Autophosphorylation of Tyr[397] and its phosphorylation by Src-family kinases are altered in focal-adhesion-kinase neuronal isoforms

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In brain, focal adhesion kinase (FAK) is regulated by neurotransmitters and has a higher molecular mass than in other tissues, due to alternative splicing. Two exons code for additional peptides of six and seven residues ('boxes' 6 and 7), located on either side of Tyr[397], which increase its autophosphorylation. Using *in situ* hybridization and a monoclonal antibody (Mab77) which does not recognize FAK containing box 7, we show that, although mRNAs coding for boxes 6 and 7 have different patterns of expression in brain, FAK + 6,7 is the main isoform in forebrain neurons. The various FAK isoforms fused to green fluorescent protein were all targeted to focal adhesions in non-neuronal cells. Phosphorylation-state-specific antibodies were used to study in detail the phosphorylation of Tyr[397], a critical residue for the activation and function of FAK. The presence of boxes 6 and 7 increased autophosphorylation of Tyr[397] independently and additively, whereas they had a weak effect on FAK kinase activity towards poly(Glu,Tyr). Src-family kinases were also able to phosphorylate Tyr[397] in cells, but this phosphorylation was decreased in the presence of box 6 or 7, and abolished in the presence of both. Thus the additional exons characteristic of neuronal isoforms of FAK do not alter its targeting, but change dramatically the phosphorylation of Tyr[397]. They increase its autophosphorylation *in vitro* and in transfected COS-7 cells, whereas they prevent its phosphorylation when co-transfected with Src-family kinases.

Key words: alternative splicing, autophosphorylation, brain, hippocampus, neurons.

INTRODUCTION

Focal adhesion kinase (FAK) is a widely expressed 125-kDa non-receptor cytoplasmic protein tyrosine kinase implicated in integrin-mediated signal transduction [1,2]. In cells in culture, FAK localizes to focal adhesions. Activation of FAK by integrin clustering or by seven-transmembrane-domain receptors leads to the autophosphorylation of Tyr[397], which allows the binding of the Src-homology 2 (SH2) domain of Src [3], Fyn [4] or phosphatidylinositol 3-kinase [5]. The recruitment of Src-family kinases results in the phosphorylation of tyrosine residues in the catalytic (Tyr[407], Tyr[576] and Tyr[577]) [6] and C-terminal (Tyr[871] and Tyr[925]) regions of FAK [7,8]. Phosphorylation of Tyr[576] and Tyr[577] increases FAK catalytic activity [6]. In some cells, the SH2 domain of Grb2 binds to phospho-Tyr[925] and triggers a Ras-dependent activation of the mitogen-activated-protein-kinase (MAP kinase) pathway [7,9]. The C-terminal region of FAK also contains two proline-rich sequences (PR1 and PR2), which provide binding sites for Src-homology-3 (SH3)-domain-containing proteins, including p130-cas [10,11], HEF [12] and GRAF [13]. In addition, the C-terminal extremity of FAK contains a sequence necessary and sufficient for focal adhesion-targeting (FAT) [14] and is associated with other proteins, including paxillin [15,16] and talin [17]. The whole C-terminal region of FAK can be expressed autonomously in some cell types and play a regulatory role [18,19]. Although several of the proteins associated with FAK are its substrates *in vitro*, they are also good substrates for Src-family kinases, and it is unclear which proteins are directly phosphorylated by FAK in intact cells. The main function of FAK could rather be to recruit Src-family kinases and to serve as a regulated adapter protein. Thus activation of FAK by integrin clustering would result in the formation of active multimolecular complexes, triggering a number of signalling pathways (see [20]). In contrast with the wealth of information accumulated on the properties of FAK C-terminal region, the function of the N-terminal domain of FAK is poorly understood. Recent sequence analysis has revealed that this domain of FAK is related to the band 4.1/ERM (ezrin/radixin/moesin) domain and to the N-terminal domain of Janus kinases (JAKs) [21]. Many members of this domain superfamily, referred to as 'band 4.1/JEF' (JAK/ERM/FAK), interact with transmembrane proteins [22]. Accordingly, it is known that the N-terminal domain of FAK binds to peptides derived from β-integrins intracellular sequences *in vitro* [23], although it plays no role in focal-adhesion targeting [14]. Thus the band 4.1/JEF N-terminal domain may rather be involved in the regulation of FAK [22,24].

In brain, FAK is highly expressed [25] and is regulated by neurotransmitters (glutamate and acetylcholine), depolarization [26,27] and lipid extracellular messengers such as endocannabinoids [28] and lysophosphatidic acid (LPA) [27]. Indirect evidence suggests that FAK is implicated in synaptic plasticity

Abbreviations used: FAK, focal adhesion kinase; SH2, Src-homology 2; SH3, Src-homology 3; MAP kinase, mitogen-activated protein kinase; FAT, focal-adhesion targeting; ERM, ezrin/radixin/moesin; JAK, Janus kinase; LPA, lysophosphatidic acid; GFP, green fluorescent protein; RPTP, receptor-like protein tyrosine phosphatase; RT-, reverse transcription.

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[29], in ion-channel regulation [30] and possibly in other functions (see [24] for a recent discussion). In brain, FAK has a higher molecular mass than in other tissues [31,32], due to the presence of short peptides coded by alternative exons [32,33]. In the absence of knowledge of the complete intron/exon organization of the FAK gene, we have suggested to term 'FAK+' the isoforms which contain a three-amino-acid peptide (Pro-Trp-Arg) in the C-terminal region [28] and to refer to the isoforms with additional exons coding for peptides on either sides of Tyr^{397} by a number indicating their length in amino acids (i.e. 'boxes' 28, 6 and 7). (It should be noted that the presence of the exon coding for box 7 results also in a frameshift mutation that causes a Thr-to-Ala residue substitution [32].) In the present study we refer to the various isoforms as follows:

FAK0, isoform without additional exon

FAK+, containing the Pro-Trp-Arg insert

FAK+6, containing Pro-Trp-Arg and box 6

FAK+7, containing Pro-Trp-Arg and box 7

FAK+6,7, containing Pro-Trp-Arg and boxes 6 and 7

We have shown previously that the simultaneous presence of boxes 6 and 7 increases dramatically the autophosphorylation of FAK in transfected COS-7 cells, whereas the presence of Pro-Trp-Arg, or the additional presence of box 28, has no effect on autophosphorylation [32]. However, the respective role of boxes 6 and 7 in the increased autophosphorylation and its mechanism remained unknown. The aim of the present study was to dissect the expression pattern and the properties of the alternative exons of FAK independently. We used phosphorylation-state-specific antibodies to study in detail the phosphorylation of Tyr^{397} in transfected COS-7 cells, which do not express naturally the variants of FAK, and provide a model system to determine the precise effects of the retention of exons characteristic of neuronal FAK on the properties of the protein. The results demonstrate that boxes 6 and 7 both contribute to dramatic alterations in the phosphorylation of Tyr^{397}, increasing its autophosphorylation and preventing its transphosphorylation when co-expressed with Src-family kinases.

EXPERIMENTAL

Antibodies

Serum SL38 was prepared by immunizing a rabbit against the N-terminal fragment of rat FAK (residues 1–376) expressed in *Escherichia coli* as a hexahistidine fusion protein [28]. SL41 was raised against a synthetic peptide encompassing residues 901–911 of FAK+ [28]. Anti-phospho-Tyr^{397} antibody S625857 was raised against a phosphorylated peptide encompassing residues 392–399 of rat FAK (**SETDDYAE**T-$CONH_2$; the final T was added in place of the naturally occurring I to improve solubility without producing too short a peptide; -$CONH_2$ is the amidated C-terminus), coupled to keyhole-limpet haemocyanin and affinity-purified as described in [34], except that elution from the phosphopeptide affinity column was carried out at pH 2.6. Anti-FAK monoclonal antibody clone 77 (Mab77), raised against a peptide sequence encompassing residues 354–533, was from Transduction Laboratories, Lexington, KY, U.S.A.. Anti-FAK polyclonal antibodies A-17 directed against residues 2–18, and C-20 raised against residues 1033–1052, were from Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A. Anti-phosphotyrosine 4G10 monoclonal antibody was from Upstate Biotechnologies Inc., Lake Placid, NY, U.S.A. A hybridoma culture supernatant containing mouse monoclonal antibody VII F9 against vinculin was generously provided by Dr. M. Gloukhova (Institut Curie, Paris, France).

Subcloning and construction of FAK isoforms and mutated forms

We used a cDNA clone of FAK+6,7 obtained from a rat striatum cDNA library, encompassing 5′ leader sequences E (49–75) and C (1–114) followed by nucleotides 400–4157 of rat FAK+ sequence (GenBank® accession no. AF02077) with the additional presence of exons described as box 6 and box 7 at positions 1608 and 1665 respectively [32,33]. This FAK+6,7 clone was first placed into a pBlueScript vector (Stratagene, La Jolla, CA, U.S.A.) in which the *Cla*1 site in the polylinker had been deleted. FAK+6 was obtained by exchanging the *Cla*1–*Nsi*1 fragment of this clone for that of another FAK+ cDNA clone obtained from the library screening, which did not contain box 7. This latter FAK+ clone was also used as a template to synthesize a PCR fragment devoid of box 6, using primer oligonucleotides MF13 (5′ GACCTGATCGATGGATACTGCCGG 3′) and MF12 (5′ AGGGCATGGTGTATGTGT 3′). The amplified DNA was digested with *Sph*1 and *Cla*1 and introduced into clones FAK+6 and FAK+6,7 at the corresponding restriction sites, giving rise to FAK+ and FAK+7 subclones respectively. Using this strategy, all FAK subclones used in the present study included identical 5′ and 3′ untranslated regions. Mutated forms of FAK in which Tyr^{397} was replaced by Phe were constructed with PCR fragments generated using primers MF13 and MF12YM (5′ TCCTCATCGATGATCTCTGCAAAGTCATCTGT 3′) containing an A/T mismatch and introduced at the *Sph*1–*Cla*1 sites. For expression in COS-7 cells, the various FAK sequences were introduced into pBK-CMV2 vector, derived of pBK-CMV (Stratagene) by deletion of an *Nhe*1–*Sal*1 fragment corresponding to the bacterial promoter. Kinase-dead forms of FAK were generated by mutating Lys^{454} to Arg using the QuickChange site-directed-mutagenesis kit (Stratagene) and oligonucleotide primers FAK-KR1 (5′ TCCTCATCGATGATCTCTGCAAAGTCATCTGT 3′) and FAK-KR2 (5′ TCCTCATCGATGATCTCTGCAAAGTCATCTGT 3′). Sequences of the modified regions within all the FAK subclones and mutated forms were verified using the Thermosequenase sequencing procedure (Amersham). Fusion proteins in which green fluorescent protein (GFP) was placed at the N-terminus of FAK0, FAK+, or FAK+6,7 were constructed using pEGFP (Clontech). Mouse n-Src (a gift from Dr. D. Black, Department of Microbiology, Immunology and Molecular Genetics, University of California, Los Angeles, CA, U.S.A.) or human Fyn (a gift from Dr. S. Roche, CNRS, Montpellier, France) were placed in pBK-CMV2. c-Src was constructed from n-Src by deletion of the neuronal-specific exon.

Cell culture and transfection

For biochemical experiments, COS-7 cells were grown in uncoated 100 mm-diameter culture dishes, transfected in the presence of polyethyleneimine [35] and lysed 48 h after transfection. The amount of DNA used per dish was 8 μg when FAK isoforms were transfected alone, or 7 μg of FAK and 1 μg of Src-family kinase DNA or of empty vector DNA for co-transfection experiments. For immunocytochemistry, we used a mouse fibroblast-like line generated as control for FAK-deficient cells [36,37] and kindly provided by Dr. D. Ilic (Department of Stomatology and Anatomy, University of California, San Francisco, CA, U.S.A.). Cells were grown on uncoated 14 mm-diameter coverslips and transfected with 0.5 μg of plasmid DNA as described above. Primary cultures of neurons and astrocytes were as described previously [38].

Immunoprecipitation, *in vitro* kinase assays and immunoblotting

Transfected COS-7 cells were homogenized in ice-cold non-denaturing buffer [20 mM Tris/HCl (pH 7.4)/2 mM EDTA], containing 1 mM sodium orthovanadate, 1 % (v/v) Nonidet P40 and protease inhibitors (Complete; Boehringer). For *in vitro* kinase assays, homogenates prepared in the absence of sodium orthovanadate were incubated for 10 min with the catalytic domain of the receptor-like protein tyrosine phosphatase (RPTP)-β phosphatase in fusion with glutathione S-transferase (GST–RPTP-β; generously given by Dr. Janine Ragab, INSERM, Toulouse, France) coupled to glutathione–Sepharose beads. This treatment permitted removal of endogenous phosphate from tyrosine residues and possible associated SH2-containing proteins. Beads were removed by centrifugation, and FAK+ was immunoprecipitated from the supernatants as described in [26,28]. Immune precipitates kinase assays were carried out for 5 min at 25 °C in 50 μl of buffer containing 50 mM Hepes, pH 7.4, 10 mM $MnCl_2$, 5 μM ATP and 5 μCi of [γ-^{32}P]ATP (3000 Ci/ mmol; NEN Life Science Products). To study kinase activity of FAK, 50 μg of poly(Glu,Tyr) (4:1; Sigma) was added and samples were preincubated for 4 min at 25 °C prior to the addition of ATP. In some experiments the concentration of ATP was increased to 1 mM with non-radioactively labelled ATP. Samples were analysed by SDS/7 %-(w/v)-PAGE and autoradiography. Quantification was achieved by direct measurement of the radioactivity with an Instant Imager (Packard). Immunoblotting was carried out either from homogenates or from immune precipitates as described previously [26,28]. In some experiments, tissue pieces or COS-7 cells were directly homogenized in a solution of 1 % SDS in water containing 1 mM sodium orthovanadate and immediately placed in a boiling-water bath. Following immunoblotting, antibodies were detected with a peroxidase–chemiluminescence method and quantified by computer-assisted scanning of the autoradiograms. Statistical analysis of data was carried out with one-way ANOVA followed by pairwise comparisons with Neuman–Keul's test.

Immunocytochemistry

At 17 h after transfection, cells on coverslips were fixed in 3.6 % paraformaldehyde, washed in PBS containing 0.2 % Triton X 100, and twice in PBS. Immunostaining for vinculin was carried out with VII F9 hybridoma culture supernatant diluted 1:2, affinity-purified biotinylated anti-mouse IgG (H+L) antibodies 1/750 (Vector Laboratories, Burlingame, CA, U.S.A.) and Texas Red–streptavidin 1:100 (Molecular Probes, Eugene, OR, U.S.A.). Staining of filamentous actin was carried out with 1 unit per coverslip of Alexa 594–phalloidin (Molecular Probes). Coverslips were mounted in 50 % Mowiol 4-88 (Calbiochem; from France Biochem, Meudon, France). Images were acquired with a Provis Olympus fluorescence microscope and a digital charge-coupled-device camera (Princeton Instruments, Trenton, NJ, U.S.A.).

In situ hybridization

Horizontal sections of adult rat brains were prepared and hybridized as described previously [25]. Oligonucleotides were for FAK common sequence (GGACCGTTCCGTGCCAGTA-CCCAGGTGAGTCTTAGTACTCGAACTTGG [25]), for the exon coding for box 6 (GTCTCGTCCCCACTAATTTC) and for the exon coding for box 7 (GGCTTCATCTATTCCAT-AGC). Oligonucleotides were labelled with [α-^{33}P]dATP (Amersham) and terminal transferase (Boehringer). In order to test the specificity of labelling, some sections were incubated with a 10-fold excess unlabelled oligonucleotide.

RESULTS

Alternative exons coding for boxes 6 and 7 are not uniformly distributed in rat brain

Studies with RT-PCR followed by Southern hybridization with exon-specific probes showed that the exon coding for box 7 is more widely expressed than that coding for box 6, since, besides brain, it is found at high levels in testis [32]. In brain, the two exons were detected in similar proportions in all regions, with the exception of cerebellum, in which the exon for box 7 was more abundant than the exon for box 6 [32]. To compare the expression patterns of these exons with more accuracy, we examined the distribution of FAK mRNA isoforms in rat brain using *in situ* hybridization with ^{33}P-labelled oligonucleotides, assuming that, if the two exons were always retained simultaneously, they should give identical patterns. A 49-mer oligonucleotide complementary to a sequence of rat FAK mRNA common to all isoforms showed a widespread distribution of FAK transcripts in

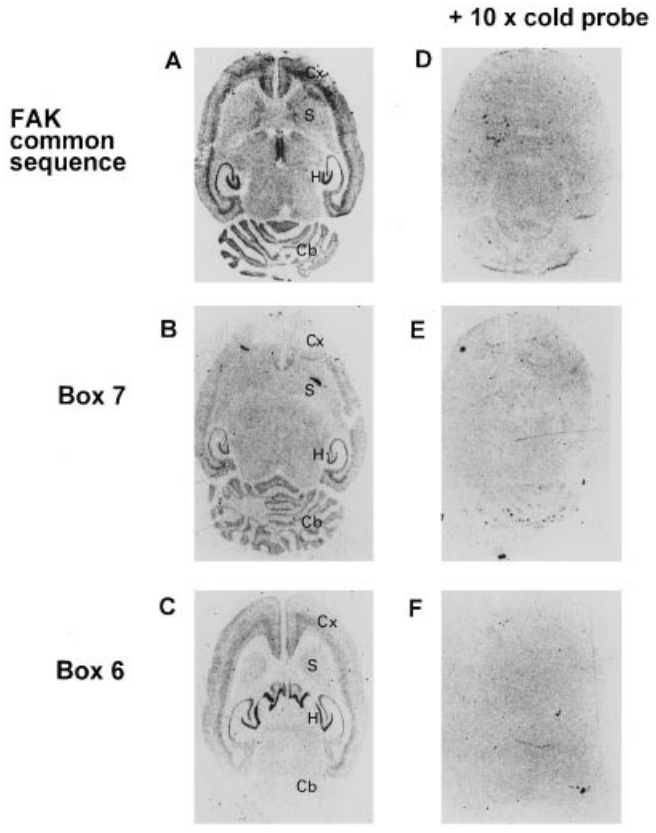

Figure 1 Expression of alternatively spliced FAK

In situ hybridization of horizontal brain sections from adult rat with ^{33}P-labelled oligonucleotides. (**A**) Hybridization with an antisense oligonucleotide complementary to a sequence of rat FAK mRNA common to all isoforms. (**B**) Hybridization with an oligonucleotide antisense probe specific for the exon coding for box 7. (**C**) Hybridization with an oligonucleotide antisense probe specific for the exon coding for box 6. (**D**, **E** and **F**) For specificity control, hybridization of the same ^{33}P-labelled oligonucleotides as in (**A**), (**B**) and (**C**) was carried out in the presence of a 10-fold molar excess of the corresponding unlabelled oligonucleotide ('+10 × cold probe'). Abbreviations: Cx, cerebral cortex; S, striatum; H, hippocampus; Cb, cerebellum.

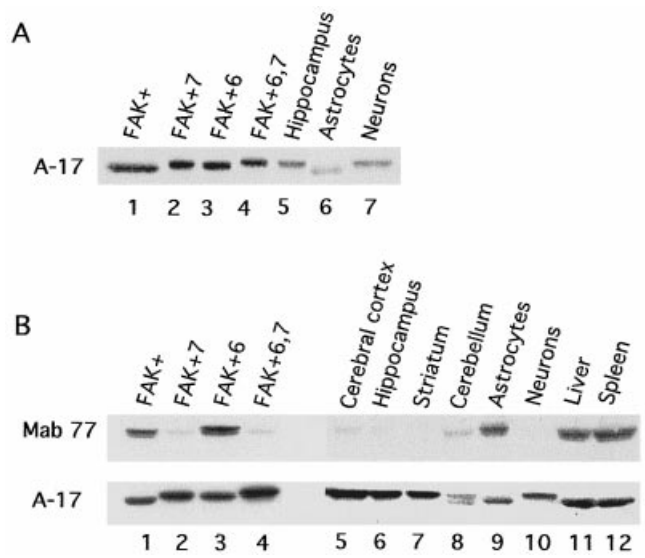

Figure 2 Immunoblot analysis of FAK in tissues and in transfected COS-7 cells

(**A**) Lanes 1–4, COS-7 cells transfected with FAK+, FAK+ containing box 7 alone (FAK+7), box 6 alone (FAK+6), or boxes 6 and 7 (FAK+6,7) were homogenized in 1% SDS and immunoblotted with polyclonal antibody A-17. Lanes 5–7, samples from rat hippocampal tissue, astrocytes and neurons in primary culture homogenized in 1% SDS were analysed on the same gel. (**B**) In a different experiment, SDS homogenates from transfected COS-7 cells, rat brain regions (lanes 5–8), astrocytes and neurons in primary cultures (lanes 9 and 10), liver (lane 11), and spleen (lane 12) were immunoblotted with Mab77 or polyclonal antibody A-17. The presence of box 7 decreased dramatically the immunodetection of FAK with Mab 77. Proteins were separated by electrophoresis and transferred to nitrocellulose. Antibodies were revealed with peroxidase-coupled secondary antibodies, and detected by chemiluminescence and autoradiography.

adult rat brain, and their relative enrichment in the hippocampus and the cerebral cortex (Figure 1A), as previously reported [25]. In contrast, hybridization with oligonucleotides specific for box 7 exon (Figure 1B) or box 6 exon (Figure 1C) gave distinct patterns. Box 7 exon appeared to be expressed rather diffusely in all brain regions (Figure 1B), whereas the oligonucleotide specific for box 6 exon generated a more contrasted pattern: hybridization was very high in the hippocampus, high in the cerebral cortex, intermediate in the striatum and not detectable in cerebellum (Figure 1C). In all cases, hybridization disappeared in the presence of a 10-fold molar excess of unlabelled oligonucleotides, demonstrating the specificity of the labelling (Figures 1D, 1E and 1F). Thus *in situ* hybridization revealed contrasts between the expression patterns of boxes 6 and 7 that were not detectable with reverse transcription (RT)-PCR, which averages the levels of transcripts in all cells. In addition, the differences between the levels of expression of boxes 6 and 7 in the cerebellum, although detected by RT-PCR [32], appeared more striking, as visualized by *in situ* hybridization. These results reveal that, in some brain regions, box 7 is likely to be retained in the absence of box 6.

Expression of FAK splice isoforms in brain structures and in neurons and astrocytes in culture

Although migration of FAK0 and FAK+ are identical [32], three groups of FAK isoforms can be separated by size following electrophoresis in the presence of SDS on 7% polyacrylamide gels: FAK+, FAK+6/FAK+7 and FAK+6,7 (Figure 2A, and see the other Figures below). Careful comparison of the apparent molecular mass of endogenous FAK in brain tissue and in neurons with that of transfected FAK isoforms in COS-7 cells demonstrated that, in hippocampal and neuronal extracts, FAK migrated as FAK+6,7 (Figure 2A, lanes 5 and 7), whereas in astrocytic extracts it migrated as FAK0/+ (lane 6).

Our attempts to raise specific antibodies against boxes 6 and 7 were unsuccessful. However, immunoblotting analysis revealed that a commercially available monoclonal antibody, Mab77, raised against amino acids 354–533 of FAK reacted very poorly with FAK isoforms containing either box 7 (FAK+7) alone or boxes 6 and 7 (FAK+6,7) (Figure 2B, lanes 1–4). Therefore we used the comparison of the immunoreactivity with Mab77 and with a polyclonal antibody reacting with all FAK isoforms (A-17) as an index of the presence of box 7 in FAK from various tissues and cell types (Figure 2B, lanes 5–12). A strong signal for FAK was obtained in all samples with A-17 (Figure 2B, lanes 5–12). In contrast, Mab77 reacted with FAK in liver and spleen (Figure 2B, lanes 11 and 12), but not in brain samples (Figure 2B, lanes 5–7), demonstrating the expression of FAK proteins containing box 7 in brain. In cerebellum, two bands with different molecular masses were observed, only the lower one being recognized by Mab77 (Figure 2B, lane 8). Although polyclonal antibody A-17 generated a good signal for FAK in astrocytes and neurons in primary culture, Mab77 detected readily FAK in astrocytes, but not in neurons (Figure 2B, lanes 9 and 10). Altogether these results provide strong evidence that, in forebrain tissue and in neurons in culture, the main form of FAK contains boxes 6 and 7, as indicated by its size and its lack of reactivity with Mab77. In combination with previous results showing that the vast majority of brain FAK contains the Pro-Trp-Arg insertion [28,39], we conclude that the main isoform of FAK in forebrain neurones is FAK+6,7.

FAK isoforms have a similar intracellular targeting

One of the most characteristic properties of FAK is its targeting to focal adhesions [1,2]. The FAT sequence responsible for this localization has been mapped to the C-terminus of the protein, between residues 853 and 1012 of the chicken sequence [14]. The Pro-Trp-Arg insertion of FAK+ isoforms is located between residues 903 and 904 (rat sequence) [28,33] and encompasses charged or bulky residues that could potentially disrupt the structure or the interactions of the FAT region. Therefore we examined whether FAK+ could still be targeted to focal adhesions using fusion with GFP. In transfected fibroblast-like cells, FAK0–GFP was highly enriched in the peripheral part of focal adhesions, identified by simultaneous labelling for vinculin (Figures 3A and A′) or for filamentous actin (Figures 3D and 3D′). A similar location was observed for FAK+–GFP (Figures 3B, 3B′, 3E and 3E′) and for FAK+6,7–GFP (Figures 3C, 3C′, 3F and 3F′). These results demonstrate that the presence of additional exons does not prevent the targeting of FAK neuronal isoforms to focal adhesions.

Phosphorylation-state-specific antibodies as a tool for measuring the phosphorylation of Tyr^{397}

To study autophosphorylation of FAK, an important step in its activation, we used affinity-purified antibodies (S625857) reacting only with FAK phosphorylated on Tyr^{397}. Since these antibodies are a critical tool in the experiments presented below, we have characterized them in detail. The time course of *in vitro* autophosphorylation of FAK+6,7 in immune precipitate kinase assays was similar whether it was measured by immunoblotting with affinity-purified S625857 antibodies or by detection of ^{32}P

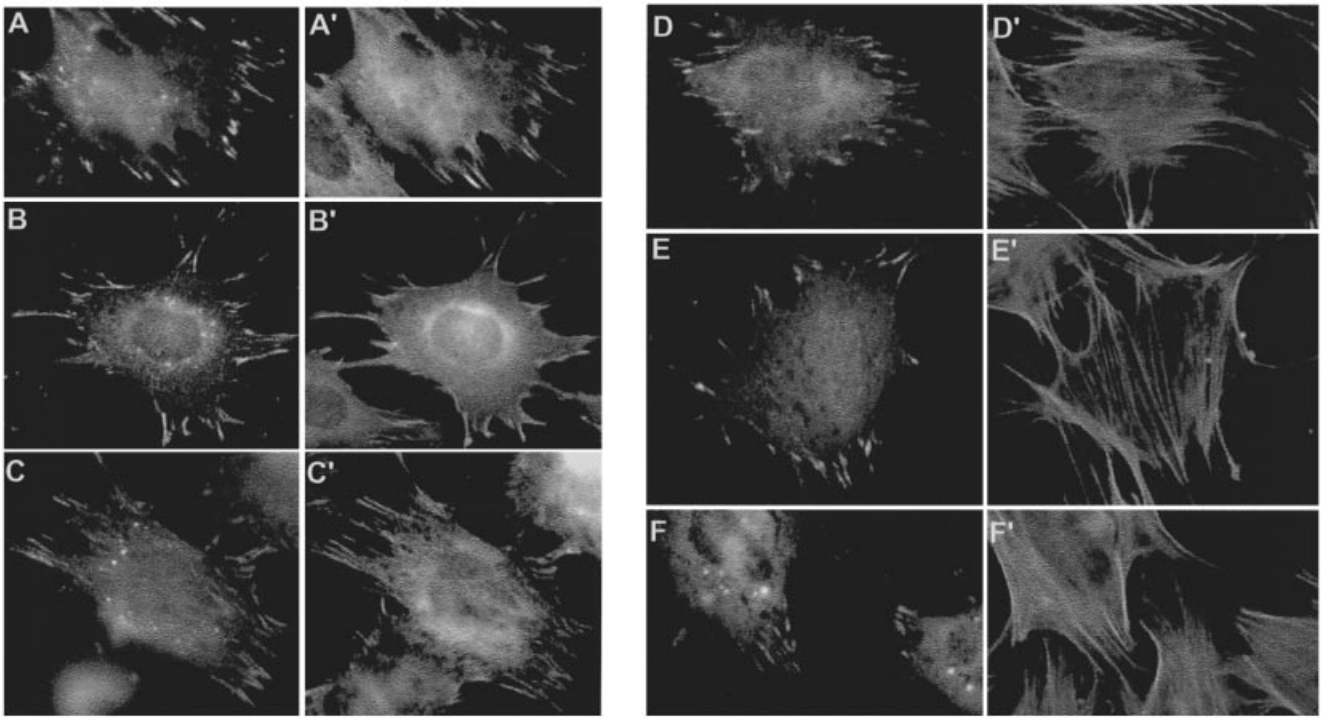

Figure 3 Intracellular targeting of the various isoforms of FAK

Mouse fibroblast-like cells were transfected with fusion proteins FAK0–GFP (**A**, **A′**, **D**, and **D′**), FAK+–GFP (**B**, **B′**, **E** and **E′**) or FAK+6,7-GFP (**C**, **C′**, **F** and **F′**). Intracellular localization of GFP was analysed 17 h after transfection (**A**–**F**). Double labelling was carried out with a monoclonal antibody against vimentin, revealed with biotinylated anti-mouse antibodies and Texas Red–streptavidin (**A′**–**C′**) or Alexa 594–phalloidin to label filamentous actin (**D′**–**F′**).

incorporation in the same samples (Figures 4A and 4B). Conversely, incubation of transfected COS-7-cell homogenates with the phosphatase RPTP-β catalytic domain resulted in a very rapid decrease in phospho-Tyr[397] immunoreactivity, whereas the total amount of FAK+6,7 remained constant (Figure 4C). Finally, in homogenates from COS-7 cells transfected with various FAK isoforms, although numerous bands were labelled by anti-phosphotyrosine antibody 4G10, the affinity-purified S625857 antibodies reacted with a single 125 kDa band (Figure 4D). The observed immunoreactivity was increased by the presence of boxes 6 and 7, and unaltered by the presence of box 28 (Figure 4D), as already reported [32].

Boxes 6 and 7 both contribute to the increased autophosphorylation of FAK neuronal isoforms in immune precipitates without marked alteration in their intrinsic kinase activity

The different patterns of expression of boxes 6 and 7 revealed by *in situ* hybridization prompted us to study their effects on FAK properties independently. We used transfected COS-7 cells to examine the properties of these isoforms in intact cells and as a source of material for *in vitro* studies. Since these isoforms all contained the Pro-Trp-Arg insertion defining FAK+, which is absent from COS-7-cell endogenous FAK, they could be specifically recovered by immunoprecipitation with serum SL41 specific for FAK+ [28]. In these experiments, cell lysates were pretreated with phosphatase RPTP-β to allow the comparative study of the intrinsic ability of FAK isoforms to autophosphorylate, independently of their level of phosphorylation in COS-7 cells. This phosphatase pretreatment resulted in a complete disappearance of FAK immunoreactivity for phospho-Tyr[397] (Figure 4C) and for total phospho-Tyr (results not shown). Immune-precipitate-kinase assays showed that the presence of box 7 alone or box 6 alone increased ^{32}P incorporation, as well as immunoreactivity for phospho-Tyr[397] (Figure 5A, lanes 3 and 4). The effects of the two boxes were additive (Figure 5A and 5C). These experiments were carried out with a low concentration of ATP (5 μM) to provide a high ^{32}P specific radioactivity. The phospho-specific antibodies allowed us to test whether the increased autophosphorylation could result from an increased affinity for ATP of FAK+ with boxes 6 and/or 7. The results obtained with 1 mM ATP (Figure 5B) were identical with those with 5 μM (Figure 5A), demonstrating that this was not the case.

We examined the effects of the presence of box 6 and/or 7 on the intrinsic kinase activity of FAK, using an exogenous substrate, poly(Glu,Tyr) (4:1). Although the activity of FAK+6,7 was significantly increased as compared with FAK+, this increase was very modest ($\approx$ 2-fold, Figure 5D) as compared with the increase in autophosphorylation of the same isoform ($\approx$ 9-fold, Figure 5C). To verify that the observed effects resulted from the autophosphorylation of FAK on Tyr[397] and not from the presence of a contaminating kinase and/or the phosphorylation of other tyrosine residue(s), we used mutated forms of FAK as controls. Mutation of Tyr[397] to Phe suppressed the autophosphorylation of FAK+ and FAK+6,7 (Figure 5A, lanes 1 and 6). Under our experimental conditions, in which FAK was dephosphorylated prior to the kinase assay, this mutation had no significant effect on the ability of FAK+ to phosphorylate poly(Glu,Tyr) (Figure 5D). As expected, mutation of Lys[454] to Arg suppressed both autophosphorylation (Figures 5A and 5C, lane 7) and phosphorylation of poly(Glu,Tyr)

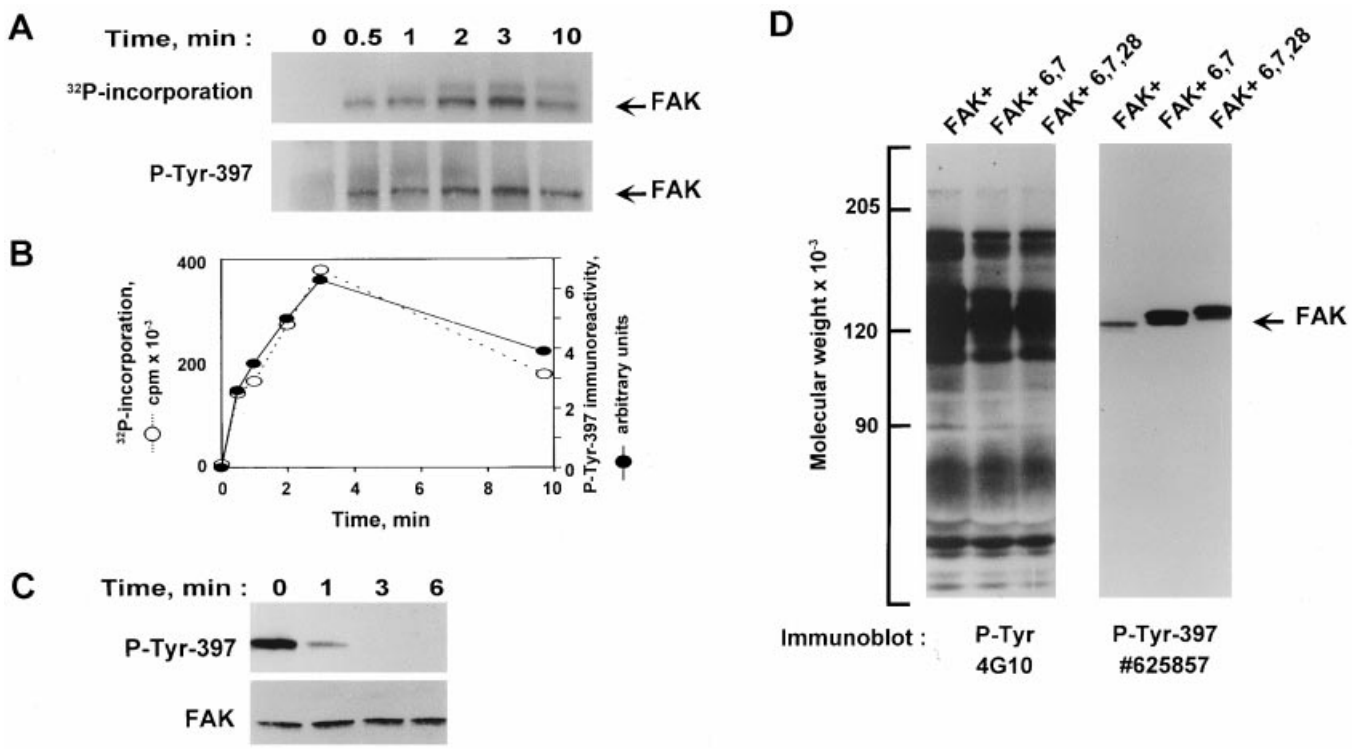

Figure 4 Characterization of affinity-purified antibodies S625857 reacting specifically with FAK phosphorylated on Tyr^{397}

(**A**) Transfected COS-7 cells were homogenized in non-denaturing buffer and incubated for 10 min with RPTP-β. FAK + 6,7 was immunoprecipitated with serum SL41. Equal amounts of immune precipitate were incubated for various times in the presence of 5 μM [γ-^{32}P]ATP. Samples were resolved by SDS/PAGE, transferred to nitrocellulose and the same membrane was analysed by autoradiography ('^{32}P-incorporation') and immunoblotting with affinity-purified S625857 antibodies and chemiluminescence detection ('P-Tyr-397'). Note that, in these experiments, a dephosphorylation occurred at the 10 min time point, due to the presence of a contaminating phosphatase. (**B**) ^{32}P incorporation was quantified with an Instant Imager and phospho-Tyr^{397} immunoreactivity was quantified by densitometry. (**C**) Transfected COS-7 cells with FAK + 6,7 were homogenized in non-denaturing buffer and incubated during the indicated times with GST–RPTP-β catalytic domain coupled to glutathione–Sepharose beads. Samples were analysed by immunoblotting with affinity-purified S625857 antibodies ('P-Tyr-397') or A-17 ('FAK'). (**D**) Homogenates of COS-7 cells transfected with the different isoforms of FAK ('FAK + ', 'FAK + 6,7' and 'FAK + 6,7,28') were immunoblotted either with a monoclonal antiphosphotyrosine antibody ('P-Tyr 4G10') or with S625857 ('P-Tyr-397 #625857').

(Figure 5D, bar 7). Altogether these results show that Tyr^{397} is the only tyrosine residue of FAK autophosphorylated in FAK + 6,7.

Effects of boxes 6 and/or 7 on the phosphorylation of FAK + by Src-family kinases

Tyrosine phosphorylation of FAK is thought to occur generally in two steps: autophosphorylation of Tyr^{397}, followed by the recruitment of Src-family kinases that phosphorylate additional tyrosine residues [3,6]. We examined the consequences of the presence of boxes 6 and/or 7 on this cascade in intact cells. In transfected COS-7 cells, the total tyrosine phosphorylation of immunoprecipitated FAK + 6,7 was much higher than that of FAK + (Figure 6A, lanes 1 and 4). Phosphorylation of FAK + 6 or FAK + 7 was intermediate between that of FAK + and of FAK + 6,7 (Figure 6A, lanes 2 and 3). Mutation of Tyr^{397} to Phe or of Lys^{454} to Arg (kinase-dead mutant) decreased the total tyrosine phosphorylation of FAK + 6,7 (Figure 6A, lanes 5 and 6). However, in both mutants a low level of tyrosine phosphorylation was still observed, indicating that endogenous tyrosine kinases in COS-7 cells are capable of phosphorylating FAK in the absence of phosphorylation of Tyr^{397}. Moreover, when c-Src was co-transfected with FAK, a strong tyrosine phosphorylation of all FAK isoforms was apparent (Figure 6A, lanes 7–10), as previously reported [32]. Interestingly, however, the Tyr^{397} → Phe or Lys^{454} → Arg mutants were also highly tyrosine-phosphorylated under these conditions (Figure 6A, lanes 11 and 12). These results indicated that c-Src has the ability to phosphorylate FAK even in the absence of autophosphorylation of Tyr^{397}.

We studied the phosphorylation of Tyr^{397} under the same experimental conditions (Figure 6B). When FAK was transfected alone, phosphorylation of Tyr^{397} was higher in FAK + 6,7 than in FAK + and intermediate in FAK + 6 or FAK + 7 (Figure 6B, lanes 2–5). Phosphorylation of Tyr^{397} was not detected in Tyr^{397} → Phe or kinase-dead (Lys^{454} → Arg) mutants (Figure 6B, lanes 1, 6 and 7). When c-Src was co-transfected with FAK, phosphorylation of Tyr^{397} increased (lanes 9–12). As expected, no signal was detected for the Tyr^{397} → Phe mutant (Figure 6B, lane 13). However, a surprising result was observed with kinase-dead mutants: Tyr^{397} was phosphorylated in the kinase-dead FAK +, but not in kinase-dead FAK + 6,7 when they were co-transfected with c-Src (Figure 6B, lanes 7 and 14). This suggested that the presence of boxes 6 and/or 7 prevented phosphorylation of Tyr^{397} by Src.

To explore further the effects of the alternative exons on phosphorylation of Tyr^{397} by c-Src, we used kinase-dead mutants of all isoforms. The kinase-dead mutants of the various FAK isoforms did not appear to be phosphorylated on Tyr^{397} when transfected alone in COS-7 cells (Figure 6C, lanes 1–4). When co-

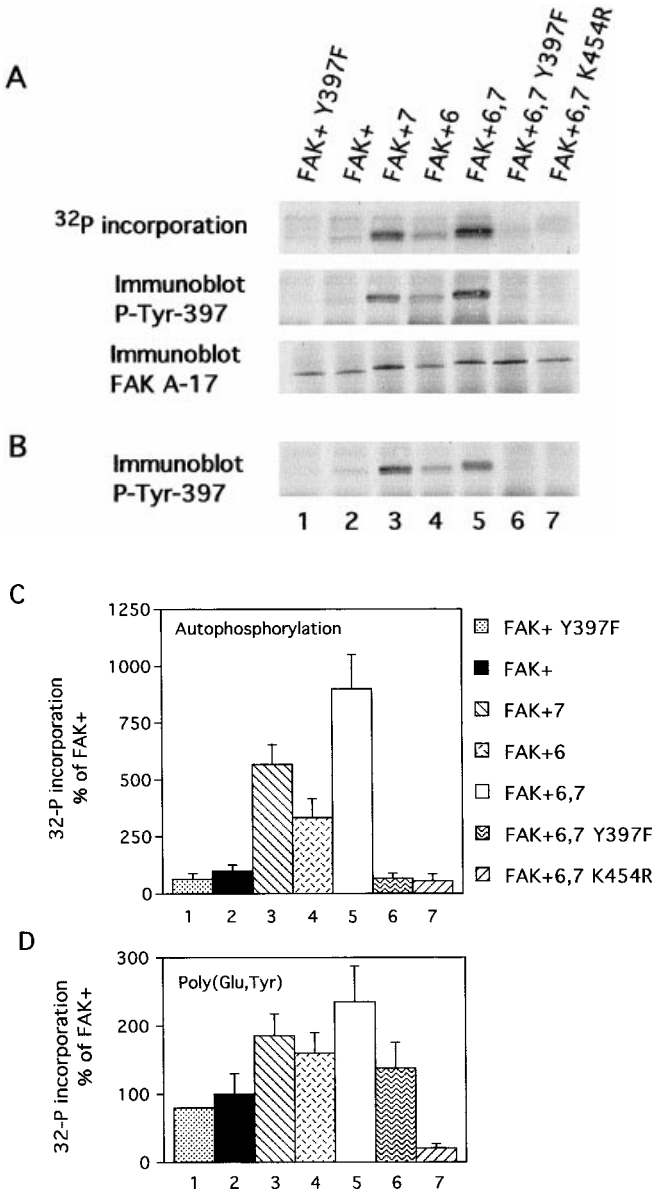

Figure 5 Autophosphorylation and kinase activity of FAK+ isoforms expressed in COS-7 cells

COS-7 cells were transfected with FAK+, FAK+7, FAK+6, or FAK+6,7 with wild-type sequence (lanes 2–5) or point mutations of Tyr[397] to Phe (Y397F) (lanes 1 and 6) or of Lys[454] to Arg (kinase-dead mutant, K454R, lane 7). Homogenates were dephosphorylated with GST–RPTP-β and FAK+ was immunoprecipitated. Pooled immune precipitates for each form of FAK were divided into several fractions used for the various assays described below. (**A**) One fraction was incubated for 5 min with 5 μM [γ-^{32}P]ATP (3000 Ci/ mmol). Samples were transferred to nitrocellulose and analysed for ^{32}P incorporation by autoradiography and for phosphorylation of Tyr[397] by imunoblotting with affinity-purified S625857 antibodies ('Immunoblot P-Tyr-397'). A second fraction from each immune precipitate was used for determining the total amount of FAK ('Immunoblot FAK A-17'). (**B**) A third fraction from each immune precipitate was incubated for 5 min in the presence of 1 mM unlabelled ATP and analysed by immunoblotting with affinity-purified S625857 antibodies ('Immunoblot P-Tyr-397'). (**C**) Quantification of ^{32}P incorporation in the various FAK+ isoforms and mutants during *in vitro* kinase assays, as in (**A**). Data correspond to the mean ± S.E.M. for four experiments. Values were significantly different by ANOVA ($F6,14 = 13.36$, $P < 10^{-4}$). Pairwise comparisons by Student–Newman–Keul's test showed that autophosphorylation of FAK+7 and FAK+6,7 was significantly higher than that of FAK+ ($P < 0.05$) and that autophosphorylation of FAK+6,7 was higher than that of FAK+6 or FAK+7 ($P < 0.01$). (**D**) One fraction of each immune precipitate was incubated with poly(Glu,Tyr) (4:1) and [γ-^{32}P]ATP. Data correspond to the mean ± S.E.M. for four experiments. Values were significantly different by ANOVA ($F6,12 = 19.3$, $P < 10^{-4}$). Pairwise comparisons showed that phosphorylation of poly(Glu,Tyr) by FAK+6,7 was significantly higher than that by FAK+ ($P < 0.05$).

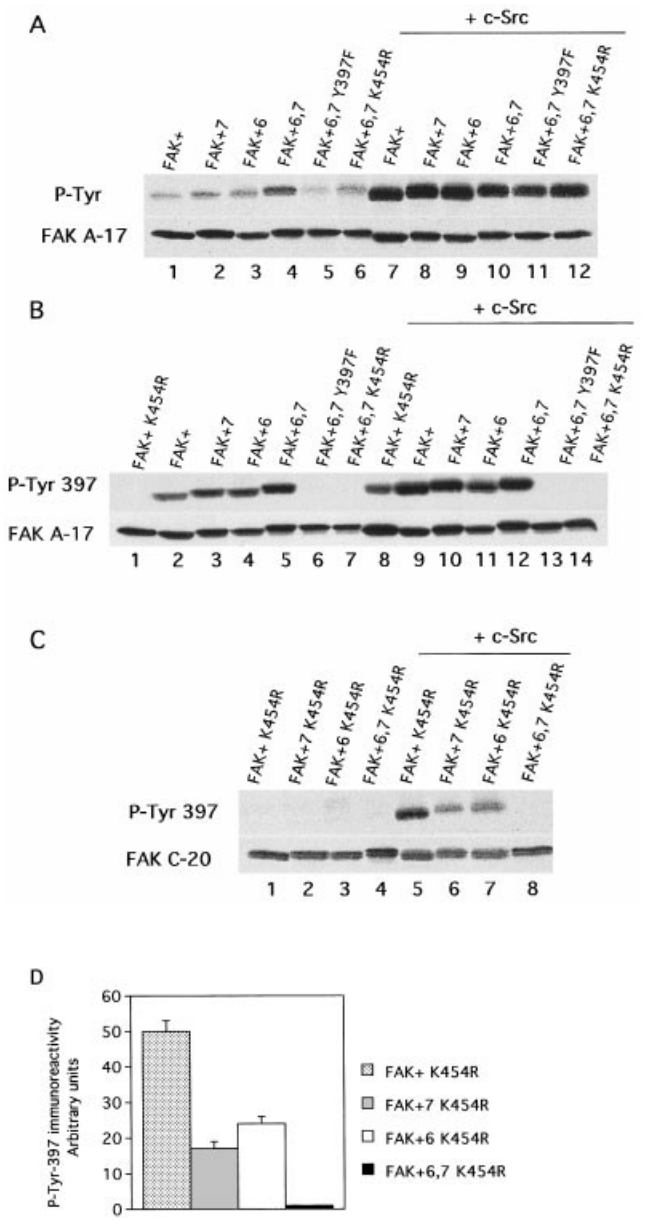

Figure 6 Phosphorylation of various FAK+ isoforms by c-Src

COS-7 cells were co-transfected with wild-type or mutated isoforms of FAK (see the legend to Figure 5) and vector alone or c-Src. (**A**) Transfected isoforms were immunoprecipitated with serum SL41, which reacts specifically with FAK+, and analysed by immunoblotting with monoclonal anti-phosphotyrosine antibody 4G10 ('P-Tyr'). The total amount of FAK was verified by immunoblotting with A-17 antibodies ('FAK A-17'). (**B**) Phosphorylation of Tyr[397] was studied by immunoblotting with affinity-purified S625857 ('P-Tyr 397') and the total amount of FAK was verified by immunoblotting with A-17 antibodies ('FAK A-17'). (**C**) COS-7 cells were co-transfected with kinase-dead mutants ('K454R') of various splice isoforms of FAK, and vector alone or c-Src. Homogenates were immunoblotted either with affinity-purified S625857 antibodies ('P-Tyr 397') or with antibodies C-20 to control for the total amount of FAK ('FAK C-20'). (**D**) Results were quantified by computer-assisted scanning of the autoradiograms. Data correspond to the mean ± S.E.M. for five experiments. Values were significantly different by ANOVA ($F3,16 = 117.3$, $P < 10^{-4}$). Pairwise comparisons by Student–Newman–Keul's test showed that all differences were significant ($P < 0.05$).

transfected with c-Src, kinase-dead FAK+ was phosphorylated on Tyr[397] (Figure 6C, lane 5). Phosphorylation of Tyr[397] was weaker in kinase-dead FAK+6 or FAK+7, and was virtually abolished in kinase-dead FAK+6,7 (Figure 6C, lanes 6–8, and

Figure 6D). Identical results were observed with the neuronal isoform of Src, n-Src, and with Fyn, which are both highly expressed in neurons (results not shown).

DISCUSSION

In vertebrates a single gene codes for FAK. The closest related kinase, PYK2/CAKβ, has only 45% amino acid sequence identity [40,41] and a markedly distinct expression pattern [39,42]. The absence of functional redundancy of FAK and PYK2/CAKβ is demonstrated by the fact that FAK knockout mice die early during development [36]. A variety of FAK isoforms can be generated by the use of several promoters and of multiple alternatively spliced exons [18,32,33]. Several short exons coding for peptides of 3–28 amino acids are retained in FAK in brain. This may be linked to the preferential inclusion of small exons which has been suggested to occur during mRNA splicing in brain [43]. These exons are conserved in several species, indicating their functional importance: FAK+ has been found in rat, mouse [33], human [44] and *Xenopus* [45]; boxes 6, 7 and 28 exist in rat and mouse [32], and boxes 6 and 7 have also been reported in *Xenopus* [45]. We had already provided evidence that two exons coding for Pro-Trp-Arg, defining FAK+, and for box 7, respectively, are expressed in brain and testis, whereas those coding for box 6 and box 28 were found only in brain [28,32,33]. Isoforms containing box 28 appear to be expressed at very low levels in adult brain [32]. Additional alternative exons within the coding sequence may exist, but have not been characterized [33,46]. The results reported here, in combination with our previous work [28,32,33], show that although box 7 appears to be more widely expressed than box 6, the major isoform of FAK in rat forebrain, as well as in neurons in culture, is FAK+6,7. Interestingly, whereas FAK+ is also found in astrocytes [33], FAK+6, FAK+7 and FAK+6,7 were not detected in astrocytes (the present study).

These observations raise the question of the specific properties of neuronal FAK. Our data demonstrate that neither the presence of Pro-Trp-Arg in the FAT sequence, nor the insertion of peptides on either side of the autophosphorylated tyrosine, prevented targeting of FAK to focal adhesions in transfected cells. Although the functional consequences of the presence of Pro-Trp-Arg remain unknown, we show here that the presence of either box 6 or box 7 has dramatic consequences on the properties of FAK. The presence of these boxes increases FAK autophosphorylation in immune-precipitate assays and in transfected cells, in an additive manner. This effect is most likely due to a better accessibility of Tyr^{397} to the catalytic site and not to a change in the intrinsic catalytic properties of the kinase domain, whose activity was only marginally increased in FAK+6,7 as compared with FAK+.

The present study demonstrates that the presence of boxes 6 and 7 altered the interactions of FAK with Src-family kinases. These interactions are complex, since Src can bind to FAK by both its SH2 and its SH3 domains. The SH2 domain binds to phosphorylated Tyr^{397} [3,47,48], and the SH3 domain binds to a peptide encompassing Pro^{371} and Pro^{374} [49]. Src binding results in its activation, leading to the phosphorylation of several additional tyrosine residues in the FAK sequence [6,8] and in neighbouring proteins, triggering several signalling cascades. Thus, the interaction between FAK and Src has important consequences (see [20] for a discussion). Our results with an antibody specific for phospho-Tyr^{397} and kinase-dead mutants of FAK clearly demonstrate that Src-family kinases, c-Src, n-Src, and Fyn, are capable of phosphorylating Tyr^{397} in intact cells. This is perhaps not surprising, since Tyr^{397} is located within a relatively good Src substrate sequence [50]. Interestingly, phosphorylation of Tyr^{397} by Src was completely prevented by the simultaneous presence of boxes 6 and 7 in transfected cells. This effect could be due to a conformational change in neuronal isoforms of FAK, or to their interaction with other proteins, preventing the phosphorylation of Tyr^{397} by Src-family kinases. It should be pointed out that recombinant Src added to FAK immune-precipitate assays phosphorylated Tyr^{397} equally well in all isoforms (M. Toutant and J.-A. Girault, unpublished work). This indicates that the effects observed in transfected cells are due either to a conformational change in FAK, which is labile and lost in immune precipitates, or that the effect of Src-family kinases on phosphorylation of Tyr^{397} is indirect and mediated by another kinase, a phosphatase or an interacting protein.

The physiological meaning of the direct phosphorylation of FAK by Src-family kinases is not known. It is possible that two pathways of interaction between Src and FAK exist in cells. In the 'classical' scenario, FAK autophosphorylation is first activated by integrin engagement and/or stimulation of a G protein-coupled receptor, and, secondarily, quiescent Src becomes activated by its dual SH2 and SH3 interaction with FAK. In an alternative scenario, suggested by the present results, Src could be activated by another pathway, and could recruit FAK, not only by phosphorylating several residues in the catalytic and C-terminal domains, as previously reported [6], but also by phosphorylating Tyr^{397}, either directly or indirectly. This could lead either to a stronger interaction between Src and FAK, or to the recruitment and activation of additional Src molecules by providing binding sites for their SH2 domain. In this respect it should be kept in mind that FAK was originally identified as a protein highly phosphorylated on tyrosine in v-Src-transformed cells [1]. Our results suggest that, in neurons which express mostly (if not only) FAK+6,7, regulation of Tyr^{397} phosphorylation by Src may not be possible, pointing towards an additional difference with the ubiquitous isoform.

In transfected COS-7 cells, as well as in immune-precipitate assays, FAK+6,7 is highly phosphorylated on tyrosine, whereas the phosphorylation of FAK0 or FAK+ is very low. This high degree of phosphorylation of FAK+6,7 suggests that this isoform behaves as a constitutively activated form of FAK. However, it is clear that FAK+6,7 is still tightly regulated in cells. First, the high degree of phosphorylation of FAK+6,7 in COS-7 cells was dependent on cell attachment, since it was markedly decreased 30 min after cells had been placed in suspension (M. Toutant, M. Gelman and J.-A. Girault, unpublished work). Secondly, in hippocampal slices, phosphorylation of FAK+6,7 is low under basal conditions and increases dramatically in response to neurotransmitters or neuromodulators [26,28]. The contrast between the levels of phosphorylation of FAK+6,7 in transfected non-neuronal cells and in nervous tissue suggests that specific regulatory mechanisms controlling FAK activation may exist in neurons. One such mechanism could be the existence of protein tyrosine phosphatase(s) lowering the level of tyrosine phosphorylation of FAK under basal conditions and that could be subject to regulation by extracellular messengers. Thus a high turnover rate of the phosphate on Tyr^{397}, resulting from very active autophosphorylation and dephosphorylation, could account for the high responsiveness of FAK to neurotransmitters or endocannabinoids and may provide an original pathway for coupling rapid events to changes of longer duration in the nervous system.

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