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The Pyk2 FERM regulates Pyk2 complex formation and phosphorylation

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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 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.

Keywords

Pyk2; focal adhesion kinase; tyrosine kinase; invasion; adhesion signaling

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

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5. Conflict of interest:
None

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 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 Quickchange 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 3X 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 p3XFLAG-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 p3XFLAG-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 incubation on ice and cleared by centrifugation at 20,000x*g* for 5 minutes. During centrifugation and subsequent processing, the samples were kept on ice or at 4 °C. Five hundred microgram 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 hrs. 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 over-expressed 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 cotransfected with HA-Pyk2 indicating that full length Pyk2 forms an intracellular complex of at least dimer complexity (Fig. 2A). No FLAG-tagged Pyk2 was present in the anti-HA immunoprecipitate from control cells co-transfected with FLAG-Pyk2 and empty vector indicating antibody specificity.

Previously, we demonstrated that expression of an autonomous Pyk2 FERM domain inhibited Pyk2 tyrosine phosphorylation and glioma cell migration [29,37]. To determine if the Pyk2 FERM domain could also form a complex with full length Pyk2, cells co-transfected with a HA-tagged Pyk2 FERM domain and FLAG-tagged full length Pyk2 were lysed and immunoprecipitated with anti-HA antibody. Immunoblotting confirmed that the full length FLAG-Pyk2 co-immunoprecipitated with the Pyk2 FERM domain (Fig. 2A) indicating that the Pyk2 FERM domain associated with full length Pyk2. In contrast, only a trace amount of FLAG-Pyk2 was detected in the anti-HA immunoprecipitates obtained from cells co-transfected with FLAG-tagged Pyk2 and HA tagged-FAK FERM domain.

To determine which domains of full length Pyk2 mediate its interaction with the Pyk2 FERM domain, HA-Pyk2 FERM was co-expressed with various FLAG-tagged Pyk2 constructs and the extent of complex formation assessed by co-immunoprecipitation (Fig. 2B). The Pyk2 FERM interacted strongly with the Pyk2 FERM domain but did not co-immunoprecipitate with the FAK FERM domain. Similarly, the Pyk2 FERM domain interacted only weakly with the FERM domain of moesin with which it shares a similar structure but is significantly different in primary sequence. The Pyk2 FERM domain had additional, although much weaker, interactions with other regions of Pyk2 aside from the FERM domain. Only a trace amount of the Pyk2 FERM domain co-immunoprecipitated with the Pyk2 variants Pyk2 Δ376 (which lacks the FERM domain) or a chimeric construct in which the Pyk2 FERM domain was replaced

with the corresponding FAK FERM domain in full length Pyk2 (Pyk2FF). Similarly, the Pyk2 FERM domain interacted very poorly with the C-terminal domain of Pyk2 (PRNK). These results indicate that the Pyk2 FERM domain interacts most strongly with the partner FERM domain and to a much lesser extent with other domains in full length Pyk2.

Sasaki and co-workers recently described the formation of Pyk2 FERM domain homodimers [38]. The Pyk2 FERM oligomers they examined apparently were formed *de novo* in cell lysates in a calmodulin/Ca^{2+} dependent process. Interestingly, the Pyk2 FERM domain complexes we immunoprecipitated from transfected cell lysates were obtained following cell lysis in a buffer that lacked Ca^{2+}. Therefore, if Ca^{2+} is required for intracellular Pyk2 oligomer formation, it is possible that Ca^{2+} derived from the intracellular stores released during lysis was sufficient for complex formation. Alternatively, once the Pyk2 oligomers have been assembled, Ca^{2+} may no longer be required for maintenance of the complex. To examine these possibilities, 293 cells were co-transfected with HA-tagged and FLAG-tagged full length Pyk2, the cells were lysed, and the lysates were incubated in the presence or absence of EGTA. After incubation, FLAG-Pyk2 was immunoprecipitated with anti-FLAG antibody and the precipitate immunoblotted with anti-HA and anti-FLAG antibodies to determine the effect of EGTA on complex formation. Exposure to EGTA did not affect the formation of the Pyk2 oligomeric complexes as an equivalent amount of HA-Pyk2 was present in the anti-FLAG immunoprecipitates from the EGTA and non-EGTA treated cell lysates (Fig. 3A). Thus, it does not appear that the Pyk2 oligomers observed in the immunoprecipitates were assembled *de novo* in the lysates in a calcium-dependent manner. The presence of calmodulin in the immunoprecipitated complexes was also examined. Calmodulin was easily detected in the cell lysates prepared from cells co-expressing HA-tagged Pyk2 and FLAG-tagged Pyk2 however, calmodulin was not observed in the anti-FLAG immunoprecipitate (Fig. 3B). In contrast, c-Src which is known to bind to phosphorylated Pyk2 Tyr402, was readily detected in the Pyk2 oligomeric complexes.

3.3. Autonomously expressed Pyk2 FERM domain competes with full length Pyk2 in oligomeric complex formation

Intracellular expression of an autonomous Pyk2 FERM domain reduced Pyk2 tyrosine phosphorylation and Pyk2-dependent glioma cell migration [29]. To better understand how the Pyk2 FERM domain inhibited Pyk2 phosphorylation, we first examined the effect of the autonomously expressed Pyk2 FERM domain on the assembly of full length Pyk2 oligomers. HA-tagged and FLAG-tagged full length Pyk2 were co-expressed in 293 cells along with increasing FLAG-Pyk2 FERM or FLAG-FAK FERM. The oligomers containing HA-Pyk2 were immunoprecipitated with anti-HA antibody and blotted to detect the co-precipitating FLAG-Pyk2 (figure 4). As in earlier experiments, HA-tagged and FLAG-tagged Pyk2 were co-precipitated from cell lysates with the anti-HA antibody. In the lysates from cells also expressing the FLAG-Pyk2 FERM, the recovery of HA-Pyk2 was unchanged however, the amount of FLAG-Pyk2 that co-precipitated with HA-Pyk2 was progressively reduced concomitant with the appearance of the autonomous FLAG-Pyk2 FERM in the anti-HA immunoprecipitate (Fig. 4, lanes 1 to 3). This competition was specific for the Pyk2 FERM domain as the expression of an autonomous FAK FERM domain did not significantly change the ratio of HA-Pyk2 and FLAG-Pyk2 present in the immunoprecipitate and only a trace amount of FAK FERM was present in the anti-HA immunoprecipitate when expressed at higher levels (Fig. 4, lanes 4 to 6). As expected, expression of the Pyk2 FERM domain reduced Pyk2 tyrosine phosphorylation (Fig. 4A). Consistent with its inability to significantly inhibit Pyk2 complex formation, co-expression of the FAK FERM domain did not alter Pyk2 tyrosine phosphorylation (Fig. 4A). These data indicate that the FLAG-Pyk2 FERM competes with full length FLAG-Pyk2 for inclusion into a complex with HA-Pyk2 leading to a reduction in Pyk2 complex formation and reducing Pyk2 phosphorylation.

3.4. Tyrosine phosphorylation state of Pyk2 in complexes

The inhibition of full length Pyk2 complex assembly by expression of a Pyk2 FERM domain and the concomitant decrease in Pyk2 tyrosine phosphorylation levels lead us to more directly interrogate the phosphorylation state of Pyk2. To measure specific tyrosine phosphorylation of Pyk2, we co-expressed full length wild-type HA-Pyk2 with either FLAG tagged wild-type Pyk2, a kinase dead and autophosphorylation deficient Pyk2 variant (FLAG-Pyk2 Y402F K457A), or a FLAG tagged Pyk2 FERM domain. Pyk2 was immunoprecipitated from resulting cell lysates with anti-FLAG antibody and the immunoprecipitates analyzed by western blotting with specific anti-phospho tyrosine antibodies (Fig. 5). Pyk2 complexes containing FLAG- and HA-tagged wild-type Pyk2 had high levels of phosphorylation on Tyr402. These complexes also contained Src and accordingly exhibited significant phosphorylation on Tyr579/580. In the complexes containing FLAG-Pyk2 Y402F K457A and HA-Pyk2, the only potential source of the pTyr402 signal is the HA-tagged wild-type Pyk2. In these heteromeric complexes, Tyr402 phosphorylation was significantly reduced consistent with trans-phosphorylation occurring in these complexes. The significant reduction in phosphorylation of Tyr402 in these complexes markedly reduced the recruitment of Src and the phosphorylation of Tyr579/580. The autonomously expressed Pyk2 FERM domain had a similar effect on the phosphorylation of HA-Pyk: phosphorylation of Tyr402 and Tyr579/580 was significantly reduced and Src was absent from the complexes. Collectively, these results suggest that the Pyk2 FERM-mediated blockage of Pyk2 complex formation, subsequent loss of Tyr402 phosphorylation, and the lack of Src recruitment, is responsible for the observed Pyk2 FERM inhibition of Pyk2 activity.

3.5. Deletion of the Pyk2 FERM domain enhances complex formation

As demonstrated above, the Pyk2 FERM domain can form a heteromeric complex with full length Pyk2 as well as FERM:FERM homomeric oligomers. This raises the question of whether these FERM interactions are required to initiate oligomer formation, and secondly, do they significantly contribute to the stability of the Pyk2 oligomers. To address this, we measured complex formation between wild-type Pyk2 and the Pyk2 variant Pyk2Δ376 in which the entire FERM domain has been deleted. 293 cells were co-transfected with HA- and FLAG-tagged wild-type Pyk2 or HA- and FLAG-tagged Pyk2Δ376. Immunoblotting of whole cell lysates indicated a similar level of expression of the epitope tagged wild-type Pyk2 and Pyk2Δ376 (Fig. 6A, right panels). The co-transfected cells were lysed, immunoprecipitated with anti-HA antibody, and the precipitates analyzed for the presence of FLAG-Pyk2. While similar amounts of HA-Pyk2 and HA-Pyk2Δ376 were immunoprecipitated (Figure 6A, top left panel), there was a six-fold increase in the quantity of FLAG-Pyk2Δ376 co-precipitating with the HA-Pyk2Δ376 compared to the amount of wild-type FLAG-Pyk2 co-precipitating with wild-type HA-Pyk2 (Fig. 6B, bottom right panel). These results suggest that residues outside of the FERM domain are capable of mediating complex formation and that loss of the FERM domain can facilitate these interactions.

4. Discussion

We have previously described a role for the N-terminal FERM domain of Pyk2 in the regulation of Pyk2 activity. Selected mutations within the FERM domain significantly inhibit Pyk2 phosphorylation and expression of the FERM domain as an autonomous fragment inhibits Pyk2 activity. In the present study, we sought to investigate the mechanism that underlies these observations. The major findings of this report are as follows: (1) Pyk2 forms oligomeric complexes in cells (2) complex formation correlates with tyrosine phosphorylation, (3) the autonomous FERM domain can be incorporated into a complex with Pyk2 resulting in reduced Pyk2 phosphorylation, and (4) Pyk2 complex formation is enhanced by deletion of the FERM domain. 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.

FAK and Pyk2 are activated following cellular interaction with a variety of agonists however, the intrinsic mechanism of activation of these kinases remains to be fully elucidated. Notably, we and others have observed that increased expression of Pyk2 in cells is accompanied by increased Pyk2 phosphorylation. In contrast, increased expression of FAK is accompanied by a more modest increase in phosphorylation suggesting intrinsic differences in the regulatory mechanisms for these two related kinases. It is well appreciated that Pyk2 activity is stimulated following cellular interaction with ligands resulting in increased intracellular Ca^{2+} [22]. Kohno and colleagues [38] recently provided insights into the potential mechanism for this effect by demonstrating that in lysates of transfected cells, exogenously expressed Pyk2 could be pulled down by calmodulin agarose in the presence of Ca^{2+} but not in the presence of EGTA. In the current study, we also demonstrated that exogenously expressed Pyk2 formed intracellular complexes that could be directly immunoprecipitated from cell lysates. Interestingly, these complexes were stable in the presence of EGTA and although camodulin was readily detectable in the cell lysates, calmodulin was not detected in the immunoprecipitated complexes. These results might be explained due to differences in the experimental approach. Alternatively, it is possible that the complexes we immunoprecipitated from cells represent mature complexes. As such, these complexes may have required calcium initially to form the complexes however, once formed these complexes apparently no longer required calcium. The suggestion that the immunoprecipitated Pyk2 complexes represented mature complexes is supported by the observation that the immunoprecipitates contained Src that is known to bind to Pyk2 following phosphorylation of Tyr402.

The FERM domain is a conserved protein module found in a number of proteins that can mediate protein-protein interactions and protein-membrane targeting. In the canonical FERM domain containing proteins ezrin, radixin, and moesin, the FERM domain interacts directly with the C-terminal tail domain to mask binding sites for protein interactants [39–42]. Interaction with PIP2 molecules at the membrane induces conformational changes that unmask the full length proteins and allow its interaction with the cytoplasmic domains of transmembrane proteins, other cytoplasmic proteins, and the interaction of the C-terminal tail with actin [26]. We previously demonstrated that expression of an autonomous FERM domain significantly decreased Pyk2 phosphorylation and inhibited Pyk2 stimulated glioma cell migration [29] although how the autonomous FERM domain was able to exert this inhibitory effect was not understood. The results of the present study indicate that the autonomously expressed FERM interacts with full length Pyk2 to form a FERM:Pyk2 complex that can be immunoprecipitated from cells. Utilizing different epitope-tagged Pyk2 constructs, we demonstrated that expression of the Pyk2 FERM domain competed with Pyk2 for inclusion into a Pyk2:Pyk2 complex. The reduction in Pyk2:Pyk2 complex formation correlated with a reduction in Pyk2 phosphorylation. Therefore, it is likely that the capacity of the autonomously expressed Pyk2 FERM domain to inhibit Pyk2 function is related to its capacity to form a complex with Pyk2. This effect was specific for the Pyk2 FERM domain. An autonomously expressed FAK FERM did not interact with the Pyk2 FERM domain nor did it interact with full length Pyk2. As such, it was unable to compete with Pyk2 for the formation of a Pyk2:Pyk2 complexes and did not alter Pyk2 phosphorylation. Together, these data indicate that the Pyk2 FERM domain and the FAK FERM domain regulate the activity of Pyk2 and FAK respectively, but do so in different ways.

Autonomously expressed Pyk2 FERM domains interact to form FERM:FERM complexes that can be immunoprecipitated from cells. This interaction is specific as the autonomous Pyk2 FERM domain did not interact with the FAK FERM domain either alone or in the context of intact Pyk2 no greater than the extent to which it interacted with the unrelated moesin FERM

domain. The autonomously expressed Pyk2 FERM domain also interacted with full length Pyk2 and could be co-immunoprecipitated from cells. The major site for this interaction appears to be the FERM domain as deletion of the FERM domain from full length Pyk2 significantly inhibited the capacity of the FERM domain to interact with Pyk2. Similarly, only a minor interaction was observed between the Pyk2 FERM domain and the C-terminal PRNK domain. Although the autonomous FERM interacted with the FERM domain in full length Pyk2 and inhibited complex formation between full length Pyk2, removal of the FERM domain from Pyk2 facilitated complex formation. Thus, the Pyk2Δ376 variant more readily formed complexes and was constitutively phosphorylated in cells relative to the wild-type Pyk2. These results indicate that regions in addition to the FERM domain ultimately contribute to the formation of Pyk2 complexes in agreement with a previous study [36].

The results of the current study suggest a complex mechanism for the regulation of Pyk2 activity (Fig. 7). For endogenous Pyk2, agonist stimulation results in Pyk2 complex formation that is likely facilitated through a Ca^{2+}/calmodulin dependent mechanism [38]. Pyk2 complex formation is mediated initially by FERM domain interactions although other regions are likely to participate in stabilizing and strengthening the interaction. It is proposed that the transition of this initial FERM based oligomer to a complex capable of transphosphorylation requires FERM domain mediated re-arrangement. This might be accomplished via conformational changes resulting from the formation of the complex. Alternatively, FERM domain rearrangement might be mediated by interaction of the complex with another protein in the cells. The latter possibility is suggested by the effect of select mutations within the Pyk2 FERM domain to inhibit Pyk2 phosphorylation [29,32]. Thus, the I308E substitution in the Pyk2 FERM domain does not effect Pyk2 complex formation (unpublished results) but could still significantly inhibit Pyk2 phosphorylation by preventing a protein-protein interaction required for efficient transphosphorylation of Pyk2 in the complex. Similarly, intracellular expression of a scFv targeting the Pyk2 FERM domain inhibited Pyk2 phosphorylation [32] further suggesting the interaction of an interacting protein in the formation of the stable complex. Deletion of the FERM domain obviates the need for the FERM domain rearrangement and leads directly to the formation of a Pyk2 complex. Formation of the complex leads to transphosphorylation of Pyk2 in the complex, recruitment of Src and full activation of Pyk2. Collectively, the data indicate that the FERM domain of Pyk2 plays a role in the regulation of Pyk2 activity although it utilizes a mechanism that is distinct from the intramolecular association observed for the FAK FERM domain. Given the diverse range of protein interactions reported for FERM domains it is likely that the Pyk2 FERM domain may mediate protein-protein interactions with a variety of as yet unidentified proteins that contribute to the regulation of Pyk2 activity. The Nir family of proteins have been reported to interact with the Pyk2 FERM domain [43] but the relationship of this interaction to Pyk2 complex formation and activity is unknown. In the past 5 years, there has been significant progress in the identification, characterization, and development of small molecules that inhibit protein-protein interactions through direct binding to the contact surface or through allosteric sites [44,45]. Hence, the current results suggest that FERM domain mediated protein-protein interactions may represent a novel approach to target specific regulation of Pyk2 activity for therapeutic intervention in cancer, inflammation, and osteoportic disease.

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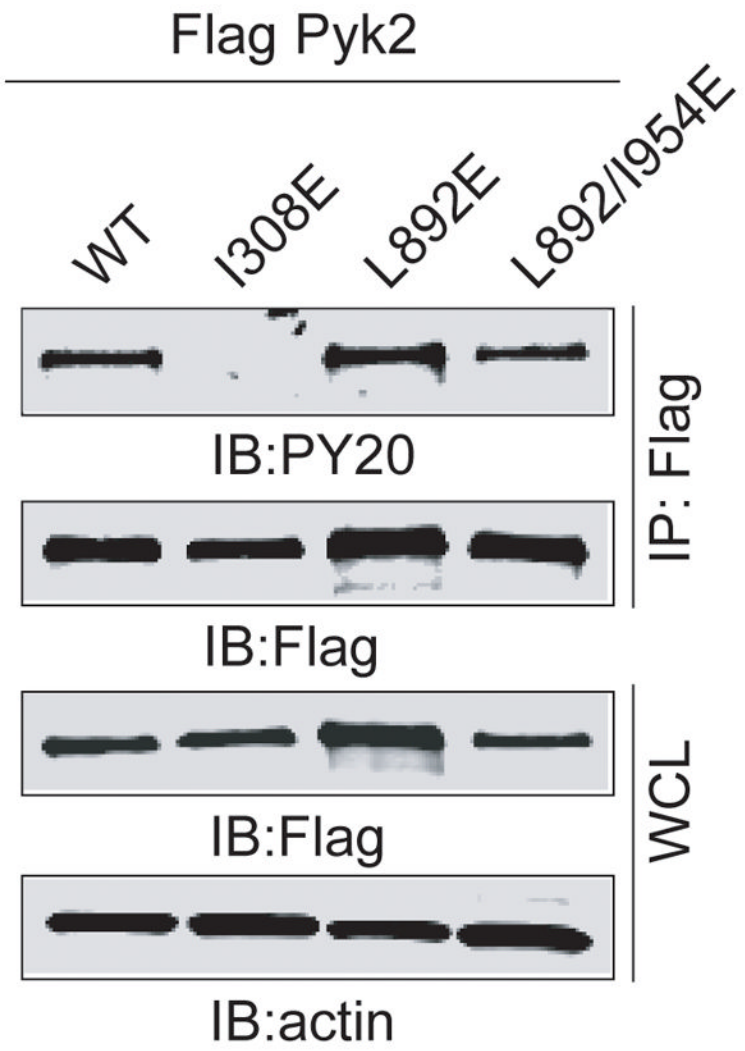

Figure 1.

Differential effect of FERM and FAT domain mutations. 293 cells were transfected with FLAG epitope-tagged wild-type (WT) Pyk2 or the indicated Pyk2 variant. The cells were lysed, immunoprecipitated (IP) with anti-FLAG antibody, and the immunoprecipitates immunoblotted (IB) with anti-FLAG or anti-phosphotyrosine antibody PY20. Whole cell lysates were immunoblotted with anti-FLAG or anti-actin as a loading control.

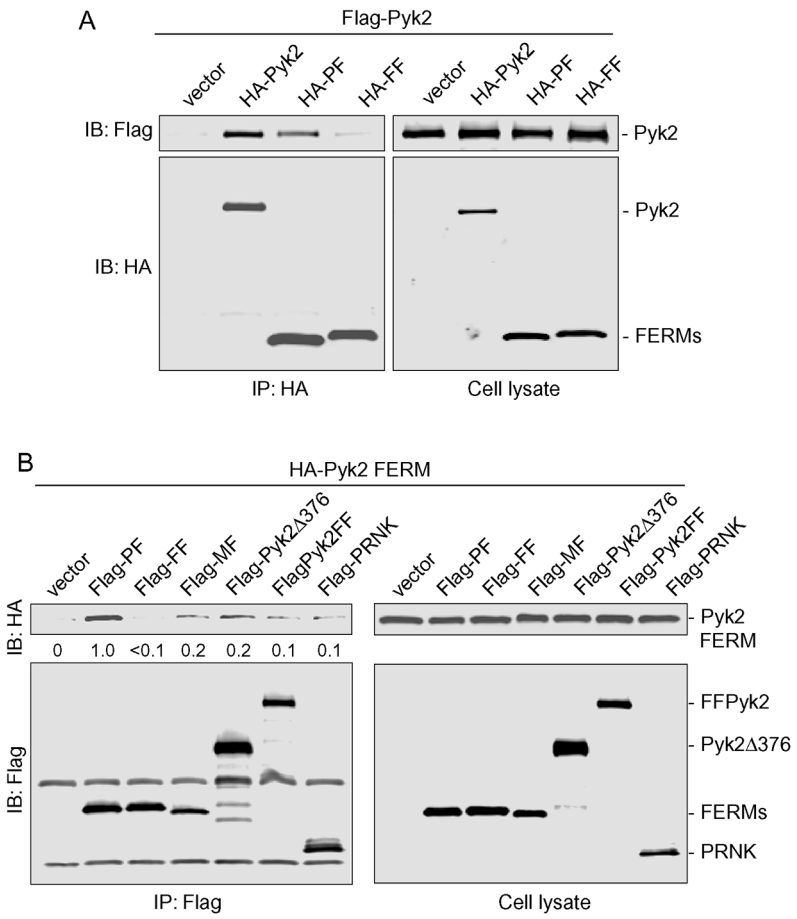

Figure 2.

Pyk2 and Pyk2 FERM oligomeric complexes are present in cell lysates. **A**. 293 cells were co-transfected with FLAG-epitope tagged Pyk2 and either empty vector, HA-tagged Pyk2, HA-Pyk2 FERM (HA-PF), or HA-FAK FERM (HA-FF). The cells were lysed, immunoprecipitated (IP) with anti-HA antibodies, and the immunoprecipitates immunoblotted (IB) with anti-FLAG or anti-HA antibodies. Right panel, immunoblotting of whole cell lysates. **B**. 293 cells were co-transfected with HA-Pyk2 FERM and the indicated FLAG-tagged constructs. Pyk2 FERM (PF), FAK FERM (FF), moesin FERM (MF), Pyk2 lacking the first 376 amino acids (Pyk2Δ376), full length Pyk2 with the Pyk2 FERM domain replaced with the FAK FERM domain (Pyk2FF), or the Pyk2 C-terminal domain (PRNK). Cell lysates were immunoprecipitated with anti-FLAG antibody and the precipitates blotted with anti-FLAG and anti-HA antibodies (left panel). The relative amount of HA-Pyk2 FERM that co-immunoprecipitated with the FLAG-tagged proteins is indicated below the HA immunoblot. Right panel; immunoblotting of whole cell lysates.

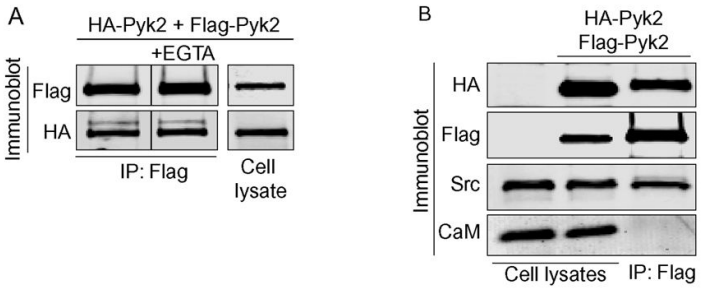

Figure 3.

A. Pyk2 complexes are stable in the presence of EGTA. 293 cells cotransfected with HA-Pyk2 and FLAG-Pyk2 were lysed and the lysates were incubated in the presence or absence of 2 mM EGTA. The lysates were immunoprecipitated with anti-FLAG antibody and the immunoprecipitate immunoblotted with anti-FLAG and anti-HA antibodies. Expression of HA-Pyk2 and FLAG-Pyk2 was confirmed by immunoblotting of the cell lysates. **B**. c-Src, but not calmodulin, was detected in the Pyk2 complexes. 293 cells cotransfected with HA-Pyk2 and FLAG-Pyk2 were lysed and immunoprecipitated with anti-FLAG mAb. Whole cell lysates and the anti-FLAG immunoprecipitate were immunoblotted with the indicated antibodies.

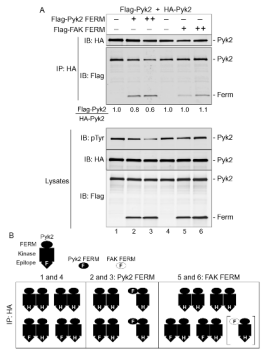

Figure 4.

Pyk FERM competes with full length Pyk2 for incorporation into complexes. **A**. 293 cells were transfected with equal amounts of FLAG- and HA-tagged full length Pyk2 and increasing amounts of either FLAG-Pyk2 FERM or FLAG-FAK FERM. Cell lysates were immunoprecipitated with anti-HA antibody and the immunoprecipitates immunoblotted with anti-FLAG and anti-HA antibodies. The FLAG-Pyk2:HA-Pyk2 ratio in each immunoprecipitate is indicated below the FLAG blot. Bottom panel, cell lysates were immunoblotted with the indicated antibodies. **B**. Scheme of the oligomers present in the anti-HA immunoprecipitate. The FAK FERM-Pyk2 complex recovered in trace amounts under conditions of high FAK FERM expression is bracketed

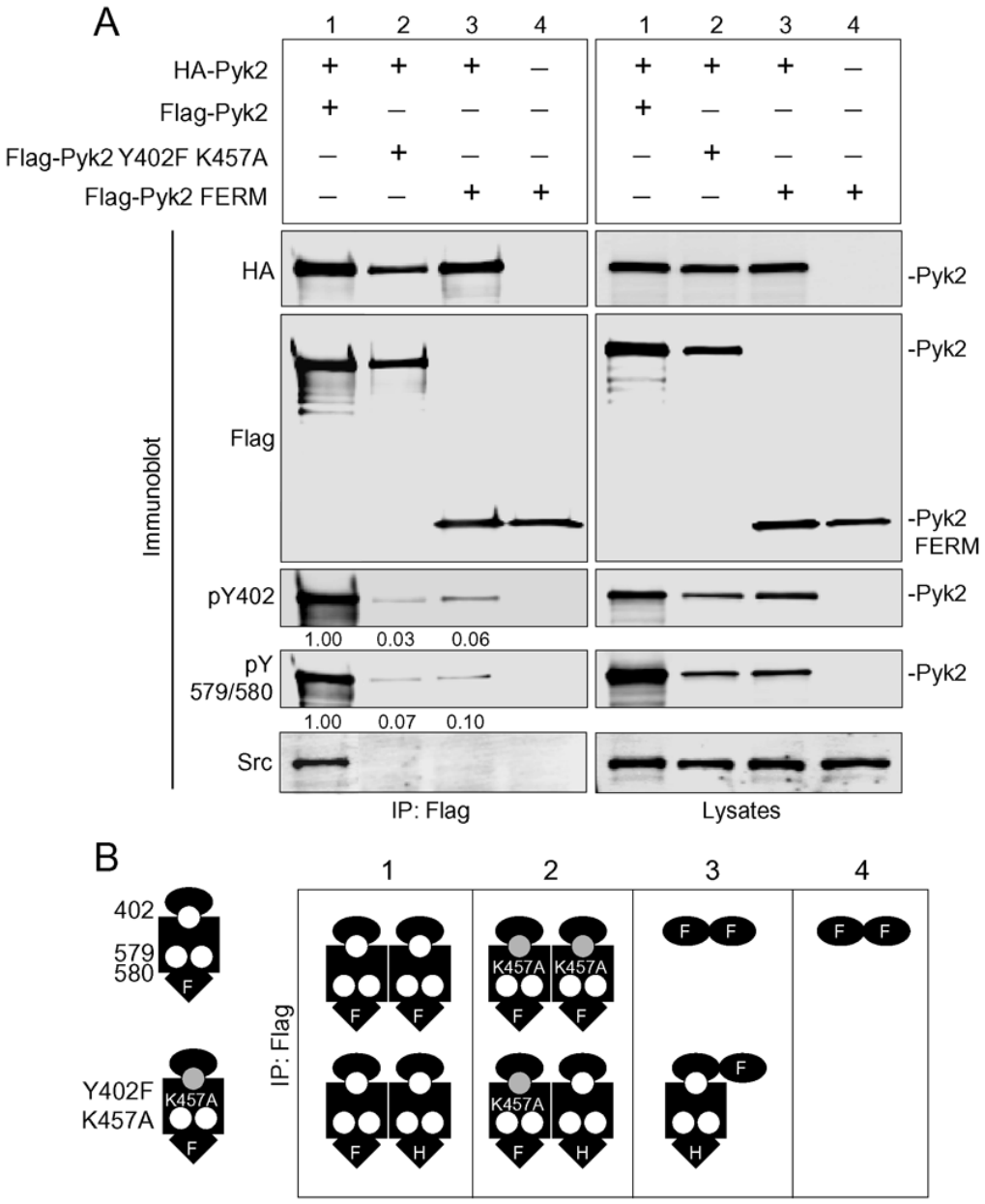

Figure 5.

Pyk2 tyrosine phosphorylation occurs in the oligomeric complexes. **A**. 293 cells were transfected with the indicated epitope-tagged Pyk2 or Pyk2 FERM constructs. Cell lysates were immunoprecipitated with anti-FLAG antibody and the immunoprecipitates immunoblotted with the indicated antibodies (left panels). Below each phospho-tyrosine blot is the relative phospho-tyrosine signal, normalized to the wild-type Pyk2 signal in lane 1. Cell lysates were also blotted to confirm equivalent expression (right panels). **B**. Scheme of the immunoprecipitated FLAG-tagged Pyk2 oligomers and their possible tyrosine phosphorylation sites. Three tyrosine phosphorylation sites (Tyr402 and Tyr579 and Tyr580) are indicated by the white circles and the Y402F mutation is represented by the grey circle.

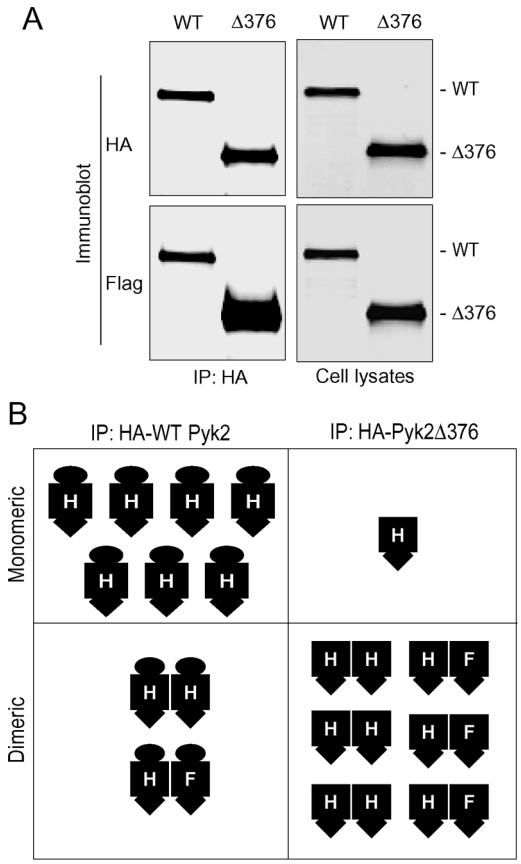

Figure 6.

Deletion of the Pyk2 FERM domain enhances complex formation. 293 cells were co-transfected with differentially epitope-tagged (HA or FLAG) wild-type Pyk2 or Pyk2 Δ376 constructs and expression verified by immunoblotting (right panels, cell lysates). The cell lysates were immunoprecipitated with HA antibody and the precipitate was immunoblotted with anti-FLAG or anti-HA antibodies (left panels).

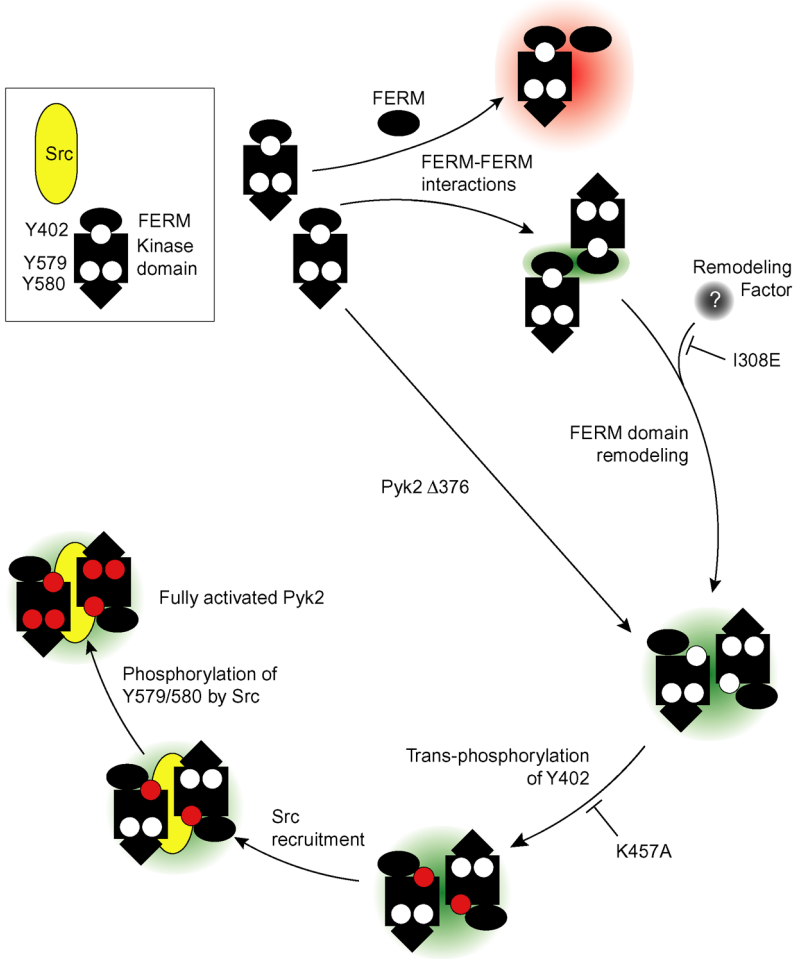

Figure 7.

Potential model of the Pyk2 activation pathway. An early step of complex formation involves FERM-FERM interactions. Autonomously expressed FERM domain can trap full length non-phosphorylated Pyk2 in an inactive, dead-end complex (indicated by red background). Following the initial FERM-FERM interaction, which might involve calmodulin, remodeling of the FERM domains intrinsically or through accessory protein interaction enables transphosphorylation of Y402. Deletion of the Pyk2 FERM domain facilitates complex formation bypassing the requisite initial FERM-FERM interactions and FERM remodeling. Phosphorylation of Tyr402 enables interaction with Src, the phosphorylation of Tyr579 and Tyr580 and full activation. Dimeric complexes are illustrated in a head-to-tail orientation for simplicity, but Pyk2 might oligomerize into higher order structures possibly with other proteins.