Differential Regulation of Pyk2 and Focal Adhesion Kinase (FAK)

THE C-TERMINAL DOMAIN OF FAK CONFFERS RESPONSE TO CELL ADHESION*

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Pyk2 is a recently described cytoplasmic tyrosine kinase that is related to focal adhesion kinase (FAK) and can be activated by a variety of stimuli that elevate intracellular calcium. In this report, we showed that Pyk2 and FAK tyrosine phosphorylation are regulated differentially by integrin-mediated cell adhesion and soluble factors both in rat aortic smooth muscle cells, which express endogenous Pyk2 and FAK, and in transfected Chinese hamster ovary cells. We also found that Pyk2 is diffusely present throughout the cytoplasm, while FAK is localized in focal contacts as expected, suggesting that the different localization may account for their differential regulation. By analyzing a chimeric protein contain N-terminal and kinase domains of Pyk2 and C-terminal domain of FAK, we provided evidence that the distinctive C-terminal domains of Pyk2 and FAK were responsible for their differential regulation by integrins and soluble stimuli as well as their subcellular localization. Finally, we correlated FAK, Pyk2, and the chimeric protein binding to talin, but not paxillin, with their regulation by integrins and focal contact localization. These results demonstrate that the distinct C-terminal domain of Pyk2 and FAK confer their differential regulation by different subcellular localization and association with the cytoskeletal protein talin.

Proline-rich tyrosine kinase 2 (Pyk2; also known as CARβ, RAFTK, and CADTK) is a recently described cytoplasmic tyrosine kinase that is related to the focal adhesion kinase (FAK) (1–4). Recent studies have shown that Pyk2 is involved in calcium-induced regulation of ion channel and mitogen-activated protein kinase activation (1), stress-induced c-Jun N-terminal kinase activation (5), and Src-mediated activation of the mitogen-activated protein kinase signaling pathway in PC12 cells (6). Although these studies indicate that Pyk2 is involved in several signal transduction pathways, many questions concerning the regulation and function of Pyk2, especially the role of Pyk2 in cell adhesion, remain unanswered.

Pyk2 and FAK share a similar structural organization with a tyrosine kinase domain flanked by non-catalytic domains at both the 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 (1–3). Because of the high sequence homology and similar overall organization between Pyk2 and FAK, it is especially interesting to compare the regulation of Pyk2 with that of FAK, in particular their responses to integrin-mediated cell adhesion. Several recent reports have presented somewhat conflicting data regarding regulation of Pyk2 by integrin-mediated cell adhesion in different cell types. It has been reported that Pyk2 displays an integrin-dependent phosphorylation and is localized in focal contacts in B lymphocytes, megakaryocytes, and transfected COS cells (7, 8); however, in transfected 3Y1 cells, Pyk2 phosphorylation is not increased upon plating on fibronectin (FN) and Pyk2 has been shown to localize in intercellular junctions (2). In addition, Siciliano et al. (9) reported that, in rat hippocampal slices and cortical synaptosomes, Pyk2 and FAK are regulated differentially by pathways involving calcium and protein kinase C.

To determine whether induction of Pyk2 phosphorylation by integrins is as effective as that by soluble factors and how it compares with FAK phosphorylation in cell adhesion in the same cells, we investigated regulation of Pyk2 and FAK in rat aortic smooth muscle (RASM) cells, which express both kinases. Using a CHO cell transfection system, we also analyzed regulation of Pyk2, FAK, or a chimeric protein containing the N-terminal and kinase domains of Pyk2 fused to the C-terminal domain of FAK. Our results demonstrated that Pyk2 and FAK were regulated differentially in both RASM cells and transfected CHO cells and that the distinctive C-terminal domains are responsible for the differential regulation by directing different subcellular localization and association with the cytoskeletal protein talin.

EXPERIMENTAL PROCEDURES

Antibodies and Other Materials—Antiserum against human Pyk2 was prepared in rabbits using a GST fusion protein containing the C-terminal 250 residues (GST-Pyk2.C). Anti-Pyk2 antibodies were then affinity-purified from the antiserum using GST-Pyk2.C immobilized on a blue-Sepharose 4B (Pharmacia, Piscataway, NJ). Anti-FAK antibodies were affinity-purified from the antiserum using GST-FAK.C immobilized on a blue-Sepharose 4B (Pharmacia, Piscataway, NJ). Anti-Pyk2 polyclonal antibody 12CA5 against an epitope of the hemagglutinin (HA) protein were affinity-purified from the antiserum using GST-Pyk2.C immobilized on a blue-Sepharose 4B (Pharmacia, Piscataway, NJ).

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1 The abbreviations used are: Pyk2, proline-rich tyrosine kinase 2; FAK, focal adhesion kinase; FN, fibronectin; RASM, rat aortic smooth muscle; HA, hemagglutinin; PLL, poly-L-lysine; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; CHO, Chinese hamster ovary; GST, glutathione S-transferase; PDGF, platelet-derived growth factor; PAGE, polyacrylamide gel electrophoresis; phosphate-buffered saline.
Cell Culture—RASM cells were obtained from ATCC (Rockville, MD) and grown in Dulbeco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Inc.), 10 μg/ml penicillin, and 0.25 μg/ml streptomycin. Confluent cells were incubated overnight in DMEM with 0.2% FBS and then subjected to different treatments as described. In some experiments, prior to sorbitol addition, serum-starved cells were plated onto dishes that had been coated with 10 μg/ml FN or 50 μg/ml PLL and blocked with 2 mg/ml heat-inactivated bovine serum albumin, as described previously (13). Transient transfections of CHO cells were performed using LipoXfектAMINE (Life Technologies, Inc.) according to the manufacturer’s guidelines.

Construction of cDNA Expression Vectors—pKH3-FAK has been described previously (14). The full-length human Pyk2 cDNA was isolated from a placenta cDNA library by polymerase chain reaction based on an EST clone generously provided by Dr. Y. Li of HGS, Inc.2 Sequence analysis showed that our clone is identical with the published Pyk2 sequence, except for the presence of threonine rather than alanine at position 612. It was then cloned into the EcoRI site of the pKH3 vector with the N terminus fused in frame to the triple HA tag to generate plasmid pKH3-Pyk2. The Pyk2/FAK chimERIC (designated PFFhy1) was created by polymerase chain reaction with the “gene splicing by overlap extension” method as described previously (15). Correct fusion was confirmed by DNA sequencing, and this fragment was then inserted into either the EcoRI or SalI site of pHKS.

Immunoprecipitation and Western Blot—Cells were lysed with Nonidet P-40 lysis buffer (20 mM Tris, pH 8.0, 137 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM NaVO4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 2 μg/ml leupeptin) as described previously (12, 14). Immunoprecipitation was carried out at 4 °C by incubating cell lysates for 2 h with indicated antibodies followed by incubation for 1 h with Protein A-Sepharose or Protein G-Plus. After washing, the beads were then resuspended in SDS-PAGE sample buffer, boiled for 5 min, and resolved by SDS-PAGE. Western blotting was performed with appropriate antibodies as indicated using the ECL system (Amersham Life Science), as described previously (12, 14). In some experiments, whole cell lysates were analyzed directly by Western blotting.

Immunofluorescence—Cells grown overnight on FN-coated glass coverslips were washed in PBS supplemented with 1 mM CaCl2 and 1 mM MgCl2, fixed in 4% paraformaldehyde in PBS for 15 min at room temperature, and then washed in PBS and permeabilized in 0.5% Triton X-100 in PBS for 15 min at room temperature. Cells were washed twice in PBS and stained with primary antibody in 10% normal goat serum in PBS for 1 h at room temperature. After two washes in PBS, they were incubated with either fluorescein isothiocyanate- or rhodamine-conjugated secondary antibody in 10% normal goat serum in PBS for 1 h at room temperature. After washing three times in PBS, coverslips were mounted on SlowfLM (Molecular Probes, Eugene, OR) and examined and photographed using a Zeiss Axiosplan (magnification, ×100) universal microscope.

RESULTS

Differential Tyrosine Phosphorylation of Pyk2 and FAK in Response to Cell Adhesion and Soluble Stimuli in RASM Cells—Because they express both endogenous Pyk2 and FAK, RASM cells provide an ideal system to compare regulation of tyrosine phosphorylation of Pyk2 and FAK by cell adhesion and by soluble factors in the same cells, which may clarify the somewhat contradicting results on Pyk2 regulation by integrins in previous reports (2, 7, 8). As described under “Experimental Procedures,” lysates were prepared from suspended, attached cells or attached cells that had been treated with various soluble factors shown to stimulate Pyk2 previously (1, 4–6). They were immunoprecipitated with either anti-Pyk2 or anti-FAK antibodies, resolved by SDS-PAGE, and then analyzed by Western blotting using either a monoclonal antibody against phosphotyrosine (anti-pY), PY20, to detect tyrosine phosphorylation levels, or anti-Pyk2 or anti-FAK antibodies to verify similar expression levels. As shown in Fig. 1A, there is a low level of Pyk2 phosphorylation in serum-starved, attached RASM cells compared with almost non-detectable Pyk2 phosphorylation in suspended cells. Stimulation of the attached cells by angiotensin II, PDGF, sorbitol, calcium ionophore A23187, and KCl all caused further increases in Pyk2 phosphorylation (approximately 5–10-fold as determined by densitometric analysis). In contrast, FAK phosphorylation in unstimulated attached RASM cells was near the maximum level and showed no further increase after stimulation with A23187 or KCl and only a small increase (≤1-fold as determined by densitometric analysis) upon treatment with angiotensin II, PDGF, and sorbitol (Fig. 1B). These results suggested a differential regulation of Pyk2 and FAK in the same cells; while FAK phosphorylation is primarily controlled by cell adhesion, Pyk2 phosphorylation is influenced more by soluble factors that elevate intracellular calcium.

To determine the specificity of the cell adhesion effect, we examined Pyk2 and FAK phosphorylation in RASM cells plated on FN or PLL. Little tyrosine phosphorylation was detected for either Pyk2 or FAK in cells plated on PLL (Fig. 2). Cell adhesion to FN increased Pyk2 phosphorylation slightly, while sorbitol treatment caused a more dramatic (8–10-fold) increase, as shown in Fig. 2A. In contrast, cell adhesion to FN enhanced FAK phosphorylation more significantly, to a nearly maximum level, and sorbitol treatment caused only a minimal additional increase in FAK phosphorylation (Fig. 2B). These results confirmed the differential responses of FAK and Pyk2 to integrin-mediated cell adhesion and to soluble factors in RASM cells.

Differential Localization of Pyk2 and FAK in RASM Cells—To explore potential mechanisms of the differential response of Pyk2 and FAK to cell adhesion, we examined cellular localization of these two kinases in RASM cells upon their adhesion to FN. Cells plated on FN-coated coverslips were stained by double-label immunofluorescence using either rabbit polyclonal anti-FAK or anti-Pyk2 antibodies and mouse monoclonal anti-vinculin antibody, as described under “Experimental Procedures.” Fig. 3 shows the typical distribution of FAK in focal contacts of cells plated on FN (panel A), which colocalizes extensively with vinculin, a major component of focal contacts (panel C). In contrast, Pyk2 was detected as predominantly diffuse staining throughout the cytoplasm (panel B), exhibiting no colocalization with the vinculin staining of

**Fig. 1.** Pyk2 and FAK phosphorylation in response to different stimuli in RASM cells. Quiescent RASM cells were stimulated with various factors as indicated at 37 °C (0.5 μM angiotensin II (AII) for 2 min; 20 ng/ml PDGF for 5 min; 300 mM sorbitol for 5 min; 6 μM calcium ionophore A23187 for 10 min, 50 mM KCl for 5 min). Suspended cells (susp) were prepared by first detaching cells with trypsin, washing with PBS containing 0.5 mg/ml soybean trypsin inhibitor, and then incubating with serum-free DMEM at 30 min at 37 °C. Cell lysates were then collected and immunoprecipitated (IP) with either anti-Pyk2 (panel A) or anti-FAK (panel B) antibodies. The immune complexes were Western-blotted either with PY20 (anti-pY IB) to assay for Pyk2 or FAK phosphorylation or with anti-Pyk2 (anti-Pyk2 IB) or anti-FAK (anti-FAK IB) to verify expression levels.

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Tyrosine phosphorylation of Pyk2 and FAK in response to cell adhesion and sorbitol. Quiescent RASM cells were plated onto plates that had been coated with PLL (50 µg/ml) or FN (10 µg/ml). After 45 min plating, one plate of cells on FN were stimulated with sorbitol (300 mM) for 5 min. Lysates were immunoprecipitated (IP) using anti-Pyk2 antibody (A) or anti-FAK antibody (B) and assayed for tyrosine phosphorylation (anti-pY IB), Pyk2 (anti-Pyk2 IB), or FAK (anti-FAK IB) to verify expression levels, as described in Fig. 1.

The C-terminal Domain of FAK Confers Its Regulation by Integrin and Subcellular Localization—The C-terminal domain of FAK contains the focol adhesion targeting sequence that has been shown to be both necessary and sufficient for FAK localization to focal contacts (16). Even though the C-terminal domain of Pyk2 is ~40% identical to that of FAK and, in fact, exhibits a higher degree of homology within the focal adhesion targeting sequences, the lack of focal contact localization of Pyk2 in RASM cells raised the possibility that the differences in the C-terminal domains of these two kinases are responsible for their differential regulation by integrins.

The differential localization of Pyk2 and FAK might be responsible for their differential regulation by integrins.
autophosphorylation was detected for Pyk2 despite a similarly high expression level (bottom panel). Therefore, the regulation of PFHy1 resembled that of FAK, but not Pyk2 in transfected CHO cells.

To determine if the C-terminal domain of FAK conferred integrin-regulation of PFHy1 by targeting it to focal contacts, we compared subcellular localization of PFHy1 with that of Pyk2 and FAK in transfected CHO cells (Fig. 7). Both the transfected FAK and PFHy1 were colocalized with vinculin in focal contacts as detected by double-label immunofluorescence using rabbit polyclonal anti-FAK (panels A and B) and mouse monoclonal anti-vinculin antibody (panels D and E). In contrast, Pyk2 was present in the transfected cells in a diffuse pattern throughout the cytoplasm as detected by anti-Pyk2 antibody (panel C), which is clearly distinct from the vinculin staining in focal contacts (panel F). To exclude the possibility that different staining patterns of FAK and PFHy1 versus Pyk2 were due to the use of different antibodies (our Pyk2 antibody was generated against the C-terminal part of Pyk2 and therefore does not recognize the chimeric protein PFHy1), we stained the transfected cells with the anti-HA antibody (12CA5) which recognizes the epitope tag on all three transfected proteins. This analysis confirms the focal contact localization of FAK (panel G) and PFHy1 (panel H) as well as the diffuse cytoplasmic staining of Pyk2 (panel I).

Taken together, these data demonstrate that replacing the C-terminal domain of Pyk2 with that of FAK results in a chimeric protein PFHy1, which resembles FAK in both regulation of tyrosine phosphorylation and subcellular localization. They suggest that the C-terminal domains of Pyk2 and FAK are functionally distinct even though they share 40% homology.

**FIG. 6. Differential phosphorylation of transfected FAK, Pyk2, and PFHy1 in response to cell adhesion and sorbitol.** CHO cells were transfected with vectors encoding the proteins as indicated. Two days after transfection, cells were plated onto dishes that had been coated with PLL (50 μg/ml) or FN (10 μg/ml). After 45 min of plating, one plate of cells on FN were stimulated with sorbitol (300 mM) for 5 min. Lysates were immunoprecipitated (IP) using anti-HA antibody and assayed for tyrosine phosphorylation (anti-pY IB) or anti-HA to verify expression levels.

**FIG. 7. Analysis of Pyk2 and FAK localization in transfected CHO cells.** CHO cells were transfected with vectors encoding epitope tagged FAK (A, D, and G), PFHy1 (B, E, and H) or Pyk2 (C, F, and I). One day after transfection, cells were plated on FN-coated glass coverslips and incubated in serum-free medium overnight. They were then processed for immunofluorescence as described under “Experimental Procedures.” The primary antibodies used were anti-FAK (A and B), anti-Pyk2 (C), anti-vinculin (D–F), and anti-HA (G–I).

**DISCUSSION**

Previous studies have suggested that regulation of Pyk2 tyrosine phosphorylation by cell adhesion is cell type- and integrin-specific. Pyk2 has been shown to display an integrin-dependent phosphorylation in B lymphocytes, CMK cells, and transfected COS cells (7, 8). In contrast, Pyk2 phosphorylation has been found to be independent of either cell adhesion in transfected 3Y1 cells or integrin ligation during platelet aggregation (2, 24). It is not clear, however, whether induction of Pyk2 phosphorylation by integrins is as effective as that by soluble factors or how it compares with FAK phosphorylation in cell adhesion in the same cells. In this report, we investigated these questions using both RASM cells expressing endogenous Pyk2 and FAK, and transfected CHO cells. We showed that tyrosine phosphorylation of FAK is controlled mainly by...
integrin-mediated cell adhesion in both cell types, as expected (25). In contrast, only a small increase in Pyk2 tyrosine phosphorylation is observed upon cell adhesion in these cells. A significantly higher increase in Pyk2 tyrosine phosphorylation is obtained when the cells are stimulated with soluble factors such as angiotensin II, PDGF, sorbitol, and calcium ionophore A23187, whereas these treatments produced little change in FAK phosphorylation. These results demonstrate that Pyk2 and FAK are differentially regulated by cell adhesion and soluble factors in these cell types.

The mechanism by which cell adhesion differentially regulates Pyk2 and FAK is poorly understood at present. However, it has been suggested that aggregation of FAK with integrins and cytoskeletal proteins in focal contacts may be responsible for FAK activation and autophosphorylation by integrins in cell adhesion (25, 26). Indeed, we have observed that, in contrast to the localization of FAK in focal contacts, Pyk2 is detected diffusely in the cytoplasm in both RASM cells and the transfected CHO cells. Therefore the lack of (or relatively little) stimulation of Pyk2 by cell adhesion could be explained by the lack of focal contact localization in these cells. Interestingly, studies in several other cell types have also demonstrated a correlation between integrin-dependent Pyk2 phosphorylation and its localization in focal contact-like structures (2, 7). Finally, this hypothesis is strengthened by our analysis of a chimeric protein containing the N-terminal and kinase domains of Pyk2 and the C terminus of FAK, which is localized in the focal contacts and stimulated by cell adhesion to the same extent as FAK.

Direct interactions of FAK with the integrin cytoplasmic domain or cytoskeletal proteins such as paxillin and talin have been suggested to mediate focal contact localization and activation of FAK (25, 26). Recent studies have indicated an interaction of Pyk2 with paxillin (21–23), suggesting that binding to paxillin is unlikely to account for the differential regulation of FAK and Pyk2 observed here. Consistent with this, we have found that paxillin was associated with FAK, Pyk2, and the chimeric protein in both RASM cells and transfected CHO cells. In contrast, we have found an association of talin with FAK, but not Pyk2, in these cells. In addition, the chimeric protein PFhy1, which behaves like FAK in terms of regulation and localization, was also found to associate with talin as effectively as FAK in transfected CHO cells. Therefore, association with talin may be responsible for the differential regulation and localization of Pyk2 and FAK in these cells, although we cannot exclude the possibility that binding to the integrin cytoplasmic domain or to other, as yet unidentified, proteins are also involved.

In addition to its activation by integrins, Pyk2 has been shown to be activated by a variety of soluble factors that elevate intracellular calcium (1, 4–6). The results presented here indicate clearly that in comparison with these soluble factors, cell adhesion mediated by integrins is a relatively weaker activator of Pyk2 in both RASM cells and transfected CHO cells. The small increase in Pyk2 phosphorylation upon cell adhesion could be due to low levels of focal contact localization of Pyk2 that are below the sensitivity of the immunofluorescence method used in these studies. We have occasionally observed localization of Pyk2 in focal contacts in a small fraction (≤5%) of cells (data not shown), which could contribute to the small increase in Pyk2 phosphorylation. However, we did not find any increase in Pyk2 localization in focal contacts, even after stimulation with sorbitol or angiotensin II (data not shown). Alternatively, Pyk2 may be solely regulated by intracellular calcium and protein kinase C, as suggested in previous studies (1, 6, 27). Integrin clustering has been shown to induce calcium transients in some cell types (25, 28). It is possible that integrin-mediated cell adhesion could increase intracellular calcium (although increases may be relatively small compared with those generated by the soluble factors used) to activate Pyk2 to some extent in these cells. It will be interesting to compare directly the level of calcium increases in response to cell adhesion and to the various soluble factors used in these cells.

Results from these comparative studies of Pyk2 and FAK may also have implications for our understanding of FAK regulation by integrins. For example, the potential calcium increase upon cell adhesion, although small, could lead to a small increase in FAK phosphorylation (just like Pyk2), which may promote its aggregation in focal contacts resulting in an additional, more significant increase in phosphorylation. This two-step FAK activation model is consistent with previous reports indicating a role for calcium and protein kinase C in FAK activation (29–31), as well as with the importance of focal contact localization in the regulation as discussed above. Phosphorylation of FAK by protein kinase C and possibly by other serine/threonine kinases has been observed (32, 33), although their potential role in FAK regulation awaits further investigation.

Given the high degree of sequence homology between Pyk2 and FAK, it is somewhat surprising to find that these kinases are differentially regulated. Analysis of a chimeric protein suggests that the C-terminal domains of the kinases are responsible for these regulation differences despite their 40% identity. Although we cannot exclude a role for the N-terminal and kinase domains in the regulation of Pyk2 and FAK, it is likely that part of the amino acid differences in the C-terminal domains are critical for the differential talin binding, focal contact localization, and integrin regulation as described here. We have previously reported that deletion of amino acids 965–1012 within the focal adhesion targeting sequence of FAK ablated its binding to talin (12), suggesting that the different residues within this region may be important. Further studies are needed to identify residues that are responsible for differential regulation of Pyk2 and FAK.

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