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Identification of an Autoinhibitory Domain of p21-activated Protein Kinase 5*

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The p21-activated protein kinases (Paks) are serine/threonine protein kinases activated by binding to Rho family small GTPases, Rac and Cdc42. Recently, Pak family members have been subdivided into two groups, I and II. Group II Paks, including Pak4, Pak5, and Pak6, does not contain the highly conserved autoinhibitory domain that is found in the group I Paks members, *i.e.* Pak1, Pak2, and Pak3. In the present study, we have purified the glutathione *S*-transferase fusion form of Pak5 and shown for the first time that Pak5 autophosphorylation can be activated by GTP bound form of Cdc42. Mutation of histidine residues 19 and 22 to leucine on the p21-binding domain of Pak5 completely abolished the binding of Cdc42 and the Cdc42-mediated autophosphorylation. On the other hand, mutation of tyrosine 40 to cysteine of Cdc42 did not knockout the binding of Pak5. Analysis of C-terminal deletion mutants has identified an autoinhibitory fragment of Pak5 that is absent from other group II Pak family members. Taken together, these results suggest that Pak5, like Pak1, contains an autoinhibitory domain and its activity is regulated by Cdc42.

The Rho GTPase subfamily, which belongs to Ras small G protein superfamily, plays vital roles in diverse cellular processes including cytoskeleton reorganization, gene transcription, and cell cycle progression (1–3). The Rho GTPase family, such as RhoA, Cdc42, and Rac1, exists in either the active or GTP bound state or the inactive or GDP bound state dependent on the cellular signals. The p21-activated protein kinases (Paks),[1] which are serine/threonine protein kinases important for the Cdc42 and Rac1 signaling, contain a well defined p21-binding domain (PBD). There are six members of this kinase family and they are subdivided into two groups according to the kinase domain homology and the existence of an autoinhibitory domain (AID) (4). Group I Paks (Pak-I), including Pak1, Pak2, and Pak3, contain a highly conserved AID important for its regulation. Binding of the active form of Cdc42 or Rac1 to the PBD domain of Pak1 induces a conformational change, which causes the kinase inhibitor (KI) fragment within AID to dissociate from the kinase active site. The releasing of KI allows the autophosphorylation of T-loop and the subsequent activation of the kinase to take place (5).

Because of the absence of an identifiable AID in the Pak-II family members, it is still unclear whether the Rho GTPases regulate the activity of Pak-II family. Recent reports have shown that Pak4 is constitutively active and mediates the actin polymerization by targeting to Golgi apparatus. However, relatively little is known about the regulation of Pak5, apart from the finding that it is predominantly expressed in brain and stimulates the neurite outgrowth in mouse neuroblastma cell line by the down-regulation of RhoA activity (8). In the present study, the regulation of Pak5 activity in relation to its autophosphorylation was studied. We showed that Cdc42, but not Rac1 or RhoA, was able to activate the autophosphorylation of the purified GST fusion form of Pak5 in a GTP-dependent manner. Mutations of the residues on Pak5, which is important for the association with Cdc42, abolished the effect of Cdc42 on the Pak5 autophosphorylation. Using the truncation mutants of Pak5, an inhibitory fragment of about 120 amino acids was mapped.

EXPERIMENTAL PROCEDURES

Plasmids and Constructs—The full-length human Pak5 cDNA was amplified from the KIAA1264 clone obtained from Kazusa DNA Research Institute, Japan, using forward primer 5′-CCGAATTCATGTTTGGGAAGAAAAAGAAAAAGA and reverse primer 5′-CCGGGTCGACTCAGTGATGCCTGTATTGTCTC. The cDNA was subcloned into the pGEX-4T vector (Amersham Biosciences) and pCMV-Tag2 vector (Stratagene). The site-directed mutagenesis of histidines 19 and 22 to leucine was carried out using H19L/H22L primer 5′-GTCCAACTTTGAACTCAGGGTTCTTACTGGGTTTGATCC (mutations are underlined). C-terminal truncation mutants (N60/N180/N222) of the Pak5 were constructed by double digesting the pGEX-4T-Pak5 with *Nsi*I/*Sal*I, *Stu*I/*Sal*I, and *Xho*I/*Sal*I followed by fill-in with Klenow fragment and re-ligation.

The autoinhibitory domain of Pak1 (aa 70–150) was amplified by forward primer 5′-CCGGATCCGAGCGGCCAGAGAT and reverse primer 5′-CCGAATTCATCTTCAGCTGACTT and was subcloned into the pGEX-4T vector (Amersham Biosciences). All the constructs were confirmed by double-stranded DNA sequencing.

Kinase Assay—Different amounts of GST-Pak5 and its mutants were incubated with GST-Cdc42, GST-Rac1, or GST-Rho in kinase buffer (50 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 0.2 mM DTT) containing [γ-^{32}P]ATP (10 μCi) and 5 μg of myelin basic protein (MBP) as substrate. The kinase reaction was performed at 30 °C for 10 min, and the reactions were terminated by addition of SDS loading buffer.

Purification of Protein—GST fusion form of Rac1, Cdc42, and Rho proteins were purified as described previously (9). GST-Pak5 and its mutants were overexpressed in BL21 (DE3) *Escherichia coli* strain. Briefly, the bacterial cells were lysed by sonication in lysis buffer containing 50 mM Hepes, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 20% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride, and 1 mM leupeptin. The GST fusion proteins were purified using glutathione (GSH)-Sepharose 4B beads (Amersham Biosciences). After washing with lysis buffer, the bound proteins were eluted in buffer containing 8 mM reduced glutathione (Calbiochem), 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, and the eluted proteins were dialyzed in buffer containing 50 mM Hepes, pH 7.5, 5 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT, and 10% glycerol overnight before storage in −80 °C.

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[1] The abbreviations used are: Pak, p21-activated protein kinase; AID, autoinhibitory domain; PBD, p21-binding domain; GST, glutathione *S*-transferase; aa, amino acids; KI, kinase inhibitor; GTPγS, quanosine 5′-(3-thiotriphosphate); DTT, dithiothreitol; MBP, myelin basic protein; PVDF, polyvinylidene difluoride.

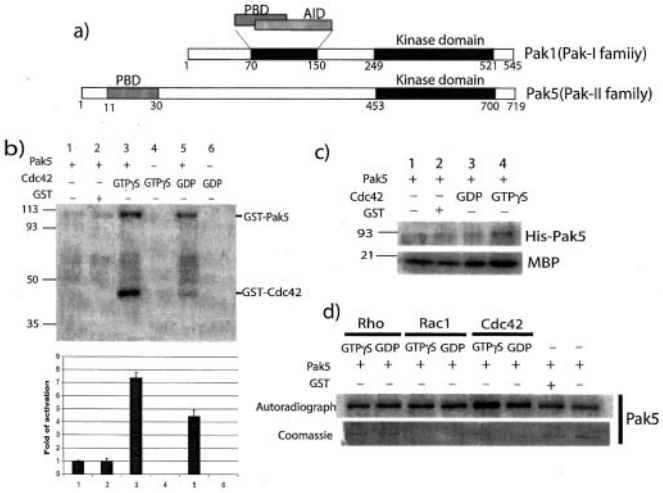

FIG. 1. **Activation of Pak5 autophosphorylation by Cdc42.** *a*, the diagram showed the common structural features of Pak1 and Pak5. PBD and AID denoted p21-binding domain and autoinhibitory domain, respectively. The numbers represent the corresponding amino acid residues of the sequence. *b*, purified GST-Pak5 (2.5 μM) was incubated with GST-Cdc42 (20 μM) preloaded with GTPγS or GDP as described under "Experimental Procedures." *Lanes*: *1*, Pak5 alone; *2*, Pak5 with GST control; *3*, Pak5 with Cdc42 loaded with GTPγS; *4*, Cdc42 loaded with GTPγS alone; *5*, Pak5 with Cdc42 loaded with GDP; *6*, Cdc42 loaded with GDP alone. The molecular weight markers are indicated on the *left* of the film. The *bottom graph* shows the quantitation with S.E. indicated of the GST-Pak5 autophosphorylated band by densitometer of at least three independent experiments. *c*, His-Pak5 (2 μM) was incubated with the GST-Cdc42 (20 μM) preloaded with GTPγS and GDP as described under "Experimental Procedures." *Lanes*: *1*, Pak 5 alone; *2*, Pak5 with GST control; *3*, Pak5 with Cdc42 loaded with GDP; *4*, Pak5 with Cdc42 loaded with GTPγS. The autophosphorylated band of His-Pak5 and phosphorylation band of MBP were indicated. *d*, activation of Pak5 autophosphorylation by Rho GTPases. GST-Rac1, Cdc42, and Rho (20 μM) preloaded with GTPγS or GDP were incubated with an equal amount of GST-Pak5 (2.5 μM). The autophosphorylated bands and the Coomassie Blue-stained GST-Pak5 bands are shown.

Cell Transfection and Affinity Binding Assay—293T cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum at 37 °C in a 5% CO_2 air atmosphere. Cells were transfected by calcium phosphate precipitated method at 40–60% confluence using 10 μg of plasmid DNA. At 24 h post-transfection, the transfected cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, and 1 mM DTT) with 1 mM PMSF, 1 mM aprotinin, and 1 mM leupetin on ice for 30 min. 20 μg of GST-Cdc42 and GST protein was first bound to the GSH-Sepharose beads and then incubated with 70 μg of transfected cell lysate for 1 h at 4 °C. The beads were then washed three times with RIPA buffer.

RESULTS

Activation of Pak5 by Cdc42-GTP—All Pak family kinases contained two common structural features, *i.e.* the kinase domain and the PBD. However, the AID, which partially overlapped with PBD domain, has only been identified in Pak-I family (Fig. 1*a*). Due to the absence of AID, it is unclear whether Pak-II family kinases are regulated by Rho GTPases. To address this question, we have purified the full-length Pak5 in GST fusion form and tested the activation of the Pak5 by *in vitro* reconstitution kinase assay. As shown in Fig. 1*b*, incubation of purified bacterially expressed GST-Pak5 with GST control or by itself had a very low level of basal autophosphorylation. However, incubation of GST-Pak5 with Cdc42 preloaded with GTPγS (Cdc42-GTPγS), a non-hydrolyzable form of GTP, greatly enhanced the autophosphorylation of Pak5 by more than 7-fold, whereas Cdc42 preloaded with the GDP (Cdc42-GDP) also activated the autophosphorylation of Pak5, but to a much lesser extent, by about 4-fold. Cdc42 protein appeared to be phosphorylated by Pak5 in kinase assay, because there is a

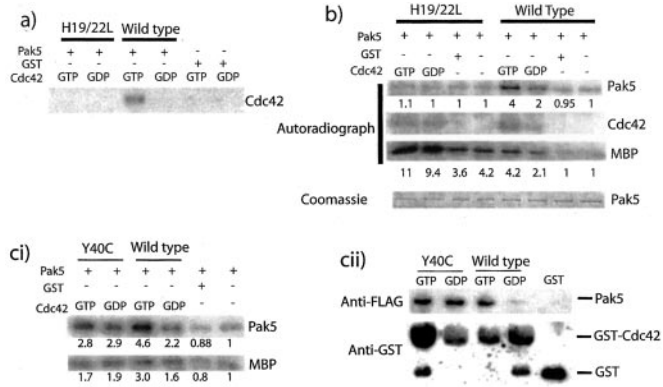

FIG. 2. **Associations of Cdc42 and Pak5.** *a*, Cdc42 was prepared by removing the GST tag from the GST-Cdc42. An equal amount of GST-Pak5 (H19L/H22L (*H19/22L*)) mutant, GST-Pak5, or GST was incubated with Cdc42 preloaded with GTPγS and GDP. The proteins were allowed to bind for 1 h and precipitated by GSH-Sepharose beads. The beads were washed three times with kinase assay buffer, and the precipitates were separated by SDS-PAGE, transblotted to PVDF membrane, and probed with anti-Cdc42 antibody (Santa Cruz Biotechnology). *b*, activation of wild type and mutant Pak5 autophosphorylation and kinase activity by Cdc42. An equal amount (2.5 μM) of wild type and H19L/H22L mutant of Pak5 was incubated by itself, with GST, or GST-Cdc42 (20 μM) preloaded with GTPγS or GDP. The autophosphorylation of Pak5 and phosphorylation of MBP was shown. The *bottom* of the figure shows the relative intensity of the phosphorylated bands. *ci*, wild type and Y40C mutant of GST-Cdc42 (20 μM) preloaded with GDP or GTPγS were incubated with GST-Pak5 (2.5 μM) for 10 min at 30 °C. The GST protein and Pak5 alone were included as control. The autophosphorylation of Pak5 and phosphorylation of MBP are shown. *cii*, an equal amount of the GST control, wild type, and Y40C mutant of GST-Cdc42 (50 μM) was mixed with FLAG-Pak5-transfected cell lysate as described under "Experimental Procedures." The GSH beads precipitate was detected by Western blot analysis using anti-FLAG and anti-GST antibodies.

kinase phosphorylation site at the linker region between the GST-tag and Cdc42. The up-regulation of autophosphorylation and activation of Pak5 by Cdc42-GTPγS was also observed in purified His-tagged form of Pak5 (Fig. 1*c*), ruling out the possibility that the two proteins were brought together by GST dimerization.

Since both Cdc42 and Rac activate Pak1, the activation of Pak5 by other Rho GTPase was examined. Fig. 1*d* shows that the activation of Pak5 autophosphorylation was specific for Cdc42. Neither Rac1 nor RhoA preloaded with GTP activated Pak5 autophosphorylation. This observation is in agreement with other reports (6, 8), which suggested that Pak5 binds to Cdc42 preferentially.

Mutation of PBD of Pak5 Completely Abolished Binding of Cdc42—To examine whether the stimulation of Pak5 autophosphorylation requires the binding of Cdc42, the histidine residues 19 and 22 of Pak5, corresponding to the conserved histidine residue 83 and 86 of Pak1, were mutated to leucine (H19L/H22L). These two residues within PBD have already been shown to be essential for the association of Cdc42 and Rac1 (10). As shown in Fig. 2*a*, the H19L/H22L mutant, but not the wild type, of Pak5 was unable to bind to Cdc42-GTPγS in the affinity binding assay. To further confirm that the Cdc42 binding is required for the activation of Pak5, the H19L/H22L mutant of Pak5 was incubated with Cdc42-GTPγS and tested for autophosphorylation. As indicated in Fig. 2*b*, the H19L/H22L mutant autophosphorylation was not enhanced by incubation with Cdc42. However, the basal kinase activity of H19L/H22L was about 4-fold higher than that of the wild type, and the activity was even higher by about 9- and 11-fold in the presence of Cdc42 preloaded with GDP and GTPγS, respectively (Fig. 2*b*). The reason for this activation remains to be determined.

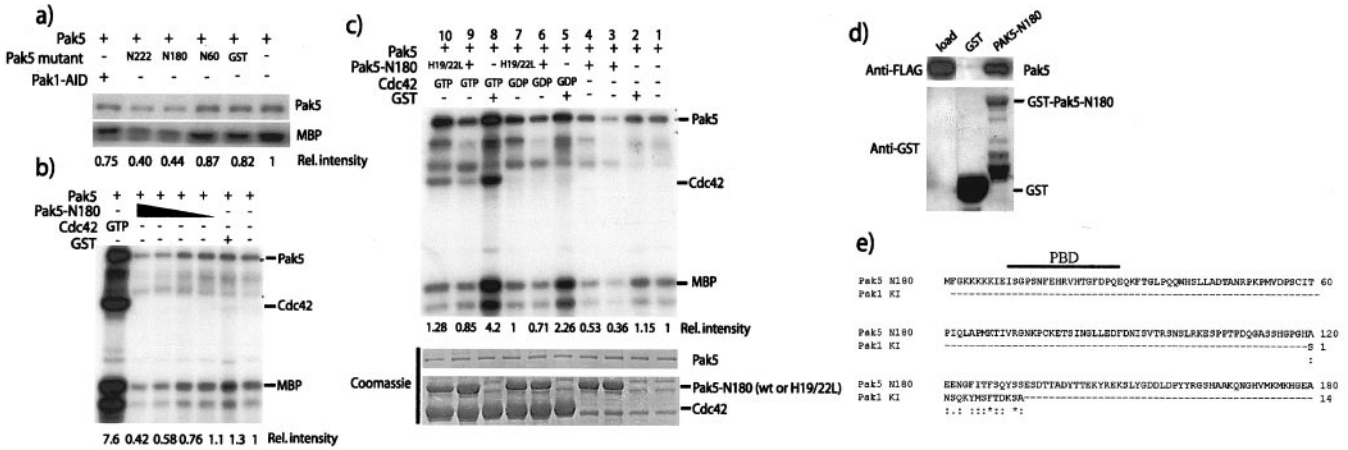

FIG. 3. **Mapping of the autoinhibitory domain of Pak5.** *a*, GST-Pak5 was incubated with (1 μM) C-terminal truncation mutants of Pak5, including N222, N180, and N60, or Pak1 autoinhibitory domain (Pak1-AID), and the GST protein was included as control. The *upper strip* shows the autophosphorylation of Pak5, and the *lower strip* shows the phosphorylation of MBP and the quantitation of the phospho-MBP bands. *b*, increasing amounts of N180 mutant (0.1, 0.5, 1, and 2 μM) were incubated with Pak5. GST and GST-Cdc42 preloaded with GTPγS were used as negative and positive controls, respectively. The *bottom* of the figure shows the relative intensity of the phospho-MBP bands. *c*, an equal amount of GST-Pak5 was incubated with GST-Pak5 N180 and N180 H19L/H22L fragments in the presence and absence of the GST-Cdc42 preloaded with GTPγS and GDP. GST was included as a control. The autoradiograph shows the phosphorylation of Pak5, Cdc42, and MBP and the relative intensity of the phospho-MBP bands. The Coomassie-stained bands of Pak5, Cdc42, N180 and N180 H19L/H22L bands are shown. *d*, an equal amount of the FLAG-Pak5-transfected cell lysate (50 μg) was mixed with the GST and GST-Pak5 N180 fragment. The precipitate was separated on SDS-PAGE, transferred to PVDF, and probed with anti-FLAG and anti-GST antibody. *e*, the KI fragment from Pak1 was aligned to the N180 fragment of the Pak5 using the ClustalW program. PBD denotes the putative p21-binding domain of Pak5. The *asterisks*, *double dots*, and *single dots* represent identical, similar, and related amino acids, respectively.

To investigate the binding of Pak5 to Cdc42, the tyrosine residue at 40 position of Cdc42 was mutated to cysteine (Y40C), which has been shown to abolish the Cdc42 binding to Pak1 (11). Incubation of the GTP and GDP bound forms of Y40C mutant with Pak5 both enhanced Pak5 autophosphorylation and kinase activity to a similar extent, whereas the GTP and GDP forms of wild type Cdc42 had a remarkable difference in the Pak5 activation (Fig. 2*ci*). To confirm whether the Y40C mutant can still bind to Pak5, an affinity pulldown assay was performed. While the GST control and wild type Cdc42-GDP were unable to precipitate Pak5 from the transfected cell lysate, the wild type Cdc42-GTPγS, and both the GDP- and GTP-bound forms of Y40C mutants, were able to precipitate Pak5 (Fig. 2*cii*) from the transfected cell lysate.

Mapping of the Autoinhibitory Domain of PAK5 by Deletion Mutants Analysis—The activation of Pak5 by Cdc42 suggests that there may be an autoinhibitory domain within the Pak5 sequence, like that in Pak1. To map this domain, several truncation mutants of Pak5 were constructed and their inhibitory activity was examined. As shown in Fig. 3*a*, incubation of the N-terminal 180 and 222 aa (N180 and N222) fragment of Pak5 inhibited about 60% of the kinase activity and autophosphorylation of GST-Pak5. However, if the N-terminal fragment was shortened to only 60 (N60) aa, the inhibitory activity was lost, indicating that the 120-aa fragment (from residues 60–180) was essential for the inhibition. The inhibition was specific, since neither the GST control nor the autoinhibitory domain (aa 70–150) of Pak1 blocked the kinase activity of Pak5. Furthermore, the N180 fragment was able to inhibit the Pak5 activity in a dose-dependent manner. Incubation of increasing amount of N180 fragment with Pak5 reduced its kinase activity up to about 40%, whereas incubation of Cdc42-GTPγS with Pak5 increased its kinase activity more than 7-fold (Fig. 3*b*). To further examine whether the N180 fragment was able to block the activation of Pak5 induced by Cdc42, the N180 fragment was added to a mixture of Pak5 and Cdc42 preloaded with GTPγS and GDP. As shown in Fig. 3*c*, both autophosphorylation and kinase activity of Pak5 was inhibited by the N180 fragment (Fig. 3*c*, *lanes 3*, *6*, and *9*). Furthermore, the same fragment from the H19L/H22L mutant of Pak5 was also able to inhibit the basal and Cdc42-induced Pak5 kinase activity, but almost had on effect on the autophosphorylation (Fig. 3*c*, *lanes 4*, *7*, and *10*). The inhibitory activity of N180 H19L/H22L fragment was, however, slightly lower than the wild type (Fig. 3*c*, *lanes 3* and *4*). Since the N180 H19L/H22L fragment is defective in the binding of Cdc42-GTP, this data strongly suggested that the N180 inhibits Pak5 kinase activity not by sequestering the Cdc42-GTP. To demonstrate that the N180 inhibitory fragment can still bind to Pak5, an affinity pull-down assay was performed. The GST-Pak5 N180 fragment, but not the GST control, was able to precipitate the FLAG-tagged full-length Pak5 from the transfected cell lysate, indicating that the autoinhibitory domain was able to bind Pak5 (Fig. 3*d*). Interestingly, when the KI fragment, *i.e.* residues 137–149, of Pak1 (5) was aligned to the N180 fragment, it was mapped to the residues 119–123 of Pak5 sequence (14% identity and 71% similarity), suggesting that this region might have similar inhibitory function (Fig. 3*e*).

DISCUSSION

Pak5 is a recently identified Pak-II family member, whose regulation is still largely unknown. Pak5 contains a highly conserved PBD, but lacks an identifiable autoinhibitory domain. In the present study, we have shown that active form of Cdc42, but not Rac1 and Rho, protein was able to activate the purified GST-Pak5 autophosphorylation and kinase activity. Mutations of Pak5, which disrupted the interaction of Cdc42 and Pak5, also abolished the induction of autophosphorylation. Using the deletion mutants, a 180-aa fragment at the N terminus (N180) of Pak5 was found to contain inhibitory activity toward Pak5 autophosphorylation and kinase activity. The N180 fragment also blocks the Cdc42-mediated Pak5 activation. The mechanisms by which N180 fragment inhibits Cdc42-induced Pak5 activity can be 2-fold. First the N180 fragment, containing PBD, may simply compete with the full-length Pak5 for Cdc42 protein, leading to the inhibition of autophosphorylation. Second, the fragment may contain an inhibitory domain that directly binds to full-length Pak5 and inhibits its activity.

However, since incubation of the N180 fragment with Pak5 in the absence of Cdc42 inhibits its autophosphorylation and activity (Fig. 3*b*) and the N180 H19L/H22L fragment, which is defective in the binding of Cdc42-GTP, also inhibits the Cdc42-induced Pak5 kinase activity (Fig. 3*c*), these data demonstrate conclusively that the N180 contains a distinct inhibitory domain, and the inhibition is not mediated by sequestering Cdc42-GTP. Taken together, these results suggest that Pak5 contains a previously unidentified autoinhibitory domain and may be regulated by a mechanism similar to Pak1.

In this report, we have identified a novel inhibitory fragment of Pak5 that may potentially be important in the regulation of Pak5 activity. So far the inhibitory fragment that we mapped is about 120 aa (residues 60–180). As hinted from the sequence alignment data, the amino acids that are essential for the inhibition may fall within the residues 119–123 of Pak5 sequence (Fig. 3*d*). By sequence alignment analysis, it revealed that the residues 117–140 of Pak5 sequence were not conserved in the Pak-II family (data not shown). Noticeably, this region is included in the Pak5 inhibitory fragment that we mapped. Interestingly, the residues 119–123 can only be found in Pak5, but not in Pak4 or Pak6. This observation has raised the possibility that the activity of at least Pak4 and Pak6 is not regulated by autoinhibition. This speculation is consistent with the prevailing view that Pak4 is constitutively active and its activity is independent on the Cdc42 protein (7). An attempt to express the wild type GST-Pak4 protein was unsuccessful, because the yield of the protein is extremely low. This may be due to some unidentified bactericidal effect of GST-Pak4, similar to Pak1. Although it remains to be determined whether Pak4 and 6 also contain an inhibitory fragment, our data have proposed that the regulation of the activity within Pak-II family members can be quite different. In terms of Pak5 regulation, one report has suggested that Pak5 activity was independent on Cdc42 (6). However, the discrepancy in data may be attributed to the difference in the system and mutation that employed in the studies.

The H19L/H22L mutant of Pak5 was insensitive to the Cdc42-induced autophosphorylation. However, its basal activity seems to be higher than that of the wild type, suggesting that the wild type is in a sub-optimal activity state. This is likely due to the presence of autoinhibitory domain, and the mutations of residues that are important for the binding of the Cdc42 may have disrupted the binding of AID to the Pak5. Unexpectedly, the addition of the Cdc42 protein to the H19L/H22L mutant increased the Pak5 kinase activity without affecting the autophosphorylation of Pak5. The reason for this is warrant for further investigation.

Cdc42 Y40C mutant has been well documented to be defective in binding of Pak-I family members. However, evidences have shown that the Y40C mutant is still able to induce the actin polymerization and formation of filopodia (12). Thus, in addition to Pak1, there may be other downstream targets of Cdc42 that regulate actin polymerization. Our data have indicated that both GDP and GTP bound form of the Y40C mutant can bind to Pak5 of similar affinity. The mutation has only led to a loss of the specificity on GTP bound form of Cdc42 but not the association. Another member of Pak-II family, Pak4, has also been shown to associate with Y40C mutant (7). Thus, these data suggest that the binding site of Cdc42 to Pak-II may be different from Pak-I, and it may reveal a potential interesting capacity of Cdc42 in control actin polymerization by simultaneously regulating both Pak-II and Pak-I family members activity.

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