Cellular Signalling 23 (2011) 288–296

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

The Pyk2 FERM regulates Pyk2 complex formation and phosphorylation

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ARTICLE INFO

Article history:
Received 16 August 2010
Accepted 6 September 2010
Available online 16 September 2010

Keywords:
Pyk2
Focal adhesion kinase
Tyrosine kinase
Invasion
Adhesion signaling

ABSTRACT

The focal adhesion kinase Pyk2 integrates signals from cell adhesion receptors, growth factor receptors, and G-protein–coupled receptors leading to the activation of intracellular signaling pathways that regulate cellular phenotypes. The intrinsic mechanism for the activation of Pyk2 activity remains to be fully defined. Previously, we reported that mutations in the N-terminal FERM domain result in loss of Pyk2 activity and expression of the FERM domain as an autonomous fragment inhibits Pyk2 activity. In the present study, we sought to determine the mechanism that underlies these effects. Utilizing differentially epitope-tagged Pyk2 constructs, we observed that Pyk2 forms oligomeric complexes in cells and that complex formation correlates positively with tyrosine phosphorylation. Similarly, when expressed as an autonomous fragment, the Pyk2 FERM domain formed a complex with other Pyk2 FERM domains but not the FAK FERM domain. When co-expressed with full-length Pyk2, the autonomously expressed Pyk2 FERM domain formed a complex with full-length Pyk2 preventing the formation of Pyk2 oligomers and resulting in reduced Pyk2 phosphorylation. Deletion of the FERM domain from Pyk2 enhanced Pyk2 complex formation and phosphorylation. Together, these data indicate that the Pyk2 FERM domain is involved in the regulation of Pyk2 activity by acting to regulate the formation of Pyk2 oligomers that are critical for Pyk2 activity.

1. Introduction

The focal adhesion kinases FAK and Pyk2 are uniquely situated to function as a point of convergence to integrate signals from cell adhesion receptors, growth factor receptors, and G-protein–coupled receptors leading to the activation of signaling pathways that regulate the proliferation, migration, and survival of numerous cell types [1–3]. These two related non-receptor kinases share a conserved domain structure consisting of an N-terminal FERM domain, a central kinase domain, a number of proline-rich sequences, and the C-terminal focal adhesion targeting (FAT) domain. Despite the shared domain structure and conserved sequence identity, FAK and Pyk2 possess a number of significant differences. FAK exhibits a wide tissue distribution while Pyk2 is expressed in a more limited number of cell types with expression being highest in cells of hematopoietic lineage and in the CNS [1,4]. Intracellular distribution is also markedly different. FAK is localized predominately within the focal adhesion while Pyk2 characteristically exhibits a more diffuse cytoplasmic localization often with some enrichment in peri-nuclear regions. Interestingly, while only a small proportion of Pyk2 is typically found localized in focal contacts, the C-terminal FAT domain of Pyk2 exhibits strong focal adhesion targeting when expressed as an autonomous fragment [5]. This suggests that other sequences in Pyk2 may play a more dominant role in Pyk2 localization potentially by interacting with a different set of proteins within the cells that determine the ultimate localization. Indeed, FAK and Pyk2 interact with a similar set of focal adhesion proteins including paxillin but interact differentially with talin [6]. Notably, the structure of the FAT domain of FAK [7,8] and Pyk2 [9] have been solved and found to be very similar. Subcellular localization is a critical determinant of FAK activity and mutations in the FAT domain of FAK result in loss of targeting and subsequent loss of activity [7,10,11]. While subcellular localization is likely important for Pyk2 activity, localization to focal contacts does not appear to be required. Interestingly, Pyk2 expression has been observed to increase following loss of FAK expression and can compensate for some, but not all, of FAK regulated functions [12–15]. On the other hand, the expression of FAK or Pyk2 has been reported to differentially regulate cell cycle progression [16,17]. Thus, the relationship between FAK and Pyk2 signaling is complex and can be either compensatory or antagonistic depending on the cell type and cellular context.

In addition to differences in tissue and intracellular distribution, differences have been reported in the mechanisms of stimulation of kinase activity. FAK is primarily activated in response to integrin ligation to ECM [2,18], although FAK can also be activated in response to a number of other agonists [19]. Stimulation of Pyk2 activity in response to integrin ligation has been noted in a number of cell types [13,20,21]; however, it is well appreciated that Pyk2 is activated following increases in intracellular Ca^{2+} following interaction with a number of agonists [1,22,23]. The intrinsic mechanism for the

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doi:10.1016/j.cellsig.2010.09.015

regulation of cellular FAK activity is not completely understood; however, recent studies have provided compelling evidence that FAK activity is regulated by an intramolecular interaction. Structural studies demonstrated that the N-terminal FERM domain of FAK binds directly to the FAK kinase domain inhibiting access to the catalytic cleft and preventing phosphorylation of the activation loop [24]. The mechanism of how this interaction is disrupted has not been defined but it has been postulated that it may be induced by interaction with an activating protein [24] or through the interaction with membrane phospholipids [25] such as is the case with the canonical FERM domain proteins [26,27].

Despite the similarity in structure between the FAK FERM domain [28] and the Pyk2 FERM domain [29], we and others [30] have failed to identify an interaction between the Pyk2 FERM domain and the Pyk2 kinase domain suggesting that a different mechanism is likely to regulate cellular Pyk2 activity. Nevertheless, a number of studies have indicated that the Pyk2 FERM domain is involved in the regulation of Pyk2 activity. Notably, deletion of the Pyk2 FERM domain results in constitutive phosphorylation of Pyk2. Similarly, replacement of the Pyk2 FERM domain with the FAK FERM domain resulted in the enhancement of Pyk2 autophosphorylation, increased substrate phosphorylation, and altered cellular localization [5]. Interestingly, overexpression of Pyk2 [31] or the overexpression of chimeric proteins containing the Pyk2 N-terminus [5] was associated with the induction of changes in cell morphology. We previously demonstrated that expression of the Pyk2 FERM domain as an autonomous fragment inhibited Pyk2 phosphorylation [29]. Similarly, intracellular expression of a scFv fragment of a monoclonal antibody specifically targeting the Pyk2 FERM domain inhibited Pyk2 phosphorylation [32], further supporting a role for the Pyk2 FERM domain in the regulation of Pyk2 activity. In the present study, we sought to examine the mechanisms for the regulation of Pyk2 activity and the role of the Pyk2 FERM domain in this process.

2. Materials and Methods

2.1. Antibodies

The anti-FLAG monoclonal antibody M2 was from Sigma (St. Louis, MO). Anti-HA, anti-c-Src, and anti-Pyk2 pY579/pY580 rabbit polyclonal antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit polyclonal antibody to Pyk2 pY402 was from Calbiochem (Gibbstown, NJ). The monoclonal antibody to calmodulin was from Millipore (Temecula, CA). The anti-phosphotyrosine monoclonal antibody pY20 was from BD Biosciences (San Jose, CA).

2.2. Expression constructs

The HA-epitope tagged Pyk2 and the FLAG-epitope tagged PRNK [33]; the HA-epitope tagged Pyk2 FERM domain, the FLAG-epitope tagged Pyk2, and the FLAG-epitope tagged Pyk2Δ376 variant [34]; the FLAG-epitope tagged Pyk2 I308E variant [29]; and the HA-epitope tagged FAK FERM domain [32] were constructed as previously described. The FLAG-Pyk2 Y402F/K457A, FLAG-Pyk2 L892E, and FLAG-Pyk2 892/I954E variants were generated using the Quick-change site-directed mutagenesis kit (Stratagene, La Jolla, CA). The FLAG-epitope tagged Pyk2 FERM domain was constructed by amplifying Pyk2 residues R39-A367 by polymerase chain reaction and ligating the product in-frame downstream of the 3× FLAG epitope in p3XFLAG-CMV (Sigma, St. Louis, MO). Similarly, the FAK FERM domain (residues R35-P362) and the moesin FERM domain (residues M1-R310) were amplified and cloned in-frame into p3× FLAG-CMV. In the FLAG-Pyk2 FAK FERM construct, the Pyk2 FERM domain (Pyk2 residues R39-K372) was replaced by the corresponding FAK FERM domain (FAK residues R35-R368) by splice overlap extension PCR and cloned into the p3× FLAG-CMV vector.

2.3. Cell culture, transfection, immunoprecipitation, and immunoblotting

HEK 293 cells were passaged in DMEM containing 10% bovine calf serum, 1% non-essential amino acids, 2 mM glutamine, 100 units/ml penicillin, and 10 mg/ml streptomycin. For transfection, subconfluent cultures were transfected with Effectene reagent (Qiagen, Chatsworth, CA) as previously described [29]. Twenty-four hours after transfection, cells were washed in cold PBS and lysed in IPB buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Igepal CA-630, 0.05% Tween 20) containing protease and phosphatase inhibitors. Cell lysates were collected from the dishes after 5 minute of incubation on ice and cleared by centrifugation at 20,000×*g* for 5 minutes. During centrifugation and subsequent processing, the samples were kept on ice or at 4 °C. Five hundred micrograms of cleared cell lysate was precleared with protein G-agarose beads for 1 hr. Specific antibodies were added to the precleared lysate and the reaction was incubated for at least 3 hr. Protein G-agarose was then added and incubation continued for an additional 1 hr. The protein G-agarose immunoprecipitate was washed three times with lysis buffer and once with 20 mM Tris-HCl (pH 7.5). Antibody complexes were eluted in denaturing SDS-PAGE gel loading buffer, resolved by SDS-PAGE, and transferred to Immobilon FL membrane (Millipore, Billerica, MA). Western blots were processed and visualized on a Li-Cor Infrared Imaging System (Lincoln, NE) as recommended. Antibody signals on blots were quantified using the Li-Cor Odyssey 3.0 software. When feasible, two secondary antibodies labeled with different infrared dyes were used to detect two different primary antibodies on the same blot. All blots are representative of results from at least two independent experiments.

3. Results

3.1. Mutations in the FERM domain but not the FAT domain inhibit Pyk2 phosphorylation

Studies of both endogenous and overexpressed Pyk2 in glioma cell lines indicated that there is a positive correlation between Pyk2 activity and glioma cell migration rates [33,34]. To investigate the basis for the pro-migratory effect of Pyk2 in glioma cells, we sought to examine the molecular mechanism of Pyk2 activation. As localization to focal adhesion sites is critical for FAK activity, we first examined whether a similar requirement could be observed for Pyk2. The localization of FAK or Pyk2 to the focal adhesion has been linked to an interaction between the FAT domain and the LD motifs of paxillin [1,35]. The FAT domain of Pyk2 is a four-helix bundle that binds paxillin LD motifs through two hydrophobic patches on opposite sides of the bundle [9]. The double mutation I936E/I998E in the FAT domain of FAK completely abolished binding to paxillin [7]. Introduction of similar substitutions at the corresponding residues in the Pyk2 FAT domain, L892E or L892E/I954E did not alter Pyk2 phosphorylation relative to phosphorylation of wild-type Pyk2 in transfected cells (Fig. 1). In contrast, Pyk2 phosphorylation was nearly completely abolished by substitution of I308 in the Pyk2 FERM domain consistent with a regulatory role for the Pyk2 FERM domain.

3.2. Pyk2 forms intracellular oligomeric complexes

Park et al. [36] previously reported that an early step of Pyk2 activation is the trans-phosphorylation of Tyr402. We first examined if oligomeric complexes of full-length Pyk2 could be immunoprecipitated from cell lysates. 293 cells, which lack detectable endogenous Pyk2, were co-transfected with plasmids encoding full-length Pyk2 with an HA-epitope tag (HA-Pyk2) or a FLAG-epitope tag (FLAG-Pyk2). The HA-Pyk2 was immunoprecipitated from lysates of the transfected cells with a polyclonal anti-HA antibody and the immunoprecipitate was probed for the presence of the co-transfected FLAG-Pyk2. Immunoblotting demonstrated that the FLAG-tagged Pyk2 was present in the anti-HA immunoprecipitate from cells co-transfected with HA-Pyk2 indicating