INTRODUCTION

Proline-rich tyrosine kinase 2 (Pyk2; also known as CAKβ, RAFTK, FAK2, and CADTK) is a recently reported cytoplasmic tyrosine kinase that is closely related to the focal adhesion kinase (FAK) (Avraham et al., 1995; Herzog et al., 1996; Lev et al., 1995; Sasaki et al., 1995; Yu et al., 1996). Pyk2 and FAK share a similar structural organization with a tyrosine kinase domain flanked by non-catalytic domains at both N- and C-termini. These two kinases are approximately 60% identical in the central catalytic domain and share approximately 40% identity in both the N- and C-terminal domains (Avraham et al., 1995; Lev et al., 1995; Sasaki et al., 1995). The major autophosphorylation site corresponding to Y397 in FAK is conserved in Pyk2 (Y402) and activated Pyk2 has been shown to bind to Src through its Src homology 2 (SH2) domain (Dikic et al., 1996). Recent studies also indicated constitutive association of Pyk2 with paxillin (Li and Earp, 1997; Salgia et al., 1996) and p130cas (Astier et al., 1997), two other FAK-binding proteins (Guan, 1997).

Despite the similar structures of these two kinases, there are a number of differences between Pyk2 and FAK. Expression of Pyk2 is more restricted (mainly in the central nervous system, hematopoietic and a few other cells) whereas FAK is widely expressed in almost all tissues and most cell lines (Avraham et al., 1995; Lev et al., 1995; Salgia et al., 1996; Schlaepfer et al., 1994; Schwartz et al., 1995). Some cell types express only FAK or Pyk2 (Li et al., 1998; Salgia et al., 1996; Tokiwa et al., 1996) while others express both kinases (Lev et al., 1995; Matsuya et al., 1998; Salgia et al., 1996; Yu et al., 1996). Furthermore, FAK, but not Pyk2, is colocalized with integrins in focal contacts in adherent cells (Hanks et al., 1992; Schlaepfer et al., 1994; Zheng et al., 1998). While tyrosine phosphorylation of FAK is controlled mainly by integrin-mediated cell adhesion, Pyk2 tyrosine phosphorylation is much more dependent on stimulation with soluble factors that elevate intracellular calcium in the same cells (Zheng et al., 1998). Finally, FAK, but not Pyk2, has been shown to bind to talin (Chen et al., 1995; Zheng et al., 1998) whereas Pyk2, but not FAK, associated with Hic-5, Nirs and Paps (Andreev et al., 1999; Lev et al., 1999; Matsuya et al., 1998).

Although Pyk2 has been shown to mediate activation of Erk in cell adhesion whereas FAK and PFhy1 stimulated it, suggesting a role for Erk activation in mediating differential regulation of cell cycle by Pyk2 and FAK. A role for Erk and JNK pathways in mediating the cell cycle regulation by FAK and Pyk2 was also confirmed by using chemical inhibitors for these pathways. Finally, we showed that while FAK and PFhy1 were present in focal contacts, Pyk2 and FPhy2 were localized in the cytoplasm. Interestingly, both Pyk2 and FPhy2 (to a greater extent) were tyrosine phosphorylated and associated with Src and Fyn. This suggested that they may inhibit Erk activation in an analogous manner as the mislocalized FAK mutant ΔC14 described previously by competing with endogenous FAK for binding signaling molecules such as Src and Fyn. This model is further supported by an inhibition of endogenous FAK association with active Src by Pyk2 and FPhy2 and a partial rescue by FAK of Pyk2-mediated cell cycle inhibition.

Key words: Pyk2, FAK, Cell cycle, Inducible expression, MAP kinase
(extracellular signal-regulated kinase) and JNK (c-Jun N-terminal kinase) in response to a variety of stimuli (Dikic et al., 1996; Lev et al., 1995; Tokiwa et al., 1996; Yu et al., 1996), the cellular function of Pyk2 is largely unknown at present. It has been shown recently that overexpression of Pyk2 induced apoptosis in several cell lines (Xiong and Parsons, 1997). These results suggested possible antagonistic cellular functions for Pyk2, and FAK, which has been shown to protect cells from detachment-induced apoptosis (Frisch et al., 1996). However, expression of Pyk2 was up-regulated in FAK−/− fibroblasts, suggesting a possible compensation of at least some of the FAK functions by Pyk2 (Sieg et al., 1998).

Using a tetracycline-regulated expression system and other approaches, we have recently identified FAK as a mediator of cell cycle regulation by integrins (Zhao et al., 1998). In this report, we investigated a potential role and mechanisms of Pyk2 in cell cycle regulation using the inducible expression systems and chimeric molecules between Pyk2 and FAK. We found that, in contrast to FAK, Pyk2 plays a negative role in regulation of cell cycle progression by its differential activating JNK signaling pathways and by competing with endogenous FAK in focal adhesions for binding signaling molecules Src and/or Fyn thus inhibiting Erk activation in cell adhesion.

MATERIALS AND METHODS

Antibodies

The mouse mAb 12CA5 (α-HA) and the rabbit polyclonal α-FAK serum have been described previously (Chen et al., 1994; Chen and Guan, 1995). The clone28 antibody against activated e-Src (Kawakatsu et al., 1996) was a generous gift from Dr H. Kawakatsu (UCSF, San Francisco, CA). The following antibodies were purchased as indicated: mouse mAb α-BrdU, mouse mAb α-vinculin from Sigma (St Louis, MO); mouse mAb α-phosphotyrosine PY20 from Transduction Laboratories (Lexington, KY); mouse mAb α-Pyk2, rabbit α-Src, α-Fyn, α-Erk, α-JNK1, α-HA probe from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); and rabbit α-phospho-MAPK and α-phospho-JNK from New England Biolabs, Inc. (Beverly, MA).

Generation of cell lines with inducible expression of Pyk2, FAK, PFhy1 and FPhy2

The cell line with inducible expression of FAK was described previously (Zhao et al., 1998). The full-length human Pyk2 cDNA and the chimeric construct PFhy1 in the mammalian expression vector pKH3 have been described previously (Zheng et al., 1998). The primary antibodies used were α-HA (1:50), α-HA probe (1:200), α-HA (1:2000), α-Pyk2 (1:2000), α-JNK1 (1:1000), α-Erk (1:2000), PY20 (1:2000), or clone28 (1:2000) using the Amersham ECL system, as described previously (Zhao et al., 1998). In some experiments, equal amounts of protein lysates were analyzed directly by western blotting.

Immunofluorescence staining

Cells were processed for immunofluorescence staining as described (Zhao et al., 1998). The primary antibodies used were α-HA (1:50), α-HA probe (1:200), α-BrdU (1:300), and α-vinculin (1:50). The secondary antibodies used were FITC-conjugated anti-mouse or anti-rabbit antibodies (1:300, Sigma), Rhodamine-conjugated anti-mouse or anti-rabbit antibodies (1:200, Sigma), or Texas Red-conjugated phallolidin (1:300, a generous gift from Dr Wannian Yang, Cornell University, Ithaca, NY). In α-BrdU staining experiments, cellular DNA was digested with DNease I (0.5 units/ml; New England Biolabs, Inc.) for 30 minutes at 37°C prior to staining with the primary antibodies and nuclei were stained with Hoechst (0.5 μg/ml) for 10 minutes at room temperature before mounting for analyses by immunofluorescence microscopy.

In vitro MAP kinase assays

For MAP kinase (Erk1/2 or JNK) assays, cell lysates were immunoprecipitated with α-phospho-MAPK or α-phospho-JNK as described above. The immune complexes were washed 3 times with ice-cold modified RIPA buffer and twice with Erk kinase buffer (50 mM Tris pH 7.4 and 10 mM MgCl2) or JNK kinase buffer (20 mM Hepes pH 7.6, 20 mM MgCl2, 0.1 mM Na3VO4, 4 mM NaF, 20 mM Hepes, 0.5 mg/ml G418 and 0.4 μg/ml tetracycline) to suppress expression of the exogenous FAK, Pyk2, PFhy1 and FPhy2 until experiments as indicated.

Analysis of 5-bromodeoxyuridine (BrdU) incorporation

Subconfluent cells were serum-starved for 48 hours in DMEM with 0.5% CS, 0.5 mg/ml G418 and 0.4 μg/ml tetracycline. They were then washed twice with DMEM and incubated for 16 hours with 100 μM BrdU (Sigma) in DMEM plus 10% CS, 0.5 mg/ml G418 and with (uninduced) or without (induced) 0.4 μg/ml tetracycline. In some experiments, various concentrations of the chemical inhibitors Curcumin, PD98059 or the control SB202474 (Calbiochem, San Diego, CA) were included in the incubation. Cells were then processed for immunofluorescent staining with α-BrdU and Hoechst (Sigma), as described below. The percentage of BrdU (+) cells was determined for approximately 500 cells in multiple fields in each independent experiment. Statistical analyses were performed by Minitab Release 10.5 Xtra (Minitab Inc.).
p-nitrophenyl phosphate, and 2 mM dithiothreitol). They were then incubated in 48 μl kinase buffers containing 5 μg myelin basic protein (MBP) or 4 μg GST-c-Jun(1,79) (a kind gift from Dr Xinmin Zheng, Cornell University, Ithaca, NY), 25 μM ATP and 10 μCi [γ-32P]ATP for 30 minutes at 30°C. The kinase reactions were stopped by addition of SDS sample buffer, boiled for 5 minutes, and then resolved on 15% SDS-PAGE. The gels were dried and the phosphorylated MBP or GST-c-Jun bands were subjected to phoshomage quantitative analyses by using the scanner model Storm 840 and the software named ImageQuant IQMac v1.2 (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Regulation of cell cycle progression by FAK, Pyk2 and their chimeric molecules

To investigate the potential role of Pyk2 in the cell cycle regulation, we generated NIH3T3 cells with inducible expression of Pyk2, FAK, PFhy1 and FPhy2 using the tetracycline-regulated expression system (Zhao et al., 1998). PFhy1 is a chimeric protein containing Pyk2 N-terminal and kinase domain fused to FAK C-terminal domain, as described previously (Zheng et al., 1998). FPhy2 is a reciprocal chimeric molecule containing the FAK N-terminal and kinase domain fused to Pyk2 C-terminal domain, as described in the Materials and Methods (Fig. 1A). Fig. 1B shows the regulated expression of these proteins by removal of tetracycline from the media. Cells were incubated in growth media with (uninduced) or without (induced) tetracycline for 16 hours. Lysates were then prepared and subjected to western blot analysis by mAb α-HA which recognizes the triple HA epitope tags fused to the N terminus of FAK, Pyk2 or the chimeric proteins. No expression of the exogenous Pyk2, FAK or the chimeric proteins was detected under the uninduced conditions (lanes U). Upon induction of expression by removal of tetracycline, all four exogenous proteins were expressed at a similarly high levels (lanes I). Western blotting with α-FAK (recognize the C terminus) detected the expression of exogenous FAK and PFhy1 as expected. Comparison with the endogenous FAK revealed an approximately 6- to 8-fold increase in expression levels (Fig. 1C). Western blotting with anti-Pyk2 (for its C terminus) detected the expression of exogenous Pyk2 and FPhy2 as expected and no expression of endogenous Pyk2 (Fig. 1D). Time course experiments indicated that the exogenous proteins could be detected by α-HA at about 6 hours after tetracycline removal and reached maximal levels 12 hours after induction (data not shown; Zhao et al., 1998).

The effect of induced expression of Pyk2, FAK, PFhy1 and FPhy2 on cell cycle progression was examined by measuring BrdU incorporation in response to serum stimulation of quiescent cells (Fig. 2). Consistent with previous observations, induction of FAK expression resulted in a small, but statistically significant increase in new DNA synthesis. In contrast, induction of Pyk2 expression caused a decrease in BrdU incorporation by approximately 30% compared with uninduced cells. Interestingly, these analyses showed that expression of chimeric protein PFhy1 (with Pyk2 N-terminal and kinase domains fused to FAK C-terminal domain, see Fig. 1A) led to a similar small increase of BrdU incorporation as FAK. Conversely, expression of the other chimeric protein FPhy2 (with FAK N-terminal and kinase domains fused to Pyk2 C-terminal domain, see Fig. 1A) resulted in an approximately 50% decrease of BrdU incorporation compared with uninduced cells. All cells under uninduced conditions as well as Mock cells under uninduced or induced conditions showed similar BrdU incorporation (Fig. 2, open bars; data not shown). Together, these results demonstrated that despite its sequence homology with FAK, Pyk2 functioned in an opposite manner to FAK to inhibit cell cycle progression. They also suggested that the C-terminal domains of these two related kinases are the key determinants of their functions in cell cycle regulation.

![Fig. 1](image1.png)

**Fig. 1.** Inducible expression of Pyk2, FAK, PFhy1 and FPhy2 in NIH3T3 cells. (A) The schematic structures of Pyk2 (open) and FAK (filled) are shown. The chimeric protein PFhy1 is composed of Pyk2 N-terminal and kinase domain (aa 1-665) fused to FAK C-terminal domain (aa 663-1052). The chimeric protein FPhy2 consists of FAK N-terminal and kinase domain (aa 1-665) fused to Pyk2 C-terminal domain (aa 669-1009). (B-D) α-HA (B), α-FAK (C) and α-Pyk2 (D) immunoblot of whole cell lysates prepared from cells expressing the indicated proteins under uninduced (lanes U) and induced (lanes I) conditions.

![Fig. 2](image2.png)

**Fig. 2.** Effects on cell cycle progression by expression of Pyk2, FAK, PFhy1 and FPhy2. The percentage of BrdU (+) cells expressing the indicated proteins at 16 hours after serum stimulation under uninduced (open bars) and induced (filled bars) conditions. The results show mean ± s.d. for at least three independent experiments.
Differential effects of FAK and Pyk2 on Erk and JNK activation

While FAK is a mediator of Erk activation in cell adhesion (Schlaepfer et al., 1994; Schlaepfer and Hunter, 1996; Zhao et al., 1998), Pyk2 has been shown to induce activation of Erk and JNK in response to a variety of stimuli (Dikic et al., 1996; Herzog et al., 1996; Lev et al., 1995; Tokiwa et al., 1996). To determine whether differential activation of JNK by Pyk2 and FAK plays a role in their opposite effects on cell cycle progression, we examined their effects on the activation of JNK in cell adhesion using the inducible NIH3T3 cells. Twelve hours after induction of exogenous genes, cells were removed from the plates and either maintained in suspension (lanes Sus) or replated on fibronectin (FN; lanes FN). They were then lysed and tested for FN adhesion-induced JNK activation, as shown in Fig. 3. Consistent with previous observations (Dolfi et al., 1998; Oktay et al., 1999), cell adhesion to FN caused an increase in JNK activity (compare lanes 1-5 and 6-10). Whereas induction of FAK expression had no effect on the activation of JNK (compare lanes 6 and 7), induction of Pyk2 expression further increased JNK activity significantly (compare lanes 6 and 8). Interestingly, overexpression of FPhy2 also increased JNK activity while PFhy1 did not cause any change in JNK activity (compare lanes 9 and 10 with lane 6). Little JNK activity was detected under suspended condition for all cells (lanes 1-5), suggesting that expression of the exogenous genes affected JNK activation by cell adhesion. Western blotting of aliquots of cell lysates with α-JNK verified similar levels of JNK in all samples (Fig. 3A). Quantitation of the results from three independent experiments indicated an approximately 150% increase of JNK activity by Pyk2 and FPhy2 in comparison to Mock cells (Fig. 3C). These results suggested that activation of JNK by Pyk2 might play a role in inhibition of cell cycle progression in NIH3T3 cells. They also suggested that the C-terminal regions of these two kinases specify their ability to activate JNK.

Although they stimulated JNK activation to a similar extent (Fig. 3C), induction of FPhy2 expression led to a more significant inhibition of cell cycle progression than that of Pyk2 (Fig. 2). This suggested that other mechanisms besides differential activation of JNK might also contribute to cell cycle regulation by these two kinases and the chimeric molecules. Pyk2 has recently been reported to activate the p38 MAPK (Pandey et al., 1999) whereas it is not known whether FAK can activate p38. However, we found that induction of expression of Pyk2, FAK, PFhy1 and FPhy2 did not exhibit any significant effects on the activation of p38 in the inducible NIH3T3 cells (data not shown).

We also investigated Erk activation in these cells although previous studies have indicated that both FAK and Pyk2 can activate Erk in various systems (Dikic et al., 1996; Lev et al., 1999; Schlaepfer et al., 1994, 1996; Tokiwa et al., 1996; Xiong and Parsons, 1997). Mock cells or cells with induced expression of FAK, Pyk2, PFhy1 or FPhy2 were removed from the plates and either maintained in suspension (lanes Sus) or replated on FN (lanes FN). The lysates were then prepared and the endogenous Erk activity was tested as described in Materials and Methods. Fig. 4A shows that, as expected, cell adhesion to FN induced Erk activation (compare lanes 1-5 and 6-10). Also consistent with several previous observations (Schlaepfer et al., 1994; Schlaepfer and Hunter, 1996; Zhao et al., 1998), induction of FAK expression further increased Erk activation in the NIH3T3 cells (compare lanes 6 and 7). Surprisingly, induction of Pyk2 expression reduced the increase of Erk activation in cell adhesion when compared with Mock cells (compare lane 6 and 8). Expression of the chimeric protein PFhy1 increased Erk activation to a similar level as that by FAK (compare lanes 7 and 9). Conversely, expression of the other chimeric protein FPhy2 caused a decrease in Erk activation, which was even more than that by Pyk2 (compare lanes 6, 8 and 10). Little Erk activity was detected under suspended condition for all cells (lanes 1-5), suggesting that expression of the exogenous genes affected Erk activation by cell adhesion. Western blotting of cell lysates with α-Erk verified similar levels of Erk in all samples (Fig. 4A). Quantitation of Erk activity from three independent experiments indicated an approximately 50-60% increase in Erk activation by FAK and PFhy1, about 35% reduction in Erk activation by Pyk2, and approximately 60% reduction in Erk activation by FPhy2 (Fig. 4C). All these cells under the
uninduced conditions showed similar Erk activation as the Mock cells (data not shown). Taken together, these results suggested that their opposite effects on Erk activation in cell adhesion may also contribute to (together with or in addition to their opposite effects on JNK) stimulation vs inhibition of cell cycle progression by FAK vs Pyk2, respectively. The greater decrease of Erk activity by FPhy2 than Pyk2 may explain the stronger inhibition of cell cycle progression by the former (see Fig. 2).

To further evaluate the roles of JNK and Erk signaling pathways in mediating regulation of cell cycle by Pyk2 and FAK, we employed specific chemical inhibitors to block these pathways upon induced expression of Pyk2 or FAK. As observed previously, induction of FAK expression caused a small increase in BrdU incorporation (compare without inhibitors, Fig. 5A and B). At a concentration of $1 \times IC50$, the MEK inhibitor PD98059 completely reverted this increase whereas the JNK inhibitor curcumin or the control SB202474 had little effect (Fig. 5A). At higher doses ($3 \times$ and $10 \times IC50$), PD98059 showed a significantly more inhibition and curcumin also exhibited some inhibition of BrdU incorporation in these cells. These results suggested that Erk activation played a major role in mediating FAK-stimulated cell cycle progression while JNK was also involved. Similar results were observed for both FAK and Pyk2 cells under the uninduced conditions, where the endogenous FAK was presumably mediating the cell cycle regulation by cell adhesion (Fig. 5B and D). Induction of Pyk2 expression resulted in a decrease of BrdU incorporation (compare without inhibitors, Fig. 5C and D). Inhibition of Erk signaling pathway by PD98059 further decreased it in a dose-dependent manner. In contrast, inhibition of the JNK pathway by curcumin at $1 \times 3 \times IC50$ caused a partial reversal of the inhibitory effect of Pyk2 on cell cycle progression. At higher dose ($10 \times IC50$), curcumin showed a slight inhibition compared with the untreated cells or cells treated with the control SB202474. These results suggested that while some level of JNK activation is required for cell cycle progression, additional activation of JNK by Pyk2 is responsible (in part) for the Pyk2-mediated cell cycle inhibition.

**Mechanism of Pyk2 inhibition of Erk in cell adhesion**

To investigate potential mechanisms by which Pyk2 and FPhy2 inhibited Erk in our NIH3T3 inducible expression systems, we examined the subcellular localization of FAK, Pyk2 and the chimeric proteins in the induced cells (Fig. 6). Immunofluorescent staining with polyclonal $\alpha$-HA probe

**Fig. 4.** Effects of Pyk2, FAK, PFhy1 and FPhy2 on Erk activation in cell adhesion. Quiescent Mock or Pyk2, FAK, PFhy1 and FPhy2 cells under induced conditions were detached by trypsin/EDTA and either maintained in suspension (Sus) or replated on fibronectin (FN) for 20 minutes, as described in Materials and Methods. Whole cell lysates were immunoblotted with $\alpha$-Erk1/2 (A) or immunoprecipitated by $\alpha$-phospho-Erk followed by in vitro kinase assay with MBP as substrate (B). The average and standard deviation of relative Erk activities was obtained from three independent experiments. The relative activities were normalized to Mock cells plated on FN (C).

**Fig. 5.** Effects of inhibitors for MEK or JNK on cell cycle progression by FAK and Pyk2. The percentage of BrdU (+) cells expressing FAK (A,B) or Pyk2 (C,D) at 16 hours after incubation in media with serum plus the indicated inhibitors (SB 202474 as negative control, PD 98059 for MEK and curcumin for JNK) under induced (A,C) and uninduced (B,D) conditions. The results show mean ± s.d. for at least three independent experiments.
showed a typical focal contact localization of FAK (panel a), as expected. Consistent with our previous analysis in other cell types (Zheng et al., 1998), Pyk2 showed a diffuse cytoplasmic distribution in the NIH3T3 cells (panel c) and the chimeric protein PFhy1 (with the C-terminal domain from FAK) was localized in focal adhesions (panel e). Interestingly, the chimeric protein FPhy2 (with the C-terminal domain from Pyk2) exhibited a diffuse staining pattern in the cytoplasm similar to that of Pyk2 (panel g). Co-staining with α-vinculin mAb showed normal focal adhesions of all the cells with induced expression of FAK, Pyk2, PFhy1 or FPhy2 (panels b,d,f,h). Furthermore, double staining of actin stress fiber with phalloidin and the exogenous FAK, Pyk2 or the chimeric molecules with α-HA showed apparently normal cell spreading and actin cytoskeleton in cells with induced expression of the exogenous kinases (Fig. 7). Together these results demonstrated that, consistent with our previous observation in other cells (Zheng et al., 1998), the C-terminal domains of FAK and Pyk2 determined their presence or absence in focal adhesion. They also indicated that overexpression of FAK, Pyk2 or the chimeric kinases did not affect cell spreading or the integrity of actin cytoskeleton, implying that the exogenous kinases did not regulate cell cycle progression by inducing changes in these cellular properties.

Our previous studies showed that a mislocalized FAK mutant (ΔC14, lacking the C-terminal 14 residues) inhibited Erk activation upon cell adhesion by competing with endogenous FAK in focal adhesions for binding Src family kinases (Zhao et al., 1998). The cytoplasmic localization of Pyk2 and FPhy2 therefore suggested the possibility that they could inhibit Erk activation in a similar manner to the FAK ΔC14 mutant. To test this possibility, we analyzed tyrosine phosphorylation of the exogenous kinases and their association with intracellular signaling molecules. Fig. 8 shows that FAK was tyrosine phosphorylated and associated with Src and Fyn at a high level, as expected (lanes 2). PFhy1 also exhibited a comparable level of phosphorylation and association with Src and Fyn as FAK (compare lanes 2 and 4). Similar biochemical activity and subcellular localization between PFhy1 and FAK may explain their similar effects on Erk activation and cell cycle progression despite the apparent different sequences on their N-terminal and kinase domains. Although not localized in focal contacts, both Pyk2 and FPhy2 also showed tyrosine phosphorylation.
phosphorylation and association with Src and Fyn (lanes 3 and 5). Interestingly, the chimeric molecule FPhy2 showed a higher level of phosphorylation and association with Src and Fyn compared with Pyk2 (compare lanes 3 and 5). Together, these data supported our hypothesis that the cytoplasmic localization of Pyk2 and FPhy2 may contribute to their inhibitory effect on cell cycle progression by competing with endogenous FAK in focal adhesions for binding Src and/or Fyn (Zhao et al., 1998). This competition model could also explain the greater inhibition of Erk activation and cell cycle progression by FPhy2 than Pyk2 because the chimeric molecule showed stronger binding to Src and Fyn.

To further evaluate this competition model, we examined the effect of FAK overexpression on Pyk2-mediated cell cycle inhibition. Fig. 9 shows that transient transfection of FAK into cells with induced Pyk2 expression partially rescued the inhibition of BrdU incorporation by Pyk2. These results are consistent with the competition model because increased FAK expression could revert the inhibition of Erk activation by Pyk2 by recruiting signaling molecules to focal contacts. However, increase expression of FAK is not expected to reduce the JNK activation by Pyk2, which may explain the partial (instead of full) rescue.

To test this competition model directly, we also examined the effects of expression of FAK, Pyk2 and their chimeras on the association of endogenous FAK with Src. Lysates prepared from uninduced or induced cells were immunoprecipitated by anti-FAK (C-terminal specific) and then analyzed by western blotting with mAb clone28 recognizing the activated Src (Kawakatsu et al., 1996). Fig. 10 shows association of active Src with endogenous FAK in uninduced cells (lanes U). Induction of exogenous FAK or PFhy1 expression increased the amount of active Src in the FAK immune complex (compare lanes 2 and 6 with lanes U). The increased Src may be associated with the exogenous FAK and PFhy1, that were also recognized by the anti-FAK. The increase is not proportional to the increased expression levels of total FAK, suggesting that the pool of cellular Src may be limited. Interestingly, induction of Pyk2 or FPhy2 expression resulted in a significant decrease of the active Src associated with the endogenous FAK (compare lanes 4 and 8 with lanes U). These results provide further support for our hypothesis that Pyk2 and FPhy2 could compete with the endogenous FAK for binding signaling molecules such as Src. Surprisingly, both Pyk2 and FPhy2 caused almost complete and similar dissociation of active Src from endogenous FAK (lanes 4 and 8) although they showed different efficiency in binding Src and Fyn (see Fig. 8, lanes 3 and 5). This leaves open the possibility that FPhy2 may inhibit Erk activation and cell cycle progression to a greater extent than Pyk2 by some alternative, yet unknown mechanisms.

**DISCUSSION**

Recent studies have shown that FAK and its associated signaling pathways play important roles in cell spreading, migration, cell survival and apoptosis, and cell cycle regulation (Cary and Guan, 1999; Guan, 1997). In contrast, little is known about the cellular functions of the closely related tyrosine kinase Pyk2. In this paper, we examined the possible roles and mechanisms of Pyk2 in cell cycle regulation using a tetracycline-regulated inducible expression of exogenous Pyk2 and FAK as well as chimeric molecules between these two kinases. We found that induction of Pyk2 expression inhibited cell cycle progression whereas comparable induced expression

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**Fig. 8.** Tyrosine phosphorylation of Pyk2, FAK, PFhy1 and FPhy2 and their association with Src and Fyn. Whole cell lysates were prepared from cells that had been induced to express the indicated proteins. They were immunoprecipitated by α-HA (A,B), α-Src (C), or α-Fyn (D). The immune complexes were immunoblotted with α-HA (A,C,D) or PY20 (B).

**Fig. 9.** Effect of overexpression of FAK on Pyk2-induced cell cycle inhibition. Cells with induced Pyk2 expression (Pyk2/I) were transiently transfected with pKH3-FAK. One day after transfection, the cells were subjected to BrdU incorporation assays as described in Materials and Methods. The percentage of BrdU (+)/positively transfected cells (as defined by the presence of the transfected HA-FAK in focal contacts) was determined by analyzing 40-50 positively transfected cells for each transfection in multiple fields. The results show mean + s.d. for at least three independent experiments. *P<0.01 in comparison to value from Pyk2/I.

**Fig. 10.** Effects of Pyk2, FAK, PFhy1 and FPhy2 on endogenous FAK association with active Src. Whole cell lysates were prepared from cells expressing the indicated proteins under uninduced (lanes U) and induced (lanes I) conditions. They were immunoprecipitated with α-FAK and the immune complexes were immunoblotted with α-FAK (upper panel) or clone 28 (lower panel).
of FAK stimulated it as we reported previously (Zhao et al., 1998). Our findings are reminiscent of previous reports showing stimulation of apoptosis by Pyk2 (Xiong and Parsons, 1997) and prevention of apoptosis by FAK signaling (Frisch et al., 1996; Sieg et al., 1998). Together, these studies suggested that Pyk2 and FAK might function in an antagonistic manner in regulation of apoptosis and cell cycle progression despite their structural similarity.

The mechanisms by which Pyk2 and FAK differentially regulated cell cycle progression are not clear at present. Previous studies have demonstrated that FAK functioned to promote cell cycle progression through G1 (Gilmore and Romer, 1996; Zhao et al., 1998) by regulating activation of Erk in cell adhesion (Zhao et al., 1998). Pyk2 has been reported to mediate Erk activation in response to agonists which increase intracellular Ca\(^{2+}\) concentration and/or trigger G-protein-coupled signaling pathway in PC12 cells (Dikic et al., 1996; Lev et al., 1995). However, Pyk2 did not play a role in mediating Erk activation by growth factors in the same cells (Dikic et al., 1996). Furthermore, tyrosine phosphorylation of Pyk2 and Erk activation have been dissociated in response to certain stimuli in rat liver epithelial cell line GN4 (Yu et al., 1996) or rat aortic smooth muscle cells (Guo et al., 1998). These observations suggested that Pyk2 may mediate Erk activation in a stimuli-dependent manner and perhaps also cell type-dependent manner. Although a number of studies showed Erk activation upon transient transfection of Pyk2 into 293 cells (Lev et al., 1995; Sieg et al., 1998), we found that induction of Pyk2 in NIH3T3 cells inhibited Erk activation in cell adhesion (Fig. 4). The apparent discrepancy could be due to cell type differences in these studies as discussed above. Alternatively, transient transfection into 293 cells may lead to a much high overexpression of the exogenous gene whereas the use of tetracycline inducible system allows controlled expression of the exogenous gene at levels more closely related to physiological conditions. In any case, our results suggested that inhibition of Erk activation by Pyk2 might be responsible for its inhibition of cell cycle progression.

A role of Erk in mediating cell cycle regulation by FAK and Pyk2 is also supported by analysis of the two chimeric molecules between FAK and Pyk2. We found that the chimeric protein PF\(^{1}\) stimulated Erk activation as well as cell cycle progression to a similar extent as FAK. The other chimeric molecule PF\(^{2}\) inhibited Erk activation to a greater extent than Pyk2 and it also inhibited cell cycle progression more than that by Pyk2 (see Figs 2 and 4). Interestingly, a construct similar to our PF\(^{1}\) (N-terminal and kinase domains of Pyk2 fused to the C-terminal region of FAK) functioned more like Pyk2 than FAK in inducing apoptosis upon transfecting into fibroblasts (Xiong and Parsons, 1997). This apparent difference could be due to the use of different cell lines in these two studies. Xiong and Parsons (1997) showed that Pyk2 can induce apoptosis in several cell lines including Rat1, 10T1/2, Swiss3T3, quail QT6, and HEK293 cells, but they did not examine the effect in NIH 3T3 cells. We, however, found that overexpression of Pyk2 did not result in apoptosis in the inducible NIH3T3 cells (data not shown). Alternatively, this could be due to the different cellular activities examined in these two studies. The N-terminal segment of Pyk2 could play a key role in its induction of apoptosis, as suggested by Xiong and Parsons (1997). Therefore the PF\(^{1}\) construct would be expected to function like Pyk2 in the apoptosis assay. In contrast, the C-terminal regions of FAK and Pyk2 could be more important in the regulation of cell cycle progression as shown in our studies, which would make the same construct functioning more like FAK. Lastly, this could be due to the slight differences of the constructs used in these two studies. Our construct contained slight more FAK C-terminal sequence (1-665 from Pyk2, 663-1052 from FAK, Zheng et al., 1998) than that by Xiong and Parsons (1-695 from Pyk2, 692-1052 from FAK, Xiong and Parsons, 1997). Further experiments will be necessary to clarify these possibilities.

We have previously observed that a mislocalized FAK mutant \(\Delta C14\) functioned in a dominant negative manner to inhibit Erk activation in cell adhesion by competing with endogenous FAK in focal adhesions for binding signaling molecules such as Src and/or Fyn (Zhao et al., 1998). Interestingly, Pyk2 and the chimeric molecule PF\(^{2}\), which inhibited Erk activation, also showed a diffuse cytoplasmic distribution (Figs 6 and 7). Furthermore, both were tyrosine phosphorylated and associated with Src and Fyn (Fig. 8), and a stronger phosphorylation of PF\(^{2}\) and its association with the signaling molecules correlated with its greater inhibition of Erk than Pyk2 (see Figs 2 and 8). Lastly, we showed that expression of Pyk2 and PF\(^{2}\) decreased association of active Src with the endogenous FAK (Fig. 10). It is therefore possible that Pyk2 (and the chimeric molecule PF\(^{2}\)) inhibited Erk activation in cell adhesion in a similar manner as the FAK \(\Delta C14\) mutant by competing with endogenous FAK in focal adhesions. Indeed, simultaneous expression of wild type FAK partially rescued the cell cycle inhibition by Pyk2 (Fig. 9). Also consistent with this model, FAK and the chimeric protein PF\(^{1}\) were found to be localized in focal adhesions in these cells (as observed previously in other cell types, Zheng et al., 1998) and both stimulated Erk activation. These results suggested that differential subcellular localizations of Pyk2 and FAK might play a crucial role in their function in Erk activation in cell adhesion and cell cycle regulation.

Consistent with previous reports (Dikic et al., 1996; Yu et al., 1996), we found that Pyk2 expression significantly increased JNK activation whereas FAK did not have any effect on JNK in our inducible NIH3T3 cell lines (Fig. 3). These results suggested a potential role for JNK activation in inhibition of cell cycle progression by Pyk2. This possibility is also supported by the analyses of the chimeric molecules, which showed that expression of PF\(^{2}\), but not PF\(^{1}\), induced JNK activation and inhibited cell cycle progression (see Figs 2 and 3). Interestingly, it has been shown recently that JNK can phosphorylate tumor suppressor protein p53 (Frisch et al., 1996), which may lead to its reduced ubiquitination and increased stability (Fuchs et al., 1998b). Therefore the activation of JNK signaling pathways may increase the expression of cdk inhibitor p21, a well-characterized p53 target, thereby inhibit cell cycle progression. Although there are data both in favor and against a role for JNK pathway in cell cycle regulation (Ip and Davis, 1998), our results suggested that differential activation of JNK might contribute to the antagonistic functions of Pyk2 and FAK in cell cycle regulation.

Recently, it has been reported that inhibition of FAK signaling by dominant negative mutants resulted in a decrease in JNK activity and cell cycle progression (Oktay et al., 1999).
However, these are transient transfection assays done in different cells (inhibition of JNK in 293 cells and cell cycle effect in NIH3T3 cells). Furthermore, the potential effects of the dominant negative mutants on Erk activation by FAK were not assessed (in particular in the NIH3T3 cells), making it possible that a reduction in Erk signaling pathway (rather than or in addition to inhibition of JNK activity) is responsible for cell cycle inhibition. Indeed, we have observed that expression of dominant negative FAK mutants using a tetracycline inducible system in NIH3T3 cells inhibited Erk activation, but had little effect on JNK activation (Zhao et al., 1998; data not shown). Further studies using specific inhibitors of Erk and JNK pathways suggested that Erk activity is required for FAK stimulated cell cycle progression. However, the effect of JNK activity on cell cycle progression is more complex. Expression of Pyk2 induced JNK activation and cell cycle inhibition, suggesting that activated JNK play a negative role in cell cycle progression. Consistent with this, treatment of these cells with a JNK inhibitor partially reverted the inhibition of cell cycle progression by Pyk2 (Fig. 5C). However, treatment of these cells at high doses of the inhibitor also reduced cell cycle progression to some extent. Together these results suggested that while some level of JNK activity may be necessary for cell cycle progression, additional JNK activity (stimulated by Pyk2) could inhibit it. Alternatively, activation of JNK may stimulate cell cycle progression only when Erk pathway is also activated by cell adhesion and growth factors. While it induced JNK activation, Pyk2 inhibited Erk activation by cell adhesion (Fig. 4). Induction of Pyk2 expression also slightly inhibited Erk activation by PDGF in these cells (data not shown). This may reconcile the apparent discrepancy between our results and those reported by Oktyay et al. (1999). Future studies using the well-controlled inducible systems where the biochemical analysis and cell cycle phenotypes are examined in the same cells should further clarify a potential role for JNK signaling pathways in cell cycle regulation by Pyk2 and FAK. It will also be desirable to examine the potential role of Pyk2 in cell cycle regulation in cells with endogenous expression of Pyk2 such as rat aortic smooth muscle cells or other blood cells.

Previous studies in our laboratory have suggested that the distinctive C-terminal domain of Pyk2 and FAK confer their differential localization in focal adhesions as well as regulation by integrins (Zheng et al., 1998). Analyses of these molecules as well as an additional chimeric protein FPhy2 (with N-terminal and kinase domain of FAK and C-terminal domain of Pyk2) in NIH3T3 cells confirmed the role of the FAK C-terminal domain in targeting to focal adhesions (Figs 6 and 7). Surprisingly, however, while FPhy1 was highly phosphorylated in NIH3T3 cells as observed previously in CHO cells (Zheng et al., 1998), the complementary chimeric molecule FPhy2 also showed high phosphorylation levels even though it was not localized in focal adhesions (Fig. 8). Together with other recent reports (Lyman et al., 1997; Zhao et al., 1998), these results indicated that focal adhesion localization and induction of tyrosine phosphorylation by integrins are two separate (although coordinated in many cases) events. While a particular focal adhesion targeting sequence has been identified within the C-terminal domain of FAK (Hildebrand et al., 1993), the mechanisms by which FAK or Pyk2 (or the chimeric molecules) is activated by integrins remain elusive.

Our present analyses with the chimeric molecules suggested that potential interactions of the N-terminal (or kinase) domain with the C-terminal domain might play a role in the regulation of tyrosine phosphorylation of these kinases. It is possible that specific interaction of these two domains in Pyk2 prevented its activation by integrins, but they may dissociate to allow activation upon stimuli that elevate intracellular calcium. Such interaction may be disrupted in both chimeric molecules (and not exist in FAK), allowing activation of the kinases by integrins through some as yet unknown mechanisms. Future study will be necessary to test this possibility and to identify the molecular basis of the interaction.

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