Signal Transduction from the Extracellular Matrix
— A Role for the Focal Adhesion Protein-tyrosine Kinase FAK

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The proliferation and differentiation of cells in higher organisms requires specific external signals. These can be presented to target cells in the form of soluble peptide hormones, proteins retained on the surface of adjacent cells, or as part of the extracellular matrix (ECM). Many growth factors activate membrane-associated receptor protein-tyrosine kinases (PTKs), which in turn stimulate intracellular signal transduction pathways. Whereas much progress has been made in characterizing growth factor receptor PTKs signal transduction, less is known about the mechanisms by which signals from the extracellular matrix are transduced into the interior of the cell.

Extracellular matrix proteins such as fibronectin, vitronectin, laminin, and collagen bind to a family of heterodimeric transmembrane proteins called integrins. The integrin receptors cluster at distinct sites of cell contact with the ECM termed focal adhesions, and one of their roles is to link the interior actin cytoskeleton of cells with ECM proteins. This structural linkage of the actin cytoskeleton with integrins is believed to be mediated by cytoskeletal-associated proteins such as talin and α-actinin which can bind to integrin cytoplasmic domains (for a review see 1). In addition to a structural role, integrin receptor binding to ECM results in the activation of a number signal transduction pathways similar to those of stimulated by growth factors and cytokines (for a reviews see 2, 3, 4, 5, 6, 7). Integrin induced signals include calcium influx (8, 9), proton exchange (10), changes in phosphoinositide metabolism (11, 12, 13, 14, 15), stimulation of enhanced protein-tyrosine phosphorylation (16, 17, 18), and can lead to the activation of mitogen-activated protein (MAP)-ERK (19, 20, 21, 22) and c-jun N-terminal (JNK) MAP kinase cascades (23). Integrin-induced cellular responses in the nucleus can stimulate specific gene expression (24, 25, 26, 27, 28), prevent apoptotic cell death (29, 30, 31, 32), and can promote DNA synthesis and cell growth (33, 34, 35).

Integrins are composed of α and β type transmembrane subunits, among which 16α and 8β subunits can heterodimerize to form more than 20 different integrin receptors (1). The relatively short cytoplasmic domains of the α and β integrin subunits do not have any intrinsic enzymatic activity and likely transduce intracellular signals by promoting the formation of multi-protein complexes containing both cytoskeletal and catalytic signaling proteins (23, 36). Protein phosphorylation is one of the earliest events detected in response to integrin stimulation. Some of the first evidence that integrin receptors could induce protein-tyrosine phosphorylation came from studies in platelets (for reviews see 6, 37). Tyrosine phosphorylation of 50–72 kDa platelet proteins occur on immobilized fibrinogen (38, 39), whereas subsequent increased tyrosine phosphorylation of other proteins (120–140 kDa) require platelet-platelet aggregation or cell spreading (40, 41). Subsequent studies have specifically identified the protein-tyrosine kinase, Syk, as being rapidly activated in response to fibrinogen binding to the integrin receptor, αIIbβ3, in platelets (42).

Increased tyrosine phosphorylation has been shown to be a common response to integrin engagement in many cell types including fibroblasts, carcinoma cells, and cells of hematopoietic lineages (for a review see 7). In fibroblasts, several protein-tyrosine kinases have been implicated in integrin signaling events by virtue of their integrin-dependent activation or their localization to focal contacts. Members of the Src-family of protein-tyrosine kinases have been implicated in integrin signaling events due to the fact that v-Src or constitutively activated forms of c-Src localize to focal contacts (43, 44). In addition, in cells which are deficient in the Csk protein-tyrosine kinase which phosphorylates and negatively regulates c-Src, c-Src is activated and localized to focal adhesions (45, 46, 47, 48).

Another protein-tyrosine kinase that appears to play a central role in integrin-mediated signal transduction is the cytoplasmic focal adhesion protein-tyrosine kinase (FAK). In fibroblasts, FAK normally localizes with surface integrin receptors at sites where cells attach to the ECM (49, 50), and is activated by cell binding to fibronectin (16, 18, 21, 49, 50) or by extracellular antibody cross-linking of β1 integrins (16, 51). In vitro, FAK has been shown to interact with short peptides from several β integrin cytoplasmic domains (52) and can bind to the cytoskeletal protein talin, which also can associate with β integrin cytoplasmic domains (53). Since the transmembrane integrin receptors lack endogenous catalytic activity and need to recruit signaling molecules, recent research interest has been focused on FAK as a likely...
candidate for a key signaling molecule which is recruited and activated by integrin receptors in order to promote intracellular signal transduction.

**Mechanisms of Integrin-mediated Signaling/A Role for the FAK PTK Family**

FAK was originally identified as the 125 kDa target of a monoclonal antibody produced against a panel of v-Src associated tyrosine phosphorylated proteins (54). Subsequently, the FAK cDNA was cloned both by using this monoclonal antibody to screen a chicken expression library (50) and independently cloned from a mouse cDNA library using degenerate PCR primers to conserved motifs found in PTK catalytic domains (49). FAK homologues also have been cloned from human (55, 56) and Xenopus cDNA libraries (57, 58) and the predicted protein sequences from human, mouse, chicken, and Xenopus FAK share over 90% identities. FAK is ubiquitously expressed in many tissues, found in focal adhesions as its name implies, and is unusual among non-receptor protein-tyrosine kinases in that it does not contain Src-homology 2 (SH2) or SH3 domains. FAK is comprised of a central kinase domain flanked by two large non-catalytic domains.

The FAK C-terminal domain contains sequences required for focal adhesion localization (59), as well as binding sites for other cellular proteins. The cytoskeletal protein, paxillin, binds to residues in the tail of the FAK C-terminal domain (60) and has been shown to be phosphorylated by FAK in vitro (61). Conserved tyrosines residues in the FAK C-terminal domain have been shown to be phosphorylated in vivo (62) and in particular, FAK Tyr925 (YENV motif) serves as a binding site for the SH2 domain of the GRB2 SH2/SH3 adaptor protein (21, 63). The FAK C-terminal domain also contains conserved proline-rich motifs. It has been shown that the SH3 domain of p130Cas (64), the major phosphoprotein associated with the v-Crk oncprotein (65), bound to a proline-rich motif (residues 711-717) in FAK. The kinetics of increased p130Cas/SH3 phosphorylation on tyrosine after integrin stimulation by fibronectin in intact cells (66, 67, 68) are very similar to that of FAK with the implication being that p130Cas may be a physiological substrate of FAK. In addition, the SH3 domain of the p85 subunit of P13-kinase has been shown to bind FAK in vitro (13). P13 kinase activity associated with FAK immunoprecipitates is enhanced after fibronectin and growth factor stimulation of cells (12, 69).

Specific cellular binding protein the FAK N-terminal non-catalytic domain also has been demonstrated in vitro and in vivo. FAK Tyr397 (YAEI motif) is one of the major FAK autophosphorylation sites (62, 70), and also serves as a binding site for the SH2 domain of Src-family protein-tyrosine kinases (70, 71, 72). Mutation of FAK Tyr397 to Phe disrupts Src binding to FAK in vivo (70), results in a significant reduction of FAK phosphotyrosine content (70), but only results in a modest decrease in the ability of FAK to transphosphorylate exogenous substrates (62). Mutation of autophosphorylated tyrosine residues (Tyr576 and Tyr577) in the kinase domain activation loop or mutation of a conserved lysine residue in the predicted ATP-binding loop of the FAK catalytic domain (Lys454) results in a loss of FAK kinase activity (62). The autophosphorylation of FAK at Tyr397 outside of the catalytic domain may be due to either inter or intra-molecular FAK phosphorylation events. Evidence for intra-molecular FAK phosphorylation of Tyr397 was obtained using recombinant FAK chimeras that were transmembrane-anchored with the addition of CD2 extracellular sequences (73). These FAK chimeras exhibited enhanced kinase activity in vitro and tyrosine phosphorylation in vivo with CD2 antibody-mediated clustering of the recombinant FAK constructs.

The significance of FAK Tyr397 phosphorylation may be a means by which FAK can recruit Src-family tyrosine kinases to focal adhesion structures. In Src-transformed cells, a stable association between activated Src and FAK can be detected (71). We have shown that integrin stimulation by replating cells on fibronectin promotes the transient association of c-Src and FAK in vivo in non-transformed mouse NIH3T3 fibroblasts (21). Interestingly, the Src SH2 domain can also bind strongly to FAK Tyr397 in vitro in serum starved fibroblasts where no in vivo co-immunoprecipitation of FAK and c-Src can be detected. These results indicate that FAK and c-Src may be independently regulated and that their association in vivo may be mediated by other factors such as the dephosphorylation of the negative-regulatory tyrosine in the c-Src C-terminal domain (for a review see 74) or by allosteric activation of FAK molecules that lead to the exposure of the phosphorylated Tyr397 residue as a high-affinity Src SH2 binding site. As we will discuss below, this formation of a FAK/Src bipartite kinase complex is intimately involved in the processes of integrin-mediated signal transduction.

**PYK2/CAKβ — New Member of the FAK Family**

The recent discovery of a structurally related PTK, PYK2/CAKβ, now defines a FAK family (75, 76). The cDNA encoding human PYK2 was cloned by virtue of its amino acid similarity to another novel PTK, PYK1, which was identified by screening expression libraries with the GRB2 adaptor protein (75). The rat homologue of PYK2, termed CAKβ, was independently isolated using degenerate PCR primers designed to recognize conserved elements of PTKs (76). Like FAK, PYK2/CAKβ contains a central kinase domain (60% identity shared with FAK) that is flanked by large N and
C-terminal domains (~40% identities with FAK). Interestingly, the first 100 residues of FAK and PYK2/CAKβ are completely divergent. Overall, PYK2/CAKβ is slightly smaller than FAK (112 kDa) and is highly expressed in tissues of the central nervous system. PYK2/CAKβ autophosphorylates on tyrosine in vitro and when over-expressed in COS-7 cells, it is found localized in junctions of cell to cell contact but not in focal adhesions (76). PYK2/CAKβ may not be regulated by cell adhesion to fibronectin (76), but instead it may be activated by calcium-mediated signaling events (75). Various stimuli that increase intracellular calcium levels, such as carbachol, calcium ionophores, and bradykinin, result in increased levels of PYK2/CAKβ tyrosine phosphorylation in vivo and increased PYK2/CAKβ in vitro kinase activity (75).

Comparisons between FAK and PYK2/CAKβ also reveal that they both contain conserved tyrosine residues and sequence motifs for SH2 binding sites in similar regions of the two proteins. The Src SH2 binding site on FAK Tyr397 (YAEI motif) has been completely conserved in PYK2/CAKβ at Tyr402 (YAEI). In addition, the GRB2 SH2 binding site on FAK Tyr925 (YENV) has been conserved at an analogous site on PYK2/CAKβ Tyr881 (YHNV). Although Tyr881 of PYK2/CAKβ has not yet been proven to be the GRB2 SH2 binding site, GRB2 has been shown to bind directly to phosphorylated PYK2/CAKβ immobilized on a membrane (75). The potential conservation of both the Src and GRB2 SH2 binding sites on FAK and PYK2/CAKβ reinforce the hypothesis that these tyrosine kinases are central regulators of similar signal transduction pathways.

**GRB2 Binding to FAK and PYK2/CAKβ—Linkage to Ras-MAPK Activation**

In general, transmembrane receptor PTK autophosphorylation can create specific high affinity binding sites for the Src homology 2 (SH2) domains of cytoplasmic proteins and lead to the formation of multimeric signaling complexes (for reviews see 77, 78). For example, the binding of the modular adaptor protein, GRB2, couples receptor PTKs to the Ras guanine nucleotide exchange factor, SOS, which in turn is able to activate Ras by converting it from the inactive GDP-bound state to the active GTP-bound form (79). GRB2 binds to specific phosphotyrosine sites on activated receptor PTKs through its SH2 domain, and to proline-rich motifs in Sos through its SH3 domains, thereby serving as a linker on "adaptor" between the two enzymes.

Our research has been focused on elucidating the molecular mechanisms involved in fibronectin-mediated signal transduction involving GRB2 as outlined in the model below (Fig. 1).

We have found that fibronectin stimulation of NIH3T3 mouse fibroblasts leads to the transient association of a FAK/c-Src bipartite signaling complex. Both increased FAK and c-Src in vitro kinase activities have been detected after fibronectin stimulation (18, 21, 80). Within the first 60 min as the fibroblasts spread rapidly,

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**Fig. 1. Integrin Signal Transduction Model.**
the transient association of GRB2 with FAK has been detected. GRB2 binds to FAK directly through its SH2 domain and mutation of FAK Tyr925 (YENV motif) abolishes fibronectin-mediated GRB2 binding in vitro and in vivo (Schlaepfer and Hunter, submitted). Mutation of the c-Src binding site on FAK (Tyr397) also disrupts GRB2 binding to FAK in vivo after fibronectin stimulation (Schlaepfer and Hunter, submitted). The conclusion from this result is that c-Src kinase association and activity is necessary for the tyrosine phosphorylation of FAK Tyr925.

Fibronectin stimulation of NIH3T3 cells also activates the ERK-2 MAP kinase with a time course similar to those of c-Src and GRB2 association with FAK. Activation of the ERK/MAP kinase pathway possibly occurs through the induction of integrin-aggregated multiprotein complexes (23). Fibronectin stimulation has been shown also to promote the nuclear translocation of MAPK (19). Similarly, over-expression of PYK2 /CAKβ in human embryonic kidney 293 cells has been shown to increase ERK-2/MAPK activity in a PYK2 /CAKβ concentration-dependent manner (75). Fibronectin-stimulated ERK-2/MAPK activation is gradual and sustained over a period of approximately sixty minutes and has been correlated with cell spreading (19, 22). We have found that fibronectin-stimulated MAPK activation is inhibited by the tyrosine kinase inhibitors genistein, herbimycin A, tyrphostin AG213 as well as cytochalasin D which disrupts the integrity of the actin cytoskeleton (Schlaepfer and Hunter, submitted). Both the fibronectin-stimulated increase in FAK and c-Src in vitro kinase activities are reduced in cells treated with cytochalasin D or with the tyrosine kinase inhibitors. Other drug treatments such as wortmannin, which inhibits PI-3 kinase activity, and protein kinase C inhibitors, calphostin C and chronic TPA treatment which downregulates PKC, did not affect fibronectin-stimulated ERK-2/MAPK activation. These results suggest that increased tyrosine kinase activity is necessary and sufficient for fibronectin-stimulated signal transduction to MAPK.

In order to determine the role that c-Src plays in fibronectin-stimulated ERK-2/MAPK activation, experiments were performed with Src-deficient fibroblasts isolated from the c-Src knock-out mice (81). Fibronectin-induced GRB2 binding to FAK and ERK-2/MAPK activation was significantly lower in Src-deficient cells compared to the same cells re-expressing c-Src (Schlaepfer and Hunter, submitted). In addition, the over-expression of FAK in human embryonic kidney 293 cells enhances the fibronectin-stimulated ERK-2/MAPK activation. In the same cells, over-expression of a dominant-negative form of Ras, N17Ras which remains bound to GDP and inactive, completely blocks the FAK and fibronectin-stimulated ERK-2/MAPK activation (Schlaepfer and Hunter, submitted). We propose that the integrin-mediated FAK activation creates a binding site for c-Src at Tyr397; bound c-Src then phosphorylates FAK Tyr925 creating a binding site for GRB2/SOS. The binding of GRB2/SOS to FAK could in turn activate Ras and the ERK-2/MAPK pathway, providing one mechanistic pathway for integrin-mediated signaling.

The Role of FAK in Cell Migration

Since FAK is a protein-tyrosine kinase localized to focal adhesion structures and that phosphotyrosine-staining is most intense in new more peripheral focal adhesions than in central, older focal adhesions (2), it was generally assumed that FAK would be involved in the formation of focal contacts. Recently, both FAK and fibronectin-deficient mice have been generated and individually both deficiencies yielded similar embryonic lethal phenotypes (82, 83). For both fibronectin and FAK deficiencies, mutant embryos displayed a general defect of mesodermal development. A number of interesting conclusions can be made from these knock-out results. Primarily, the fact the both the fibronectin and FAK deficiencies yielded strikingly similar phenotypes, indicates that fibronectin signaling through FAK activation is critical in development. Surprisingly, FAK-deficient cells were isolated from mutant embryos and these cells exhibited enhanced focal adhesion contact formation and reduced rates of cell motility and migration when stimulated by fibronectin (83).

These results suggest that FAK-mediated processes may be involved in the turnover of focal adhesion contacts during cell migration. In support of the role for FAK activation with the processes of cell motility, recent medical studies have correlated increased levels of FAK expression with human tumors exhibiting enhanced metastatic and invasive properties (84, 85). It is interesting to speculate on the role of FAK-mediated signal transduction to ERK-2/MAPK with respect to cell migration events. Many growth factors and mitogens that can stimulate cell migration also enhance the level of FAK tyrosine phosphorylation (86). In tissue wounding and cell migration assays, the increased level of FAK tyrosine phosphorylation has been correlated with migrating cells (87, 88). In Xenopus developmental systems, dominant-negative ERK-2/MAPK constructs have been shown to interfere with proper FGF/activin-induced mesoderm development (89). In mammalian cells, micro-injected antibodies to Ras have been shown to inhibit endothelial cell migration events (90). Clearly, future research endeavors will be aimed at elucidating a more precise role of the FAK-GRB2-Ras-ERK-2 /MAPK signal transduction pathway in mediating cell migration processes.
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REFERENCES

