Direct transmembrane clustering and cytoplasmic dimerization of focal adhesion kinase initiates its tyrosine phosphorylation

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Abstract

We investigated mechanisms for inducing focal adhesion kinase (FAK) tyrosine phosphorylation and their ability to trigger MAP kinase signaling using transmembrane chimeras that localize FAK and its mutants to the plasma membrane. We tested whether tyrosine phosphorylation was triggered by FAK transmembrane aggregation using antibodies against the chimeric extracellular domain. Experimental clustering of chimeras containing integrin β cytoplasmic domains or FAK induced FAK tyrosine phosphorylation and trans-phosphorylation of endogenous FAK, as well as strong ERK activation. Next, we examined whether lower-order molecular proximity, namely dimerization, could regulate FAK tyrosine phosphorylation. We found that even relatively low-affinity FAK dimerization (Kd = 3.9 × 10−5 M), in either of two different orientations, could induce FAK tyrosine phosphorylation. However, this cytoplasmic FAK dimerization could not induce MAP kinase activation or trans-phosphorylation of endogenous FAK. We conclude that dimerization of FAK is sufficient to induce its tyrosine phosphorylation, but that higher-order molecular proximity (clustering) at the cell membrane is apparently needed for additional biochemical events. This study identifies a proximity mechanism for regulating the initiation of FAK-mediated biochemical signaling.

Keywords: FAK; Tyrosine phosphorylation; Clustering; Dimerization; MAP kinase

1. Introduction

Functions of integrin receptors and of the cytoskeleton in signal transduction are receiving increasing attention as evidence for their roles in triggering or modulating cellular signaling pathways has accumulated (for recent reviews, see Refs. [1–15]). Transmembrane signal transduction by integrins can regulate cellular functions as diverse as cell shape, migration, gene expression, growth, differentiation, and apoptosis. Rapid signaling processes activated by integrins include tyrosine phosphorylation of focal adhesion kinase (FAK), activation of mitogen-activated protein kinase (MAPK) pathways involving both extracellular signal-regulated kinase (ERK) and c-Jun amino-terminal kinase (JNK), and changes in membrane fluxes of divalent cations and hydrogen ions.

The precise mechanisms by which integrins transduce events such as the activation of MAPKs remain to be established. Previous studies have indicated that MAPKs can be activated by integrin aggregation, in the absence of integrin occupancy [16,17]. Leading candidates for mechanisms of activation include FAK phosphorylation by Src family members and binding of Grb2, or alternatively, the linkage of Grb2 to integrins via Shc in a FAK-independent process [18–21]. Integrin-mediated activation of the ERK pathway has been described as Ras-dependent, Ras-independent, and/or Rho-dependent [22–24], with some differences possibly due to cell type. A major factor contributing to the complexity of integrin-mediated signaling is the fact
that integrin-mediated cell contact with extracellular molecules results in the accumulation of a number of proteins into large complexes of cytoskeletal and signaling molecules [3,5,6,25]. These multimolecular complexes that form early after cell contact with ligand- or anti-integrin-coated beads can include not only cytoskeletal proteins such as talin, tensin, α-actinin, and F-actin, but also a total of over 20 signaling molecules such as FAK, Src family kinases, phosphatidylinositol 3′-kinase, and Ras.

An initial approach to dissecting integrin signaling mechanisms was to study the function of isolated integrin cytoplasmic domains. When expressed as chimeras with extracellular reporter domains such as the IL-2 receptor α (tac) subunit or CD4, integrin β subunit cytoplasmic tails can localize to focal contacts, stimulate FAK phosphorylation especially when clustered, and even function as dominant-negative inhibitors of cell adhesion [26–29]. Their effects vary depending on the type of cytoplasmic domain. They are likely to mediate interactions with many cytoplasmic structural and signaling molecules (for review see Ref. [30]), which makes understanding the pathways of activation more difficult.

A theoretically more focused and systematic approach would be to express an individual cytoplasmic molecule that is a candidate for a key signaling intermediate, then to examine whether localizing it to the plasma membrane or clustering it is sufficient for its activation, and for inducing downstream signaling events. Simple membrane localization of Raf-1 can promote its activation [31], and overexpression of FAK linked to the extracellular and transmembrane domains of CD2 can result in its phosphorylation, suppression of apoptosis, and anchorage-independent cell growth [32–34]. Although one interpretation of the latter results is that localization adjacent to the plasma membrane is itself sufficient to activate FAK, an alternative explanation is that local high concentrations of FAK close to the membrane leads to activation and other downstream effects. Indeed, it has been demonstrated that overexpression of FAK can induce MAPK activation [35,36].

We previously demonstrated that FAK could be detected in substantial quantities in integrin-induced adhesion complexes, if integrins were aggregated in the absence of ligand occupancy, even if tyrosine phosphorylation was selectively suppressed, if integrins were aggregated in the absence of ligand [35,36]. These results suggest that integrins can include not only cytoskeletal proteins such as talin, tensin, α-actinin, and F-actin, but also a total of over 20 signaling molecules such as FAK, Src family kinases, phosphatidylinositol 3′-kinase, and Ras.

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We previously demonstrated that FAK could be detected in substantial quantities in integrin-induced adhesion complexes, if integrins were aggregated in the absence of ligand occupancy, even if tyrosine phosphorylation was selectively inhibited by genistein or herbimycin A [17]. In this study, we tested directly the regulation of FAK by membrane translocation and clustering using transmembrane chimeras of FAK and its mutants. We also addressed questions related to the regulation of its tyrosine phosphorylation and downstream signaling by low-order aggregation utilizing a novel dimerization system based on modified leucine zippers [37]. We found that simple cell surface expression and membrane localization of FAK in NIH 3T3 cells, tethered to the membrane by the IL-2 receptor extracellular and transmembrane domains, neither induces its phosphorylation nor MAPK activation involving the ERK pathway. In contrast, clustering of the FAK chimera with beads coated with an antibody to the IL-2 receptor extracellular domain produced ERK activation, prolonged substantially beyond the typical integrin-induced response. We also observed trans-phosphorylation of endogenous FAK by clustered, activated chimeric FAK.

Next, we examined directly whether low-order molecular proximity (dimerization) could also promote FAK phosphorylation. We found that relatively low-affinity dimerization of FAK (Kd = 3.9 × 10⁻⁵ M) in parallel or tandem orientations could promote FAK phosphorylation. We conclude that molecular proximity can control the levels of FAK phosphorylation, and that higher-order transmembrane clustering can induce MAPK activation and FAK trans-phosphorylation, all independent of cell adhesion.

2. Materials and methods

2.1. Construction of chimeric receptors

Chimeric transmembrane receptors were constructed using standard methods [38]. The sequences of all constructs were confirmed by nucleotide sequencing, and protein expression from the constructs described in this study was confirmed after transfection using immunoprecipitation or Western immunoblotting. The general approach to generate a chimera with each type of cytoplasmic domain was to insert partial or full-length cDNA molecules into HindIII–XhoI or HindIII–XbaI restriction sites of the plasmid vector pCMV/IL-2R. This vector is driven by the CMV promoter, and it expresses the extracellular and transmembrane domains of the non-signaling α subunit of the interleukin-2 receptor (IL-2R) as a fusion protein with any molecule of interest as the cytoplasmic domain [28].

Mouse FAK cDNA clone (pT7-7-FAK) was obtained from American Type Culture Collection (Manassas, VA). Full-length mouse FAK cDNA was inserted into pCMV/IL-2R as follows: a PCR product encoding the first 500 bp of FAK cDNA was generated using pT7-7-FAK as template with a primer containing a HindIII site followed by 15 nt encoding the first 5 amino acids of FAK (GenBank MUSFAK, residue 110): 5′-GGCTAGACGCTTTATGG-CAGCTGTATTATG-3′, and the anti-sense primer 5′-GGTGCT-TTCTAGAGCTACTTTGACG-3′ coding for nt 586–602 followed by an XhoI site. The PCR product was ligated into CMV/IL-2R using its HindIII and XhoI sites. An AccI–XhoI fragment was then removed from the ligation product and replaced by an AccI–XhoI fragment from pT7-7-FAK, creating a full-length IL-2R/FAK chimera that included the FAK 3′ non-translated region. The FAK454R mutant was generated by converting codon 454 encoding lysine to an arginine residue, and FAK397F and FAK925F were obtained by replacing the tyrosine resi-
due corresponding to amino acid positions 397 or 925 with a phenylalanine residue, using PCR-directed mutagenesis [38]. Mutations were confirmed by DNA sequencing.

2.2. Construction of expression vectors encoding leucine zipper fusion proteins

Full-length murine FAK cDNA was incorporated into pcDNA3 expression vectors (Invitrogen, Carlsbad, CA) containing sequences coding for different epitope tags. An expression vector containing full-length chicken vinculin was kindly provided by B. Geiger (Weizmann Institute of Science, Rehovot, Israel). Leucine zippers were amplified by PCR using Vent polymerase (New England Biolabs, Beverly, MA) using previously described templates [37]. Leucine zipper PCR products were ligated into the appropriate expression vectors using standard molecular biology techniques [37,38].

2.3. Cells and transfections

NIH 3T3 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 1 mM glutamine, 50 µg/ml streptomycin, 50 U/ml penicillin, and 10% heat-inactivated bovine serum (BioWhittaker, Walkersville, MD). Electroporation of these cells was performed as previously described at 170 V and 960 µF with a Bio-Rad GenePulser (Hercules, CA), but without any thymidine block [28]. Transfections were performed using 30 µg DNA for each chimeric IL-2R/cytoplasmic protein construct, 5 µg/ml pcDNA3 containing HA-ERK2 DNA, or 5 µg/ml pcDNA3 containing Ras N17 DNA [39]. For expression of leucine zipper fusion proteins, sub-confluent cultures of NIH 3T3 cells in 100 mm tissue culture dishes were transfected with 3 µg of purified DNA and 30 µl of Lipofectamine (Life Technologies, Grand Island, NY), according to the manufacturer’s protocol.

2.4. Immunological reagents

For clustering chimeras, latex beads were coated with mouse monoclonal antibody 7G7/B6 against human IL-2 receptor (Upstate Biotechnology, Lake Placid, NY). Mouse monoclonal antibody 12CA5 to HA epitope was purchased from Boehringer Mannheim (Indianapolis, IN). Mouse monoclonal antibody against FAK was purchased from Transduction Laboratories (Lexington, KY), and mouse monoclonal antibody against AU5 epitope was from BabCO (Berkeley, CA). For immunoprecipitation, agarose-conjugated anti-phosphotyrosine antibody 4G10 was obtained from Upstate Biotechnology. Polyclonal anti-human ERK2 antibody used in Western blotting to determine levels of expression of HA-ERK2 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.5. Measurement of ERK activation

Latex beads (1 × 10⁷; mean diameter 11.9 µm; Sigma, St. Louis, MO) were coated with 10 µg anti-human IL-2 receptor antibody 7G7/B6 as described previously [17]. After electroporation and cell culture of NIH 3T3 cells for 2 days, the cells were detached with trypsin-EDTA, allowed to recover in complete medium containing 10% bovine serum and 25 µg/ml cycloheximide (to block fibronectin synthesis and secretion) for 20 min at 37 °C, then washed and incubated in serum-free medium containing 25 µg/ml cycloheximide for 30 min. Cells (1 × 10⁶) in 500 µl were incubated with 1 × 10⁷ beads at 37 °C. For measurement of ERK activity, cells were extracted at the indicated times with 1% NP-40, 20 mM HEPES, pH 7.5, 10 mM EGTA, 40 mM β-glycerophosphate, 2 mM Na3VO4, 1 mM DTT, 2.5 mM MgCl₂, 20 µg/ml aprotinin, 20 mg/ml leupeptin, 1 mM PMSF. HA-ERK2 protein was immunoprecipitated at 4 °C using anti-HA monoclonal antibody 12CA5 for 2 h, followed by protein G-Sepharose for 1 h. The complexes were washed once with PBS containing 1% NP-40 and 2 mM Na3VO4, once with buffer (100 mM Tris, pH 7.5, 500 mM LiCl), then once with kinase reaction buffer (12.5 mM MOPS, pH 7.5, 12.5 mM β-glycerophosphate, 7.5 mM MgCl₂, 0.5 mM EGTA, 0.5 mM NaF, 0.5 mM Na3VO4).

For measuring ERK activity, HA-ERK2 immunocomplexes were incubated with kinase reaction buffer containing 1 µCi γ32P-ATP, 20 mM unlabeled ATP, 3.3 mM DTT, and 1.5 mg/ml myelin basic protein (MBP; Sigma) as a substrate for ERK at 30 °C for 20 min. The reaction mixtures were then suspended in Laemmli sample buffer, heated at 100 °C for 5 min, and analyzed by SDS-PAGE. Using gel slices, radioactivity was determined with a liquid scintillation counter (LS 6000IC, Beckman, Palo Alto, CA). Alternatively, ERK activation was also evaluated using anti-phospho-ERK polyclonal antibody, according to the manufacturer’s instructions (Promega, Madison, WI).

2.6. Flow cytometry

In order to quantify the expression of the chimeras, transfected cells were harvested by gentle trypsinization, allowed to recover in DMEM with 10% bovine serum and 25 µg/ml cycloheximide for 20 min at 37 °C, washed with PBS, incubated with a 1/50 dilution of FITC-conjugated rat anti-human IL-2 receptor antibody MCA 350F (Serotec, Raleigh, NC) for 30 min at ambient temperature, washed with PBS, fixed with 2% paraformaldehyde in PBS, and then analyzed using a Becton Dickinson FACScan flow cytometer.

2.7. Immunoprecipitation and Western immunoblotting

Chimeric receptors expressed on transfected cells were clustered with anti-IL-2R antibody coated on beads, then the cells were rinsed with PBS containing 1 mM sodium
orthovanadate and lysed with RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris pH 8.0, 20 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin A, 2 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium orthovanadate). The protein extracts were subjected to SDS polyacrylamide gel electrophoresis and Western immunoblotting. Immunoprecipitation and Western immunoblotting for leucine zipper fusion proteins were performed in a similar manner. In order to compare relative expression levels of HA-ERK2, cells were extracted with RIPA buffer and then subjected to SDS gel electrophoresis and Western immunoblotting with anti-HA antibody.

3. Results

3.1. ERK activation mediated by clustering of chimeric molecules

Cell adhesive interactions with tissue culture substrates or with beads coated with extracellular matrix ligands such as fibronectin induce the transmembrane aggregation of a variety of cytoskeletal and signaling molecules, as well as initiating biochemical signaling that includes tyrosine phosphorylation of FAK and the activation of serine/threonine MAP kinases. Integrin activation of ERK occurs in a pattern that is transient, but which generally appears more prolonged than that induced by growth factor receptors [16–18,40,41]. Molecular proximity (clustering or dimerization) of cytoplasmic domains controls the activities of growth factor receptors [42]. We therefore examined whether integrin-mediated MAP kinase signaling could be triggered by simple clustering of integrin cytoplasmic tails.

We first examined the ability of single-subunit chimeric receptors containing different individual integrin β cytoplasmic domains to mimic integrin-mediated induction of ERK activation. Chimeric receptors consisting of the integrin β1, β3, or β5 cytoplasmic domains [28] connected to the extracellular and transmembrane domains of the small, non-signaling α subunit of the human IL-2R were transiently expressed in NIH 3T3 fibroblasts. ERK2 containing a hemagglutinin tag (HA-ERK2) was co-transfected to monitor activation of the ERK MAPK signaling pathway. The doubly transfected cells were then incubated with beads coated with anti-IL-2R antibodies to cluster the chimeric receptors, and the activation of ERK was examined with an in vitro kinase assay for activation of the co-transfected HA-ERK2 precipitated with anti-HA antibody. All of the experiments were performed using suspended cells, in order to avoid complications of signaling events mediated by cell adhesion.

ERK2 activation triggered by clustering of each of these integrin β chimeric receptors was observed consistently at 10 min, became maximal at 30 min, and then dissipated by 60 min (Fig. 1A for β1 and additional data not shown for β3 and β5). This pattern appeared identical to that previously observed with intact integrins (e.g., see Ref. [17]). The same time course and activity of these transfected β1, β3, or β5 intracellular domain chimeras observed in NIH 3T3 cells was observed using primary human foreskin fibroblasts (data not shown). As described previously for intact integrins [17], co-clustering of FAK was observed by immunofluorescence (not shown). Control experiments in NIH 3T3 cells and human foreskin fibroblasts examining for effects of aggregation of IL-2R consisting of the extracellular and transmembrane domains, but lacking any cytoplasmic domain, could not induce ERK activation (Fig. 1A and data not shown).

We next examined directly whether FAK clustering might trigger signal transduction in the form of ERK activation. We generated a chimeric molecule consisting of the IL-2 extracellular and transmembrane domains fused to cytoplasmic FAK. The chimera was transiently expressed in NIH 3T3 cells. Forty-eight hours after transfection, the cells were trypsinized and suspended, and the ability of the chimeric FAK to activate ERK was measured before and after aggregation by beads coated with anti-IL-2R antibody. Chimeric receptors consisting of IL-2R and intracellular FAK displayed little activation of ERK until they were aggregated with antibody-coated beads (Fig. 1A). The time course of activation was rapid, but then became prolonged, with high activation of ERK observed from 10 min to periods as long as 120 min or longer (Fig. 1A). Effective expression of each of the transfected chimeric receptors was confirmed by Western immunoblot analysis and quantitated by flow cytometry. The percentage of expression of each chimeric receptor according to flow cytometry ranged from 25% to 35% of the cells, and the magnitude of each was similar regardless of the molecules present as the cytoplasmic domain (Fig. 1B). We also routinely confirmed that the expression of HA-ERK2 was at similar levels in each type of transfected cell by Western immunoblotting (data not shown). These results indicate that (a) chimeric receptors consisting of the extracellular IL-2R portion and different β integrin cytoplasmic domains induce the activation of ERK with similar time courses as native integrins, and (b) chimeric receptors with FAK as the cytoplasmic domain induce activation of ERK after clustering.

3.2. Tyrosine phosphorylation mediated by chimeric receptors with FAK mutations

In order to explore which elements of the FAK protein were required for the induction of its tyrosine phosphorylation upon clustering, chimeric receptors containing intracellular domains consisting of mutated forms of FAK were compared. The mutants included the mutation FAK454R which replaces the well-conserved, catalytically essential lysine 454 residue within its kinase domain, mutant FAK397F which lacks the tyrosine phosphorylation site.
involved in binding of Src, and mutant FAK\(^{925F}\) which lacks the tyrosine phosphorylation site that mediates binding of the adapter protein Grb2 (Fig. 2A). All of these FAK mutants were used in previous studies [18,20,32,33]. Forty-eight hours after transfection of NIH 3T3 cells, the phosphorylation of normal and mutated FAK chimeric proteins were compared with each other and with endogenous FAK molecules (Fig. 2). Tyrosine phosphorylation of the chimeric FAK and mutated FAK\(^{454R}\) proteins was clearly observed from 10 to 120 min of clustering. Endogenous FAK was also tyrosine phosphorylated from 10 to 120 min, in parallel with exogenous chimeric FAK and FAK\(^{454R}\) protein phosphorylation (Fig. 2B). At 10 and 60 min, tyrosine phosphorylation of the mutated FAK molecules FAK\(^{397F}\) and FAK\(^{925F}\) were also detected, accompanied by weak endogenous FAK tyrosine phosphorylation (Fig. 2C). In these experiments, the expression of each chimeric receptor ranged from 25% to 35% of total cells according to flow cytometry (not shown). These results indicate that membrane translocation of moderate levels of FAK are not sufficient to induce FAK phosphorylation, unless accompanied by transmembrane clustering. The clustering-mediated induction of FAK phosphorylation and trans-phosphorylation of the endogenous FAK do not depend on the well-conserved residue lysine 454.

3.3. FAK dimerization efficiently induces its tyrosine phosphorylation, independent of cell adhesion

The experiments described above clearly indicate that high-order molecular proximity (clustering) can induce FAK tyrosine phosphorylation. It has been previously demonstrated that low-order molecular proximity, namely dimerization, of cytoplasmic molecules such as ZAP70 or Raf-1 can also regulate biochemical responses [43,44]. We examined directly the role of dimerization in the regulation of FAK phosphorylation. Two major strategies have been used.
previously to manipulate molecular proximity of membrane receptors and cytoplasmic proteins: (a) chemical inducers of dimerization (CIDs) \[44,45\], and (b) direct dimerization based on leucine zippers \[46\]. We utilized modified leucine zippers based on the vitellogenin gene binding protein (VBP) as mediators of FAK dimerization \[37\].

The homo- and hetero-dimerization affinities of a pair of VBP leucine zippers were manipulated by introducing point mutations in their e and g positions (Fig. 3A). Thus, a variety of leucine zippers with a wide range of homo- and hetero-dimerization affinities were generated \[37\], and some of them were utilized in the current study to mediate FAK dimerization. The amino acid sequences and the heptad organization of VBP leucine zipper forms E\(_{1}\)/C\(_{1}\)E34, E\(_{1}\)/C\(_{1}\)R34, and R\(_{1}\)/C\(_{1}\)R34 are shown in Fig. 3B. These leucine zipper forms were selected because of their different affinities for homo-dimerization (\(K_d = 8.1 \times 10^{-4} \) M and a melting temperature of 21.6 °C for the E\(_{34}\) form; \(K_d = 3.9 \times 10^{-5} \) M and a melting temperature of 27.1 °C for the R\(_{34}\) form; and \(K_d = 7.0 \times 10^{-9} \) M and a melting temperature of 52.0 °C for the E\(_{34}\) form) \[37\]. The E\(_{34}\) and the R\(_{34}\) leucine zippers form heterodimers with an affinity of \(K_d = 7 \times 10^{-9} \) M and a melting temperature of 51.5 °C \[37\]. Different combinations of leucine zippers were fused to FAK molecules in the following experiments.

First, we examined whether leucine zippers can mediate effective FAK dimerization. Two FAK constructs, each containing a different epitope tag (HA or AU5), and both fused to leucine zippers designed for high-affinity dimerization were co-transfected into NIH 3T3 cells (Fig. 4A, part 2). Control cells were transfected with a mixture of two FAK forms, each containing a different epitope tag, but only one fused to the high-affinity leucine zipper (Fig. 4A, part 1). Forty-eight hours after transfection, the cells were trypsinized and incubated in suspension for 1 h. In both sets of transfections, the cells expressed equal amounts of HA or AU5-tagged FAK molecules (Fig. 4B). When only one type of tagged FAK was fused to the leucine zipper E\(_{34}\), no co-
3.4. FAK phosphorylation can be induced by relatively low-affinity dimerization, even in two different orientations

The wide range of available leucine zipper affinities enabled us to examine whether high affinity or specific orientation of dimerization is needed to induce FAK phosphorylation. As shown in Fig. 6A, HA-tagged FAK was fused to different types of leucine zippers with different homo-dimerization affinities. The plasmids were individually expressed in NIH 3T3 cells. Forty-eight hours after transfection, the cells were incubated in suspension for 1 h, then immunoprecipitation for the HA epitope was performed. The levels of FAK tyrosine phosphorylation were high when fused to the leucine zippers E-R34 and R-R34, with affinities of $K_d = 7.0 \times 10^{-9}$ M and only $K_d = 3.9 \times 10^{-5}$ M, respectively. On the other hand, a zipper pair with 20-fold lower affinity could not induce autophosphorylation (Fig. 6). This experiment indicates that even a relatively low affinity of dimerization (lower than $10^{-5}$ M, but higher than $\sim 10^{-3}$ M) is sufficient to promote FAK tyrosine phosphorylation. Interestingly, we did not detect precipitation of both types of tagged FAK was observed (Fig. 4B, part 1). However, when both types of tagged FAK contained the leucine zipper E-R34 (designed to homo-dimerize with high affinity, $K_d = 7.0 \times 10^{-9}$ M) immunoprecipitation of the AU5-tagged FAK resulted in the co-precipitation of the HA-tagged FAK (Fig. 4B, part 2). This experiment demonstrates that leucine zippers can mediate FAK dimerization. We also examined the localization of the different forms of FAK within the cell. We found that both HA-tagged FAK and HA-tagged FAK fused to leucine zipper E-R34 localized to focal contacts of NIH 3T3 cells plated on fibronectin in a manner similar that of endogenous FAK (not shown).

Next, we examined whether dimerization could modulate the levels of FAK phosphorylation. NIH 3T3 cells were transfected with control HA-tagged FAK, or with HA-tagged FAK fused to leucine zipper E-R34, which can mediate FAK dimerization as established above. Forty-eight hours after transfection, the cells were trypsinized and incubated in suspension for 1 h. Then, immunoprecipitation for HA was performed, followed by Western blot analysis for the HA epitope and phosphotyrosine (pTyr). As shown in Fig. 5A, the cells expressed equal amounts of HA-tagged FAK, but the pTyr levels of the dimerized form were much higher. We observed nearly 4-fold increases in FAK tyrosine phosphorylation upon dimerization (Fig. 5B). This experiment demonstrated that low-order molecular proximity (dimerization) is sufficient to induce FAK phosphorylation.
any ERK activation after transfecting the cells with highly phosphorylated dimerized FAK/E-R34 fusion (Fig. 6C).

Both leucine zipper-mediated dimerization and transmembrane clustering of FAK chimeras form aggregates in which FAK molecules are positioned in a parallel orientation. Other studies have shown that activation of ZAP-70 by molecular proximity may depend on a single orientation of the individual molecules within the dimer [43]. We examined whether FAK hyper-phosphorylation mediated by molecular proximity depends on a single, parallel orientation. We utilized a unique combination of leucine zipper/FAK fusions to promote tandem FAK dimerization in order to examine whether an orientation other than parallel can also promote its phosphorylation. Two types of FAK molecules were co-expressed in NIH 3T3 cells: one was HA-tagged and fused to the R-C1R34 leucine zipper at its C-terminus, and the other was Flag tagged and fused to the E-C1E34 leucine zipper at its N-terminus (Fig. 7A, part 4).

The leucine zippers effectively mediated FAK dimerization (Fig. 7B, lane 4) in a head-to-tail tandem orientation of FAK molecules. As shown in Fig. 7B, tyrosine phosphorylation levels of FAK molecules fused to leucine zippers mediating tandem dimerization (lane 4) were higher (~4-fold increase as evaluated by densitometry) compared to controls (Fig. 7B, lanes 1–3 and 5).

We utilized the following controls in this experiment: expression of FAK fused to the very low-affinity E-E34 leucine zipper (Fig. 7A and B, lane 1); dimerization of FAK and a control high molecular weight molecule, vinculin (Fig. 7A and B, lane 2); co-expression of two types of FAK molecules, only one fused to the low-affinity E-E34 leucine zipper (Fig. 7A and B, lane 3); and co-expression of two types of FAK molecules, both fused to the low affinity E-E34 leucine zipper (Fig. 7A and B, lane 5).

![Fig. 5. Leucine zipper-mediated dimerization induces FAK hyper-phosphorylation. (A) NIH 3T3 cells were transfected either with HA-tagged FAK (lane 1) or with HA-tagged FAK fused to the E-R34 leucine zipper (lane 2). Forty-eight hours following transfection, the cells were trypsinized and incubated in suspension for 1 h, and then immunoprecipitation for HA-tagged FAK was performed. Samples were analyzed by Western blotting for FAK or pTyr. Note that the dimerized form of FAK (lane 2) contained a much higher level of pTyr (bottom panel) compared with control, non-dimerized FAK (lane 1). (B) Quantitative evaluation of hyper-phosphorylation of FAK induced by its dimerization. The phosphorylated FAK bands were quantitated by densitometry using NIH Image software. The data shown represent the average of five independent experiments and their S.E. Note the nearly 4-fold increase in tyrosine phosphorylation of dimerized FAK (right bar) compared with the non-dimerized control (left bar).](image1)

![Fig. 6. Relatively low-affinity FAK dimerization can induce its hyper-phosphorylation. (A) HA tagged FAK molecules were expressed alone or fused to modified leucine zippers E-R34 (with homo-dimerization affinity of \(K_d = 8.1 \times 10^{-4} \) M), R-R34 (with homo-dimerization affinity of \(K_d = 3.9 \times 10^{-5} \) M), or E-R34 (with homo-dimerization affinity of \(K_d = 7.0 \times 10^{-9} \) M). HA-tagged FAK without a zipper was used as a control. (B) NIH 3T3 cells were transfected with FAK/leucine zipper fusions or control non-fused FAK as indicated in panel A. Forty-eight hours following transfection, the cells were trypsinized and incubated in suspension for 1 h, followed by immunoprecipitation for the HA tag on FAK. Samples were analyzed by Western blotting for HA or pTyr. A notable increase in FAK phosphorylation was detected with both the R-R34 and E-R34 fusion proteins (lanes 3 and 4, respectively). (C) NIH 3T3 cells were transfected with FAK/E-R34 fusion (lane 3), constitutively activated Ras V12 (lane 2) or non-transfected control (lane 1). Forty-eight hours later, ERK activation was measured as described in Materials and methods.](image2)
We could not observe MAPK activation or trans-phosphorylation of endogenous FAK in any of the experiments where dimerized forms of FAK were expressed. These experiments indicate that molecular proximity, not necessarily of high affinity or in a single orientation, can induce FAK tyrosine phosphorylation. However, dimerization and FAK phosphorylation alone were not sufficient to induce additional biochemical signaling.

4. Discussion

This study has investigated mechanisms that can induce FAK tyrosine phosphorylation. Since integrin-dependent signaling via both the ERK and JNK pathways of MAPK signaling, as well as FAK tyrosine phosphorylation, could be induced by integrin aggregation even in the absence of integrin receptor occupancy [17], we predicted that trans-
membrane or cytoplasmic aggregation of FAK could regulate cytoplasmic biochemical responses. Our major new conclusions are as follows:

(a) ERK2 signaling mediated by integrin aggregation involves a transient pattern of response with a peak at 30 min, which was mimicked exactly by transmembrane aggregation of chimeras containing integrin β1, β3, and β5 cytoplasmic domains.

(b) Transmembrane aggregation of full-length FAK resulted in ERK2 signaling with similar initial kinetics, but often with prolonged activation.

(c) Transmembrane clustering of FAK resulted in its tyrosine phosphorylation, and in trans-phosphorylation of endogenous FAK.

(d) Molecular proximity of a lower-order (dimerization) also resulted in FAK phosphorylation. FAK phosphorylation was induced even with relative low-affinity dimerization, and by FAK molecules in either parallel or tandem (head-to-tail) orientations.

These studies establish a direct mechanism for the induction of FAK tyrosine phosphorylation and triggering of MAPK activation in fibroblasts. Simple aggregation of FAK close to the plane of the membrane triggers a robust and prolonged level of ERK activation. In fact, the simple aggregation of endogenous β1 integrins in the absence of ligand occupancy can induce the clustering of a number of signal transduction molecules, among them FAK [17]. This same condition results in FAK tyrosine phosphorylation and ERK signal propagation. Although co-clustering with integrins of nearly all signal transduction molecules was blocked by certain selective inhibitors of tyrosine kinase activity, such as herbimycin A, FAK still co-accumulated readily [17]. These experiments suggested that clustering may be an important regulator of FAK activation. Here, we demonstrated directly that clustering of FAK can induce both its tyrosine phosphorylation and ERK signaling. These studies indicate that a major regulatory mechanism controlling FAK phosphorylation and other activities may be mediated by its position within the cytoplasm relative to other FAK molecules, but with the potential involvement of some other kinase (e.g., Src or Pyk2 [47]). FAK must obviously be a proximal trigger of the ERK signaling process in this study because it was the sole intracellular component of our FAK chimeras. We frequently observed prolonged (e.g., 120 min) ERK activation induced by FAK clustering, whereas integrin-(or integrin tail-) induced ERK activation dissipated after 60 min. This result suggests that integrins might cluster additional molecules that can down-regulate ERK activation induced by molecules such as FAK. As previously described, integrin aggregation results in the accumulation of more than 20 signaling and cytoskeletal molecules [17,48], and some of them may serve as negative regulators of signal-

Expressing substantial levels of FAK close to the plasma membrane in a chimera with CD2 was sufficient to activate it in terms of constitutive tyrosine phosphorylation [32]; nevertheless, it did not stimulate MAPK activation in one cell type [33]. Although high overexpression of FAK constructs can promote FAK activation [36], we generally did not observe constitutive activation of FAK phosphorylation in our system of chimeras. However, we readily induced FAK phosphorylation by expressing very high levels of the FAK chimeras in 293 cells (B.-Z. Katz, unpublished results). We suggest that substantial overexpression of membrane-associated FAK is sufficient to permit autophosphorylation, but that activation otherwise requires intermolecular aggregation. Overexpression may in fact mimic aggregation due to enhanced lateral interactions of membrane-intercalated molecules.

A transmembrane chimeric receptor system with more modest levels of membrane expression, such as the IL-2R system that we used in this study, combined with antibody-coated beads, can allow tests both for the necessity of membrane proximity and then for intermolecular clustering in triggering downstream signaling. Both FAK autophosphorylation and ERK activation were minimal in our system until we induced transmembrane clustering of FAK chimeras by beads coated with antibodies against the extracellular domain. Low-order molecular proximity (dimerization) also resulted in FAK hyper-phosphorylation in two different orientations. The fact that the induction of FAK phosphorylation did not depend on a single orientation of the constituents of the dimers suggests that the
molecule may be flexible. The idea of “flexible” FAK may be supported by the fact that Src binds to the Y937 residue at the N-terminus of FAK, but is able to phosphorylate the Y925 residue in its C-terminus, thereby creating a docking site for Grb2 [18,20]. Moreover, molecular proximity may induce the formation of multimolecular complexes, thereby enhancing the probability of interactions between FAK and FAK-associated molecules and inducing tyrosine phosphorylation. Therefore, high molecular density and intra-complex molecular flexibility may act as the trigger for induction of cytoplasmic tyrosine phosphorylation in our experimental models, as well as within cell-ECM attachment sites.

In conclusion, we suggest that FAK tyrosine phosphorylation is regulated by its relative position and its intermolecular associations within the cytoplasm (Fig. 8). Low amounts of FAK localized close to the inner face of the plasma membrane may not be sufficient to induce its tyrosine phosphorylation or downstream biochemical signaling. Dimerization of FAK molecules can induce its tyrosine phosphorylation, but not additional signaling events, including ERK signaling and trans-phosphorylation of cytoplasmic FAK. We suggest that only higher-order molecular proximity, generated by clustering at the plasma membrane, can induce additional biochemical signaling such as FAK trans-phosphorylation and activation of the ERK pathway (Fig. 8). Massive clusters of FAK molecules may be formed during its incorporation into newly formed focal contacts of cells attaching to extracellular matrix substrates. We demonstrate here that molecular proximity can trigger both FAK tyrosine phosphorylation and downstream biochemical events such as MAP kinase activation and FAK trans-phosphorylation. Thus, molecular proximity may serve as an activation trigger for FAK, in a similar manner to other signaling molecules [43,44]. After FAK is clustered, additional molecules such as Src may be required for phosphorylation of FAK and then for downregulating activation of signaling pathways activated by FAK, with possible involvement of other molecules such as Shc and pp130Cas.

The multimolecular complexes associated with cell adhesion sites probably contain a number of multifunctional proteins likely to participate in many complex three-dimensional molecular interactions. Experimental tests of the functions of single molecules or of pairs of molecules by the transmembrane clustering or dimerization approaches described in this study should help to identify the essential or sufficient components needed for integrin-mediated cellular responses.

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References


