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Differential regulation of cell migration and cell cycle progression by FAK complexes with Src, PI3K, Grb7 and Grb2 in focal contacts

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Abstract Focal adhesion kinase (FAK) is a key mediator of integrin signaling, which has been implicated in the regulation of cell migration and cell cycle progression. Using chimeric molecules that fuse the focal adhesion targeting (FAT) sequence directly to several signaling molecules, we investigated the potential role of FAK recruitments of signaling molecules to focal contacts in the regulation of cell migration and cell cycle progression. We found that fusion of FAT to Src, the p85 subunit of phosphatidylinositol 3-kinase, Grb7 and Grb2 resulted in the efficient focal adhesion targeting of these signaling molecules. We showed that expression of Src-FAT, p85-FAT, or Grb7-FAT, but not Grb2-FAT, each stimulated cell migration. Interestingly, tyrosine phosphorylation of paxillin, but not p130cas, was induced by expression of Src-FAT, suggesting a potential role of paxillin in mediating stimulation of cell migration by the chimeric molecule. In contrast, targeting of Grb2, but not Src, p85, or Grb7, to focal contacts increased cell cycle progression. Biochemical analyses correlated Erk activation by Grb2-FAT with its stimulation of cell cycle progression. Together, these results suggest that at least part of the role of FAK interaction with these signaling molecules is to recruit them to focal contacts and that distinct FAK signaling complexes are involved in the regulation of cell migration vs. cell cycle progression.

Key words: Focal adhesion kinase; Focal contact targeting; Cell migration; Cell cycle regulation; Signal transduction

1. Introduction

Focal adhesion kinase (FAK) is a cytoplasmic tyrosine kinase that has been implicated in integrin-mediated signal transduction pathways [1–4]. In adherent cells, FAK colocalizes with integrins in focal adhesions. FAK activation and tyrosine phosphorylation have been shown in a variety of cell types to be dependent on integrins binding to their extracellular ligands [2]. Integrin signaling through FAK has been proposed to play a role in the regulation of integrin-mediated cell migration. Increased endothelial cell migration into wounded monolayer was correlated with increased tyrosine phosphorylation and kinase activity of FAK [5], and the rapid migration of keratinocytes in epidermal wound healing was shown to coincide with FAK expression in the migrating cells [6]. Furthermore, FAK−/− fibroblasts derived from FAK knockout mouse embryos showed a significant decrease in cell migration compared with the cells from wild-type mice. The defects in cell migration have been suggested to be responsible for the general mesodermal deficiency and embryonic lethal phenotype of the knockout mice [7]. Similarly, inhibition of FAK by the FAK C-terminal recombinant protein (i.e. FRNK) caused decreased motility of both fibroblasts and endothelial cells [8] as well as a reduced rate of fibroblast spreading [9]. Lastly, overexpression of FAK in a number of cell lines including the FAK−/− cells promoted their migration on fibronectin (FN) [10–12].

Recent studies also suggested a role for FAK and its associated signaling pathways in the regulation of cell survival and cell cycle progression. Expression of a membrane-anchored FAK that is constitutively active in suspended cells prevented cell detachment-induced apoptosis of MDCK cells [13]. Conversely, inhibition of FAK by either treatment of tumor cell lines with FAK antisense oligonucleotides [14] or by microinjection of CEF cells with an anti-FAK monoclonal antibody [15] induced apoptosis. In addition, microinjection of the C-terminal fragment of FAK into either fibroblasts or endothelial cells decreased their DNA synthesis [8]. Disruption of FN matrix assembly by treating cells with FN fragments suppressed FAK tyrosine phosphorylation and resulted in the delay of the G1 to S transition, suggesting a role for FAK in cell cycle progression [16]. Using a tetracycline-regulated expression system, we have shown recently that expression of wild-type FAK accelerated G1 to S transition whereas expression of a dominant negative FAK mutant inhibited cell cycle progression at the G1 phase [17].

FAK interactions with other intracellular signaling molecules have been proposed to be responsible for its regulation of cell migration and cell cycle progression, although the exact mechanisms involved are not very clear at present. Upon its activation, FAK is autophosphorylated at Y397 which mediates FAK association with a number of SH2 (Src homology 2) domain-containing signaling molecules including Src family kinases [18–21], the p85 subunit of phosphatidylinositol 3-kinase (PI3K) [22], phospholipase C-γ [23] and Grb7 [24]. FAK binding to Src family kinases has been proposed to allow phosphorylation of Y925 of FAK by Src, which binds to the SH2 domain of Grb2 [25]. Associations of FAK with these (and potentially other) signaling molecules may result in the activation of the binding partners necessary for triggering downstream signaling pathways. Alternatively, such associations may function to recruit these FAK binding molecules to focal contacts that facilitate the downstream signaling events.

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In this paper, we investigated the role of the latter mechanism in signal transduction by FAK by creating fusion proteins that directly target Grb2, Src, p85, or Grb7 to focal contacts. We found that targeting of Src, p85, or Grb7, but not Grb2, to focal contacts each stimulated cell migration. In contrast, targeting of Grb2, but not Src, p85, or Grb7, to focal contacts increased cell cycle progression. These results suggest that at least part of the role of FAK interaction with these signaling molecules is to recruit them to focal contacts and that distinct FAK signaling complexes are involved in the regulation of cell migration vs. cell cycle progression.

2. Materials and methods

2.1. Reagents

Protein A-Sepharose 4B, human plasma FN, mouse monoclonal antibody (mAb) anti-BrdU, mouse mAb anti-vinculin and Ni beads were purchased from Sigma. Lipofectamine was purchased from Life Technologies, Inc. The mouse mAb 12CA5, which recognizes the hemagglutinin (HA) epitope tag, has been described previously [26]. The mouse anti-phosphotyrosine mAb PY-20 and mAb anti-paxillin were purchased from Transduction Laboratories (Lexington, KY, USA). The rabbit polyclonal anti-HA (HA probe), polyclonal anti-FAK (A-17), polyclonal anti-Erk, and mouse mAb 9E10, which recognizes the c-Myc epitope tag, were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The rabbit anti-phospho-MAPK was from New England Biolabs, Inc. (Beverly, MA, USA).

2.2. Construction of expression vectors

The pEGFP-C3 plasmid encoding the green fluorescence protein (GFP) was as described (Clontech, CA, USA). pKH3 and pKH3-FAK have been described previously [27]. The pRc/CMV-Cas(Myc) plasmid encoding Myc-tagged p130cas has been described previously [28]. The expression vector encoding His-tagged Erk was a generous gift of Dr. Mark Roberson (Cornell University).

The DNA segment encoding the focal adhesion targeting (FAT) sequence of FAK (residues 890–1052 of FAK) was excised from pEGFP-FAT [29] by digestion with *Sma*I and *Eco*RI. The sticky end of the fragment was filled in with Klenow and then inserted into pKH3 at the *Sma*I site to generate pKH3-FAT. The expression vector pEVX-cSrc encoding c-Src was a generous gift of D. Shalloway (Cornell University). The cDNA insert was amplified from this plasmid by PCR using sense (5′-CGGGATCCATGGGGAGCAGCAAGAGC-3′) and antisense (5′-GAATTCACGTCGACAGGTTCTCTCCAGGCTG-3′) oligonucleotides. The PCR fragments were then digested with *Bam*HI and *Sal*I. A *Sal*I to *Eco*RI fragment encoding FAT was excised from pEGFP-FAT. These two fragments were ligated together into pKH3 that had been digested with *Bam*HI and *Eco*RI to generate the expression vector pKH3-Src-FAT.

Similar strategies were used to create expression vectors encoding the Grb7-FAT, p85-FAT, and Grb2-FAT chimeras. A cDNA fragment encoding Grb7 was amplified from pKH3-Grb7 [24] by PCR using sense (5′-GGAATTCATATGGAGCTGGATCTGTCTCCAC-3′) and antisense (5′-GGTCGACAGAGGGCCACCCGCGTGC-3′) oligonucleotides. It was then digested with *Eco*RI and *Sal*I, and ligated together with the *Sal*I to *Eco*RI fragment encoding FAT into pKH3 that had been digested with *Eco*RI, resulting in the expression vector pKH3-Grb7-FAT. A cDNA fragment encoding p85 was amplified from pKH3-p85 [30] by PCR using sense (5′-CGGGATCCCATATGAGTGCTGAGGGGTACC-3′) and antisense (5′-GGAATTCCTCGCCTCTGCTGTGCATATACTG-3′) oligonucleotides. A cDNA fragment encoding Grb2 was amplified from pGEX-2T-Grb2 (generous gift of Dr. Wei Li, University of Chicago) by PCR using sense (5′-CGGGATCCATGGAAGCCATCGCCAAATATGACTTC-3′) and antisense (5′-GGAATTCCGACGTTCCGGTTCACGG-3′) oligonucleotides. The PCR fragments were then digested with *Bam*HI and *Eco*RI, ligated together with an *Eco*RI fragment encoding FAT (excised from pEGFP-FAT) into pKH3 that had been digested with *Bam*HI and *Eco*RI, resulting in the expression vectors pKH3-p85-FAT and pKH3-Grb2-FAT, respectively. Each chimeric construct was confirmed by DNA sequencing (BioResource Center, Cornell University).

2.3. Immunofluorescence staining

NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DME) supplemented with 10% calf serum (CS). They were transfected with pKH3-Grb2-FAT, pKH3-p85-FAT, pKH3-cSrc-FAT, or pKH3-Grb7-FAT using the LipofectAmine and PLUS™ transfection reagents (Life Technologies, Inc.) according to the manufacturer's instructions. One day after transfection, the cells were processed for immunofluorescence staining as described previously [29]. Briefly, cells were plated on 18 mm coverslips that had been coated with 10 μg/ml human plasma fibronectin. They were incubated in a 37°C incubator to allow for cell attachment and spreading. Cells were then fixed with 3% formaldehyde for 15 min at room temperature, washed three times with phosphate-buffered saline (PBS), permeabilized with 0.5% Triton X-100 at room temperature, and washed three times with PBS. They were stained with polyclonal anti-HA (1:300) and mAb anti-vinculin (1:50), or mAb anti-HA (1:100) and polyclonal anti-FAK (A-17) (1:100) in the presence of 10% goat serum for 1 h at 37°C. After washing three times with PBS, the bound primary antibody was detected using FITC-conjugated anti-rabbit IgG (1:150) and rhodamine-conjugated anti-mouse IgG (1:150) antibodies. Images of stained cells were captured using an immunofluorescence microscope and a CCD camera.

2.4. Cell migration assay

CHO cells were maintained in DME supplemented with 10% fetal bovine serum. They were transfected with a combination of the indicated expression vectors and pEGFP-C3 in a 5:1 ratio (3 μg total DNA) using LipofectAmine (Life Technologies, Inc.) according to the manufacturer's instructions. Two days after transfection, the cells were trypsinized and washed in serum-free medium. Approximately 1×10^5 cells were then replated on a 60 mm tissue culture dish that had been coated with 5 μg/ml human plasma fibronectin in PBS. After 2 h incubation at 37°C and 5% CO_2, the medium was replaced with a CO_2-independent medium (Gibco BRL) supplemented with 0.1% fetal bovine serum, and the cells were transferred into a humidified 37°C chamber at atmospheric CO_2. A fluorescent image was captured to detect GFP^+ (i.e. positively transfected) cells. Time-lapse phase-contrast images were then captured at 15 min intervals using the Image-Pro Plus software program v3.0 and its specific cell motility macro called OMAware (Image Acquisition and Object Motility Analysis) v1.1 for Windows NT v4.0. These phase-contrast images were converted to black and white images using OMAware and saved as JPEG images. The OMAware program was then used to determine the velocity, distance traveled and migration path for each cell based on its centroid as determined from the cell boundaries in the JPEG images. The mean velocity of the control (GFP^-) cells was determined and the velocity of each transfected cell (GFP^+) was calculated relative to this value. Data were collected using approximately 30 positively transfected cells in three independent assays for each expression vector.

2.5. Analysis of 5-bromodeoxyuridine incorporation

NIH 3T3 cells were transfected using the LipofectAmine and PLUS™ transfection reagents (Life Technologies, Inc.) according to the manufacturer's instructions. The subconfluent transfected cells were serum starved for 48 h in DME with 0.5% CS. They were then washed twice with DME and incubated for 16 h with 100 μM 5-bromodeoxyuridine (BrdU) (Sigma) in DME plus 10% CS. Cellular DNA was digested with 0.5 U/μl DNase I (New England Biolabs, Inc.) for 30 min at 37°C. Cells were then processed for double immunofluorescence staining with mAb anti-BrdU (1:300) and polyclonal anti-HA (HA probe) (1:300), as described above. About 50–100 positively transfected cells (as recognized by anti-HA) in multiple fields were scored for BrdU staining in each independent experiment.

2.6. Immunoprecipitation and Western blotting

Subconfluent cells were washed twice with ice-cold PBS and then lysed using ice-cold modified RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecylsulfate, 5 mM EDTA, 30 mM Na_2HPO_4, 50 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, and 20 mg/ml leupeptin), as described previously [10]. For detection of Erk activation by chimeric molecules, lysates were prepared from cells that had been suspended by trypsinization and replated on 10 μg/ml FN for 20 min. Lysates were cleared by centrifugation and total protein concentration was determined using the Bio-

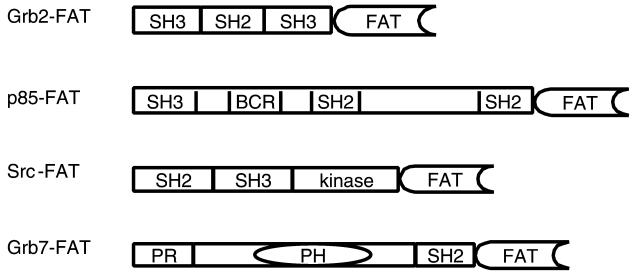

Fig. 1. Schematic diagram of chimeric signaling molecules Grb2-FAT, p85-FAT, Src-FAT and Grb7-FAT.

Rad protein assay (Hercules, CA, USA). Immunoprecipitations were carried out by incubating cell lysates with appropriate antibodies for 2 h at 4°C, followed by incubation for 1.5 h with protein A-Sepharose. After washing, immune complexes were resolved using SDS–PAGE. Western blotting was carried out using horseradish peroxidase-conjugated IgG as a secondary antibody and the Amersham ECL system for detection.

3. Results

To investigate the role of localization of specific signaling molecules to focal contacts through their association with FAK, we constructed expression vectors encoding chimeric molecules that fused the FAT sequence (residues 890–1052 of FAK) to the C-terminus of Src, p85, Grb2 and Grb7 (Fig. 1). NIH 3T3 cells were transiently transfected with these vectors, and the subcellular localization of the encoded chimeric proteins was examined by immunofluorescence staining. Fig. 2 shows that all four chimeric molecules were targeted to focal contacts (left panels), which was verified by co-staining of vinculin in the same cells (right panels). When transfected into the same cells, the corresponding signaling molecules without fusion to the FAT sequence were hardly detectable in the focal contacts (data not shown). These results suggest that addition of the FAT sequence was sufficient to target these signaling molecules to focal contacts efficiently. As observed previously [8,9], the endogenous FAK was effectively competed out of the focal contacts by expression of these chimeric molecules (Fig. 3).

We then analyzed the effects of the chimeras on cell migration. CHO cells were transiently transfected with the expression vectors encoding FAK, FAT, or the chimeric molecules together with a plasmid encoding GFP. The expression of transfected constructs was verified by Western blotting using anti-HA which recognized the triple HA epitope tag fused to

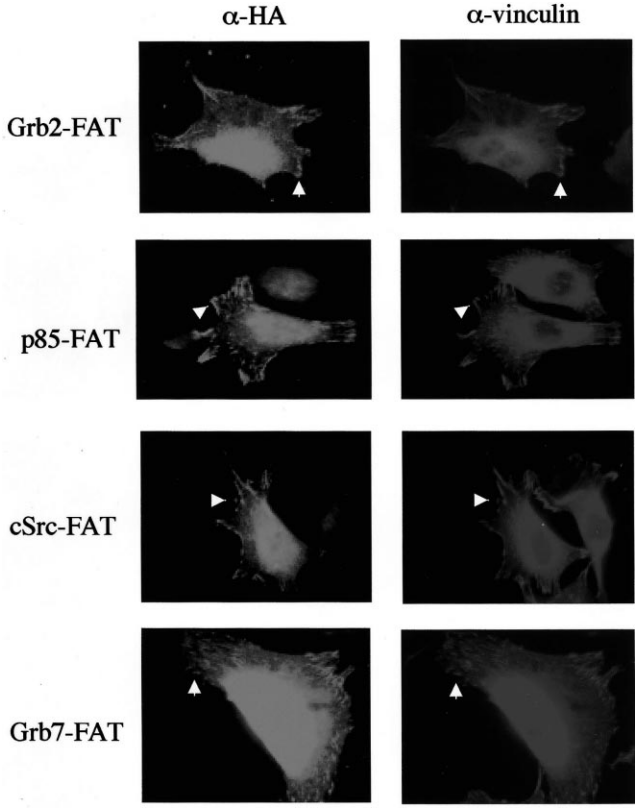

Fig. 2. Focal contact localization of chimeric signaling molecules. NIH 3T3 cells transfected with expression vectors encoding Grb2-FAT, p85-FAT, Src-FAT or Grb7-FAT were plated on fibronectin-coated coverslips. They were then fixed, permeabilized, and co-stained with polyclonal anti-HA and mAb anti-vinculin, as indicated. Representative focal contacts are marked by small white arrows.

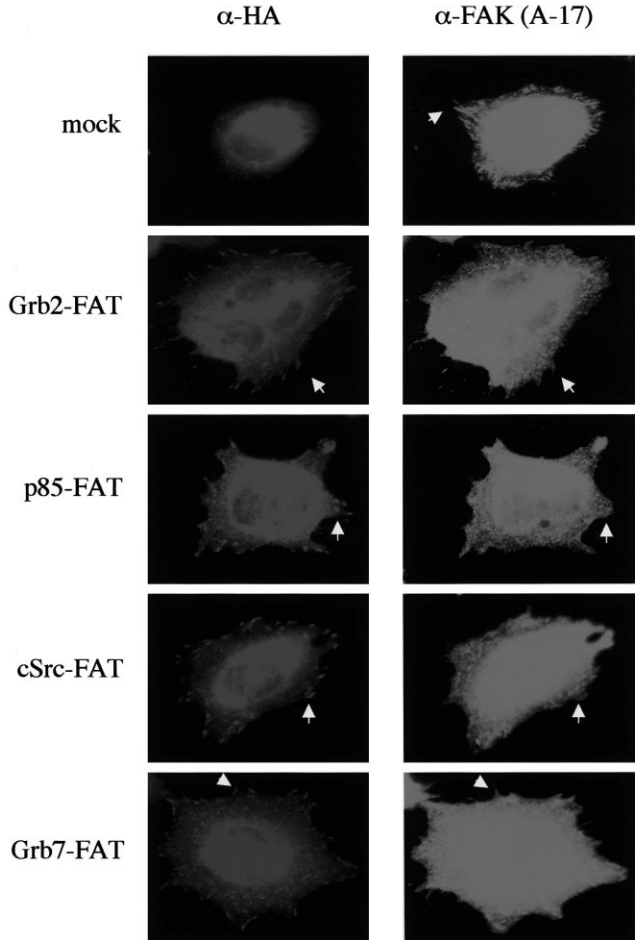

Fig. 3. Focal contact localization of chimeric signaling molecules. NIH 3T3 cells transfected with expression vectors encoding Grb2-FAT, p85-FAT, Src-FAT, Grb7-FAT, or mock control were plated on fibronectin-coated coverslips. They were then fixed, permeabilized, and co-stained with mAb anti-HA and polyclonal anti-FAK (A-17 recognizing the N-terminal domain of FAK which is not present in the chimeric molecules), as indicated. Representative focal contacts are marked by small white arrows.

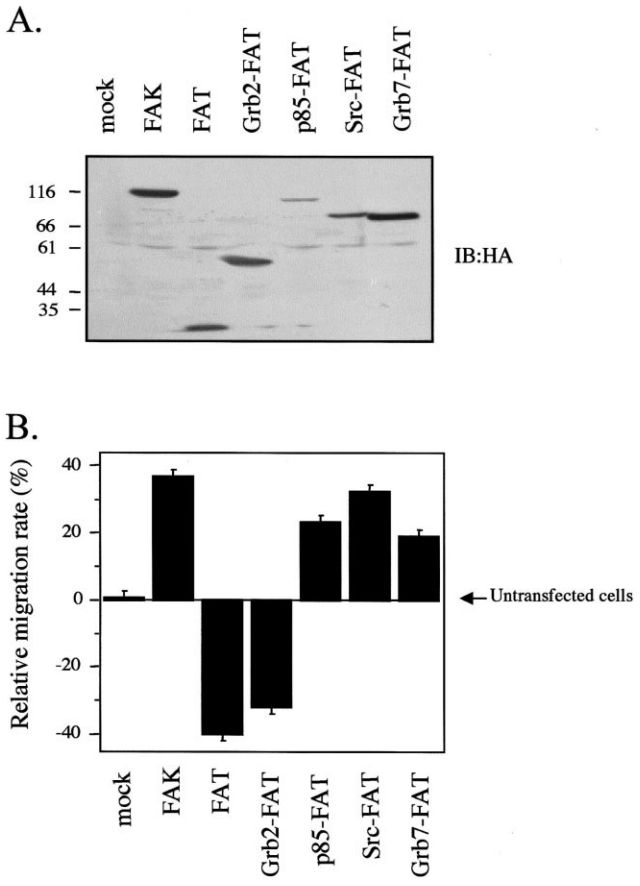

Fig. 4. Regulation of cell migration by the chimeric molecules. CHO cells were transfected with expression vectors encoding FAK, FAT, Grb2-FAT, p85-FAT, Src-FAT or Grb7-FAT, or the pKH3 vector alone control (mock), as indicated. Lysates from a fraction of the transfected cells were analyzed by Western blotting using anti-HA (A). They were then subjected to the cell migration assays as described in Section 2. The mean and standard deviation of relative migration rate (normalized to untransfected cells as 1.0) from three independent experiments are shown (B).

the N-terminus of the encoded proteins (Fig. 4A). Migration of the transfected cells on fibronectin was then evaluated using the time-lapse imaging-based computerized motility analysis method OMAware, as described previously [30]. Fig. 4B shows that, consistent with previous results [10], expression of FAK increased cell migration by about 35% whereas expression of FAT inhibited it by approximately 40%. Interestingly, expression of the Src-FAT, p85-FAT, and Grb7-FAT chimeras each stimulated cell migration by approximately 20% when compared with the mock-transfected cells. In contrast, expression of the Grb2-FAT chimera inhibited cell migration by a similar extent as the FAT alone. These results suggested that recruitment of Src, p85, and Grb7, but not Grb2, by FAK to focal contacts each played a role in the regulation of cell migration.

Previous studies suggested that the FAK/Src complex stimulated cell migration through its phosphorylation of p130cas or paxillin [28,31] whereas the downstream targets for the FAK/PI3K or FAK/Grb7 complexes are unknown at present. To explore the mechanism by which the individual chimeric molecules stimulated cell migration, we examined the effects of the chimeric molecules on the tyrosine phosphorylation of p130cas and paxillin. Consistent with previous results [28,31], overexpression of FAK induced tyrosine phosphorylation of p130cas and paxillin (Fig. 5). However, none of the chimeric molecules caused increased tyrosine phosphorylation of p130cas (Fig. 5A, B). Interestingly, expression of Src-FAT, but not the other chimeric molecules, stimulated tyrosine phosphorylation of paxillin (Fig. 5C, D). Together, these results suggest that tyrosine phosphorylation of paxillin might mediate stimulation of cell migration by the Src-FAT chimeric molecule and that p130cas was not involved in the regulation of cell migration by any of the chimeric molecules.

Recruitment of signaling molecules such as Src and PI3K to focal contacts by FAK has also been suggested to play a role in the regulation of cell cycle progression [17]. Thus, we also examined the effects of the focal contact targeted chimeric molecules on cell cycle progression using the BrdU incorporation assays (Fig. 5A, B). As observed previously [17], expression of wild-type FAK did not have significant effects on BrdU incorporation under our experimental conditions. In contrast, transfection of the expression vector encoding FAT alone inhibited BrdU incorporation in these cells. This is consistent with previous results that FAT could inhibit the endogenous FAK functions by displacing it from focal contact localizations (see Fig. 3). Interestingly, expression of the Grb2-FAT chimera reversed the inhibitory effects of FAT, suggesting that targeting of Grb2 to focal contacts provided a positive signal for cell cycle progression. Expression of the other chimeric molecules Src-FAT, p85-FAT, or Grb7-FAT showed similar inhibition of BrdU incorporation as FAT under the same conditions, further highlighting the specific activities of Grb2-FAT. Consistent with these results, we also observed that expression of Grb2-FAT, but not Src-

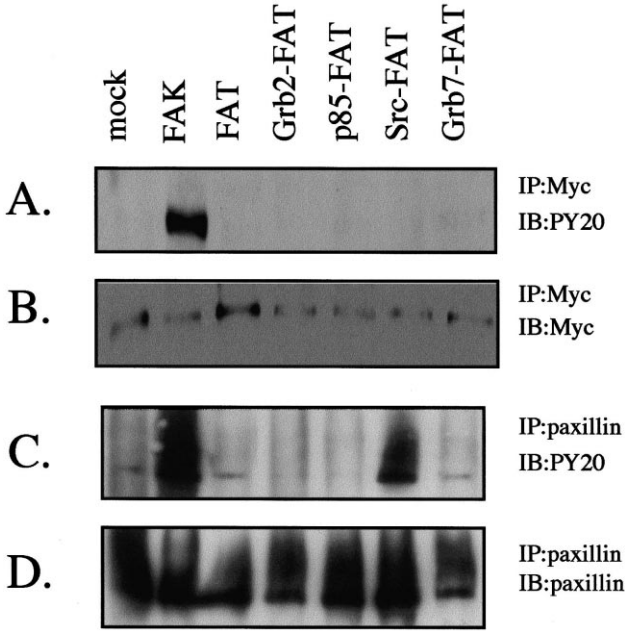

Fig. 5. Effects of the chimeric molecules on tyrosine phosphorylation of p130cas and paxillin. A, B: CHO cells were transfected with vectors encoding p130cas and FAK, FAT, or the chimeric molecules as indicated. Cell lysates were immunoprecipitated by anti-Myc antibody followed by Western blotting with PY-20 (A) or anti-Myc (B). C, D: CHO cells were transfected with expression vectors encoding FAK, FAT, or the chimeric molecules as indicated. Cell lysates were immunoprecipitated by anti-paxillin followed by Western blotting with PY-20 (C) or anti-paxillin (D).

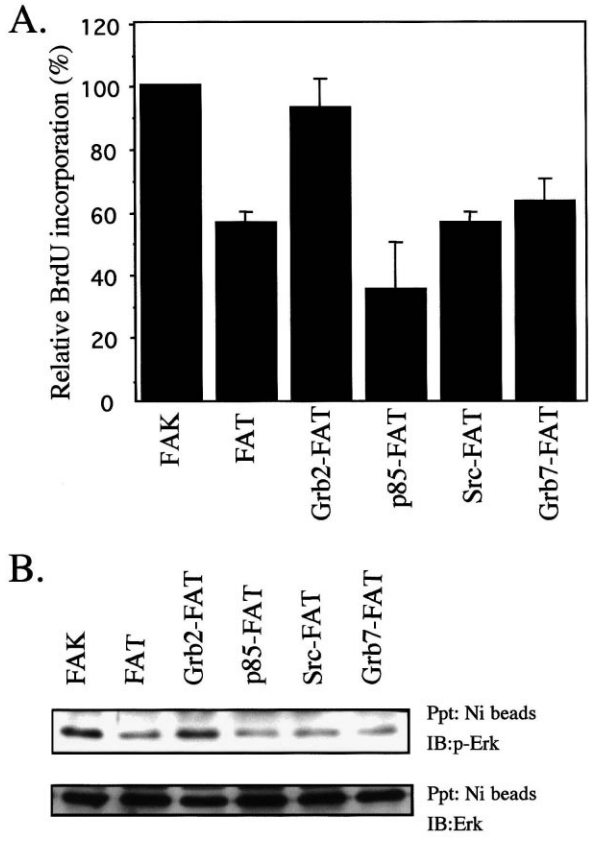

Fig. 6. Regulation of cell cycle progression and Erk activation by the chimeric molecules. A: NIH 3T3 cells were transfected with expression vectors encoding FAK or the chimeric molecules as indicated. They were then analyzed for BrdU incorporation as described in Section 2. The relative percentage of BrdU(+)/positively transfected cells was normalized to cells transfected with FAT alone. Results show the mean+S.E.M. for at least three independent experiments. B: NIH 3T3 cells were transfected with expression vectors encoding His-tagged Erk and vectors encoding FAK, FAT, or the chimeric molecules as indicated. The cells were suspended by trypsinization and replated on FN for 20 min. His-Erk was precipitated using the Ni beads and analyzed by Western blotting using anti-phospho-Erk (top) or anti-Erk (bottom).

FAT, p85-FAT, or Grb7-FAT, reversed inhibition of Erk activity by FAT in cell adhesion to FN (Fig. 6B). We did not detect any differences in the JNK activities upon transfection of any of the chimeric molecules (data not shown). Together, these results suggest that recruitment of Grb2, but not Src, PI3K, or Grb7, to focal contacts was sufficient for stimulation of cell cycle progression by FAK.

4. Discussion

Focal contacts are specialized cellular structures where cells connect the extracellular matrix (ECM) with the actin cytoskeleton through the transmembrane receptor integrins. Recent studies have also suggested that focal contacts are sites of multiple signal transduction pathways triggered by integrins [2,32]. Multiple signaling molecules have been shown to localize to the focal contacts upon integrins clustering by ECM ligands or anti-integrin antibodies [33]. FAK is a tyrosine kinase localized in the focal contacts, which also become associated with a variety of other signaling molecules such as Src family kinases, PI3K, Grb2 and Grb7 [1,3,4]. Association of FAK with these signaling molecules has been proposed to recruit them to focal contacts and/or to induce their activation. In this study, we demonstrate that direct focal contact targeting of Src, PI3K, Grb7 or Grb2 can induce signaling pathways leading to increased cell migration or cell cycle progression. These results suggest that at least part of the role of FAK interaction with these signaling molecules is to recruit them to focal contacts.

FAK has been shown to play a role in the regulation of cell migration as well as cell cycle progression [1,4]. Our findings here suggest that distinct FAK signaling complexes with other proteins are involved in the regulation of these two different cellular processes. Targeting of Src, p85 or Grb7, but not Grb2, to focal contacts stimulated cell migration (Fig. 4) whereas focal contact targeting of Grb2, but not Src, p85 or Grb7, increased cell cycle progression (Fig. 6). These results are consistent with previous reports that FAK/PI3K [30] and FAK/Grb7 complexes [24] play important roles in the regulation of cell migration while the FAK/Grb2 complex is involved in the activation of the Erk signaling pathway [25,34].

Stimulation of cell migration by each of the Src-FAT, p85-FAT, or Grb7-FAT chimeric molecules suggested that FAK complexes with these individual molecules may be sufficient to stimulate cell migration under certain conditions. The mechanisms by which FAK complexes with each of these molecules regulate cell migration are incompletely understood at present. The formation of the Grb7 complex with FAK may allow its direct phosphorylation by FAK, thus leading to the activation of potential downstream pathways in cell migration [29]. Association of p85 with FAK likely recruits the p110 catalytic subunit of PI3K to focal contacts where it may trigger downstream signals for cell migration. Indeed, we found that the p85-FAT chimeric molecule could associate with p110 as effectively as p85 itself (data not shown). Interestingly, overexpression of p85 itself did not promote cell migration (data not shown), highlighting the crucial importance of focal contact localization of the FAK/PI3K signaling complex.

The FAK/Src complex has been suggested to promote cell migration through phosphorylation of p130cas and/or paxillin [28,31]. Interestingly, Src-FAT induced tyrosine phosphorylation of paxillin, but not p130cas (Fig. 5). This may be due to the lack of p130cas binding sites (P712 and P715) in the Src-FAT chimeric molecule (Src fused to residues 890–1052 of FAK), which was shown to be necessary for induction of p130cas phosphorylation by the FAK/Src complex [28]. In any case, these results suggest that the Src-FAT chimeric molecule might stimulate cell migration mainly through its effects on paxillin. They also suggest that direct targeting of Src to focal contacts might lead to enhanced signaling in alternative pathways (e.g. paxillin phosphorylation), thus alleviating the requirement for p130cas phosphorylation in stimulation of cell migration by FAK [28].

Stimulation of cell cycle progression by the Grb2-FAT chimera suggested that the FAK/Grb2 complex and its subsequent activation of the Erk signaling pathway may play an important role in the regulation of cell cycle progression by FAK [17]. Our observation that the Src-FAT chimeric molecule did not stimulate cell cycle progression is quite surprising, however, because the FAK/Src complex has been suggested to play a role in the regulation of both cell cycle progression and cell migration [1,4]. The lack of stimulation of cell cycle progression by Src-FAT is not due to inactivation of Src kinase activity upon its fusion with FAT or targeting to focal contacts. Indeed, the chimeric molecule did promote cell migra-

tion (Fig. 4), induced paxillin phosphorylation (Fig. 5), and exhibited comparable kinase activity as wild-type Src in in vitro kinase assays (data not shown).

It is possible, however, that the Src-FAT chimeric molecule could not phosphorylate the critical targets as the FAK/Src complex in focal contacts, which were required for the stimulation of cell cycle progression by FAK. Interestingly, Src-FAT did not induce tyrosine phosphorylation of p130cas whereas overexpression of FAK did (Fig. 5). As discussed above, this may be due to the lack of p130cas binding sites (P712 and P715) in the Src-FAT chimeric molecule. Tyrosine phosphorylation of p130cas in cell adhesion has been suggested to play a role in the stimulation of Erk activation and cell cycle progression as measured by BrdU incorporation [35,36]. It has also been suggested to regulate cell cycle progression through its coupling to the JNK signaling pathway [37], although we did not detect any effects of FAK or Src-FAT on JNK pathway in our system (data not shown).

In addition, FAK/Grb2 complex formation has been shown to depend on the phosphorylation of Y925 of FAK by its associated Src [25]. Although Y925 is present in the FAT sequence of the Src-FAT chimera, it may not be accessible to the active Src directly fused to this segment of FAK to allow for phosphorylation and association of Grb2 to the chimera at the focal contacts. Indeed, binding studies suggested that the Src-FAT chimera was not associated with Grb2 (data not shown). This might provide another explanation for the lack of stimulation of cell cycle progression by the Src-FAT chimera. This is supported by our observation that Grb2-FAT, but not Src-FAT, reversed inhibition of Erk by FAT (Fig. 6). It should also be noted that the above two possible interpretations are not mutually exclusive. Indeed, phosphorylation of p130cas and Y925 of FAK (thus formation of FAK/Grb2 complex) has been proposed to mediate Erk activation by FAK in a cooperative manner [35]. Finally, we cannot exclude the possibility that the lack of phosphorylation of other key targets (other than or in addition to p130cas and Y925 of FAK) by Src-FAT accounts for its lack of stimulation of cell cycle progression.

In summary, we have shown that direct targeting of several FAK-associated signaling molecules to focal contacts stimulated cell migration or cell cycle progression, suggesting that at least part of the role of FAK interaction with these signaling molecules is to recruit them to focal contacts. Furthermore, our studies indicated that distinct FAK signaling complexes are involved in the regulation of cell migration vs. cell cycle progression. These studies provided further insights into the mechanisms by which integrin signaling through FAK regulates cell migration and cell cycle progression.

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