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ARTICLES

Focal Adhesion Kinase Is Abundant in Developing Blood Vessels and Elevation of Its Phosphotyrosine Content in Vascular Smooth Muscle Cells Is a Rapid Response to Angiotensin II

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Abstract Focal adhesion kinase (FAK) is a structurally unique nonreceptor protein-tyrosine kinase that localizes to focal adhesion plaques. Regulation of its activity has been implicated in diverse signaling pathways, including those mediated by extracellular matrix/integrin interactions, G-protein coupled receptors for mitogenic neuropeptides, and certain oncogene products. To gain evidence for specific processes in which FAK may be involved in vivo, a study was initiated to determine its expression pattern during mouse development. FAK expression was detected in early embryos and appeared to be distributed throughout all cell types at about the time of neurulation. Subsequent to neural tube closure, expression became particularly abundant in the developing vasculature. This included expression in the medial layer of arteries populated by smooth muscle cells. In vitro studies using cultured rat aortic vascular smooth muscle cells demonstrate that FAK phosphotyrosine content is dramatically elevated in response to plating cells onto the adhesive glycoprotein, fibronectin. Also, enhanced tyrosine phosphorylation of FAK is observed in these cells upon stimulation with the vasoconstrictor angiotensin II. Thus, in vascular smooth muscle cells, like fibroblasts, FAK appears to play a role in signaling mechanisms induced by extracellular matrix components as well as G-protein coupled receptor agonists. The combined results of this study suggest that signaling through FAK may play an important role in blood vessel morphogenesis and function.

Key words: protein-tyrosine kinase, embryogenesis, extracellular matrix, fibronectin

Protein-tyrosine kinases, first identified by their oncogenic potential, are now recognized as key elements in molecular pathways controlling the normal growth and differentiation of metazoan cells [reviewed by Pawson and Bernstein, 1990]. Recently a novel nonreceptor protein-tyrosine kinase, designated focal adhesion kinase (FAK), was identified from chicken [Schaller et al., 1992] and mouse [Hanks et al., 1992] sources. FAK is structurally unique among the known protein-tyrosine kinases. The catalytic domain is centrally located in the 1,052 amino acid residue polypeptide (molecular mass = 119 kDa), giving rise to large amino-terminal and carboxyl-terminal flanking domains. FAK lacks "Src homology" SH2 and SH3 domains involved in protein-protein interactions, setting it apart from most other nonreceptor protein-tyrosine kinases. Outside the catalytic domain FAK shows no significant similarity to any protein currently registered in the sequence databases.

FAK derives its name from its localization to focal adhesion plaques in cultured fibroblasts [Schaller et al., 1992; Hanks et al., 1992]. Focal adhesions [reviewed in Burridge et al., 1988] are discrete regions of the cell plasma membrane where close contacts are made, via integrin receptors, with the underlying substratum of extracellular matrix (ECM) adhesive glycoproteins. Here the small cytoplasmic tails of integrins are thought to be important for the assembly of focal adhesion protein complexes (which are comprised of a number components, including talin, vinculin, tensin, and α-actinin), as well as for the anchorage of actin filament bundles (stress fibers).

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FAK's association with focal adhesion plaques suggests that it may be involved in regulating their assembly, and subsequently affecting cell adhesion and spreading. In fact, an increase in FAK phosphotyrosine content, correlated with increased in vitro tyrosine kinase activity, has been observed in fibroblasts soon after plating onto various ECM adhesive glycoproteins, including fibronectin, vitronectin, laminin, and collagen type IV [Hanks et al., 1992; Burridge et al., 1992; Guan and Shalloway, 1992; Kornberg et al., 1992]. A similar regulation of FAK phosphotyrosine content and activity occurs following fibrinogen binding to the blood platelet integrin $\alpha_{IIb}\beta_3$ [Lipfert et al., 1992]. Thus, FAK appears to be activated in response to a variety of integrin/ligand interactions.

In addition to the integrin response pathway, at least two other routes lead to tyrosine-phosphorylation of FAK [reviewed by Zachary and Rozengurt, 1992]. First, elevated levels of FAK phosphotyrosine are found in cells transformed by Rous sarcoma virus [Kanner et al., 1990; Schaller et al., 1992], and this property is associated with abnormally high in vitro kinase activity [Guan and Shalloway, 1992]. Thus, FAK has emerged as a potential in vivo substrate for the v-Src oncoprotein. Phosphorylation by v-Src may disrupt FAK's normal activity in a way that contributes to the malignant behavior of transformed cells—for example, loss of anchorage dependency for growth. Second, treatment of fibroblasts with mitogenic neuropeptides, including bombesin, vasopressin, and endothelin, induces a rapid increase in FAK phosphotyrosine content [Zachary et al., 1992]. These agents are known to bind to a class of receptors coupled to heterotrimeric G proteins that activate phospholipase C which in turn triggers phosphoinositide turnover, calcium mobilization, and activation of protein kinase C. However, neither calcium mobilization nor PKC activation appears to contribute significantly to the bombesin-stimulated FAK tyrosine phosphorylation pathway [Sinnett-Smith et al., 1993].

From the above studies, FAK has emerged as a candidate signaling molecule in pathways that control cell adhesion, shape, mobility, and/or growth. To gain further insight into FAK's normal physiological role, we initiated an immunohistochemical investigation of its expression pattern in the developing mouse embryo. Here we report that FAK is particularly abundant in the walls of developing blood vessels shortly after or concomitant with their formation in the early embryo. FAK expression is most prominent in the medial layers of arteries populated by smooth muscle cells. Cultured vascular smooth muscle cells (VSMC) were also found to contain relatively high levels of FAK. Plating of trypsinized VSMC onto fibronectin, and treatment of quiescent VSMC with the vasoconstrictor angiotensin II, each led to a rapid increase in FAK phosphotyrosine content. These observations suggest a potential involvement of FAK in signal transduction pathways involved in blood vessel development and in processes that affect mature vessels such as regulation of vascular smooth muscle tone and mass in response to changes in blood pressure.

MATERIALS AND METHODS

Northern Analysis

Procedures for RNA isolation and Northern hybridization analysis were essentially as described previously [Hanks, 1989]. Briefly, 3 μg of total RNA from each embryo sample, 7.5–16.5 d.p.c., was separated by electrophoresis through 0.8% formamide-agarose gels and transferred to a nylon membrane. Northern blots were hybridized with a ^{32}P-labeled single-stranded DNA probe derived from a *BamHI-AccI* fragment in the 3′-untranslated region of the mouse FAK cDNA (nucleotides 3,476–4,084). The probe was prepared from a single-stranded template using Klenow polymerase with a primer that annealed to vector sequences flanking the cDNA insert.

Western Analysis

Mouse embryos and tissue samples were dissected from ICR females mated to C57.Bl6 males (testes were dissected from retired C57.Bl6 breeders). Dissections were carried out in Dulbecco's Modified Eagles Medium (DMEM) (Gibco, Gaithersburg, MD) +3% bovine serum albumin (BSA). Following dissection, embryos and tissues were rinsed thoroughly in phosphate buffered saline (PBS) before mincing and homogenization in RIPA Buffer (1% NP-40, 150 mM NaCl, 50 mM Tris-Cl (pH 7.4), 5 mM EDTA, 0.1% SDS, 0.5% Na-Deoxycholate, 50 mM NaF, 100 μm Na_3VO_4, and 1% Aprotinin). After homogenization, samples were sonicated and clarified by centrifugation. Protein insoluble in RIPA Buffer was solubilized by boiling in 1% SDS, 100 mM Tris-Cl (pH 6.8), 10 mM EDTA, and 10%

glycerol. Protein concentrations of clarified lysates and solubilized pellets were determined by the Bicinchoninic Acid protein assay (Pierce, Rockford, IL).

The monoclonal antiphosphotyrosine antibody, 4G10, was purchased from Upstate Biotechnology Incorporated (Lake Placid, NY). Antisera against FAK have been described elsewhere [Hanks et al., 1992]. Affinity purification of FAK antibodies was achieved by passing whole immune serum over a glutathione S–transferase-FAK (GST-FAK) fusion protein immobilized on sepharose beads, eluting bound antibodies with 150 mM glycine-HCl (pH 2.5), and dialyzing against Tris-buffered saline (TBS) (10 mM Tris-Cl (pH 7.4), 150 mM NaCl). The GST-FAK fusion protein contained the same antigenic carboxyl-terminal portion of the FAK protein used in raising the immune serum.

For Western analysis, 25 μg of protein from each sample was separated by SDS-PAGE on 8% polyacrylamide gels and transferred to either Immobilon-P (Millipore, Bedford, MA) for FAK protein detection or nitrocellulose (Schleicher and Schuell, Keene, NH) for phosphotyrosine detection. Blots were stained with India ink [Hancock and Tsang, 1983] to visualize protein molecular weight standards and then blocked with either 5% milk in TBST (TBS + 0.2% Tween-20) for probing with affinity-purified anti-FAK antibodies, or 1% ovalbumin and 3% BSA in TBST for probing with 4G10 antibody. Antibodies were added to Western blots at a concentration of 1 μg/ml in fresh blocking buffer for 4 h. Blots were washed extensively in either TBST (anti-FAK blots) or TBS + 0.5% NP40 (4G10 blots), followed by a brief wash in TBS. For blots probed with 4G10, a secondary rabbit anti-mouse antibody (10 μg/ml) was added for 2 h and washed as above before further processing. Finally, blots were probed with ^{125}I-protein-A (New England Nuclear, Boston, MA) (0.25 μCi/ml) for 2 h in blocking buffer and washed as above. Bound antibodies were visualized by autoradiography. In all cases, no appreciable FAK was detected in the insoluble fraction of the tissue lysates (data not shown).

Immunoprecipitation

For immunoprecipitation, 500 μg of cellular protein in 1 ml lysis buffer was precleared of proteins that bind nonspecifically to protein A by incubating for 1 h with heat killed preparations of *S. aureus* (Zymed, San Francisco, CA). Cleared lysates were then incubated with 2 μg of affinity-purified anti-FAK polyclonal antibody. Immune complexes were brought down with protein-A Sepharose (Zymed) and washed five times with NP40 Buffer before boiling in 2× SDS-PAGE sample buffer. Equal volumes of each sample were loaded onto two SDS–8% polyacrylamide gels and subjected to Western analysis using anti-FAK and antiphosphotyrosine antibodies as described above.

Immunohistochemistry

ICR × C57.Bl6 mouse embryos were dissected as described above and fixed by emersion in Bouin's fixative overnight at 4°C. Fixed embryos were dehydrated by passes through graded concentrations of ethanol. Several incubations were carried out in 70% ethanol to allow picric acid to diffuse away. After dehydration, samples were saturated with xylene and embedded with paraffin. Three and 7 μm sections were mounted on slides using Sta-On tissue section adhesive (Surgipath Medical Industries, Inc., Grayslake, IL). Sections were dewaxed by several passes in xylene and then rehydrated by passing through graded concentrations of ethanol into a final buffer of 10 mM Tris-Cl (pH 7.4) and 100 mM $MgCl_2$. Nonspecific binding sites were blocked by incubation in 10 mM Tris (pH 7.4), 100 mM $MgCl_2$, 0.05% Tween-20, 1% BSA, and 5% fetal bovine serum. Affinity-purified FAK antibodies were added at an approximate concentration of 0.1 μg/ml in TBS + 0.05% Tween-20. Washes were carried out in this same buffer, and bound antibodies were visualized by incubation with alkaline phosphatase conjugated goat antirabbit secondary antibody (Promega, Madison, WI) and reaction with 5-bromo-4-chloro-3-indolyl-phosphate (Promega) and nitroblue tetrazolium substrates (Promega) according to the manufacturer's recommendations.

VSMC Cell Culture, Plating on Fibronectin, and Treatment With Angiotensin II

VSMC were isolated from rat thoracic aorta by enzymatic dissociation according to the methods of Gunther et al. [1982] with slight modifications. Briefly, the thoracic aortae were cleaned of loosely adherent fat and then preincubated with collagenase for 30 min at 37°C. The adherent adventitia was then carefully removed, the vessels opened lengthwise, and the endothelium removed by scraping with a Q-tip. The aortae were then incubated overnight in complete

DMEM. The following morning, the vessels were subjected to complete digestion. Isolated cells were subsequently characterized by staining with a monoclonal antibody specific for smooth muscle alpha-actin (Boehringer Mannheim, Indianapolis, IN). Cells were maintained in DMEM supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 25 mM HEPES (pH 7.4), and 100 units/ml penicillin and 100 μg/ml streptomycin. Fresh media was added every 2 days, and cells were passaged at 90% confluence.

For analysis of changes in FAK phosphotyrosine content following plating on fibronectin, VSMC on 100 mm plates were trypsinized at 90% confluence, washed twice in PBS containing 0.5 mg/ml soybean trypsin inhibitor (Sigma, St. Louis, MO), resuspended in serum-free DMEM, and replated on 60 mm dishes coated with human fibronectin (Collaborative Research Incorporated, Bedford, MA). Incubations were carried out at 37°C for indicated times (10–180 min). After aspiration of media and gentle washing with cold PBS, cells were lysed by addition of 0.5 ml ice-cold NP40 Buffer (150 mM NaCl, 50 mM Tris-Cl (pH 7.4), 1% NP40, 50 mM NaF, 100 μm Na_3VO_4, and 1% aprotinin). Cell lysates were scraped from the plates and clarified by centrifugation before immunoprecipitation with affinity-purified anti-FAK antibodies and Western analysis.

For angiotensin II induction, cells between passages 5 and 15 were seeded on either 60 or 100 mm dishes. Confluent VSMC were cultured in serum-free DMEM for 72 h and stimulated with 10^{-6} M angiotensin II (Bachem, Bubendorf, Switzerland) for indicated times (30 sec to 30 min). Cells were lysed in 1 ml NP40 Buffer per 100 mm dish, clarified by centrifugation, and analyzed for FAK content and FAK phosphotyrosine content by immunoprecipitation with anti-FAK antibodies and Western analysis using anti-FAK and antiphosphotyrosine antibodies as described above.

RESULTS

Expression of FAK in the Mouse Embryo

Initially, the temporal expression of FAK mRNA during mouse embryonic development was determined by Northern analysis. The 4.5 kb FAK mRNA was detectable as early as 7.5 days post coitum (d.p.c.) and continued to be expressed through the full term of embryonic development, reaching a maximum around 9.5 d.p.c. and gradually declining after 11.5 d.p.c. (Fig. 1A). Calculations of relative levels of FAK mRNA took into account differences in RNA loading as determined by reprobing blots for the constitutively expressed glyceraldehyde 3-phosphate dehydrogenase transcript (Fig. 1B). To verify expression of FAK protein during this developmental period, whole embryo lysates were subjected to Western analysis using affinity-purified polyclonal antibodies against FAK. Expression of FAK protein closely paralleled that of the mRNA, reaching a maximum near 9.5 d.p.c. and gradually decreasing after 12.5 d.p.c. (Fig. 1C). As noted previously [Devor et al., 1993], on low percentage polyacrylamide gels, FAK resolves as a doublet of approximately 115–125 kDa. Both bands persist after phosphatase treatment and appear to represent two distinct isoforms of FAK, as V8 maps from each are virtually identical to that of in vitro transcribed FAK [A. Murry and S.K.H., unpublished *data*]. The upper FAK band is more intense than the lower band, and no change in this ratio is apparent during the developmental time course.

The spatiotemporal pattern of FAK expression in mouse embryos 8.5–17.5 d.p.c. was studied by immunohistochemistry using affinity-purified anti-FAK antibodies. In general agreement with the Western analysis, FAK appeared to be expressed abundantly in early stage embryos of 8.5 (data not shown) and 9.5 d.p.c. (Fig. 2A). Immunoreactivity was most pronounced in the neuroepithelia, branchial arches, and somites (Fig. 2A). The increased cell density in these regions may have contributed to the more intense staining levels. Staining at the basal aspect of the epithelial sheets of both neuroepithelia and somites was particularly intense (Fig. 2A, arrowheads). No staining was detected using a similar concentration of normal rabbit IgG, except for background staining in remnant deciduous tissue surrounding the embryo (Fig. 2B, arrows).

From 10.5–17.5 d.p.c., the highest levels of immunoreactivity became increasingly restricted to the vasculature. This is first evident at 10.5 d.p.c. in the dorsal aorta, which showed distinctly elevated staining compared to the surrounding mesenchyme (Fig. 2C, arrowhead). Similar intense staining was seen in smaller vessels such as the intersomitic arteries that branch from the dorsal aorta (Fig. 2C). However, blood vessels identifiable as veins, such as the posterior cardinal vein, showed little or no distinctive staining (Fig. 2C). The immunoreac-

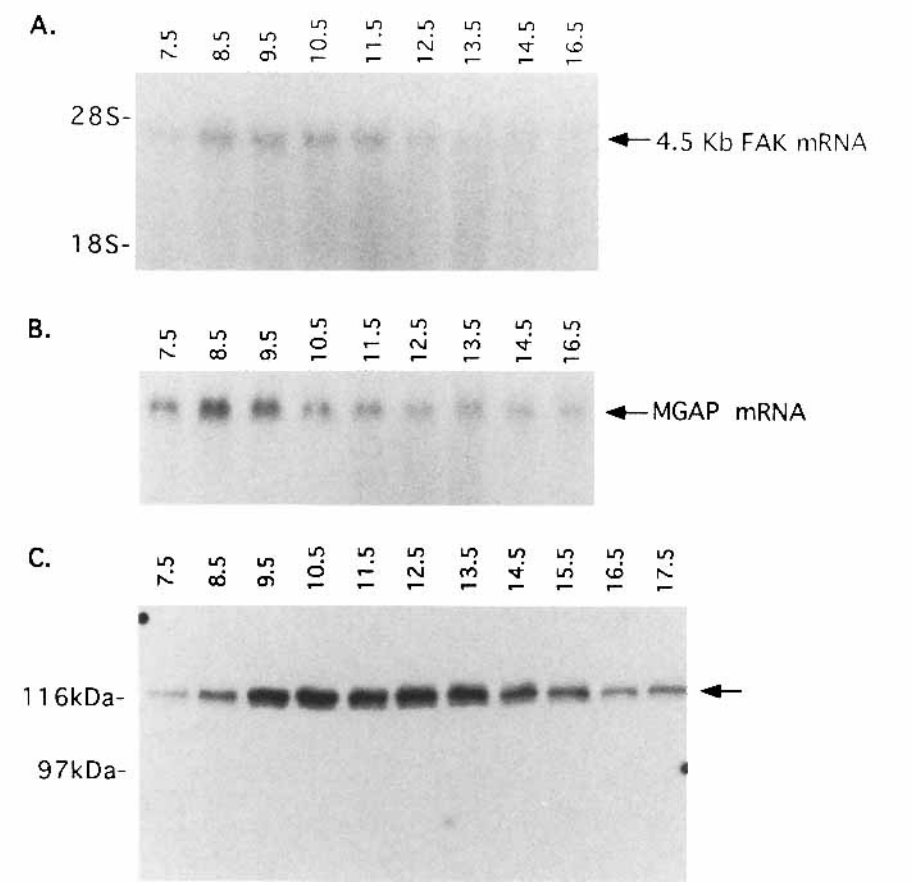

Fig. 1. FAK mRNA and protein expression during mouse embryonic development. **A:** Northern blot analysis of mouse embryo mRNA with FAK cDNA probe. Age (d.p.c.) is indicated above each lane, and the positions of 28S and 18S ribosomal RNA are marked. The arrow indicates position of the 4.5 kb FAK transcript. **B:** The same blot was reprobed with a mouse glyceraldehyde-3 phosphate dehydrogenase (MGAP) probe as a control for RNA loading. **C:** Western analysis of FAK expression in mouse embryos. Total protein (25 μg) from each sample was separated by SDS-PAGE and assayed for FAK content with affinity-purified polyclonal antibodies against FAK. The arrow indicates the position of the 115–125 kDa FAK polypeptides.

tivity of arterial walls, compared to the surrounding tissue, reached a maximum around 14.5 d.p.c., as exemplified by the descending thoracic aorta and the pulmonary artery (Fig. 3A). Control staining of an adjacent section using IgG purified from preimmune serum verified that staining of the vasculature was specific to the anti-FAK antibodies (Fig. 3B). Again, staining of venous walls was much less pronounced, as compared to arterial walls (Fig. 3C,D). The staining of vessel walls appeared to arise predominantly from the medial layer which is much thicker in arteries than in veins. Nevertheless, limited immunoreactivity was consistently detected in venous walls, possibly due to staining of endothelial cells and/or cells of the thin medial layer (Fig. 3C,D). Staining of capillaries infiltrating the central nervous system (Fig. 3E,F) is a further indication of endothelial cell immunoreactivity. Interestingly, strong staining was also observed within the glomerulus of developing (data not shown) and adult (Fig. 4A,B) kidneys. Staining within the glomerulus appeared to be confined to the capillary loops and the smooth muscle–like mesangial stalk. Staining of the efferent and afferent arterioles was particularly striking. The strong staining observed in capillaries as well as the medial layers of arteries is consistent with a view that both endothelial and vascular smooth muscle cells contribute to the immunoreactivity of the vasculature. Staining in skeletal muscle (data not shown), cardiac muscle (Fig. 3A), and nonvascular smooth muscle such as in digestive tract (Fig. 4C) did not reach the same high levels as that seen in arteries.

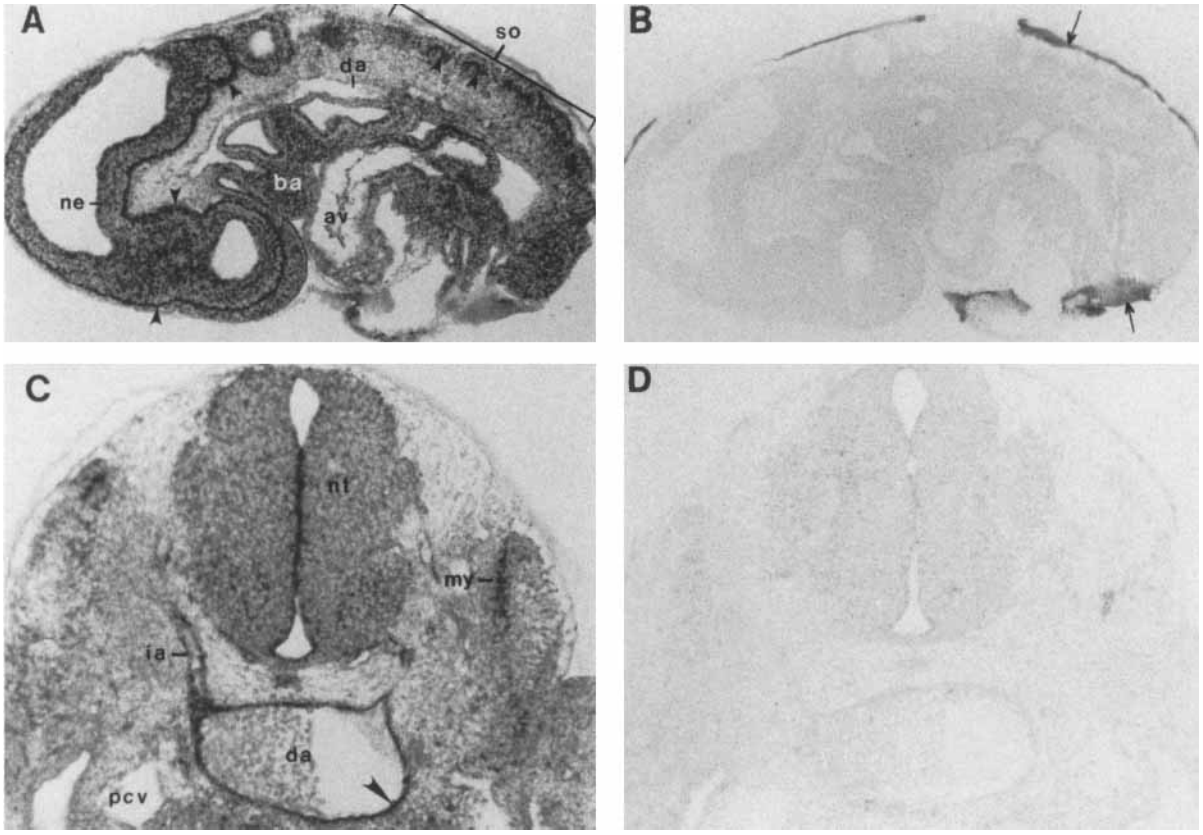

Fig. 2. Immunohistochemical detection of FAK in 9.5 and 10.5 d.p.c. mouse embryos. **A:** Parasagital section of 9.5 d.p.c. embryo showing most intense staining in neuroepithelium (ne), somites (so), and branchial arches (ba). Note strong staining at the basal surface of both neural and somitic epithelial sheets (arrowheads). Also indicated is the dorsal aorta (da) and atrioventricular canal (av). **B:** Adjacent section to A incubated with control normal rabbit IgG. Note background staining in decidual cells on surface of embryo (arrows). **C:** Transverse section of 10.5 d.p.c. embryo at the level of the budding hind limbs. Strong staining is detected in the neural tube (nt), basal surface of the dermamyotome (my), and in the wall of the dorsal aorta (da, arrowhead) and intersegmental arteries (ia). Note the lack of staining in the posterior cardinal vein (pcv). **D:** Normal rabbit IgG control of adjacent section.

Immunoreactivity was not limited to vascular structures. For example, strong staining of the germ cells of both female (data not shown) and male gonads (Fig. 4C) was observed as early as 13.5 d.p.c. Our detection system prevented assaying earlier germ cell expression patterns because of the endogenous alkaline phosphatase activity of these cells [Chiquonine, 1954]. Normal rabbit IgG controls verified that staining in the germ cells was specific for the anti-FAK antibodies (Fig. 4D). In addition, although staining of embryonic neural tissue declined to low levels by 14.5 d.p.c., strong staining reappeared in newborn (data not shown) and adult brain (Fig. 4E). Strongest staining in the adult brain was seen in neuronal layers of the neocortex and hippocampus. Subcortical regions and axon fibers displayed little or no immunoreactivity. The axon fibers are marked by fortuitous nonspecific staining of axonal tracts with normal rabbit IgG (Fig. 4E,F, arrowheads).

Western Analysis of FAK Expression in Adult Mouse Organs and Tissues

Western analysis of isolated adult mouse organs and tissues generally supported our immunostaining results (Fig. 5). Although detectable amounts of FAK were found in all samples assayed, highest levels were found in testis, lung, and brain. FAK protein was barely detectable in the kidney and small intestine, requiring a longer exposure of the blot than that shown in Figure 5. Low expression of FAK in the kidney is not inconsistent with the immunohistochemical detection of FAK in glomeruli, since these structures contribute little to the entire kidney mass.

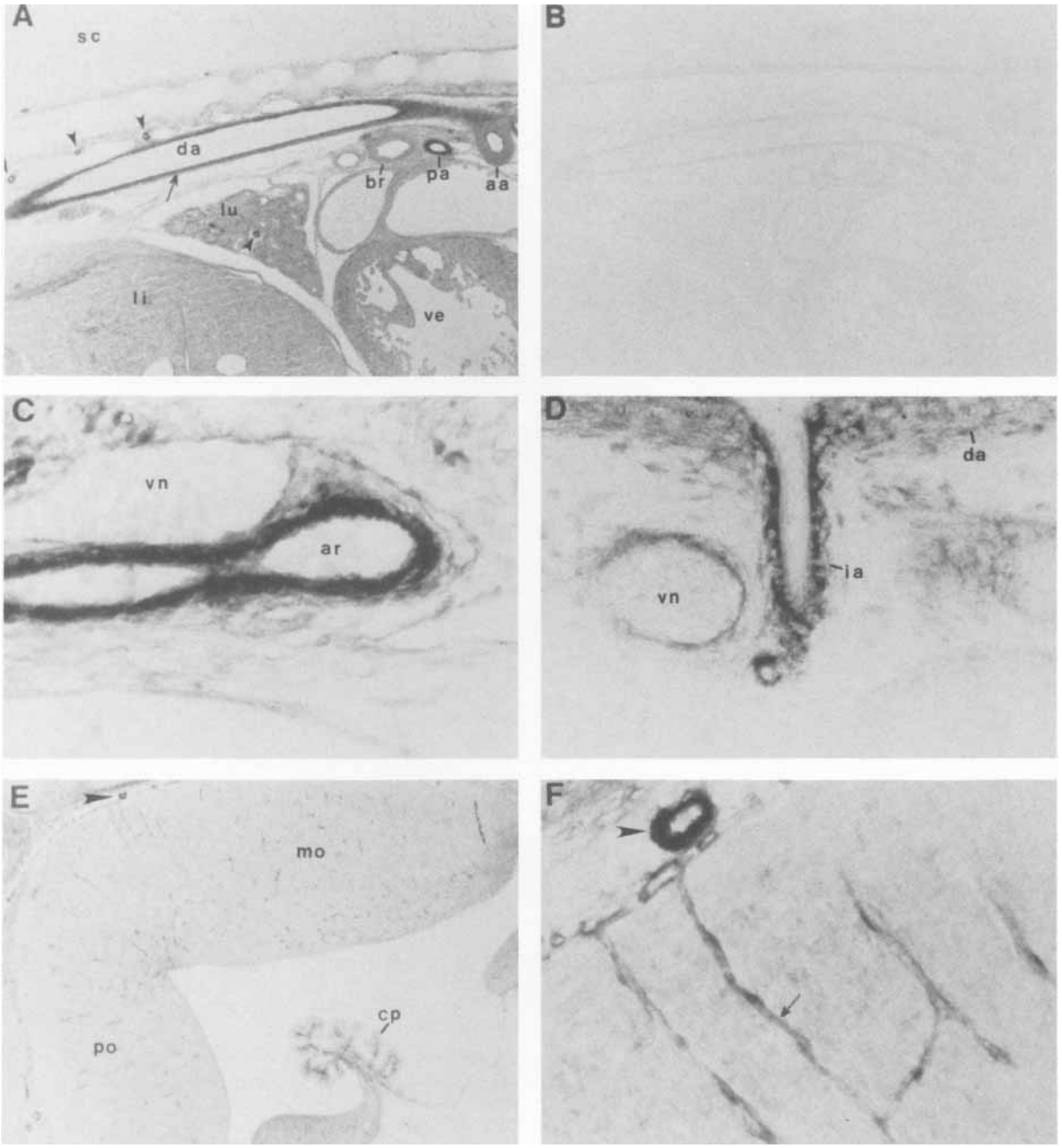

Fig. 3. Immunostaining of vasculature by anti-FAK antibodies. **A:** Immunoreactivity in 14.5 d.p.c. embryo is evident by intense staining in the descending thoracic aorta (da, arrow) and pulmonary artery (pa). Note the lesser amount of staining in the aortic arch (aa) relative to more distal aspects of the aorta. Strong staining in large arteries is contrasted by weak staining in the bronchus (br). Positive staining in smaller arteries (arrowheads) can be seen in the lung (lu) and along the developing spinal column. Small capillaries are faintly visible in spinal chord (sc) and myocardium of left ventricle (ve). The liver (li) appears devoid of intensly staining vessels. **B:** Normal IgG control of adjacent section. **C:** Medium sized artery (ar) and vein (vn) in pelvic region of 14.5 d.p.c. embryo. **D:** The intensely staining intersomitic arch (ia) is seen branching from the lightly staining wall of the descending thoracic aorta (da) of a 17.5 dpc embryo. A nearby vein (vn) is also indicated. **E:** Low magnification of the medulla oblongata (mo) and pons (po) in the developing brain of a 14.5 d.p.c. embryo. Heavy staining of arteries (arrowhead) is seen in the loosely packed mesenchyme surrounding this tissue and within the choroid plexus (cp). Capillaries are well stained within the neural tissue. **F:** High magnification of E, showing capillaries (arrow) within medulla oblongata. Arrowheads in E and F identify the same small artery.

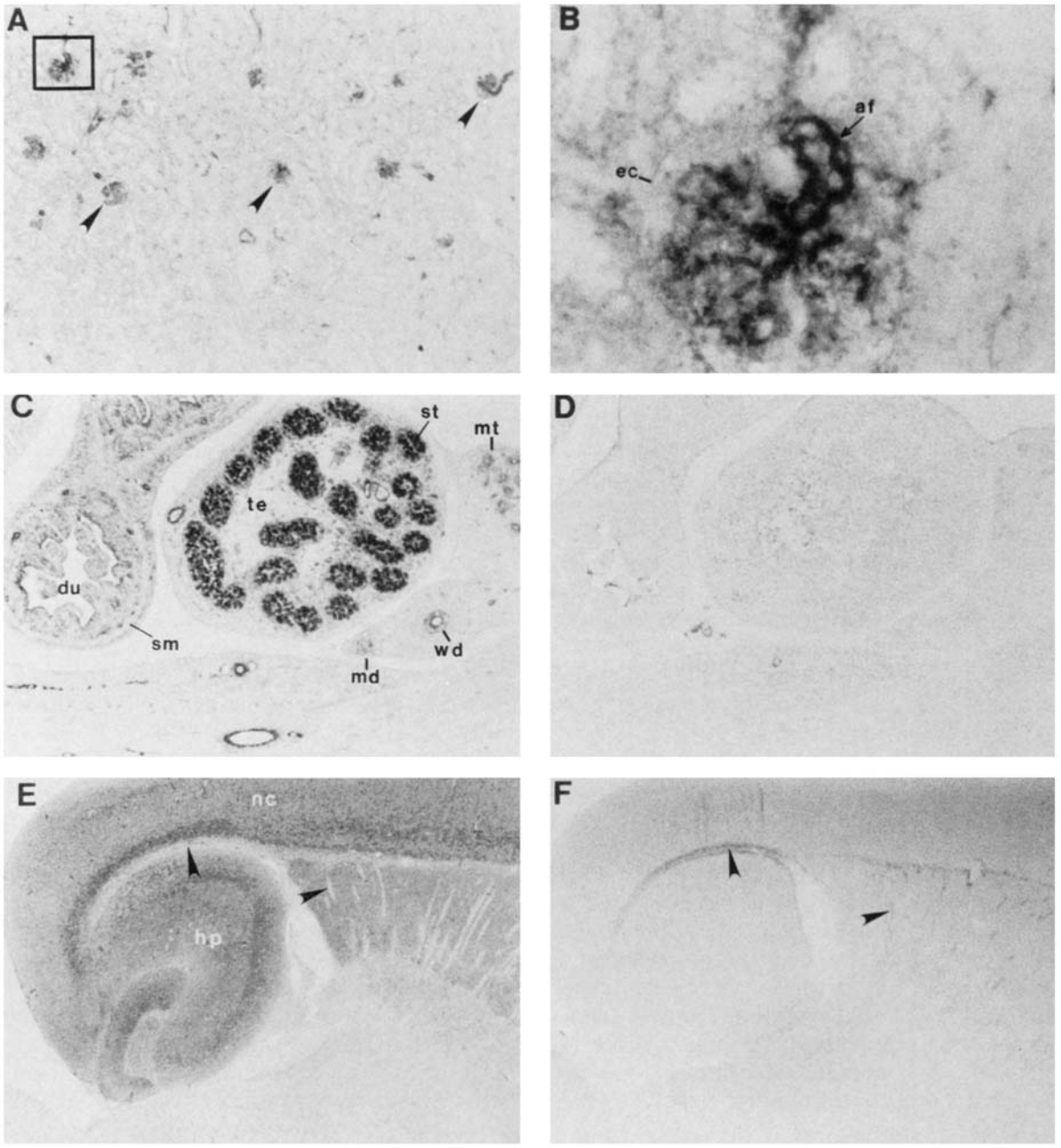

Fig. 4. Staining of other mouse tissues by anti-FAK antibodies. **A:** Staining of glomeruli (arrowheads) is evident in cortical region of adult kidney. **B:** Higher magnification of boxed region in A showing a pattern of staining that resembles capillary loops and the mesangial tree. The afferent artiole (af) also stains strongly. Note that staining is not detectable in Bowman's capsule (ec). **C:** Heavy staining of germ cells in developing seminiferous tubules (st) of testis (te) of 16.5 d.p.c. embryo. Adjacent to the testis is the duodenum (du). Note the absence of strong staining in the smooth muscle (sm) layer. Also shown are the Wolfian duct (wd), degenerating Mullerian duct (md), and degenerating mesenephric tubules (mt). **D:** Normal rabbit IgG control for staining in the testis. **E:** Immunostaining in the neuronal layers of the hippocampus (hp) and neocortex (nc) of the adult mouse brain. No staining is detected in axonal fibers (arrowheads). **F:** Normal rabbit IgG control for E. Note staining in axon fibers specific to normal rabbit IgG (arrowheads).

In the brain, anti-FAK antibodies recognize a prominent band with slightly reduced mobility-compared to that detected in other tissues, as well as a minor band of about 68 kDa. It is unclear whether these polypeptides represent modified forms of FAK or related products from a separate gene. Nonetheless, it is likely that they contribute to the pattern of immunostaining observed in the adult mouse brain.

Induction of FAK Tyrosine Phosphorylation in Cultured Rat Aortic VSMC by Fibronectin and Angiotensin II

A culture system of rat aortic VSMC was used to explore possible signaling roles for FAK in vascular tissues. Western and immunofluorescence analyses of these cells verified expression of FAK and its localization to focal adhesion plaques (data not shown). To investigate the potential for FAK's involvement in integrin-mediated signal transduction in these cells, VSMC adhesion to tissue culture plates was first disrupted by trypsinization and then cells were replated onto fibronectin-coated plates. Resulting changes in FAK phosphotyrosine content were assayed by immunoprecipitation and subsequent Western analysis using antiphosphotyrosine antibody. FAK was observed to become nearly completely dephosphorylated on tyrosine following trypsinization (Fig. 6A). FAK phosphotyrosine was partially regained by 5 min after replating and continued to increase through 1 h of culture on fibronectin (Fig. 6A). Longer incubations of up to 3 h showed that FAK phosphotyrosine content approached that of pretrypsinized cells (data not shown). FAK protein levels did not change appreciably during this period (Fig. 6B). These results are similar to those previously obtained using fibroblasts [Burridge et al., 1992; Guan and Shalloway, 1992; Hanks et al., 1992].

A large body of evidence suggests that tyrosine phosphorylation plays a role in the growth and contractile effects of small vasoactive peptides on VSMC (see Discussion). The vasoactive hormone angiotensin II has been implicated in increased smooth muscle cell growth [for review see Thyberg et al., 1990; Owens, 1991] and has been shown to induce tyrosine phosphorylation of a number proteins in VSMC [Tsuda et al., 1991; Molloy et al., 1993]. To specifically study its effect on FAK, quiescent cultures of VSMC were stimulated with angiotensin II, and the phosphotyrosine content of FAK was assayed as above. Stimulated cells showed a rapid induction of FAK phosphotyrosine content (Fig. 7A). Significant induction of FAK phosphotyrosine was apparent as early as 30 sec after stimulation, reached a maximum at about 1 min, and then gradually decreased through 30 min exposure to angiotensin II. Densitometric analysis from three separate experiments showed that the average maximal increase in FAK phosphotyrosine content is approximately fourfold. Levels of FAK protein were not affected by exposure of VSMC to angiotensin II (Fig. 7B).

DISCUSSION

Organization of the actin cytoskeleton in adherent cells is largely dependent on the formation of focal contacts between cells and the ECM. These focal contacts not only provide an anchor to which the cytoskeleton can apply stress, but also have been implicated in transducing extracellular information into the cell's interior. FAK, an intracellular PTK that localizes to points of cytoskeletal-ECM interactions, appears to be well suited for a role in regulating focal adhesion assembly and/or modulating biochemical pathways, such as those leading to gene expression, that ultimately affect cell growth and differentiation. To gain evidence as to specific physiological processes in which FAK might be involved, we undertook a study of FAK's pattern of expression in the developing mouse embryo. The major findings of this study indicate that FAK may play an important role in developing blood vessels. Subsequent in vitro studies using VSMC indicate that FAK potentially functions here in signaling pathways that involve both ECM proteins and hormonal factors.

In postneurulation embryos, one of the most striking aspects of FAK expression was observed in the developing vasculature. In particular, cells of the medial layer appeared to express much higher levels of FAK as compared to the surrounding mesenchyme. Expression of FAK in the vasculature is reminiscent of that previously observed for two other focal adhesion proteins, vinculin and talin [Duband and Thiery, 1990]. Moreover, the $\alpha_1\beta_1$ integrin receptor [Belkin et al., 1990; Defilippi et al., 1991] which binds to type IV collagen and laminin, as well as the $\alpha_5\beta_1$ integrin receptor [Muschler and Horwitz, 1991] which binds to fibronectin, have been shown to be expressed at high levels during various stages of vascular development. Based on these observations, it would appear that cells comprising

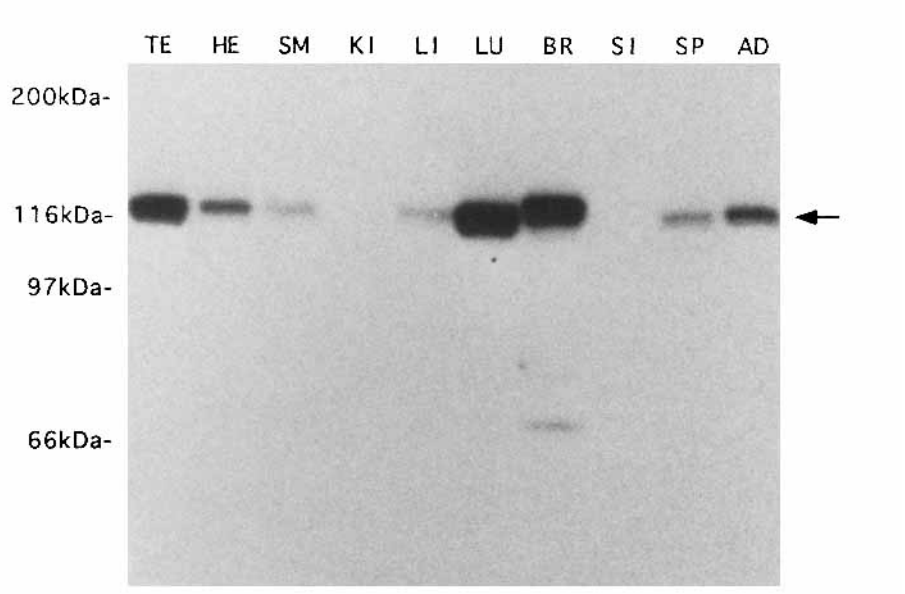

Fig. 5. Western analysis of adult organs and tissue samples. RIPA lysates of tissue samples were analyzed by 8% SDS-PAGE. Position of 115–125 kDa FAK polypeptides is indicated by arrow. TE, testis; HE, heart; SM, skeletal muscle; KI, kidney; LI, liver; LU, lung; BR, brain; SI, small intestine; SP, spleen; AD, adipose.

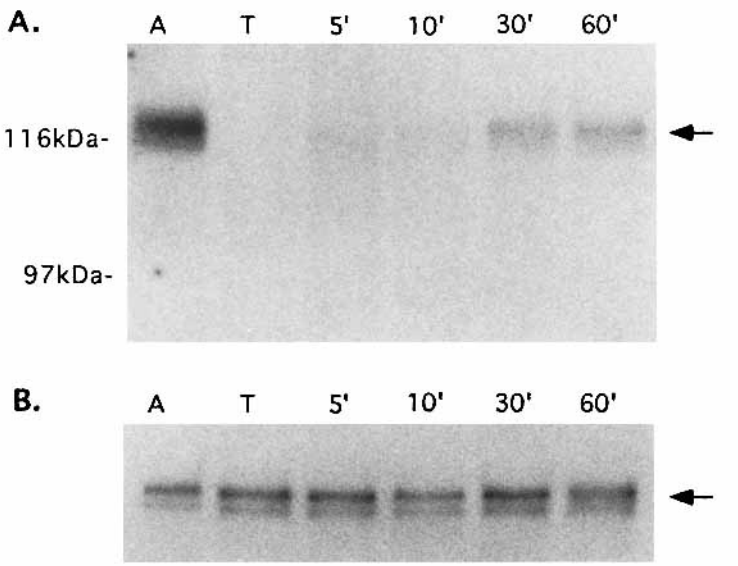

Fig. 6. Increased phosphotyrosine content of FAK in VSMC following plating onto fibronectin. FAK protein immunoprecipitated from rat aortic VSMC either growing under standard culture conditions (A), trypsinized (T), or replated on fibronectin coated dishes for various times (5', 10', 30', and 60') was subjected to Western analysis using a monoclonal antibody against phosphotyrosine (**A**), or anti-FAK antibodies to confirm equal loading of FAK protein (**B**). Arrows indicate the position of the 115–125 kDa FAK polypeptides.

the walls of developing vessels have special requirements for extensive networks of cell-matrix adherens junctions (in vivo counterparts of focal adhesions). FAK may play a role in the formation of such networks, thereby contributing to the maintenance of strong cell-matrix adhesion and/or rigid cytoskeletal architecture necessary to maintain the integrity of developing vessels.

In later stage embryos, FAK expression appeared to gradually decrease within the medial layer of the aorta, both at the level of the aortic arch and in more distal portions of descending aorta. Again, these observations are consistent

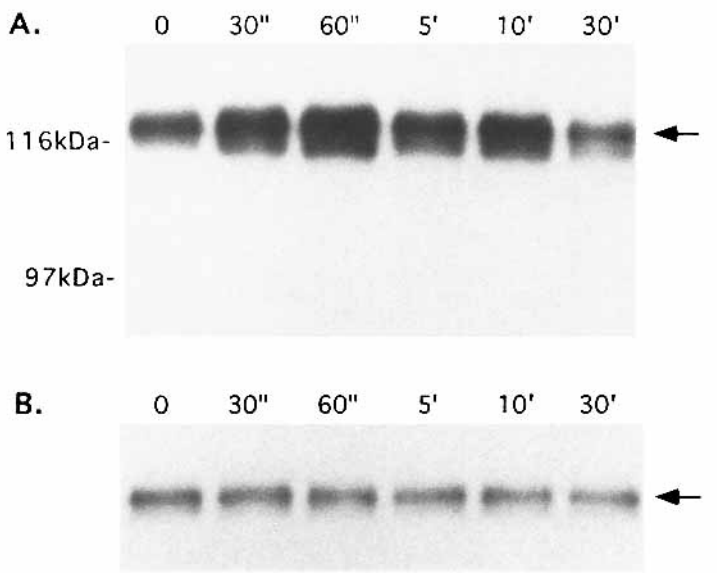

Fig. 7. Increased phosphotyrosine content of FAK in VSMC following treatment with angiotensin II. Quiescent cultures of VSMC were treated with 1 μm angiotensin II for the various times indicated. FAK protein was immunoprecipitated from cell lysates using affinity purified anti-FAK antibodies. Immunoprecipitates were subjected to Western analysis using antiphosphotyrosine antibodies (A) or FAK antibodies (B) as indicated in Fig. 6 legend. Arrows indicate position of the 115-125 kDa FAK polypeptides.

with the expression patterns of vinculin and talin, both of which have been reported to decrease substantially within the endothelial and vascular smooth muscle cells of maturing large arteries [Duband and Thiery, 1990]. The decrease in cell-matrix adherens junction proteins in maturing vessel walls occurs at a time coincident with the formation of an extensive basal lamina, further emphasizing FAK's possible involvement in maintaining the structural integrity of developing vessels. Our observation that FAK is highly expressed in the glomerulus of the kidney, a vascular bed under high blood pressure, is also consistent with this notion. Conversely, FAK does not appear to be highly expressed within the smooth muscle cells lining the digestive tract which probably are not held under the same stresses involved in maintaining tissue shape.

Extracellular matrix components that mediate cell adhesion are capable not only of inducing the formation of focal adhesion plaques and cell spreading, but also of influencing other cell characteristics such as growth and differentiation. Changes in cell shape and cytoskeletal organization that accompany cell adhesion to the ECM may influence the biosynthetic capabilities of the cell, thus affecting growth and differentiation [Ben-Ze-ev et al., 1988; Ingber and Folkman, 1989; Ingber et al., 1990]. There is also growing interest in the possibility that ECM/integrin interactions may have more direct affects on biochemical pathways, such as activation of ion channels [Banga et al., 1986; Ingber et al., 1990; Schwartz et al., 1991], induction of protein tyrosine phosphorylation [Ferrell and Martin, 1989; Golden et al., 1990; Guan et al., 1991; Hanks et al., 1992; Kornberg et al., 1991, 1992; Lipfert et al., 1992; Schaller et al., 1992] and induction of gene expression [Werb et al., 1989; Sporn et al., 1990; Streuli and Bissell, 1990; Haskill et al., 1991], that control cell growth and differentiation. To investigate FAK's potential involvement in signaling pathways that lead to these various changes, we undertook studies on cultured VSMC to determine changes in FAK's phosphotyrosine content (an indirect measure of activity) following exposure to fibronectin. As previously reported for fibroblasts, we observed that FAK phosphotyrosine content increases dramatically following plating of rat aortic VSMC onto fibronectin, thus implicating FAK in integrin/ECM-mediated signaling events in these cells. In vivo, ECM proteins are likely to play an important role during the development of blood vessels. Previous studies have shown that fibronectin and laminin are expressed at defined stages in blood vessel development [Risau

and Lemmon, 1988]. Expression of fibronectin generally correlates with the early proliferative-secretory (synthetic) state of VSMC, while laminin expression correlates with the nonproliferative contractile state of VSMC lining mature vessels. In vitro, fibronectin has been shown to promote the transition of VSMC from a contractile to a synthetic phenotype [Hedin and Thyberg, 1987]. In contrast, laminin appears to inhibit this phenotypic transition [Hedin et al., 1988]. Thus, alterations in FAK's phosphotyrosine content and activity in vascular smooth muscle may play a role in the signal transduction pathways that modulate the synthetic/contractile phenotypes.

The modulation of vascular smooth muscle phenotype is reversible, unlike the differentiation of myoblasts to myotubes. This is likely a normal adaptive mechanism that enables vessels to increase their mass in response to increases in vascular pressure. Therefore, factors that affect blood pressure may also regulate VSMC phenotype and growth. A large body of evidence suggests that the potent vasoconstrictor, angiotensin II, may be involved in the regulation of VSMC growth and differentiation [Thyberg et al., 1990; Owens, 1991; Heagerty, 1991]. This aspect of angiotensin II function has been studied predominently in the context of vascular hypertensive diseases that lead to hypertrophic or hyperplastic growth of VSMC. Angiotensin II may also play a role during embryonic development since all components of the renin-angiotensin system, including angiotensinogin, renin, and angiotensinogin converting enzyme, as well as angiotensin receptors, are found in various fetal tissues [Wilkes et al., 1985]. In particular, high levels of both angiotensin type I (AT_1) and type 2 (AT_2) receptors have been detected in embryonic tissues derived from the mesoderm including the developing vasculature [Grady et al., 1991; Viswanathan et al., 1991]. AT_2 receptors constitute approximately 80% of the angiotensin receptors in prenatal tissue [Grady et al., 1991; Viswanathan et al., 1991]. This receptor subtype predominance reverses in adult tissue, suggesting a potential functional difference between receptor subtypes [Grady et al., 1991; Viswanathan et al., 1991].

To examine a potential role for FAK in angiotensin II signaling, the phosphotyrosine content of FAK was analyzed in response to angiotensin II induction of VSMC. Previous in vitro evidence from work on both VSMC and fibroblasts suggested that tyrosine phosphorylation may play an important role in mediating the growth response to angiotensin II [Leeb-Lundberg and Song, 1993; Tsuda et al., 1991; Molloy et al., 1993]. In VSMC, at least nine proteins are known to have increased phosphotyrosine content in response to vasoactive agents, including angiotensin II, (Arg)vasopressin, 5-hydroxytryptamine, and norepinephrine [Tsuda et al., 1991; Molloy et al., 1993]. We have shown that FAK is clearly among this group of proteins phosphorylated on tyrosine in response to angiotensin II. Given the similar patterns of protein tyrosine phosphorylation induced by different contractile agonists, it is likely that FAK phosphotyrosine content is increased in response to other vasoactive peptides as well. In fact, similar studies in fibroblasts reveal increased levels of FAK phosphotyrosine content in response to the neuropeptides, vasopressin, bombesin, and endothelin [Zachary et al., 1992]. It should be noted that cultured VSMCs contain only the type 1 angiotensin receptor [Whitebread et al., 1989]; hence, it remains to be established whether FAK is also involved in signaling via AT_2 receptors which are most abundant in embryonic tissue. The mediators of the AT_2 receptor signaling pathway are currently unknown, but there is evidence that AT_2 receptor signaling involves G-protein dependent [Tsutami and Saavedra, 1992] and independent [Bottari et al., 1991; reviewed in Timmermans et al., 1992; Bottari et al., 1993] pathways.

In summary, we have demonstrated that FAK is expressed early in mouse development and becomes increasingly prominent in the developing vasculature. Furthermore, we show that FAK phosphotyrosine content is increased in response to plating VSMC on fibronectin and also by stimulation with the potent vasoconstrictor/growth factor angiotensin II. These observations support a role for FAK in the establishment of cytoskeletal structure that defines cell and tissue morphological integrity and further implicate FAK in signal transduction processes important for the differentiation and growth of VSMC.

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