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FOCAL ADHESION KINASE SIGNALING ACTIVITIES AND THEIR IMPLICATIONS IN THE CONTROL OF CELL SURVIVAL AND MOTILITY

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1. ABSTRACT

Focal adhesion kinase (FAK) was first described in 1992 as a novel nonreceptor protein-tyrosine kinase localized prominently within focal adhesions, suggesting a signaling role in regulating cell behavior resulting from integrin interaction with the extracellular matrix. Subsequent studies over the past decade have established functional roles for FAK as a positive regulator of both cell motility and cell survival, while providing considerable insight into signaling mechanisms involved. FAK signaling results from its ability to become highly phosphorylated in response to integrin-mediated adhesion on Tyr-397, permitting interactions with a number of different signaling effectors containing Src homology 2 (SH2) domains. Src-family kinases recruited to the Tyr-397 site phosphorylate two FAK-interacting proteins, Crk-associated substrate (CAS) and paxillin, which results ultimately in regulation of Rho-family GTPases contributing to cell motility. CAS phosphorylation, as well as phosphatidylinositol 3-kinase (PI3K) activation resulting from its binding to the FAK Tyr-397 site, have been implicated as downstream FAK signaling events that confer a resistance to apoptosis. This article reviews these and other aspects of FAK signaling and function.

2. INTRODUCTION

Just over a decade ago a novel cytoplasmic protein-tyrosine kinase termed "focal adhesion kinase" (FAK) was first described through molecular cloning (1-2). Initial studies showed FAK to be prominently localized within cellular focal adhesion complexes (which form at sites where clustered integrins form strong attachments to the extracellular matrix (ECM)) and to undergo adhesion-dependent tyrosine phosphorylation (1-6). These early observations provided a foundation for contemporary studies on the role of tyrosine phosphorylation in integrin control of cell behavior. Subsequent research efforts from many laboratories have yielded much information on FAK-associated signaling events and their ultimate cellular effects. This article will review key findings that led to the current understanding of FAK functional domains, signaling mechanism, and downstream pathways that promote cell survival (resistance to apoptosis) and migration (motility). These biological functions suggest that enhanced or deregulated FAK signaling may contribute to the development and progression of human cancers, and the mounting evidence in support of this pathological role is covered in the article by Hecker and Gladson in this volume.

3. DISCUSSION

3.1. Functional domains of FAK

The primary structure of vertebrate FAK is illustrated in Figure 1. The tyrosine kinase (catalytic) domain is centrally-located, giving rise to two larger flanking regions. A recent crystal structure (7) shows the unphosphorylated FAK kinase domain to adopt an "open" conformation characteristic of protein kinases in their inactive state. This suggests that FAK catalytic activity is regulated in some fashion, such as through interactions with other proteins or post-translational modification. Notably, the FAK kinase domain is subject to phosphorylation of the "activation loop" (see section 3.2) which is a common mechanism for protein kinase activation. The FAK activation loop was disordered in the crystal structure. Phosphorylation could stabilize the activation loop in a conformation that better allows substrate access.

The "focal adhesion targeting" (FAT) domain consists of a 150 amino acid region near the C-terminus

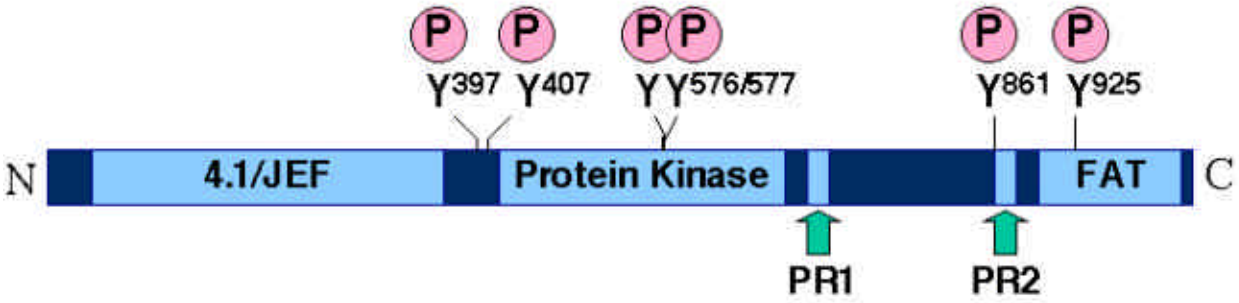

Figure 1. Linear structure of FAK showing major domains, motifs, and sites of tyrosine phosphorylation.

that folds as a four-helix bundle (8-9). The FAT domain is essential for localizing FAK to integrin adhesion sites and also for adhesion-dependent tyrosine phosphorylation (10-12). FAT domain interaction with the protein paxillin likely plays a major role in targeting FAK to adhesion sites (12-14), although paxillin binding is not the sole determinant of this localization (15) and FAT domain interactions with talin (16) may also contribute. In addition to a role in FAK targeting, paxillin functions as a FAK substrate with downstream signaling implications (see sections 3.4 and 3.6).

Between the kinase and FAT domains lie two proline-rich motifs (PR1 and PR2) that mediate interactions with Src-homology 3 (SH3) domain-containing proteins including the tyrosine kinase substrate "Crk-associated substrate" (CAS) (17). Like paxillin, tyrosine-phosphorylated CAS is found in focal adhesions (18-19). The CAS SH3 domain can interact with both PR1 and PR2 sites, although the interaction occurs most efficiently with PR1 (20-21). Not surprisingly this interaction can promote CAS tyrosine phosphorylation, an important downstream signaling event (see sections 3.4 - 3.6), but not through the obvious mechanism of direct phosphorylation by FAK (see section 3.4). In addition to CAS, the PR2 site can interact with the SH3 domain of a GTPase activating protein (GAP) called GRAF ("GAP for Rho that interacts with FAK") (22). Since GRAF is a negative regulator of Rho (23), the FAK/GRAF interaction could play a role in down-regulating Rho activity at adhesion sites. The reported interaction of ASAP1, a GAP for ARFs 1 and 6, with the PR2 site (24) is similarly intriguing.

Much of the N-terminal region is composed of a 4.1/JEF domain (also known as the FERM domain), homologous to domains found in band 4.1 protein, JAK-family kinases, and ERM-family proteins ezrin, radixin, and moesin (25). The functional role of the FAK 4.1/JEF domain is not well understood but, like the related domains in other proteins, there is evidence that it mediates interactions with proteins associated with the plasma membrane. For example the FAK 4.1/JEF domain has been shown to interact with beta-integrin cytoplasmic tails (26), although this interaction is not crucial for either FAK localization to integrin adhesion sites or adhesion-stimulation of FAK tyrosine phosphorylation (27-29). The FAK 4.1/JEF domain has also been implicated in interactions with receptors for growth factors EGF and PDGF (30), suggesting it could be important in coordinating signals from multiple inputs.

3.2. Activation of FAK signaling: tyrosine phosphorylation and partnership with Src

FAK signaling requires that FAK itself becomes phosphorylated on tyrosine, which occurs normally in response to integrin-mediated adhesion. FAK tyrosine phosphorylation is impaired if function-disrupting mutations are present in beta-integrin cytoplasmic tails (31-34), or if cells are treated with agents that either disrupt F-actin (35) or block Rho GTP loading (36). These observations suggest that FAK tyrosine phosphorylation results from the clustering of integrins maintained by Rho-mediated contraction of the actin cytoskeleton, with the resultant assembly of a protein network associated with the cytoplasmic tails of clustered beta-integrin subunits (37). Exactly how assembly of the integrin-associated cytoplasmic protein network leads to elevation of FAK phosphotyrosine levels is not entirely clear. Possible mechanisms include juxtaposition of two FAK molecules to allow intermolecular (*trans*) autophosphorylation, conformational change to expose phosphorylation sites, and/or a sequestration from tyrosine phosphatases. Recent evidence supports an intermolecular autophosphorylation mechanism, although a major isoform of FAK expressed in the brain has the additional capacity to undergo intramolecular (*cis*) autophosphorylation (38).

The identification of FAK sites of tyrosine phosphorylation provided insight into signaling mechanism and function. FAK undergoes adhesion-dependent phosphorylation on six tyrosine residues: 397, 407, 576, 577, 861, and 925 (39-42) (Figure 1). Tyr-397 is a major *in vivo* site of phosphorylation and the only apparent site of autophosphorylation (39,41). An important aspect of phosphorylated Tyr-397 (pTyr397) function is the ability to mediate an interaction with Src homology 2 (SH2) domains of Src-family tyrosine kinases (17,39,43). The Src interaction is further stabilized by the Src SH3 domain binding to an adjacent region (44). These interactions can promote the catalytic activity of Src kinases through releasing autoinhibitory interactions (45). Through this mechanism, FAK can both recruit and activate Src-family kinases at integrin adhesion sites.

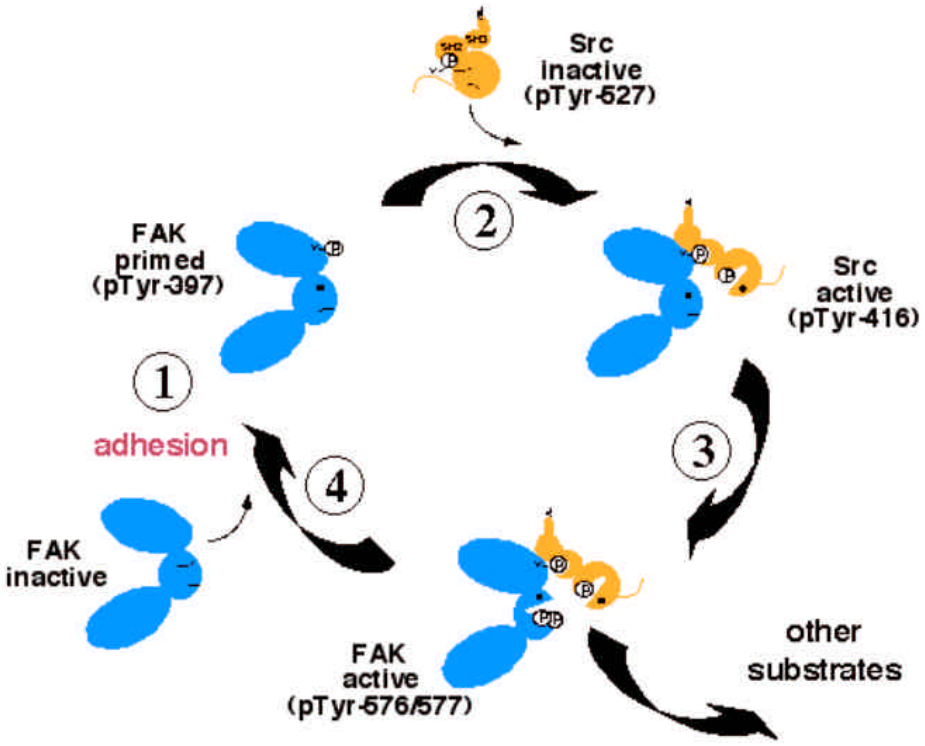

Figure 2. Model for mutual activation of FAK and Src with signal amplification. 1) Integrin clustering following cell adhesion promotes limited FAK intermolecular autophosphorylation of Tyr-397; 2) Src stably interacts with the FAK pTyr-397 site, promoting Src catalytic activation through C-terminal tail displacement, enabling tyrosine phosphatases to act on the inhibitory C-terminal phosphotyrosine, and Tyr-416 autophosphorylation; 3) FAK activation loop tyrosines phosphorylated by associated c-Src enhances FAK catalytic activity resulting in full activation of the FAK/Src complex; 4) additional FAK molecules are phosphorylated on Tyr-397, either by FAK or Src, leading to signal amplification.

Full FAK activation requires phosphorylation by a Src-family kinase. FAK tyrosines 407, 576, 577, 861, and 925 are not autophosphorylation sites but, rather, are phosphorylated by Src bound to FAK *via* the pTyr-397 site (41-42,46-48). Nor is Tyr-397 strictly an autophosphorylation site since it can also be phosphorylated by Src (41). In fibroblasts lacking Src, Fyn, and Yes, (hence, devoid of Src-family kinase activity), FAK exhibits almost no adhesion-regulated tyrosine phosphorylation (49) indicating that Src kinases make a significant contribution even to Tyr-397 phosphorylation. Among the Src-specific sites are FAK tyrosines 576 and 577 within the kinase domain activation loop. Phosphorylation of these residues could be necessary for maximal FAK catalytic activity. Supporting this, a FAK mutant with combined phenylalanine substitutions for these residues exhibits a 2-3-fold reduction of kinase activity relative to wild-type FAK (41,47,50). Thus by phosphorylating the FAK activation loop, Src can enhance FAK catalytic activity and this could be important for expanding the pool of FAK molecules initially phosphorylated on Tyr-397. The ability of FAK and Src to stimulate one another's kinase activity suggests a mechanism to amplify an initial weak signal (Figure 2).

Among the other FAK sites phosphorylated by Src, Tyr-925 has been most extensively studied. Phosphorylated Tyr-925 can bind the SH2 domain of the adaptor protein Grb2, suggesting a mechanism for integrin stimulation of "extracellular-signal regulated kinase" (ERK) *via* a FAK > Grb2/SOS > Ras pathway (40). But there is much complexity in integrin-stimulation of ERK and a number of other FAK-dependent and -independent pathways have since been identified that can be more critically involved (reviewed in 51-52, and see below). Tyr-925 lies within the FAT domain and, although it is in an exposed position (8-9), FAT domain interactions may impair the ability of this site to either be efficiently phosphorylated or to mediate an SH2 domain interaction. Compared to the other FAK tyrosine phosphoacceptor sites, Tyr-925 is poorly phosphorylated by Src (41). Since neither the Tyr-407 nor Tyr-861 site has been shown to function in mediating an interaction with an effector molecule, the functional significance of these sites has remained uncertain. Interestingly, a recent report suggests that phosphorylation of Tyr-861 could promote FAK activation by enhancing intramolecular autophosphorylation of Tyr-397 (53).

3.3. Multiple signaling proteins bind to the Tyr-397 site

While the interaction with Src-family kinases represents a well-established function of phosphorylated Tyr-397, this is not the only role for this site. The pTyr397 site can mediate interactions with SH2 domains of a

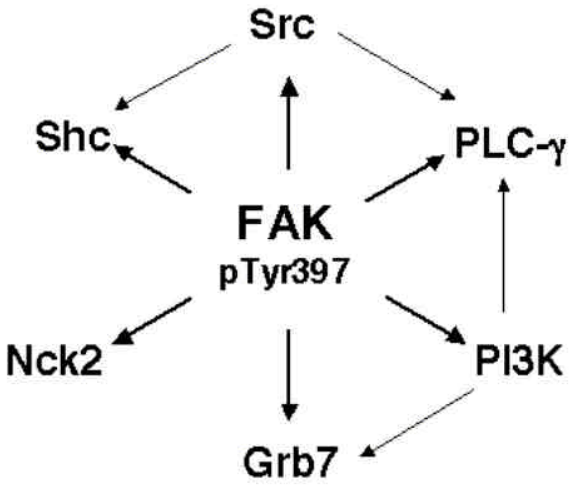

Figure 3. Proximal effectors in FAK signaling. The central thicker arrows point to six different signaling molecules that interact (or potentially interact) with the phosphorylated Tyr-397 site on FAK to promote downstream signaling events. The peripheral thinner arrows represent potential cross-talk between the FAK-bound effectors. See text for details.

number of other signaling proteins including phosphatidylinositol 3-kinase (PI3K) (54), the gamma 1 isoform of phospholipase C (PLC-gamma1) (55), Shc (56), Grb7 (57), and Nck-2 (58) (Figure 3). These observations suggest that distinct pools of activated FAK promote the assembly of different signaling complexes that exist within the larger integrin-associated protein network. This is not surprising if one considers that FAK is an abundant cellular protein that becomes phosphorylated on Tyr-397 to near stoichiometric levels and that different SH2 domains show only limited binding specificity outside the phosphotyrosine residue. Once targeted to the vicinity, many different SH2 domains would be expected to interact at the pTyr397 site, albeit with varying affinities. Competition among different SH2 domains for binding to pTyr-397 would not be a limiting factor if the pool of phosphorylated FAK is in large excess, as seems likely.

The interactions with PI3K and PLC-gamma1 suggest a role for FAK in regulating inositol lipid metabolism and phospholipid-signaling. PI3K phosphorylates the D-3 position of the inositol ring, producing phospholipids including $PI(3,4)P_2$ and $PI(3,4,5)P_3$ that interact with and activate signaling proteins that contain pleckstrin homology (PH) domains including certain isoforms of protein kinase C and the related Akt kinase (reviewed in refs. 58,59). PLC enzymes catalyze the hydrolysis of $PI(4,5)P_2$ to generate diacylglycerol, which also promotes activation of protein kinase C isoforms, and IP_3 which mobilizes intracellular Ca^{2+} (reviewed in ref. 60). The association of FAK with PI3K in response to integrin activation has been demonstrated in fibroblasts (61) and platelets (62). A phosphopeptide representing the FAK pTyr-397 site has been shown to enhance PI3K activity (54). FAK expressed in *FAK* -/- mouse fibroblasts interacts with PLC-gamma1 in an adhesion-dependent manner and stimulates IP_3 production, while these effects were not observed with expression of a FAK mutant in which Tyr-397 was changed to phenylalanine (F397) (55). FAK could activate PLC-gamma1 either through inducing a conformational change and/or by promoting its tyrosine phosphorylation. While FAK does not directly phosphorylate PLC-gamma1, this could be accomplished by nearby FAK-associated Src kinases (55). $PI(3,4,5)P_3$ produced by FAK-associated PI3K could also aid in the activation of PLC-gamma1 through interactions with the PLC-gamma1 PH domain. Such cross-talk between different FAK-associated signaling proteins is likely to be important for coordinating downstream signaling events (Figure 3).

Shc, Grb7, and Nck-2 are all “adaptor” proteins that lack enzymatic activity but function to recruit signaling enzymes to sites of tyrosine phosphorylation. Shc bound to FAK may become tyrosine phosphorylated, either directly by FAK or by a nearby Src kinase (perhaps bound to other FAK molecules), to promote Grb2 binding and this represents another FAK-dependent pathway to ERK activation through Ras (56). Grb7, in addition to its SH2 domain, is characterized by several other domains that could be involved in promoting downstream signals, including a putative Ral/Ras-associating domain, potential binding sites for SH3 domains, and a PH domain (reviewed in ref. 63). Nck-2, comprised of three SH3 domains in addition to its SH2 domain, was shown recently to colocalize with FAK in filopodia and lamellipodia of spreading cells (57). Nck-2 SH3 domains can interact with a number of proteins involved in organization of the actin cytoskeleton (reviewed in ref. 64).

3.4. CAS and paxillin: major substrates of the FAK/Src complex

CAS, first recognized as a tyrosine-phosphorylated protein in cells transformed by v-Crk or v-Src (65-67), is a docking protein consisting of multiple protein-interaction domains including an N-terminal SH3 domain, a “Src-binding domain” (SBD) near the C-terminus, and an interior “substrate domain” (SD) (67-68) (Figure 4). The CAS SH3 domain interacts not only with FAK (and the related tyrosine kinase Pyk2), but also with tyrosine phosphatases (69-70) suggesting it functions as a molecular switch regulating CAS phosphotyrosine levels. The SBD includes a proline-rich motif capable of interacting with the Src SH3 domain and a nearby tyrosine phosphorylation site (Tyr-668 or Tyr-670) that interacts with the Src SH2 domain (68,71). CAS-SD is the major region of tyrosine-phosphorylation and is characterized by fifteen tyrosine residues present in Tyr-X-X-Pro motifs which, when phosphorylated, conform to the binding consensus for the Crk SH2 domain (72). Many Tyr-X-X-Pro sites likely are phosphorylated to mediate CAS interactions with v-Crk (68,73) and its normal counterpart the SH2/SH3 adaptor c-Crk (74-75). The binding of Crk to CAS can promote downstream signaling events through proteins associated with Crk SH3 domains including SOS, C3G, and DOCK180 which stimulate guanine nucleotide exchange on Ras, Rap1, and Rac, respectively (76-80). CAS tyrosine phosphorylation also promotes SH2-

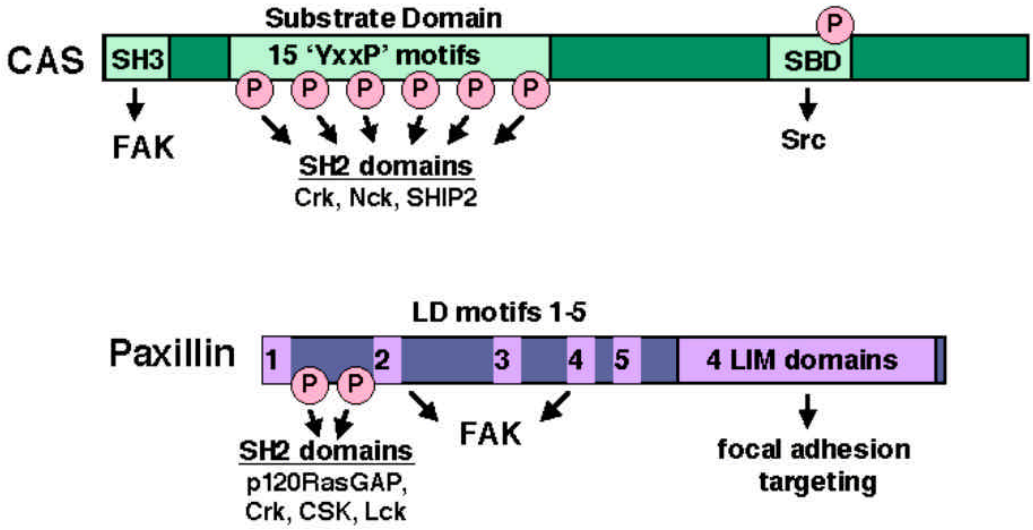

Figure 4. Linear structures of CAS and paxillin showing major domains, motifs, sites of tyrosine phosphorylation, and protein interactions.

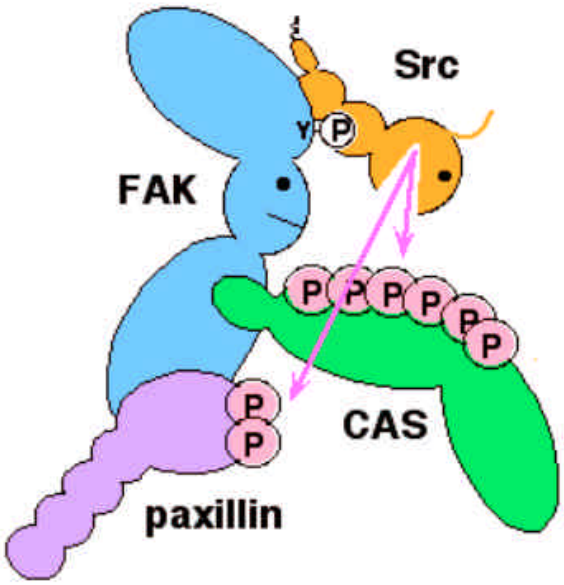

Figure 5. Model depicting FAK acting as a scaffold to recruit Src to phosphorylate FAK-associated substrates CAS and paxillin.

mediated interactions with the adaptor Nck-1 (81) and SH2-containing inositol 5'-phosphatase 2 (82), which may also act as downstream effectors in CAS signaling. Although FAK directly interacts with CAS, it is not mainly responsible for phosphorylating CAS-SD. Rather this is accomplished by Src family kinases, as evident by a lack of CAS phosphotyrosine in cells deficient in Src activity (49,75,81,83-84) and *in vitro* kinase assays showing FAK to be a very poor CAS kinase relative to Src (85). Nevertheless, FAK reexpression in *FAK* -/- cells results in a several-fold increase in adhesion-dependent CAS tyrosine phosphorylation (47), and in COS-7 cells CAS-SD phosphorylation by Src occurs more efficiently in the presence of FAK through a mechanism dependent on the FAK binding sites for Src and CAS (85). These observations indicate that FAK functions as a scaffolding protein to recruit Src to phosphorylate CAS (Figure 5).

Paxillin, like CAS, was identified as a tyrosine-phosphorylated focal adhesion protein in cells transformed by v-Src (86-87) and is a nonenzymatic docking protein containing multiple protein-interaction domains (reviewed in refs. 88-89). Within the N-terminal two-thirds of the protein are five LD motifs (eight residue leucine-rich sequences), while four tandem LIM domains (double zinc-finger motifs) are the prominent feature of the C-terminal region (Figure 4). LIM domains 2 and 3 are crucial for recruitment of paxillin to focal adhesions (the interacting proteins(s) are unknown), while FAK interacts with paxillin *via* LD motifs 2 and 4 (90). Paxillin is tyrosine-phosphorylated in response to integrin-mediated adhesion (6) and the primary sites have been identified as tyrosines 31 and 118 (91-93). Like the CAS-SD sites, paxillin tyrosines 31 and 118 fall within Tyr-X-X-Pro motifs and, upon phosphorylation, can promote a cellular interaction between paxillin and Crk (91,94). In addition to Crk, these sites can also bind to other SH2-containing signaling proteins including CSK (negative regulator of Src) (95), and p120RasGAP (96). In stimulated T cells, paxillin also interacts with the SH2 domain of the Src-family kinase Lck (97). FAK overexpression in chick embryo cells (91) or reexpression in FAK-deficient mouse fibroblasts (98) greatly elevates paxillin phosphotyrosine levels, while a FAK-binding mutant of paxillin has reduced phosphotyrosine levels (99). However, similar to its role in promoting CAS phosphorylation, FAK may primarily act as a scaffold to recruit Src kinases to phosphorylate paxillin (Figure 5). This is indicated by observations that F397-FAK is unable to promote paxillin phosphorylation (91,98). A catalytic role for Src-family kinases in paxillin phosphorylation is consistent with observations that paxillin phosphotyrosine levels are elevated in v-Src-transformed fibroblasts (86) and reduced in fibroblasts

deficient in Src-family kinases (49). It cannot be ruled out, however, that FAK kinase activity toward paxillin is upregulated through phosphorylation of FAK activation loop tyrosines by Src bound to the Tyr-397 site. Expression of activated mutants of FAK can promote paxillin tyrosine in the apparent absence of recruited Src kinases (100).

3.5. FAK signaling and cell survival

Most cells must adhere to the ECM in order to survive and proliferate in response to growth factors. Apoptosis resulting from loss of adhesion is termed "anoikis" (reviewed in ref. 101). Several studies have implicated FAK-mediated signaling as providing resistance to anoikis and other apoptotic responses. Early loss-of-function studies showed that either antisense attenuation of FAK expression (102) or FAK antibody injection (103) promoted detachment and apoptotic death in various cell types. More recently, expression of the C-terminal region of FAK containing the FAT domain (which acts as a dominant-negative inhibitor of FAK signaling) in a renal epithelial cell line was found to potentiate both anoikis and chemically-induced apoptosis (104).

Gain of function studies further implicate FAK in cell survival. Expression of a CD2-FAK fusion protein (that retains a high level of FAK phosphotyrosine even when cells are held in suspension) in Madin-Darby canine kidney (MDCK) epithelial cells confers resistance to anoikis (105). Both FAK kinase activity and the Tyr-397 site are necessary for this survival response (105), indicating that FAK signaling is involved. In another study using MDCK cells, FAK overexpression resulted in resistance to UV-induced apoptosis while a FAK mutant specifically deficient in PI3K binding (D395A) failed to provide resistance (106), thus implicating PI3K-mediated events. A role for the FAK/CAS interaction in FAK-mediated resistance to UV-induced apoptosis was also indicated (106), suggesting involvement of multiple downstream pathways. The FAK/PI3K interaction and PI3K activity were also required for FAK to rescue endothelial cells from apoptosis triggered by adenosine and homocysteine (107). The likely mechanism for PI3K-mediated cell survival is through its ability to activate the Akt protein kinase which has a well-characterized role as an inhibitor of apoptosis (reviewed in 108) by phosphorylating and regulating various components of the cell death machinery including BAD, caspase-9, forkhead family transcription factors, and IkappaB kinase (IKK) leading to enhanced NF-kappaB function. The FAK > PI3K > Akt pathway leading to NF-kappaB-mediated expression of caspase inhibitors of the IAP ("inhibitor of apoptosis") family has been implicated in protecting human leukemic HL-60 cells from oxidative stress-induced apoptosis (109).

Other studies have implicated still other FAK-stimulated pathways in survival responses. Apoptosis resulting from FAT domain expression in serum-starved fibroblasts was linked to a p53 pathway regulated by an "atypical" protein kinase C isoform potentially activated through phospholipase A_2 (110). CAS tyrosine phosphorylation leading to JNK activation through a Ras > Rac > PAK > MAP kinase 4 pathway was suggested to be involved in survival of serum-starved fibroblasts on fibronectin (111). CAS tyrosine phosphorylation has been further implicated in survival signaling in studies of anoikis-resistant lung adenocarcinoma cells (112) and serum-starved COS-7 cells held in a three-dimensional collagen matrix (113), the latter study implicating CAS/Crk coupling in the response. Finally it should be noted that FAK, CAS, and paxillin are all subject to caspase mediated cleavage (114-121) and their cleavage products could contribute to the disassembly of focal adhesion complexes and thereby interrupt survival signals from the ECM.

3.6. FAK signaling and cell motility

Integrins are critically involved in cell motility (reviewed in 122-123) and much evidence supports a role for FAK signaling as a positive effector of this process. An early suggestion of this came with the observation of upregulated FAK expression in rapidly migrating keratinocytes of repairing epidermal burn wounds (124). Genetic evidence for FAK as a positive regulator of cell motility came with the mouse knockout study showing that FAK deficiency caused early embryonic lethality characterized by severe mesodermal defects (similar to the phenotype observed for fibronectin deficiency), while mesodermal cells derived from *FAK* -/- embryos showed reduced motility relative to cells from same-stage normal embryos (125). It was later shown that reexpression of wild-type FAK, but not the F397 mutant, increased the motile capacity of the *FAK* -/- cells (47,126). Other early evidence included observations of reduced motility in endothelial or fibroblast cells expressing the dominant-negative FAK C-terminal region (127 -128), and enhanced motility in CHO cells overexpressing wild-type but not F397-FAK (129). FAK also has been strongly implicated in the motility-related process of neurite outgrowth from cultured neuronal cells (130).

Analysis of FAK variants in gain-of-function assays for cell motility (assessing either migration in Boyden or Transwell chambers or "wound healing" of confluent monolayer cultures) not only demonstrated that FAK signaling promotes cell motility, but also provided evidence for the downstream pathways involved. The requirement for the Tyr-397 site is consistently observed (47,126,129). Since Tyr-397 is an autophosphorylation site, one might expect that mutations that impair FAK kinase activity would similarly eliminate the migration response. But this is not readily evident from the published literature. When overexpressed in CHO cells a "kinase-dead" FAK mutant (R454) promotes migration equally as well as wild-type FAK (129). But the same mutant expressed in *FAK* -/- cells promoted migration at a much reduced rate relative to wild-type FAK (126). In CHO cells the R454 mutant is efficiently phosphorylated on Tyr-397 stemming perhaps from endogenous FAK in these cells, but R454-FAK expressed in the *FAK* -/- cells has much reduced phosphorylation on Tyr-397 compared to wild-type FAK. A role for FAK kinase activity in FAK-enhanced migration is further supported by the observation that a FAK mutant, in which Tyr-576/Tyr-577 residues in the

activation loop are changed to phenylalanines, is unable to promote migration when expressed in *FAK* -/- cells (47). Thus FAK's kinase activity seems to be important in motility signaling, but the strict requirement for Tyr-397 indicates that the critical role for this activity is in achieving high levels of Tyr-397 phosphorylation and that relevant downstream pathways stem from the one or more of the signaling effectors that consequently become bound to this site.

Given the multiplicity of signaling events resulting from FAK activation, it should not be surprising that multiple downstream pathways have been implicated in the mechanism by which FAK acts to enhance cell motility. Cell motility is a complex process involving the coordination of four general steps: plasma membrane protrusion at the leading edge resulting from localized actin polymerization, adhesion of leading-edge protrusions mediated by integrins, forward movement of the cell body driven by actomyosin-mediated contraction, and release of the trailing edge as contractile forces act on weakened adhesive contacts at the cell rear (see ref. 131 for a classic review). In discussing mechanisms of FAK-enhanced cell motility, it is useful to consider which of these steps are most likely to be impacted by the signaling pathways downstream of FAK.

Cells deficient in Src-family kinases have a motility defect that can be rescued by re-expressing Src (49) requiring its kinase activity (132). While many Src protein substrates could potentially contribute to its motility function, CAS and paxillin seem certain to be involved. Studies of fibroblasts derived from knockout mouse embryos support roles for CAS (133) and paxillin (14) in cell motility. Studies on FAK mutated at the PR1 site indicate that the capacity for FAK to promote cell migration is linked to its ability to bind CAS and promote Src-mediated CAS tyrosine phosphorylation (126,134). Other studies have implicated CAS-SD phosphorylation as a step leading to cell motility. When overexpressed in various cell types, wild-type CAS, but not deletion mutants lacking the SD, can stimulate cell migration (74,133,135-136). Moreover, Crk co-expression with CAS further enhances migration (74) indicating involvement of pathways downstream of Crk. One event downstream of CAS/Crk coupling likely to have a positive influence on cell motility is Rac activation mediated through DOCK180 (137-139). Consistent with this idea, Cas/Crk-enhanced migration in COS-7 cells is blocked by a dominant-negative Rac mutant (74). Activated Rac has a key role in cell motility through its ability to stimulate lamellipodium extension (protrusion) at the leading edge (reviewed in ref. 140). Paxillin may have a role similar to CAS in promoting cell motility, since Crk can associate with phosphorylated tyrosines 31 and 118, the two major sites of paxillin tyrosine phosphorylation. Expression in a carcinoma cell line of a paxillin mutant with phenylalanine substitutions for these residues resulted in impaired motility on collagen while overexpression of Crk rescued this defect (141). Others studies, however, indicate that paxillin and Crk interact poorly in some cell types (135). An alternative mechanism is suggested by recent findings that the tandem SH2 domains of p120RasGAP interact with the phosphorylated paxillin tyrosines, contributing to cell spreading and migration through release of p190RhoGAP from p120RasGAP resulting in suppression of Rho activity (96). This mechanism could underlie the observations of integrin-dependent suppression of Rho activity involving FAK (142) and Src (143), and elevated activity of Rho-kinase in *FAK* -/- cells (144). It is notable that cells deficient in either FAK, Src-family kinases, CAS, or paxillin all exhibit slower rates of cell spreading following plating on fibronectin (14, 47,132-133), consistent with a role for the signaling complexes formed by these proteins in promoting motility through stimulating the protrusion step.

There is credible evidence that the interactions of the FAK pTyr-397 site with signaling proteins other than Src-family kinases also contribute to FAK-enhanced motility. A role for PI3K is suggested by the finding that a FAK mutant capable of binding Src but not PI3K failed to promote migration of CHO cells (145). FAK-mediated PI3K signaling could also contribute to the protrusion step of motility through the ability of PI3K to promote Rac activation *via* PH-domain containing guanine nucleotide-exchange factors such as Vav1 (58,140,146). Another downstream target of PI3K relevant to FAK regulation of cell motility is Grb7. Grb7 is homologous to *C. elegans* Mig-10 protein which has a critical function in neuronal cell migration (147). In mammalian cells, overexpression of Grb7 can promote cell migration dependent on its ability to interact with both FAK and phosphoinositides including those produced by PI3K (57,148-149). The events downstream of Grb7 that lead to cell motility have not been fully elucidated, but could involve its interaction with the Rho-family member Rnd1 (150). FAK pTyr-397 interactions with PLC-gamma1 and Nck-2 could further contribute to FAK motility signaling, although to date there is no direct evidence implicating these proteins. PLC-gamma1 has a well-documented role in promoting cell motility in response to growth factor stimulation (151), a likely mechanism involving activation of actin modifying proteins (through release of inhibited form bound to PI(4,5)P2) such as gelsolin which is important for the protrusion step of cell motility (152). In addition to the FAK/Nck-2 interaction, Nck-family proteins have also been implicated in signaling events downstream of FAK by the interaction of Nck-1 with the phosphorylated CAS-SD. Nck family members can regulate a number of actin-regulatory proteins associated with the protrusion step including PAK-family kinases and Wiskott–Aldrich syndrome protein (64).

The evidence discussed above suggests that FAK, through its ability to interact with multiple signaling effectors, functions as a signaling nexus to trigger multiple downstream pathways that together act to stimulate cell motility (summarized in Figure 6). These pathways can lead to Rac activation and Rho inhibition, events associated with protrusion/lamellipodium extension in cell motility. A role for FAK in directing membrane protrusion in migrating cells is consistent with the finding that fibroblasts either lacking FAK or expressing the F397-FAK mutant

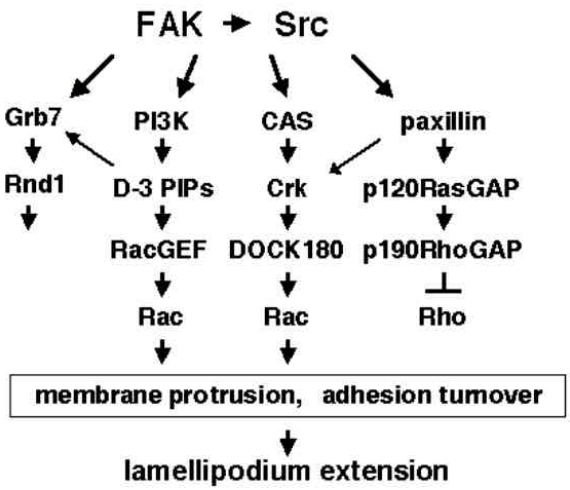

Figure 6. Signaling pathways downstream of FAK/Src implicated in promoting cell motility through directing extension of the lamellipodium. See text for details.

have poor directional persistence (that is, they more frequently change direction) in comparison to cells expressing wild-type FAK (152). In the absence of FAK signaling the cells could have a greater tendency to send out peripheral protrusions from the central cell body as opposed to the existing leading edge, then change direction as a peripheral protrusion establishes dominance. This "tug-of-war" among multiple protrusions would create a migratory instability that reduces speed. Rac activation and Rho inhibition have also been linked to the disassembly of both nascent and mature adhesions (reviewed in 140) which is important for efficient lamellipodium extension and the release of the cell rear. The early observation that FAK -/- fibroblasts tend to have unusually large and peripheral adhesions supports a role for FAK regulating cell motility through promoting adhesion turnover (125).

4. SUMMARY AND PERSPECTIVE

In a minireview written soon after FAK was first described, Zachary and Rozengurt (153) suggested potential signaling roles for this novel tyrosine kinase in regulating cell adhesion, growth, shape and/or motility, and posed paxillin and a then-uncharacterized protein p130 (now known as CAS) as candidate substrates. Although FAK does not appear to play a direct role in promoting cell adhesion (*i.e.* enhancing either integrin affinity or integrin-cytoskeletal linkages), research conducted over the past decade has shown these predictions to have been remarkably prescient. CAS and paxillin have indeed been characterized as major downstream targets of FAK. However, it is now evident that FAK acts primarily as a scaffold to recruit Src kinases to phosphorylate these proteins while FAK's own kinase activity seems to be largely reserved for autophosphorylation. While ten years ago the signaling consequences of CAS and paxillin tyrosine phosphorylation were unknown, it has since become apparent that these substrates function in the regulation of Rho-family GTPases to promote actin dynamics associated with cell motility -- in particular the membrane protrusion and adhesion turnover events associated with lamellipodium extension. Naturally, Zachary and Rozengurt could not have predicted the full complexity in FAK signaling that has emerged. FAK signaling involves interactions made with multiple SH2-containing signaling proteins that bind to the pTyr-397 site, including Src, PI3K, Grb7, PLC-gamma1, and Nck-2, all of which could contribute to cell shape and motility regulation. FAK signaling has also been shown to influence cell survival in response to various apoptotic stimuli, which in a sense confirms the speculation of Zachary and Rozengurt for a FAK role in growth regulation. The ability of FAK to stimulate ERK activity could also be important for passage through G1 phase of the cell cycle, as suggested by some studies (154-156).

What will the next ten years hold in store for FAK-related research? Certainly traditional biochemical and molecular cell biological approaches will better define how the myriad FAK-associated signaling proteins and downstream pathways act, and interact, to promote cell motility and survival responses. It can also be expected that new advances will be made in association with new experimental approaches. The crystal structure of the full FAK molecule may be determined, which will give better insight into mechanisms of FAK catalytic activation, autophosphorylation, and scaffolding function. Proteomics (mass spectrometry) techniques may be applied to characterize FAK-containing protein complexes, providing a better understanding of the critical FAK-associated signaling events. Live cell imaging techniques and FRET approaches hold promise for revealing FAK interactions and associated signaling events as they take place within the context of adhesion assembly/disassembly in a moving cell. Studies employing mouse transgenic and targeted knockout strategies can be expected to yield much information regarding the functions of FAK and FAK-activated pathways in normal developmental processes beyond early embryogenesis. Finally, the recognition of FAK orthologs in zebrafish, *Drosophila*, and *C. elegans* should lead to genetic approaches to understand FAK signaling and function.

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Abbreviations: 4.1/JEF: domain in band 4.1 protein and proteins of the JAK, ERM, and FAK families; CAS: Crk-associated substrate; ECM: extracellular matrix; ERK: extracellular-signal regulated kinase; F397: FAK mutant with Phe substituted for Tyr-397; FAK: focal adhesion kinase; FAT: focal adhesion targeting; GAP: GTPase activating protein; GRAF: GAP for Rho that interacts with FAK; IKK: IkappaB kinase; MDCK: Madin-Darby canine kidney cells; PH: pleckstrin homology; PLC-gamma1: gamma 1 isoform of phospholipase C; PI3K: phosphatidylinositol 3-kinase; PR1: proline-rich motif 1; PR2: proline-rich motif 2; pTyr: phosphorylated tyrosine residue; SBD: Src-binding domain of CAS; SD: substrate domain of CAS; SH2: Src-homology 2; SH3: Src-homology 3

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