Adhesion Localization of a Cytosolic Protein

Having demonstrated that FAK residues 853–1012 contain a

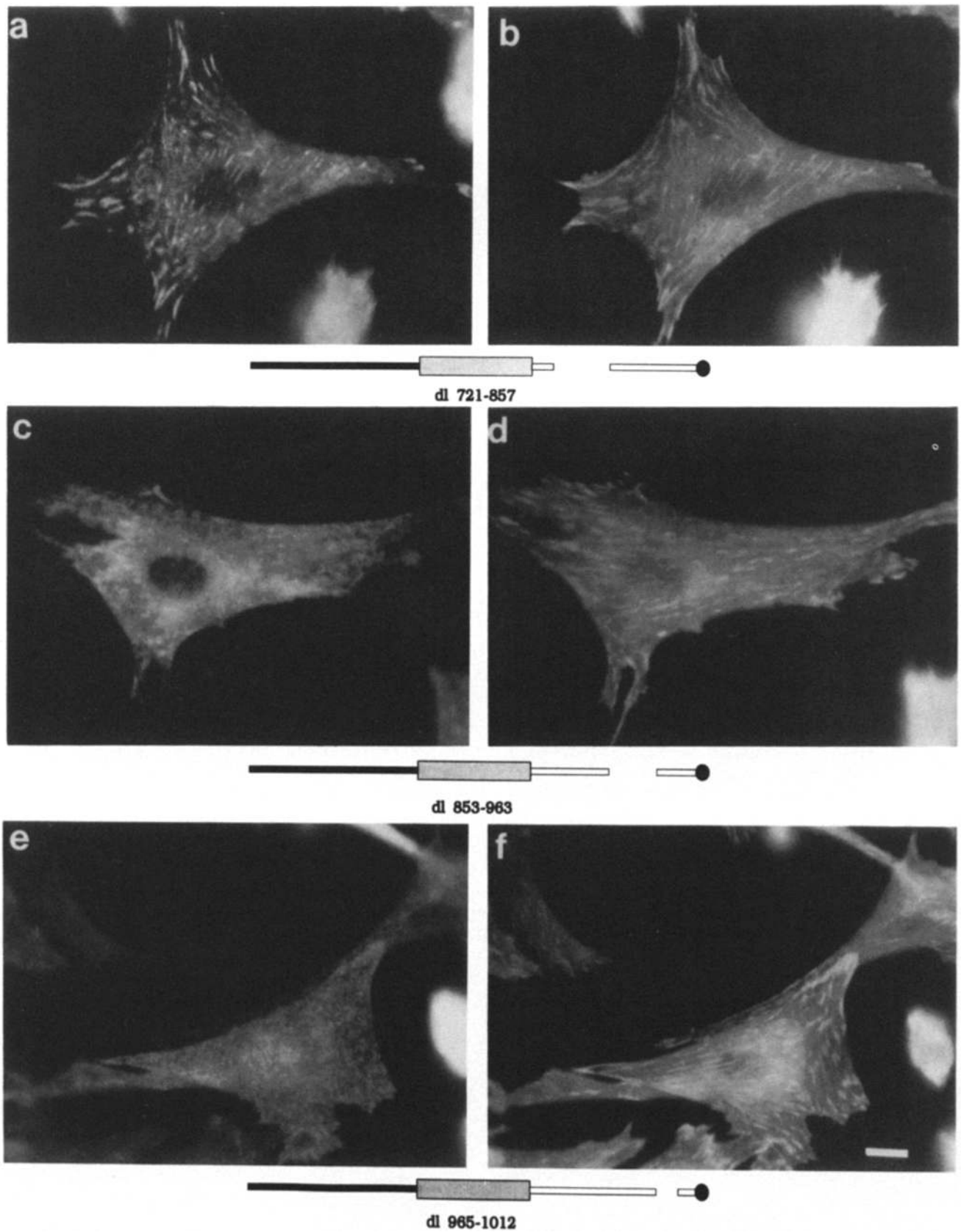


Figure 6. Fine mapping of pp125^{FAK} COOH terminus. CE cells expressing epito-tagged variants dl721-857 (*a* and *b*), dl853-963 (*c* and *d*), or dl965-1012 (*e* and *f*) were grown overnight on glass coverslips, fixed, and costained with mAb KT3 (*a*, *c*, and *e*) and a rabbit polyvalent serum specific for talin (*b*, *d*, and *f*) as described in the Materials and Methods. Bar, 3.5 μm.

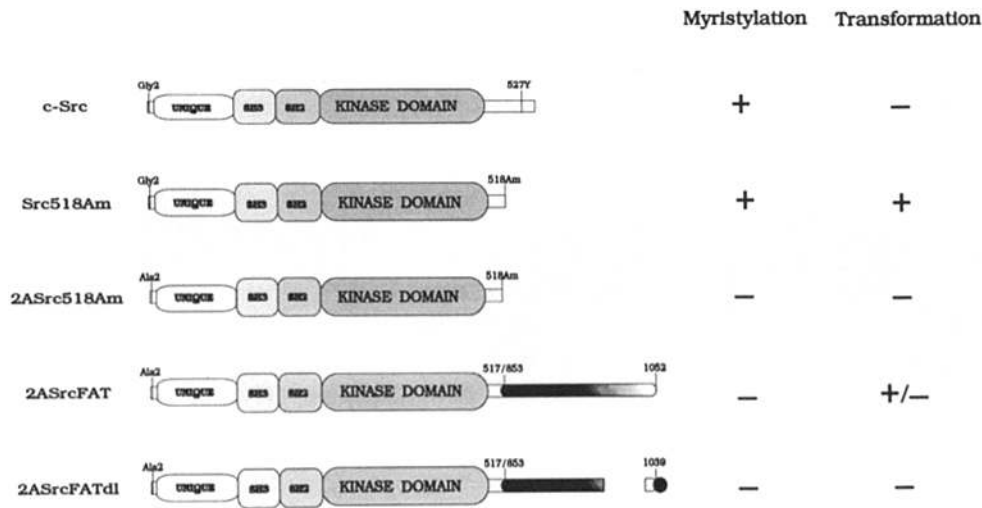


Figure 7. Schematic representation of c-Src, Src variants, and Src-FAK fusions. The cDNA encoding pp60^{c-src} was modified to encode four variants. Point mutations in codons 2 and 518 result in the generation of a glycine (Gly2) to alanine (Ala2) substitution mutation and an amber nonsense mutation (518Am), respectively. Myristylation and transforming activity of each individual variant is indicated at right (see text for discussion of the phenotype induced by each variant).

major determinant for pp125^{FAK} localization to focal adhesions, we sought to determine if the FAT sequence could function as a localization domain if placed in the context of a cytoplasmic protein. The portion of the FAK cDNA encoding the FAT sequence was ligated to the cDNA encoding an activated variant of pp60^{src} containing a Gly to Ala mutation at position 2 (Fig. 7). This mutation removes the NH₂-terminal myristylation site of pp60^{src} and consequently the mutant Src protein is cytosolic in contrast to the normal membrane-associated wild-type pp60^{src} (Reynolds et al., 1989). The resulting Src-FAK fusion protein, 2ASrcFAT, contains the first 517 residues of pp60^{src} fused to the final 199 amino acids of pp125^{FAK}. 2ASrcFAT was cloned into RCAS A (BH) and transfected into CE cells in parallel with three additional Src variants (Fig. 7). The subcellular localization of the overexpressed Src variants was assessed by dual immunofluorescence staining employing the pp60^{src}-specific mouse mAb EC10 and a rabbit antiserum directed against the focal adhesion associated protein talin (Fig. 8). Staining of cells expressing pp60^{src} with mAb EC10 showed predominantly membrane and perinuclear fluorescence, whereas the anti-talin antibodies stained focal adhesions (Fig. 8, *a* and *b*). Cells expressing 2ASrc518Am exhibit primarily cytoplasmic fluorescence with mAb EC10, while the anti-talin antibodies stained focal adhesion structures (Fig. 8, *c* and *d*). There appeared to be fewer well formed focal adhesion structures in cells expressing 2ASrc518Am than in those cells expressing c-Src, probably due to the altered cellular morphology that results from 2ASrc518Am deregulated kinase activity (see below). Cells expressing 2ASrcFAT exhibited extensive staining of focal adhesions with both EC10 and anti-talin (Fig. 8, *e* and *f*). The targeting of 2ASrcFAT to focal adhesions was dependent upon the intact FAT sequence since cells expressing 2ASrcFATdl (lacking the FAT sequence) exhibited primarily cytoplasmic fluorescence with EC10, while anti-talin revealed extensive focal adhesion staining (Fig. 8, *g* and *h*). These data establish that addition of the FAT sequence to a cytosolic protein appears sufficient to target the protein to cellular focal adhesions.

Kinase Activity of Focal Adhesion Targeted Src Variants

We anticipated that targeting an activated Src variant to

cellular focal adhesions might lead to enhanced tyrosine phosphorylation of some cytoskeletal components and subsequent alterations in cellular phenotypes as previously reported (Liebl and Martin, 1992). Since cellular transformation was not readily evident, we sought to examine the *in vivo* enzymatic activity of the Src variants 2ASrcFAT and 2ASrcFATdl. To evaluate the *in vivo* catalytic activity, total cell lysates were analyzed directly by anti-pTyr Western blotting (Fig. 9 *a*). Mock and vector-alone transfected cells showed only a 125-kD tyrosine phosphorylated protein. Cells expressing c-Src contained two tyrosine phosphorylated proteins, presumably pp125^{FAK} and pp60^{c-src} (*a*, lane 3). In contrast, cells expressing 2ASrc518Am and Src518Am contained numerous tyrosine phosphorylated cellular proteins (*a*, lanes 4 and 5, respectively), consistent with previous observations (Reynolds et al., 1989). However, cells expressing 2ASrcFAT and 2ASrcFATdl exhibited a severely restricted repertoire of tyrosine phosphorylated cellular proteins (*a*, lanes 6 and 7, respectively). The major tyrosine phosphorylated proteins appear to be the fusion proteins themselves and a protein of similar molecular weight to pp125^{FAK}. These data indicate that neither of the Src-FAK fusion proteins exhibit the ability to phosphorylate cellular proteins as efficiently as either Src518Am or 2ASrc518Am.

As shown in Fig. 9, tyrosine phosphorylation of 2ASrcFATdl was modest compared to that observed for 2ASrcFAT (*a*, lanes 6 and 7). Therefore, the level of expression and activity of individual fusion proteins were examined (Fig. 9, *b* and *c*). The Src variants and the Src-FAK fusion proteins were immunoprecipitated using EC10 and the immune complexes analyzed by EC10 immunoblotting or *in vitro* kinase assay. Western blot analysis indicated that 2ASrcFATdl was expressed at a level ~20% that of 2ASrcFAT (*b*, lanes 6 and 7). *In vitro* kinase assays indicated that each of the proteins was enzymatically active, with 2ASrcFAT and 2ASrcFATdl exhibiting *in vitro* activity comparable to the degree of expression (*b* and *c*, lanes 6 and 7, respectively).

The lack of tyrosine phosphorylation of cellular proteins in 2ASrcFAT and 2ASrcFATdl over-expressors indicates that the addition of sequences to the COOH terminus of Src may render it incapable of phosphorylating certain cellular proteins that are normally phosphorylated by Src518Am and

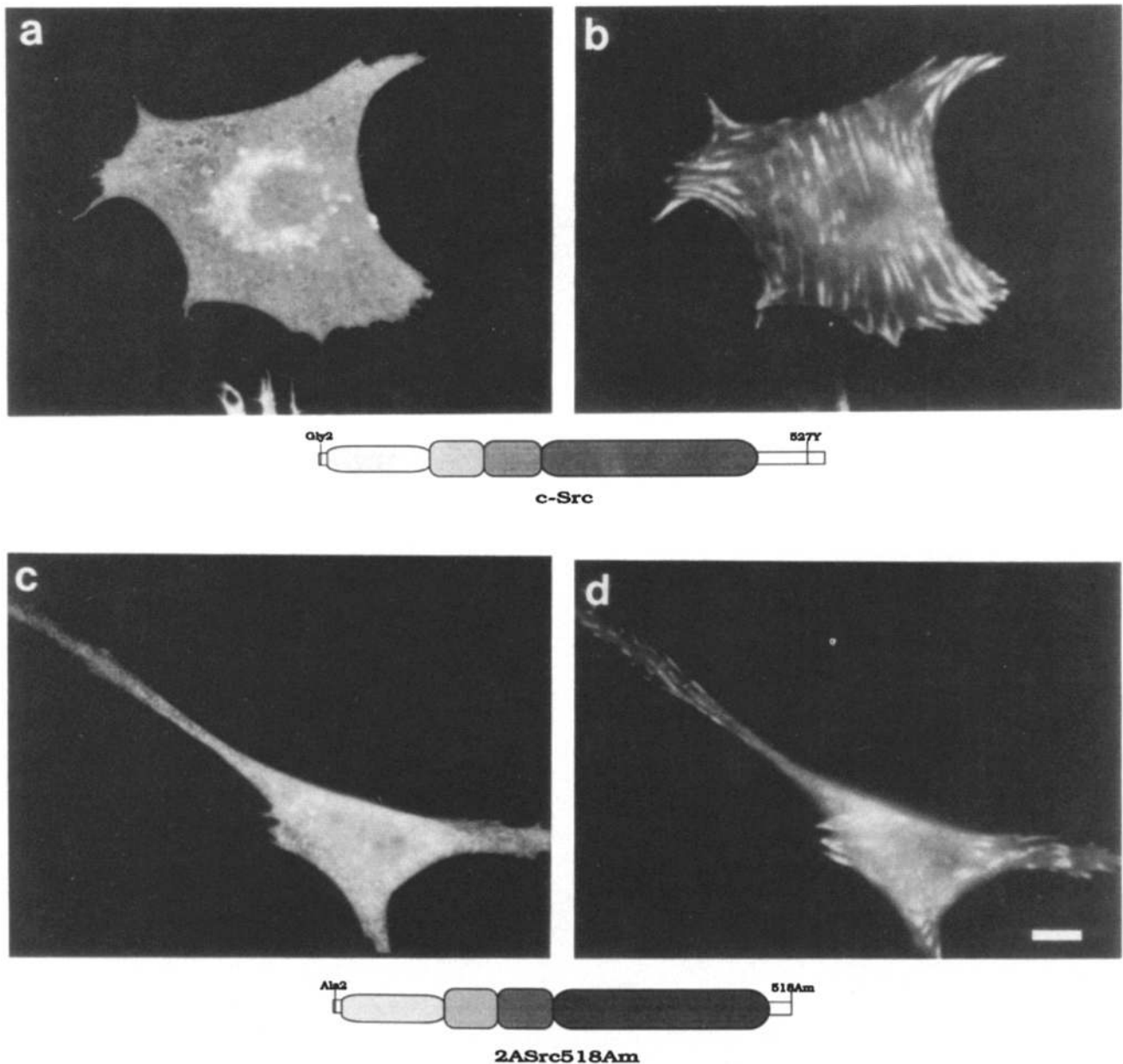


Figure 8. Immunostaining of c-Src, 2ASrc518Am, and Src-FAK fusions. CE cells expressing c-Src (*a* and *b*), 2ASrc518Am (*c* and *d*), 2ASrcFAT (*e* and *f*), and 2ASrcFATd1 (*g* and *h*) were grown overnight on glass coverslips, fixed, and costained with mAb EC10 (*a*, *c*, *e*, and *g*) and anti-talin rabbit polyvalent serum (*b*, *d*, *f*, and *h*) as described in Materials and Methods. Bar, 3.5 μ m.

2ASrc518Am. For example, the low *in vivo* enzymatic activity exhibited by 2ASrcFAT and 2ASrcFATd1 may result from steric inhibition of the catalytic activity of the kinase by intramolecular interactions between the FAT sequence and the NH₂ terminus of Src or intermolecular interaction between the FAT sequence and another cellular protein.

Discussion

The complex architecture of cellular focal adhesions is dependent upon numerous defined protein-protein interactions between focal adhesion components (Burrige et al., 1988; Turner and Burrige, 1991). However, the mechanisms and cellular components regulating these association events are unknown. Here we report the identification of the FAT se-

quence of pp125^{FAK}. The FAT sequence resides in the distal 199 COOH-terminal residues of pp125^{FAK} and is required for efficient localization to focal adhesions. The presence of tyrosine phosphorylated proteins in focal adhesions (Burrige et al., 1992; Guan et al., 1991) and the regulation of tyrosine phosphorylation of these proteins in response to cell adhesion and spreading (for review see Juliano and Haskal, 1993; Schaller et al., 1993) makes pp125^{FAK} a prime candidate for a regulatory component of focal adhesion assembly/disassembly as well as a possible mediator of signaling to the nucleus.

Experimental analysis of most focal adhesion proteins suggests that the subcellular localization of these proteins is dependent upon specific protein-protein interactions. Investigation and identification of functional domains of focal

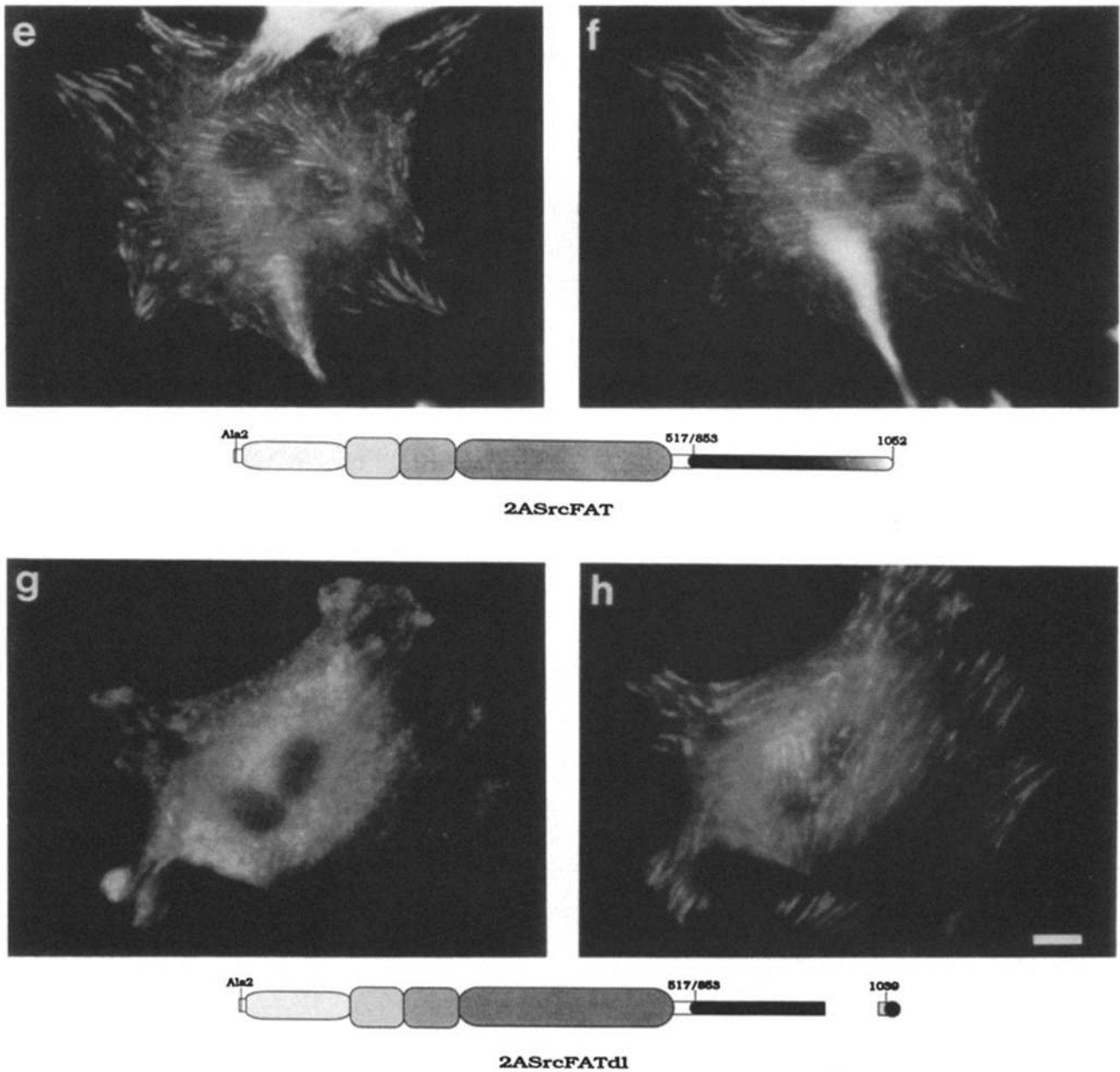


Figure 8.

adhesion proteins has relied primarily upon the biochemical study of purified focal adhesion proteins and proteolytic fragments of purified focal adhesion components (Pavalko and Burridge, 1991; Otey et al., 1990; Turner et al., 1990; Sadler et al., 1992; Nuckolls et al., 1990; Horwitz et al., 1986). Conversely, we have used a mutational analysis to investigate the domain structure of pp125^{FAK} and identify the regions that determine subcellular localization. Deletion of virtually the entire NH₂ terminus had no apparent consequence upon the localization of pp125^{FAK}. In contrast, small adjacent deletions of either 110 (dl853-963) or 47 (dl965-1012) amino acid residues in the COOH terminus abolished focal adhesion localization, resulting in cytosolic variants. A polypeptide containing residues 853-1052, when fused in frame to the COOH terminus of a normally cytosolic protein, was capable of mediating focal adhesion localization.

Therefore, this sequence directs FAK to focal adhesions and presumably contains a site that may mediate specific protein-protein interaction(s) between pp125^{FAK} and other focal adhesion components. Efforts are underway to identify the FAK-binding protein present in focal adhesions. We predict, that this protein may be a relatively abundant protein, since overexpression of pp125^{FAK} (~10-fold) results in a significant increase in focal adhesion staining. Hence, either focal adhesions contain an abundance of FAK-binding sites or FAK is capable of self-association, forming higher order aggregates in focal adhesions.

The use of specific protein-protein or protein-membrane interactions to target protein tyrosine kinases to predetermined cellular compartments is well documented. The Abl protein tyrosine kinase contains an actin-binding domain within its COOH terminus (McWhirter and Wang, 1993) and

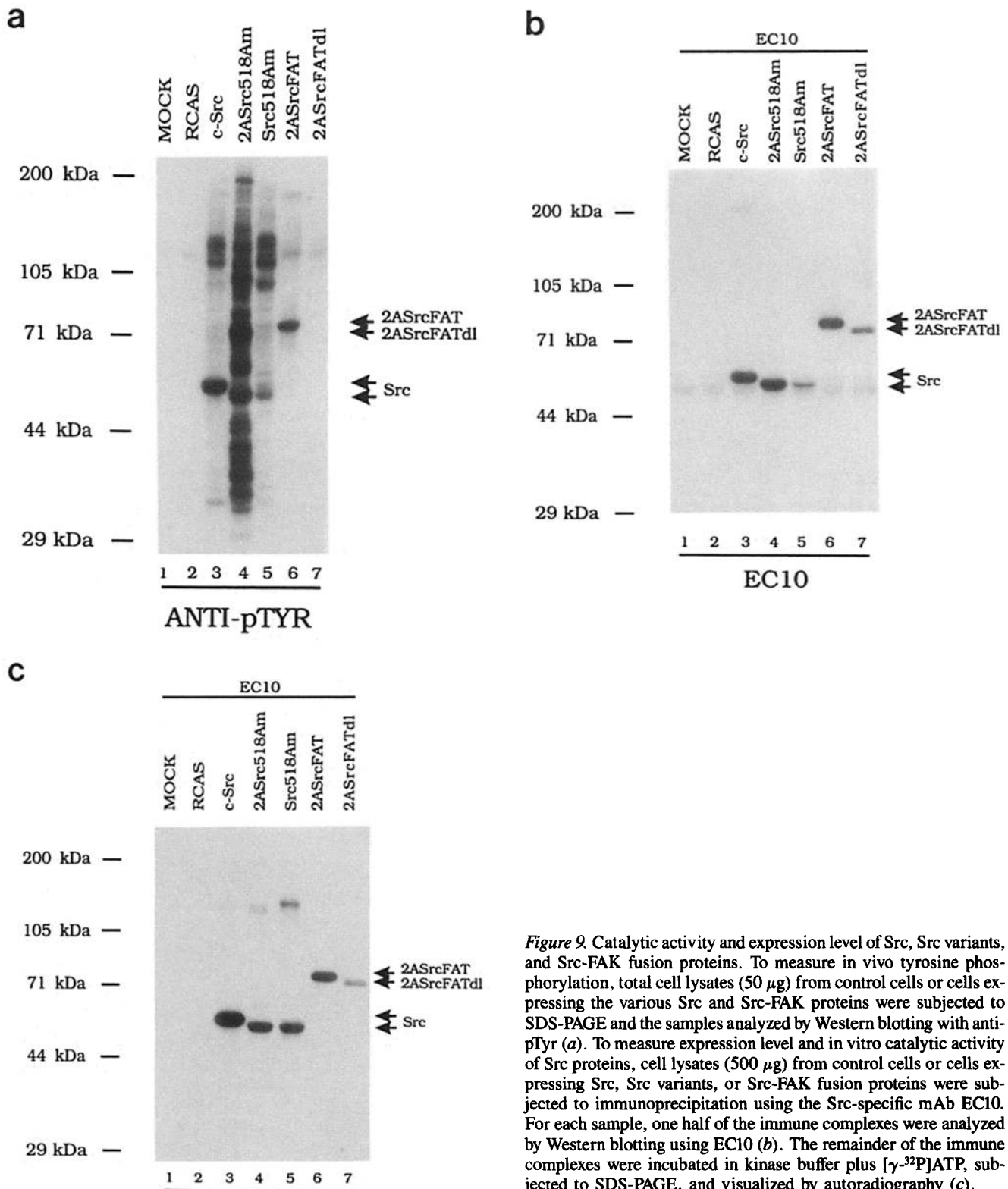


Figure 9. Catalytic activity and expression level of Src, Src variants, and Src-FAK fusion proteins. To measure *in vivo* tyrosine phosphorylation, total cell lysates (50 μ g) from control cells or cells expressing the various Src and Src-FAK proteins were subjected to SDS-PAGE and the samples analyzed by Western blotting with anti-pTyr (a). To measure expression level and *in vitro* catalytic activity of Src proteins, cell lysates (500 μ g) from control cells or cells expressing Src, Src variants, or Src-FAK fusion proteins were subjected to immunoprecipitation using the Src-specific mAb EC10. For each sample, one half of the immune complexes were analyzed by Western blotting using EC10 (b). The remainder of the immune complexes were incubated in kinase buffer plus [γ - 32 P]ATP, subjected to SDS-PAGE, and visualized by autoradiography (c).

this domain appears to be critical for both the normal function of c-Abl (Henkemeyer et al., 1990; Schwartzberg et al., 1991; Tybulewicz et al., 1991) and the transforming potential of the oncoprotein Bcr-Abl (McWhirter and Wang, 1993). The *N*-myristylation site of pp60^{src} is required for membrane association and is essential for the oncogenic potential of v-Src (Parsons and Weber, 1989), and presumably for the

normal function of its cellular homologue, c-Src (Bagrodia et al., 1993; Reynolds et al., 1989). Therefore, we speculate that the interaction of pp125^{FAK} with a specific focal adhesion protein may be essential for placing pp125^{FAK} in the correct cellular compartment, in close proximity to potential regulator and effector molecules.

Our results indicate that autophosphorylation is the pri-

mary cause of FAK tyrosine phosphorylation. The role of FAK autophosphorylation in regulating its cellular function is unknown. It is clear, however, that autophosphorylation plays no role in targeting FAK to focal adhesions since an enzymatically inactive variant of FAK properly localizes to focal adhesions. Recently we have demonstrated the stable association between FAK and activated variants of pp60^{src} in Src transformed cells and the association of FAK and c-Fyn in normal CE cells. The stable association of these kinases is dependent upon both phosphorylation of FAK and an intact Src SH2 domain (Cobb et al., 1994). We have proposed that the autophosphorylation of FAK may serve to recruit Src-family kinases or concomitantly other SH2-containing proteins, such as tensin (Davis et al., 1991), to focal adhesions. The investigation and identification of the autophosphorylation site, the role autophosphorylation may play in regulating enzymatic activity, and the possible transphosphorylation of pp125^{FAK} will provide additional clues to the functional significance of these interactions.

The ability of p41^{FRNK} and pmK454R, both of which are catalytically inactive, to localize to focal adhesions raises the possibility that either protein may function in dominant negative fashion by occupying all of the potential binding sites for enzymatically active pp125^{FAK}. To date, we have little data to suggest that overexpression of either FRNK or pmK454R effects normal cellular morphology or growth (data not shown; Schaller et al., 1993). One might predict that exclusion of pp125^{FAK} enzymatic activity from focal adhesions might alter cellular functions predicted to be dependent upon focal adhesions such as cell spreading and migration. The apparent lack of phenotype associated with cells overexpressing pmK454R or FRNK suggests that localization to focal adhesions may not be sufficient to interfere with integrin coupled signaling. Preliminary microinjection experiments using a mAb directed to the FAT sequence indicate that pp125^{FAK} is intimately involved in cellular function. Cells injected with the mAb 2A7 (Kanner et al., 1990) do not spread after trypsinization and replating onto fibronectin (Schwartz, M. A., personal communication). These observations implicate FAK as a critical regulator of cell spreading and support the model that FAK must localize to focal adhesions to be functionally active.

The localization of an activated variant of Src, 2ASrcFAT, to cellular focal adhesions did not result in significant changes in cell morphology and growth although the infected cells grew in a disorganized manner compared to normal CE cells (data not shown). Our data are in contrast to that reported by Liebl and Martin (1992), who reported that fusion of the focal adhesion targeting domain of vinculin to the NH₂ terminus of activated Src resulted in cell transformation. The experimental differences may reflect the consequences of using different domains to target proteins to focal adhesions. For example, addition of targeting domains to NH₂- or COOH-terminal regions of Src may orient the catalytic domains differently, exposing the Src kinases to different cellular substrates. Similarly, vinculin and FAK may localize to different subcompartments within a focal adhesion. This may be a critical parameter in the transforming potential, such that the catalytic domain may be more readily available to interact with cellular substrates when in the position normally occupied by vinculin than when in the position normally occupied by FAK.

Oncogenic transformation induced by most tyrosine kinases depends upon deregulated enzyme function, usually arising from the deletion or disruption of specific regulatory elements contained within the kinase itself. This paradigm applies to the oncogenic activation of both receptor and non-receptor tyrosine kinases (for review see Cantley et al., 1991; Ullrich and Schlessinger, 1990). In the case of v-erb-B, deletion of the extracellular ligand-binding domain removes the inhibitory function this structure normally imposes upon the cytoplasmic kinase domain (Downward et al., 1984). In the proto-oncogene encoded forms of Src and Src-like kinases (Cantley et al., 1991; Parsons and Weber, 1989), phosphorylated tyrosine residues located at the extreme COOH terminus of the protein interact with SH2 domains located just NH₂-terminal of the catalytic domain, functioning to maintain the kinase in an inactive state (Cantley et al., 1991; Cooper and Howell, 1993). In Src and Src-like oncoproteins transduced by retroviruses, these regulatory tyrosine residues are deleted, thus abolishing the inhibition. In the examples of both v-erb-B and Src-like kinases, disruption of the regulatory elements leads to the constitutive activation of these enzymes. In the case of Src, activation of enzyme activity is mirrored by a dramatic elevation in the tyrosine phosphorylation of other cellular proteins, presumably the effector molecules, and subsequent cellular transformation (Reynolds et al., 1989). FAK does not appear to fit the model set forth by either the Src-like kinases or the receptor tyrosine kinases. In the case of pp125^{FAK}, deletion of either NH₂- or COOH-terminal sequences does not appear to deregulate the catalytic activity of the FAK protein, as evidenced by the lack of elevated levels of tyrosine phosphorylation in cells expressing these variants. In accord, cells overexpressing pp125^{FAK} or deletion variants of pp125^{FAK} have normal growth properties and are not morphologically transformed. It is possible that the transforming capacity of FAK and FAK variants is negated by either the activity of cellular phosphatases, a narrow substrate specificity, or some other unidentified cellular mechanism.

Several cytosolic tyrosine kinases have recently been identified as signaling components of cell surface receptors that themselves contain no identified intrinsic enzymatic activity. These include the T cell receptor, the high affinity Fc receptor for immunoglobulin (for review see Weiss, 1993), and the interferon α/β receptors (Velazquez et al., 1992). These kinases, including ZAP-70 (Chan et al., 1992), p72^{syk} (Hutchcroft et al., 1991; Taniguchi et al., 1991), tyk2 (Velazquez et al., 1992; Firmbach-Kraft et al., 1990), and JAK-1 and JAK-2 (Wilks et al., 1991), like pp125^{FAK}, are structurally distinct from the Src family of protein tyrosine kinases and the receptor tyrosine kinases. Also like FAK, the mechanism by which these kinases are regulated to coordinately receive and transmit signals is unknown. These cytoplasmic kinases may not possess obvious negative regulatory domains such as those in the Src-family kinases. Therefore, the regulation of FAK may be more closely related to these cytosolic kinases than to the Src-like protein tyrosine kinases or the receptor tyrosine kinases. The FAK mutants described here should prove invaluable in the elucidation of the mechanism by which FAK is regulated.

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