Alternatively Spliced Focal Adhesion Kinase in Rat Brain with Increased Autophosphorylation Activity*

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pp125 focal adhesion kinase (FAK), a cytoplasmic tyrosine kinase transducing signals initiated by integrin engagement and G protein-coupled receptors, is highly expressed in brain. FAK from brain had a higher molecular weight and an increased autophosphorylation activity, than from other tissues. In addition to a 9-base insertion in the 3'-coding region, which defines FAK+, rat striatal FAK mRNAs contained several additional short exons, coding for peptides of 28, 6, and 7 residues, respectively (termed boxes 28, 6, and 7), surrounding the autophosphorylated Tyr-397. In transfected COS 7 cells, the presence of boxes 6 and 7 conferred an increased overall tyrosine phosphorylation, a higher phosphorylation of Tyr-397 assessed with a phosphorylation state-specific antibody, and a more active autophosphorylation in immune precipitates. The presence of box 28 did not alter further these parameters. Two-dimensional phosphopeptide maps of hippocampal FAK were identical to those of FAK+6,7. The presence of the various exons did not alter the interaction of FAK with c-Src, n-Src, or Fyn. Thus, several splice isoforms of FAK are preferentially expressed in rat brain, some of which have an increased autophosphorylation activity, suggesting that FAK may have specific properties in neurons.

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¶ The abbreviations used are: FAK, pp125 focal adhesion kinase; DOTAP, N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium methysulfate; PWR, Pro-Trp-Arg; SH2, Src-homology domain 2; SH3, Src-homology domain 3.

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(100 ng each) F-1739 and N°-371: 5'-CCATCACATACCAAGGCTGC-3', in a 40-μl reaction volume containing 15 pmol of dNTPs and 1.2 unit of BioTag (Bioprobe Systems). After a 5-min denaturation at 94 °C, 20 amplification cycles were carried out (30 s at 94 °C, 30 s at 52 °C, and 30 s at 72 °C), followed by a 5-min incubation at 72 °C. The amplification products were divided in 4 aliquots, electrophoresed in 1.2% agarose gels, transferred to Hybond-N membranes, and hybridized with oligonucleotides labeled using [γ-32P]ATP (NEN Life Science Products) and T4 kinase (Promega) (17). The oligonucleotides used as probes were: for all forms of FAK, N°-390: 5'-GACAGAAGTACGTGGCCCA-3'; for box 28: B28: 5'-TTTACAGCAGAGTGCAAGAGA-3'; for box 6: B-6: 5'-CGTGCAAGCCAAGATGTTCG-3'; and for box 7: B-7, 5'-GGCTCTCATCTATCCATAGC-3'. Hybridization was carried out at 42 °C and washes up to 50 °C in 2 × SSC containing 0.1% SDS. Autoradiographic films were exposed to membranes for 6–4 days. Mouse genomic DNA was amplified using the "Mouse genomic walker kit" (CLONTECH), and sequenced by Thermosequenase (Amersham) according to the manufacturers' instructions.

Antibodies—Serum SL38 was prepared by immunizing a rabbit against the amino-terminal fragment of rat FAK (residues 1–376) expressed in Escherichia coli as a histidine fusion protein (17). SL41 was raised against a synthetic peptide encompassing residues 901–911 of FAK+ (17). Antiphosphotyrosine 397 antibody (number 625 857) was raised against a phosphorylated peptide encompassing residues 392–399 of rat FAK, coupled to keyhole limpet hemocyanin and affinity-purified, as described (23), except that elution from the phosphopeptide affinity column was carried out at pH 2.6. This antibody reacted with the autophosphorylated form of FAK but not with other phosphopeptides.3 Anti-phosphotyrosine 4G10 and anti-FAK 2A7 monoclonal antibodies were from Upstate Biotechnologies Inc.

Expression of FAK, Src, and Fyn in COS 7 Cells—For expression in COS 7 cells, the various isoforms of FAK cloned from rat striatum, mouse n-Src (a gift of Dr. Douglas Black, Los Angeles), or human Fyn (a gift from Dr. Serge Roche, Montpellier) were cloned in pcMV2, a vector derived from pBK-CMV (Stratagene) by deletion of a NheI-Sall fragment corresponding to the bacterial promoter. c-Src was constructed by deletion of the neuronal specific exon. COS 7 cells in uncoated 100-mm Petri dishes were transfected in the presence of DOTAP (Boehringer Mannheim), according to the manufacturer's instructions, and lysed 48–72 h after transfection.

Immunoprecipitation and Immunoblotting—Immunoprecipitation and immunoblotting were carried out as described (18, 19). Tissue pieces or COS 7 cells were homogenized in ice-cold buffer (100 mM NaCl, 50 mM Tris-Cl, pH 7.4, 5 mM EDTA, 50 mM sodium fluoride) containing 1 mM orthovanadate, 1% (v/v) Nonidet P-40, and protease inhibitors (Complete, Boehringer). In vitro kinase assays following immunoprecipitation were carried out for 5 min at 25 °C in 50 μl of buffer containing 50 mM HEPES, pH 7.4, 10 mM MnCl2, and 5 μCi of [γ-32P]ATP (3000 Ci/mmol, NEN Life Science Products). Samples were analyzed by electrophoresis (7% polyacrylamide) and autoradiography after treatment of the gel in 1 M KOH for 2 h at 50 °C. Quantification was achieved by direct detection of the radioactivity with an Instant Imager (Packard). Following immunoblotting, antibodies were detected with a peroxidase-chemiluminescence method and quantified by computer-assisted scanning of the autoradiograms (18). Two-dimensional tritpic phosphopeptide mapping was carried out on Kodak cellulose thin layer chromatography plates as described (24), using conditions similar to those of Calabhi et al. (9): 70 min electrophoresis at pH 1.9 (2.2% formic acid, 7.8% acetic acid) for the first dimension and an overnight chromatography in 37.5% butanol, 25% pyridine, and 7.5% acetic acid for the second dimension.

RESULTS

Autophosphorylation of FAK from Various Tissues—When FAK was immunoprecipitated from various tissues, the amount of protein recovered from brain regions was higher than from other tissues (Fig. 1, and Ref. 18). The phosphorylation of FAK in immune precipitate kinase assays was higher in samples from brain regions than from other tissues (Fig. 1). Estimates of specific activities were (in arbitrary units, mean ± S.E. of three experiments): cortex, 85 ± 9; hippocampus, 87 ± 23; striatum, 109 ± 23; cerebellum, 181 ± 55; liver, 24 ± 8; spleen, 25 ± 7; testis, 23 ± 8. In addition, FAK from brain had a slightly slower electrophoretic mobility as compared with other tissues. This larger size was already reported by others, who found that it was not due to post-translational modifications (25). Since FAK appears to be coded by only one gene (11, 17), these observations raised the possibility that the features of FAK in brain may arise from the existence of various splice isoforms of its mRNA.

FAK mRNAs from Rat Brain Exist under Multiple Isoforms—We have reported previously that FAK cDNAs cloned from rat striatum existed under multiple isoforms, probably generated by a combination of alternative sites of transcription initiation, mRNA splicing, and polyadenylation sites (17, 18). Two of these variations predict the existence of modified proteins: a potential initiation codon 78 base upstream of the standard ATG, and a small exon in the carboxyl-terminal region defining FAK+. However, since the mRNA with an alternative initiation of translation is expressed at low levels in all tissues (17), it is unlikely that it accounts for the major properties of FAK in brain. Careful analysis of 15 independent clones obtained from a rat striatum cDNA library disclosed the existence of multiple additional variations in the coding sequence, termed boxes 28, 6, and 7 according to the number of amino acid residues for which they encoded (Fig. 2, Table I). Box 28, when present, was immediately 5' of box 6. Box 7 contained 21 bases but since it was out of frame, its presence led to the replacement of a threonine by an alanine (Fig. 2). These various modifications were observed in several independent clones (Table I), indicating that they were not due to cloning artifacts. Moreover, boxes 6 and 7 have been previously reported in one cDNA sequence from Xenopus (26), but not in another (27), and in an unpublished rat sequence (Sasaki et al. GenBank accession number U43940). In cDNAs from rat striatum, boxes 6 and 7 were always associated, while box 28 was only found in combination with the two others (Table I). Sequencing of mouse genomic DNA revealed that these boxes corresponded to exons highly conserved between rat and mouse (Fig. 2), and demonstrated that FAK isoforms are generated by alternative splicing. In addition, it should be noted that the triplet coding for Glu-365 was absent from one clone (number 30), possibly corresponding to an alternative splice acceptor site (data not shown).

FAK Splice Isoforms Are Preferentially Expressed in Brain—We studied the tissue expression of FAK boxes 28, 6, and 7 by a combination of reverse transcriptase-polymerase chain reaction and Southern hybridization of the amplified products with specific probes (Fig. 3). Although this approach did not allow a quantitative comparison of the levels of expres-
In various tissues, the ratios of the different isoforms could be compared accurately. Box 7 was expressed in brain as well as in testis, and to a lower degree in skeletal muscle, heart, and kidney, whereas boxes 28 and 6 were mostly detected in brain (Fig. 3). The ratios of the different isoforms were similar in all brain regions, with the exception of cerebellum, in which the levels of box 6 appeared lower (Fig. 3). In all brain regions the signal for box 28 was slightly weaker than for the other exons, suggesting that this exon may be expressed at lower levels, in agreement with its presence in only 2 out of 15 striatal clones (Table I).

FAK Protein Isoforms Coded by the Various cDNAs have Different Sizes and Autophosphorylation—The isoform of FAK containing no insertion, or containing boxes 6 and 7 (FAK16,7), or containing boxes 6, 7, and 28 (FAK16,7,28) were expressed in COS 7 cells. Since endogenous FAK+ was virtually absent from COS 7 cells, the use of an antibody against FAK+ (SL42) allowed the specific immunoprecipitation of transfected FAK+. In SDS-polyacrylamide gels, the apparent size of these various isoforms increased with the presence of the additional amino acid boxes (Fig. 4A). The overall tyrosine phosphorylation of FAK+6,7 or FAK+6,7,28 was higher in COS 7 cells than that of FAK+. The tyrosine phosphorylation of FAK+ in immune precipitate kinase assays was also increased by the presence of boxes 6 and 7 (Fig. 4A). Since these immune complexes did not contain significant amounts of associated Src-family kinases (as detected by their ability to phosphorylate enolase, data not shown), we concluded that the increased phosphorylation resulted from an increased autophosphorylation. On the other hand, the presence of box 28 did not result in any further change (Fig. 4A). It should also be noted that the presence of the tripeptide Pro-Tyr-Arg which defines FAK+, did not change the apparent mobility or the phosphorylation of the protein (data not shown). Thus, the presence of boxes 6, 7, and/or 28 can account for the higher molecular weight of FAK+ in brain as compared with other tissues (Fig. 4B).

To determine whether the overall increased phosphorylation
of FAK+6,7 in COS 7 cells could be accounted for an increase in phosphorylation of Tyr-397, the known autophosphorylation site (6), we used anti-phosphopeptide antibodies which reacted specifically with FAK phosphorylated on Tyr-397 (Fig. 4B). Immunoblotting with this antibody of FAK immunoprecipitated from transfected COS cells revealed that phosphorylation of Tyr-397 was increased in the isoforms containing boxes 6 and 7, alone or in combination with box 28 (Fig. 4B), whereas the presence of PWR in the carboxyl terminus had no effect (data not shown). Interestingly, phosphorylated Tyr-397 was detected in FAK immunoprecipitated from brain but not from liver (Fig. 4B), or other tissues (data not shown).

**Phosphopeptide Mapping of Autophosphorylated Rat Brain FAK**—To identify better the major isoform of FAK expressed in brain, we carried out two-dimensional tryptic phosphopeptide mapping of FAK immunoprecipitated from transfected COS 7 cells or from various tissues, and autophosphorylated in vitro as in Figs. 1 and 4. FAK+ expressed in COS 7 cells generated a single major phosphopeptide (Fig. 5A). FAK+6,7 gave rise to a more complex pattern with several phosphopeptides (Fig. 5B). Comigration experiments demonstrated that the phosphopeptide maps of FAK from hippocampus were identical to those generated by transfected FAK+6,7 (Fig. 5 C, E, and F). Mapping of FAK from testis gave a pattern similar to that of transfected FAK+ (Fig. 5D), whereas FAK from cerebellum gave an intermediate pattern (Fig. 5G). These results provide further evidence for a major contribution of the FAK+6,7 isoform in brain.

**Lack of Effect of Boxes 6, 7, or 28 on the Interaction of FAK with Src Family Kinases**—When autophosphorylated on Tyr-397, FAK becomes an excellent ligand for Src or Fyn, which, in turn phosphorylate multiple residues in FAK sequence (9). To determine whether the presence of peptidic insertions surrounding the autophosphorylated tyrosine altered these interactions, we transfected COS 7 cells with the standard isoform of FAK (no additional exon) or an isoform containing all the exons identified in brain (FAK+6,7,28), alone or in combination with c-Src, n-Src (the neuronal isoform of Src), or Fyn (Fig. 6). In all cases, cotransfection increased dramatically the overall tyrosine phosphorylation of FAK, demonstrating that the presence of the various boxes did not prevent the interaction of FAK with Src family kinases. In additional experiments we investigated the co-immunoprecipitation of Src family kinases.
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Fig. 5. Two-dimensional phosphopeptide mapping of FAK isoforms expressed in COS 7 cells and from rat tissues. Transfected COS 7 cells or rat tissue samples were homogenized, and FAK was immunoprecipitated, incubated in the presence of [γ-32P]ATP, and resolved by polyacrylamide gel electrophoresis (as in Figs. 1 and 4A). 32P-Labeled FAK was digested in the gel piece with trypsin and phosphopeptides were separated by electrophoresis at pH 1.9 in the first dimension and by chromatography in the second dimension. Phosphopeptide positions were identified by autoradiography. The origin is indicated by a circle. A, FAK+ from transfected COS 7 cells; B, FAK+ with exons 6 and 7 (FAK+6,7); C, FAK immunoprecipitated from hippocampus; D, FAK immunoprecipitated from testis; E, comigration of A and C; F, comigration of B and C; G, FAK immunoprecipitated from cerebellum. The position of the main phosphopeptide originating from FAK+ without additional exon is indicated by a solid arrowhead. The positions of the 4 major phosphopeptides originating from FAK+6,7 are indicated by open triangles.

Fig. 6. Phosphorylation of FAK isoforms expressed in COS 7 cells by Src family kinases. COS 7 cells were transfected with an isoform of FAK without exon (FAK) or FAK containing exons PWR, 28, 6, and 7 (FAK + boxes), alone (−) or cotransfected with c-Src, n-Src, or Fyn, as indicated. FAK was immunoprecipitated with specific antibodies and its phosphorylation assessed by immunoblotting with antibody 4G10. Cotransfection of Src family kinases increased markedly the phosphorylation of the two isoforms of FAK. All samples are shown in duplicate. Similar results were obtained in at least three other experiments.

with FAK, using phosphorylation of enolase to detect these kinases in the immunoprecipitate (enolase was not significantly phosphorylated by FAK itself in these conditions). We found no major difference in the ability of the various isoforms of FAK to interact with the three Src family kinases studied (data not shown). This indicated further that the presence of the insertions did not alter the interactions of FAK with Src or Fyn.

DISCUSSION

This study underlines the complexity of FAK structure and processing. Although it appears to be coded by a single gene, a number of transcriptional and post-transcriptional mechanisms generate multiple isoforms of FAK mRNA and protein. Two different polyadenylation sites and two different 5′ ends were found in rat, corresponding to those described in chick and mouse, respectively (17). A very short alternative exon, coding for 3 amino acids defining FAK+′, is found in rat, mouse (17, 18), human (28), and Xenopus (26). A short form of FAK, called FAK-related non-kinase has been described in chick (29) and may also exist in human (30). In the present study, we show that 3 additional exons can be found in rat and mouse FAK, corresponding to the region of the protein which surrounds the autophosphorylation site, at the junction between the amino-terminal and the catalytic domains of FAK. At least two of these exons (boxes 6 and 7) have been highly conserved during evolution, since they exist also in Xenopus (26). Assuming that all these variations of FAK mRNAs occur independently from the others, at least 512 different mRNAs coding for 64 different forms of full-length FAK protein could exist. This calculation does not take into account the short gene product FAK-related non-kinase and the possible additional exons which have been reported in human cDNAs (30). Although it is likely that not all combinations are expressed at significant levels, the wealth of potential isoforms, many of which, and perhaps all, have been highly conserved during evolution, provide a mechanism by which FAK expression and properties could be finely tuned.

It is noteworthy that exons which give rise to changes in the coding sequence (FAK+: boxes 28, 6, and 7) are all highly expressed in the nervous tissue. This may be linked to the preferential inclusion of small exons which has been suggested to occur during mRNA splicing in brain (31). In fact, among the other tissues examined only testis contained high amounts of FAK+ (data not shown) and box 7 (this study). An important consequence of the existence of isoforms with differences in the coding region, is that they may provide neuronal FAK with specific properties. We found that FAK and FAK+ were indistinguishable concerning their amount of tyrosine phosphorylation, their autophosphorylation, and their interactions with Src and Fyn. This is perhaps not surprising since the Pro-Trp-Arg insertion of FAK+ is located in the carboxyl-terminal region of the protein, at a distance in the primary sequence from the kinase domain and the autophosphorylation site. In contrast, we found that the presence of boxes 6 and 7 which are located on either side of the autophosphorylated tyrosine, affected dramatically the tyrosine phosphorylation in cells and in immune precipitates. Using a site- and phosphorylation-specific antibody, we could show that the increased phosphorylation of FAK+6,7 could be accounted for, at least in part, by an increased phosphorylation of Tyr-397, the major autophosphorylation site. Boxes 6 and 7 were always found associated in striatal cDNAs and we do not know whether one, or the other, or the combination of the two is responsible for the observed effect. On the other hand, the presence of box 28, located amino-terminal of box 6, did not alter the autophosphorylation rate.

Work from several laboratories has shown that autophosphorylation of FAK on Tyr-397 is a critical aspect of its regulation and function, since it is responsible for the binding of Src family kinases (6, 7) and phosphatidylinositol 3-kinase (8). Moreover, although in vitro FAK is able to phosphorylate several proteins, including paxillin and p130Cas, these are also substrates for Src.
which may be responsible for their phosphorylation in cells (see Refs. 4 and 5). The available evidence suggests that FAK may function as an adaptor regulated by autophosphorylation, rather than as a protein kinase active on different substrates. Thus, an increased ability to autophosphorylate, provided by alternative splicing, could have important functional consequences. For instance, it could increase the phosphorylation of FAK in cells which express FAK6,7 in response to integrin engagement or stimulation of G protein-coupled receptors. It may thus play a role in the high responsiveness of FAK in hippocampus to neurotransmitters or lipid messengers (18, 19).

Our studies of FAK isoforms gave important negative results concerning their interactions with Src family kinases. The presence of insertions did not alter the interaction of FAK with these kinases, at least in transfected COS 7 cells. Moreover we did not observe a preferential association of any of the isoforms of FAK with c-Src, n-Src, or Fyn.

One possible functional consequence of increased tyrosine phosphorylation of FAK is suggested by knock out experiments which have revealed that FAK is more important for the turnover of focal adhesions than for their formation (11). Interestingly, growth cones of neurons in culture do not have real focal adhesions, but display smaller structures, termed point contacts, which may be related to the high mobility of neuritic extensions (32). It will be important to determine whether the isoforms of FAK with a higher capacity to autophosphorylate are associated with these adhesion structures, which may have a high reversibility. It is striking that point contacts are also observed in transformed cells in which they may be related to an increased mobility or invasiveness (32). Since a high degree of FAK expression in malignant cells may be related to their invasiveness or their ability to create metastasis (33), our findings suggest that the expression of splice variants of FAK in such cells could have important consequences on these characteristics.

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