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# Focal adhesion protein-tyrosine kinase phosphorylated in response to cell attachment to fibronectin

(signal transduction/extracellular matrix/integrins/polymerase chain reaction)

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**ABSTRACT** **A homology-based cDNA cloning approach was used to identify a widely expressed protein-tyrosine kinase designated as "*f*ocal *ad*hesion *k*inase" (FadK). The entire mouse FadK amino acid sequence was deduced from cDNA clones, revealing a large (119-kDa) non-membrane-spanning protein-tyrosine kinase that lacks Src-homology SH2 and SH3 domains. Immunostaining of BALB/c 3T3 fibroblasts revealed that FadK is concentrated in focal adhesions. FadK is phosphorylated on tyrosine in growing cultures of BALB/c 3T3 cells but contains little or no phosphotyrosine in cells detached by trypsinization. The tyrosine-phosphorylated state is regained within minutes when the cells are replated onto fibronectin. Activation of FadK may be an important early step in intracellular signal transduction pathways triggered in response to cell interactions with the extracellular matrix.**

The metazoan protein-tyrosine kinases (PTKs) make up a large segment of the eukaryotic protein kinase superfamily (1, 2) and are known for their oncogenic potential as well as their roles in regulating aspects of normal development (reviewed in refs. 3–5). PTKs are grouped into two broad structural classes on the basis of whether or not they span the plasma membrane. Membrane-spanning PTKs include receptors for various peptide growth and differentiation factors such as epidermal growth factor and platelet-derived growth factor. Receptor PTK activity is stimulated upon ligand binding, and subsequent receptor autophosphorylation followed by phosphorylation of exogenous substrates is an initial event in the signal transduction pathway (reviewed in refs. 5 and 6). PTKs that do not span the plasma membrane (i.e., members of the Src subfamily) have also been implicated in the transduction of extracellular signals that control cell growth and differentiation (reviewed in refs. 7–9).

Given what is known about the widespread involvement of PTKs in regulating developmental processes, we sought to identify and characterize PTKs, with the ultimate goal of obtaining insight into how cells respond to environmental cues that influence their growth and differentiation. In this report, we describe a non-membrane-spanning PTK identified by a homology-based cDNA cloning strategy and present evidence that its activation may be part of a signal transduction pathway triggered in response to cellular interactions with components of the extracellular matrix (ECM).†

## MATERIALS AND METHODS

**Amplification of PTK Catalytic-Domain cDNA Fragments by PCR.** PTK cDNAs were amplified from adult rat testis poly(A)$^+$ RNA by reverse transcriptase-directed PCR (10). PCR primers were designed to recognize conserved regions in PTK catalytic domains [upstream "HRDLAA" primers, 5′-CA(C/T)-(A/C)GN-GA(C/T)-(C/T)TN-GCN-GC-3′; downstream "DVWS(FY)G" primers, 5′-CC-(A/G)(A/T)A-N(C/G)(A/T)-CCA-NAC-(A/G)TC-3′; where N = (A/C/G/T)]. The thermocycling parameters used were as follows: annealing, 2 min at 50°C; extension, 3 min at 72°C; denaturation, 0.5 min at 94°C. Amplified cDNA products of ≈210 base pairs were subcloned and nucleotide sequences were determined for 200 inserts picked at random.

**Isolation of cDNA Clones Encoding Mouse FadK and Northern Analysis of Expression.** The PCR product TK3 was used as a probe to screen (10) a mouse 8.5-day embryo cDNA library (11) constructed in λgt10. To obtain clones representative of the entire ≈4.5-kilobase (kb) transcript, it was necessary to rescreen the library with probes derived from the 5′ and 3′ ends of the initial cDNA isolates. Nucleotide sequences were determined, from both complementary strands, for four overlapping clones to obtain the composite sequence shown.

RNA isolation and Northern hybridization were done as described (12). The blots were hybridized with a probe derived from a *Bam*HI–*Acc* I fragment (nucleotides 3476–4084) in the 3′ untranslated region of the FadK cDNA.

**Production of Antiserum to FadK and Antibody Affinity Purification.** Anti-FadK antiserum was obtained from New Zealand White rabbits immunized with a bacterially expressed fusion protein consisting of the C-terminal 150 amino acid residues of mouse FadK, lying downstream of 16 vector-derived residues from the pT7-7 plasmid vector (13). Antibodies from the immune serum were affinity-purified (14) against the fusion protein blotted to Immobilon membrane (Millipore).

**Immunoprecipitation of FadK, Immune-Complex Kinase Assay, and Phospho Amino Acid Analysis.** BALB/c 3T3 mouse fibroblasts (from W. J. Pledger, Vanderbilt University) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum. Cellular proteins were metabolically labeled with [$^{35}$S]methionine/cysteine by incubating the cells for 16 hr at 37°C in methionine-free DMEM containing 5% complete DMEM, 4% calf serum, and Tran$^{35}$S-label (1000 Ci/mmol; ICN; 1 Ci = 37 GBq) at 100 μCi/ml. Phosphoproteins were labeled by incubating the cells for 4 hr at 37°C in phosphate-free medium containing 4% calf serum and [$^{32}$P]orthophosphate (9000 Ci/mmol; NEN) at 1.5 mCi/ml. Cells were lysed in RIPA buffer (50 mM Tris Cl, pH 7.4/150 mM NaCl/5 mM EDTA/1% Nonidet P-40/1% sodium deoxycholate/0.1% SDS/1% aprotinin, 50 mM NaF/0.1 mM $Na_3VO_4$) and immunoprecipitation of FadK was achieved by mixing 2 μl of antiserum with 1 ml of clarified lysate, incubating on ice for 1.5 hr, and then adding 50 μl of a 50% slurry of protein

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Abbreviations: ECM, extracellular matrix; PTK, protein-tyrosine kinase.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M95408).

A-Sepharose (Pharmacia) and incubating 1 hr at 4°C on a rocking platform. Immunoprecipitates were washed in RIPA buffer, and proteins were separated by SDS/8% PAGE and detected by fluorography ($^{35}S$) or autoradiography ($^{32}P$).

To demonstrate kinase activity, FadK was immunoprecipitated from cell lysates as described above, except that cell proteins were unlabeled and lysis was in NP-40 Buffer (50 mM Tris Cl, pH 7.4/150 mM NaCl/1% Nonidet P-40/50 mM NaF/100 $\mu$M $Na_3VO_4$/1% aprotinin). The immune complexes were washed four times in 1 ml of NP-40 buffer and once in kinase assay buffer (20 mM Tris Cl, pH 7.4/10 mM $MgCl_2$/2 mM $MnCl_2$) and suspended in 30 $\mu$l of kinase assay buffer containing 4 $\mu$M ATP and 10 $\mu$Ci of [$\gamma$-$^{32}$P]ATP (4500 Ci/mmol; ICN). After incubation for 10 min at 30°C, the phosphoproteins were separated by SDS/8% PAGE and visualized by autoradiography. After acid hydrolysis of phosphoproteins transferred to Immobilon, phospho amino acids were separated in two dimensions on thin-layer cellulose plates (15).

**Immunofluorescence Microscopy.** BALB/c 3T3 cells, grown overnight on glass coverslips coated with rat plasma fibronectin (1 $\mu$g/ml; Telios Pharmaceuticals, San Diego), were rinsed in phosphate-buffered saline (PBS) and fixed for 5 min in methanol/acetone (1:1, vol/vol). Fixed cells were preincubated with 3% bovine serum albumin in PBS for 30 min at 37°C, then incubated with primary antibodies for 2 hr at 37°C. The cells were then washed three times for 5 min in 0.5% Nonidet P-40/PBS and rinsed twice in PBS. The secondary antibodies were then applied for 30 min at 37°C. After washing as above, the coverslips were mounted, viewed in a microscope equipped for epifluorescence (Nikon Labophot), and photographed. The primary antibodies used were affinity-purified anti-FadK polyclonal antibodies, anti-talin monoclonal antibody 8d4 (16) (Sigma), and anti-phosphotyrosine monoclonal antibody py72 (17) (kindly provided by Bart Sefton, Salk Institute). Secondary antibodies were fluorescein-conjugated goat anti-rabbit IgG (Vector Laboratories) and rhodamine-conjugated goat anti-mouse IgG (Chemicon).

**Analysis of FadK Phosphotyrosine Content.** FadK immunoprecipitated from unlabeled BALB/c 3T3 cells lysed in RIPA buffer was electrophoretically separated in an SDS/polyacrylamide gel and blotted to Immobilon. The membrane was preblocked for 2 hr in Tris-buffered saline (TBS)/0.2% Tween 20 and then incubated for 2 hr in TBS/0.2% Tween 20 with anti-phosphotyrosine monoclonal antibody py72 at 2 $\mu$g/ml. This was followed by a 1-hr incubation in TBS/0.2% Tween 20 with rabbit anti-mouse IgG (Organon Teknika–Cappel) at 10 $\mu$g/ml. Finally, the blot was incubated for 1 hr in 0.2% Tween 20/0.25% gelatin with $^{125}$I-labeled protein A (low specific activity, NEN) at 0.5 $\mu$Ci/ml. After each incubation, the blot was washed three times for 5 min in TBS. Immunoreactive proteins on the blot were visualized by autoradiography.

To investigate changes in phosphotyrosine content of FadK in response to plating cells on various adhesive substrates, BALB/c 3T3 cells were harvested by trypsinization, washed twice in PBS with soybean trypsin inhibitor (Worthington), at 0.5 mg/ml, suspended in serum-free DMEM, and plated ($\approx 1.5 \times 10^6$ cells per dish) on 60-mm tissue culture dishes coated with either rat plasma fibronectin (10 $\mu$g/ml) (Telios Pharmaceuticals) or poly(L-lysine) (1 mg/ml). The dishes were incubated at 37°C and, at various times following plating, attached cells were washed in PBS and lysed in 1 ml of RIPA buffer. Protein concentrations in clarified lysates were determined and samples were standardized to equal concentrations of protein prior to immunoprecipitation of FadK. Phosphotyrosine was then detected by probing blots of immunoprecipitated proteins with py72.

## RESULTS

**Isolation of cDNA Clones Encoding a Non-Membrane-Spanning PTK.** A PCR cloning strategy (18) was used to identify 13 different PTK catalytic-domain cDNA fragments amplified from rat testis RNA. One of the cDNA fragments, TK3, was particularly abundant in the PCR library (representing 44 of 200 clones sequenced) and appeared to have no close relatives among known members of the PTK family. With the TK3 product as a probe, a mouse embryo cDNA library was screened and clones were isolated that, in composite, included the entire protein-coding sequence for a member of the PTK family (Fig. 1). The long open reading frame encodes a protein of 1052 amino acid residues with calculated molecular mass of 119.1 kDa. The ATG encoding the initiating methionine conforms to the consensus for translation initiation (19), and just upstream of this ATG are found stop codons in all three reading frames. A protein kinase catalytic domain typical of the PTKs (1, 2) and including sequences identical to those encoded by the rat TK3 PCR fragment encompasses amino acids 422–676. The deduced protein does not contain a hydrophobic transmembrane domain characteristic of growth factor receptor PTKs. Nor could we detect, aside from the catalytic domain, regions homologous to any proteins registered in the Swiss-Prot (Release 19) and Protein Identification Resource (Release 26) data bases. In particular, the deduced protein does not contain Src-homology SH2 or SH3 regulatory domains (20) as are present in members of the Src, Abl, and Fes/Fps subfamilies. On the basis of its localization in cultured fibroblasts (see below), this member of the PTK family has been designated "*f*ocal *ad*hesion *k*inase" (FadK).

**FadK Transcripts Are Widely Expressed.** Northern analysis of RNA isolated from a variety of rat and mouse adult tissues showed that FadK transcripts are widely expressed (Fig. 2). Transcripts of $\approx$4.5 kb were detected in every rat and mouse adult tissue examined. Additional transcripts were detected in the testis. The $\approx$4.5-kb transcript has also been detected in whole mouse embryos (6.5–14.5 days postcoitum) and in a variety of cell lines, including BALB/c 3T3 fibroblasts (data not shown).

**Detection of FadK and Demonstration of PTK Activity.** An anti-FadK antiserum was raised by immunizing rabbits with a bacterially expressed fusion protein. Antibodies in the immune serum specifically immunoprecipitated a protein of $\approx$120 kDa (equivalent to the calculated mass of FadK) from $^{35}$S-labeled BALB/c 3T3 cell lysates (Fig. 3*a*). When immune complexes formed by incubating the antiserum with lysates of unlabeled cells were assayed for protein kinase activity, the $\approx$120-kDa protein was found to become phosphorylated (Fig. 3*b*), and phospho amino acid analysis revealed that the phosphorylation was on tyrosine (Fig. 3*c*). These results provide strong evidence that FadK is, in fact, a PTK and that it autophosphorylates *in vitro*.

**Immunolocalization of FadK.** Affinity-purified anti-FadK antibodies were used to localize FadK in BALB/c 3T3 fibroblasts by immunostaining. [The specificity of these antibodies for the $\approx$120 kDa FadK protein was demonstrated by probing immunoblots of total cellular proteins (data not shown)]. In well-spread cells, the antibodies stained, predominantly, patchy arrowhead-shaped structures (Fig. 4*a*) reminiscent of focal adhesions, specialized regions of the plasma membrane formed at sites where cells adhere tightly to the substratum (reviewed in ref. 21). Talin, a known component of focal adhesions, is colocalized with FadK (compare Fig. 4 *a* and *b*). Diffuse perinuclear staining was also observed in cells stained with the anti-FadK antibodies. This is accounted for, at least in part, as nonspecific staining by the secondary antibody (Fig. 4*c*). Additional controls using primary antibodies obtained when preimmune serum

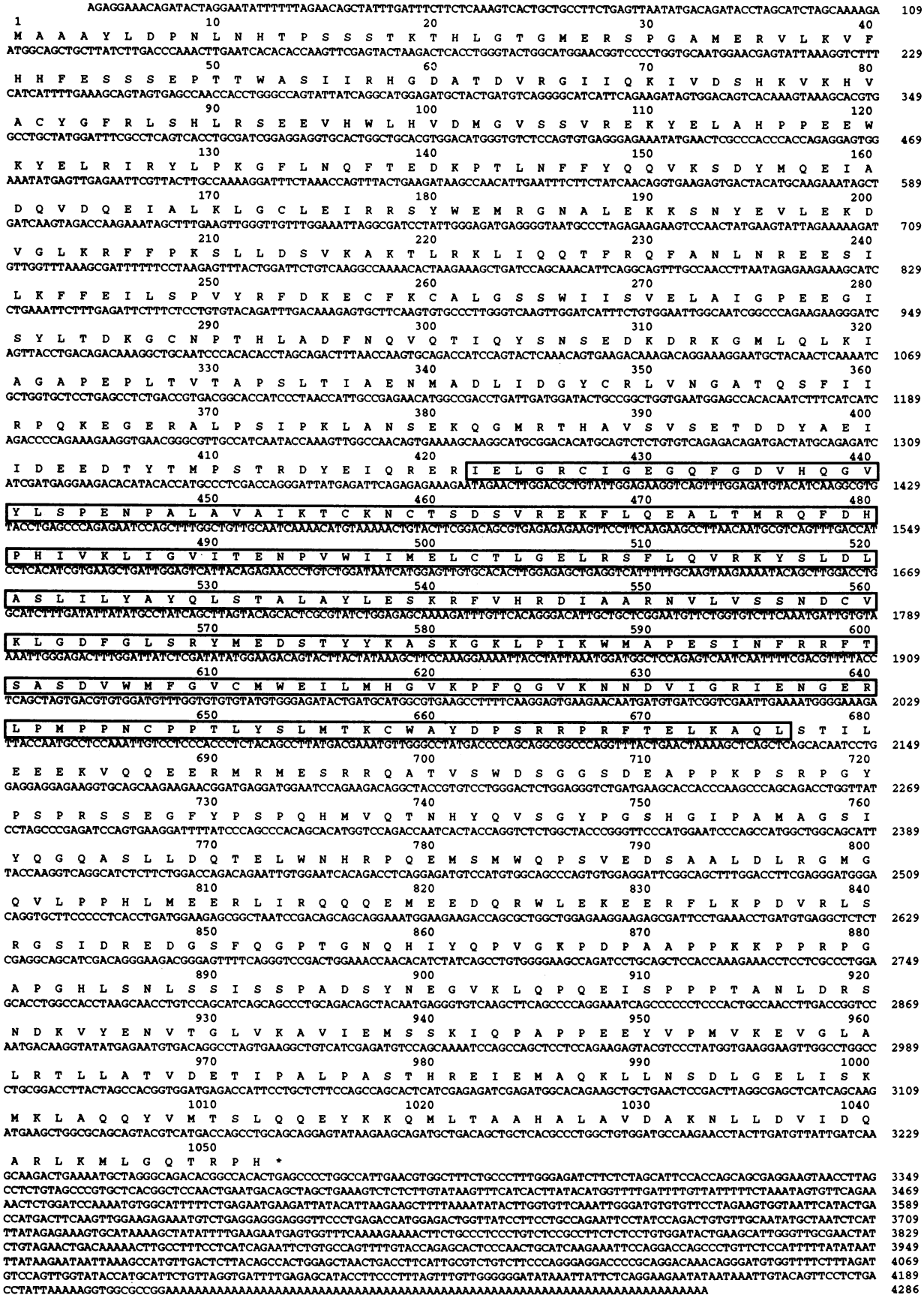

FIG. 1. Sequence of mouse FadK cDNA and deduced amino acid sequence. Numbers at right indicate nucleotide position from the 5′ end of the cDNA; numbers above amino acids indicate position from the N terminus of the encoded polypeptide. Boxed amino acid residues represent the catalytic domain.

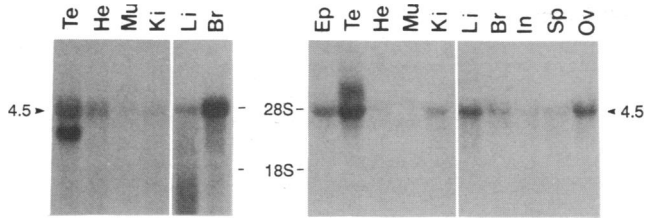


FIG. 2. Northern hybridization analysis of FadK transcripts in mouse (*Left*) and rat (*Right*) tissues. Ep, epididymus; Te, testis; He, heart; Mu, skeletal muscle; Ki, kidney; Li, lung; Br, brain; In, intestine; Sp, spleen; Ov, ovary. Arrowheads indicate the ≈4.5-kb FadK transcript. Positions of 28S and 18S rRNA are shown.

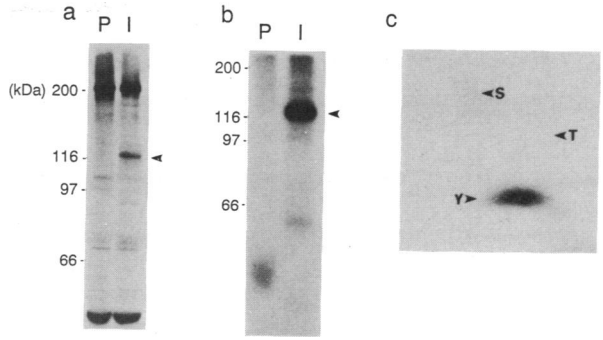


FIG. 3. Immunoprecipitation of FadK and demonstration of PTK activity. (*a*) Immunoprecipitation of FadK metabolically labeled with [$^{35}$S]methionine/cysteine. Proteins were precipitated by control preimmune serum (lane P) or by anti-FadK immune serum (lane I). (*b*) *In vitro* kinase assay of immune complexes formed using preimmune (P) or immune (I) serum. (*c*) Phospho amino acid analysis of FadK following autophosphorylation *in vitro*. Arrowheads in *a* and *b* indicate FadK. Arrowheads in *c* indicate positions of markers: S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine.

was subjected to the affinity-purification procedure also gave only the diffuse perinuclear staining (data not shown).

Immunostaining with anti-phosphotyrosine antibodies has revealed that phosphotyrosine-containing proteins in nontransformed fibroblasts are concentrated in focal adhesions (22). Double-label immunostaining of BALB/c 3T3 cells with anti-FadK and anti-phosphotyrosine antibodies showed that FadK is strikingly colocalized in focal adhesions with phosphotyrosine (compare Fig. 4 *d* and *e*). Thus the activity of FadK may contribute to the observed concentration of phosphotyrosine in focal adhesions.

**FadK Is a Phosphoprotein and Is Phosphorylated on Tyrosine in Response to Plating BALB/c 3T3 Cells onto Fibronectin.** At least a portion of the FadK pool in growing cultures of BALB/c 3T3 cells is present in a phosphorylated state, as demonstrated by immunoprecipitation from $^{32}$P-labeled cell lysates (Fig. 5*a*). The presence of phosphotyrosine in FadK was revealed by probing a blot of proteins immunoprecipitated from unlabeled cell lysates with anti-phosphotyrosine antibody (Fig. 5*b*).

The focal adhesion localization of FadK led us to investigate the possibility that its tyrosine-phosphorylated state is regulated in response to interactions with components of the ECM. To test this, BALB/c 3T3 cells were harvested by trypsinization and replated in dishes coated with either fibronectin (upon which cells attach and become well spread within 30 min) or poly(L-lysine) (upon which cells attach but do not immediately spread). At various times after plating, attached cells were lysed, FadK was immunoprecipitated, and its phosphotyrosine content was determined by probing with anti-phosphotyrosine antibody. FadK became phosphorylated on tyrosine as an early response to plating cells onto fibronectin (Fig. 5*c*). FadK immunoprecipitated from trypsinized cells contained little, if any, phosphotyrosine. But within 5 min of plating cells onto fibronectin, tyrosine-phosphorylated FadK was clearly evident. In contrast, the phosphotyrosine content of FadK immunoprecipitated from cells attached to poly(L-lysine) remained near the low levels found in trypsinized cells. A control blot using anti-FadK antiserum instead of anti-phosphotyrosine antibody verified that equal amounts of FadK were present in the lysates (data not shown). The simplest interpretation of these results is that FadK is activated and autophosphorylates as a consequence of the interaction of fibronectin with its cell surface receptor.

## DISCUSSION

ECM proteins markedly affect the growth, migration, and differentiation of eukaryotic cells, and these influences are essential for many fundamental biological processes such as

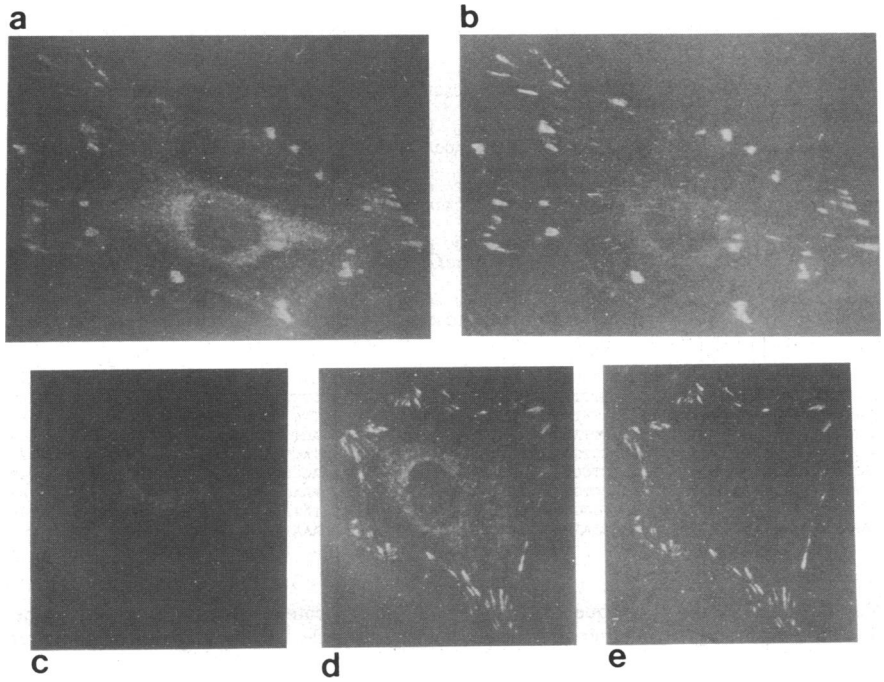


FIG. 4. Immunolocalization of FadK in BALB/c 3T3 fibroblasts. (*a* and *d*) Cells stained for FadK. (*b*) Same cell as shown in *a*, stained for talin. (*c*) Control showing staining obtained when anti-FadK primary antibodies were omitted from the procedure. (*e*) Same cell as shown in *d*, stained for phosphotyrosine.

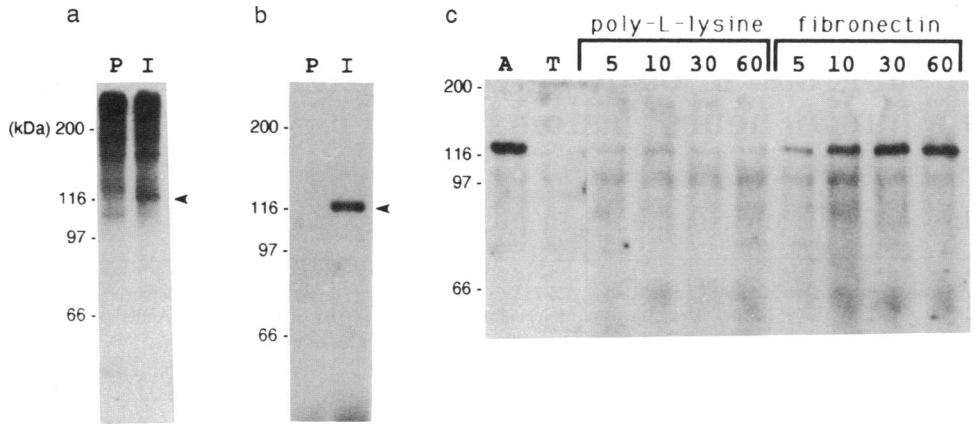


FIG. 5. Analysis of FadK phosphorylation. (*a*) Immunoprecipitation of FadK metabolically labeled with $^{32}P$ (P, preimmune serum; I, immune serum). (*b*) Detection of phosphotyrosine in immunoprecipitated FadK, using anti-phosphotyrosine antibody (P, preimmune serum; I, immune serum). (*c*) Analysis of FadK phosphotyrosine content 5, 10, 30, and 60 min after plating cells onto fibronectin or poly(L-lysine). Lane A, lysate from cells attached and growing under standard culture conditions; lane T, lysate from cells harvested by trypsinization. Arrowheads indicate FadK.

embryogenesis, wound healing, and malignant transformation (reviewed in refs. 23 and 24). Clearly, intracellular signaling processes must be activated at sites of cell–ECM contact. How are these signals mediated? Evidence pointing to a role for PTKs has accumulated. Recent studies have revealed rapid and dramatic increases in phosphotyrosine content of an ≈120-kDa protein after plating mouse NIH 3T3 fibroblasts on fibronectin (25), and of 115- to 130 kDa proteins after incubating human KB epithelial cells with antibodies to the $\beta_1$ integrin component of the fibronectin receptor (26). On the basis of the results presented here, we suggest that FadK is a major component of the phosphotyrosine-containing proteins described in these earlier studies. It will be of interest to measure changes in FadK phosphotyrosine content following cell attachment to other ECM proteins that interact with distinct integrin receptors.

Our observations suggest a model for fibronectin-induced intracellular signaling initiated by the aggregation of integrin receptors at focal adhesions, with the coincident autophosphorylation of associated FadK molecules. Subsequently, FadK may phosphorylate exogenous substrates, setting off a cascade of events that bring about alterations in cellular properties. This would be analogous to the proposed mechanisms for growth factor signal transduction across the plasma membrane involving the aggregation and intermolecular autophosphorylation of receptor PTKs (5, 6) and for the activation of the lymphocytic Src-subfamily PTKs through their interactions with transmembrane components of antigen receptor complexes (7–9). How might FadK transmit a signal within the cell? One pathway might involve the association with, and phosphorylation of, proteins that contain SH2 domains, which are known to interact with phosphotyrosine (20). In this context, the focal adhesion protein tensin, isolated and characterized on the basis of its ability to bind actin filaments (27), has been reported to contain an SH2 domain (28). The association between tensin and FadK phosphotyrosine may be an important step in the anchorage of actin filaments to the submembraneous cytoskeleton.

As this report was being prepared, we learned that a PTK similar in structure to FadK has very recently been identified as a cDNA clone isolated from a chicken embryo library (29). This chicken PTK, designated pp125$^{FAK}$, is highly similar throughout its length to mouse FadK, and their catalytic domains share 98% identical amino acid residues. Like mouse FadK, chicken pp125$^{FAK}$ was shown to localize to focal adhesions (29). Thus, these two PTKs appear to be functional homologs.

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