Regulation of the Interaction of Pak2 with Cdc42 via Autophosphorylation of Serine 141*

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Pak2, a member of the p21-activated protein kinase (Pak) family, is activated in response to a variety of stresses and is directly involved in the induction of cytostasis. At the molecular level Pak2 binds Cdc42(GTP), translocating Pak2 to the endoplasmic reticulum where it is autophosphorylated and activated. Pak2 is autophosphorylated at eight sites; Ser-141 and Ser-165 in the regulatory domain and Thr-402 in the activation loop are identified as key sites in activation of the protein kinase. The function of phosphorylation of Ser-141 and Ser-165 on the activation was analyzed with wild-type (WT) and mutants of Pak2. With S141A, the level of autophosphorylation was reduced to 65% as compared with that of WT and S141D with a concomitant 45% reduction in substrate phosphorylation, indicating that phosphorylation at Ser-141 is required for optimal activity. Autophosphorylation inhibited the interaction between WT Pak2 and Cdc42GTP. In 293T cells, WT Pak2, S141A, and S141D formed a stable complex with the constitutively active mutant Cdc42 L61, but not with the dominant negative Cdc42 N17. As shown in glutathione S-transferase pull-down assays, S141A bound to Cdc42(GTP) at a 6-fold higher level than that of S141D. In contrast, the S165A and S165D mutants had no effect on autophosphorylation, binding to Cdc42, or activation of Pak2. In summary, autophosphorylation of Ser-141 was required for activation of Pak2 and down-regulated the interaction of Pak2 with Cdc42. A model is proposed suggesting that binding of Cdc42 localizes Pak2 to the endoplasmic reticulum, where autophosphorylation alters association of the two proteins.

The p21-activated protein kinase (Pak) family members are serine/threonine protein kinases known as major targets of the small GTPases Cdc42 and Rac1. The Pak enzymes are differentially involved in many cellular processes including cytoskeletal organization, cell motility, cell growth, cell stress, and apoptosis (1, 2). Three highly conserved (78% identity) isoforms of the Pak family have been identified, namely Pak1 (α-Pak), Pak2 (γ-Pak), and Pak3 (β-Pak) (3–5). The C terminus contains the catalytic domain, and the N terminus contains the regulatory p21-binding domain and an autoinhibitory domain (AID).

The role of autophosphorylation in the activation of Pak has been analyzed with Pak1 and Pak2. Manser et al. (6) mapped the sites of autophosphorylation in Cdc42(GTPγS)-activated Pak1 in vitro; six serine residues in the regulatory domain and a single threonine residue in the catalytic domain were identified. Gatti et al. (7) identified eight autophosphorylation sites in Pak2 that are activated by Cdc42 in vitro (serines 19, 20, 55, 141, 165, 192, and 197 and threonine 402). Except for serines 19 and 165, six of the sites are equivalent to those in Pak1. Autophosphorylation of Pak2 in the absence and presence of Cdc42 showed that autophosphorylation of Ser-141, Ser-165, and Thr-402 is specific for active Pak2 (7). The conserved threonine in the activation loop of the catalytic domain, Thr-402 in Pak2 and Thr-423 in Pak1, was shown to be required for full activation of Pak (8–11). Among members of Pak family, Pak2 is the only isoform that is cleaved and activated by caspase 3 (10, 12).

In contrast to Pak1, which is activated by growth factors (6, 13–15), Pak2 is activated in response to cellular stresses such as DNA damage, serum starvation, and hyperosmolarity (16–18). Pak1 is localized to the lamellipodia and filopodia and regulates cytoskeletal structure when stimulated by platelet-derived growth factor (19). In contrast, Pak2 is translocated to the endoplasmic reticulum (ER) by Cdc42 and becomes activated, resulting in the induction of cytostasis (17, 20). Thus, activation and localization of Pak proteins are tightly regulated.

Zhao et al. (21) showed that Cdc42-mediated activation of Pak1 was inhibited 7-fold by a polypeptide containing the AID (amino acids 83–149). The inhibitory effect of AID was also observed in HeLa cells with a constitutively active mutant of Pak1 (21). When two autophosphorylated serines in the regulatory domain of Pak1, Ser-144 and Ser-149, were mutated to alanine, the activity of the S144A/S149A mutant was greatly reduced (22). Autophosphorylated Pak1 or AID phosphorylated at Ser-144 and Ser-149 completely abolishes the inhibitory effect of the AID on Pak1 activation (21, 22).

In this study, we examined the role of autophosphorylation of Ser-141 and Ser-165 in the Cdc42-dependent activation of Pak2. With S141A activated by Cdc42(GTPγS), autophosphorylation and activity measured by phosphorylation of the peptide substrate S3 were significantly reduced as compared with those of WT. The other serine mutants (S141D, S165A, and S165D) showed a similar activity to that of WT Pak2. A stable association between Pak2 and Cdc42(GTP) in vitro and in 293T cells was detected with WT Pak2 and S141A or S141D by the GST pull-down assay and coimmunoprecipitation. Binding of active Cdc42 with S141A was 5-fold higher than with S141D. In contrast, with the other mutants (T402A, T402E, K278R, S165A, S165D), no stable interaction with Cdc42 was detected. Thus, phosphorylation of Ser-141 had a negative effect on the association between Pak2 and Cdc42. These results suggest that Ser-141 is a potential site for manipulation of the cytoskeletal properties of Pak2 by regulating the localization of Pak2 to the ER.

**Experimental Procedures**

Materials—Dulbecco’s modified Eagle’s medium and cell culture reagents were purchased from Invitrogen. The insect cell culture medium EX-CELL 401 was from JRH Biosciences. Protein A/G agarose and the antibody to the N terminus of Pak2 (N-19) were purchased from Santa Cruz Biotechnology. Horseradish peroxidase-conjugated goat anti-mouse antibody was purchased from Pierce Chemical Co.
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anti-mouse IgG was from BD Pharmingen. Mouse monoclonal anti-HA antibody (HA.11) was from Covance (Berkeley, CA). [γ-32P]ATP was purchased from PerkinElmer Life Sciences. ATP, GTP·γS, and trypsin (diphenyl carbamol chloride-treated) were from Sigma. Histone 4 (H4), FuGENE6 reagent, aprotinin, leupeptin, pepstatin, and phenylmethylsulfonyl fluoride were obtained from Roche Applied Science. Okadac acid was from Calbiochem. Cellulose thin layer chromatography (TLC) sheets were purchased from Selecto Scientific (Suwanee, GA), and silica gel TLC sheets were from EM Science. P81 phosphocellulose paper was obtained from Whatman. Polyvinylidene difluoride membrane was from Millipore. Glutathione-Sepharose 4B was from Amersham Biosciences. Oligonucleotides for the preparation of mutations of Pak2 were purchased from Annovis. *Pfu* polymerase was from Stratagene. GeneClean II kit was purchased from Bio101. Miniprep and Maxiprep DNA purification kits were from Qiagen. T4 DNA ligase and restriction enzymes were from New England Biolabs. Peptide substrate S3 was prepared by PeptideGenic Research. The Bradford assay reagent was from Bio-Rad. The bacterial expression plasmid pET21 for caspase 3 was generously provided by Drs. G. S. Litwack and E. S. Alnemri, Thomas Jefferson University, Philadelphia, PA. The clones for Cdc42 L61 and Cdc42 N17 were kindly provided by Dr. J. S. Gutkind, National Institutes of Health (Bethesda, MD), and were subcloned into the pcDNA3.1+ vector as described (17). The clone for GST-Cdc42 was generously provided by Dr. Channing Der, University of North Carolina, Chapel Hill, NC. GST-Cdc42 was expressed in *Escherichia coli* and purified as described (5, 10). The DNA constructs for WT Pak2 and the mutants T402A, T402E, and K278R were described previously (8).

**Site-directed Mutagenesis**—To prepare the Pak2 mutants S141A, S141D, S165A, and S165D, site-directed mutagenesis was performed by a PCR-based megaprimers method using the *Pfu* polymerase (8, 23). To mutate Ser-141 to alanine the 5′-CTCCGAGGAGTAGGGCCAGGATCTTCAGTTCTC-3′ was used, and for aspartate the 5′-CTCCGAGGAGTAGGTTCCAGGTATCTTGGCCAC-3′ was used. The codon for the mutation site is underlined.

**Expression and Purification of Recombinant Pak2—TN5B-4**

Insect cells were maintained in EX-CELL 401 with 50 μg/ml gentamicin and incubated at 27°C. The pAcG2T plasmids for S141A, S141D, S165A, and S165D were individually transfected into insect cells and, after 2 days, the cells were collected and lysed as described previously (8). GST-Pak2 was purified by affinity chromatography on glutathione-Sepharose 4B beads and released from the beads with 10 mM reduced glutathione in 50 mM Tris-HCl (pH 8.0) or the GST was removed by cleavage with thrombin as described elsewhere (10).

**Autophosphorylation of Pak—Recombinant GST-Pak2**

[γ-32P]ATP was autophosphorylated in a 30-μl reaction mixture containing 20 mM Tris-HCl (pH 7.4), 10 mM MgCl2, 30 mM β-mercaptoethanol, and 0.2 mM [γ-32P]ATP (specific activity was 1,000 cpm/pmole) for 20 min at 30°C in the absence and presence of Cdc42 (6 μg), preloaded with GTP·γS (0.18 mM) for 10 min at 30°C as described elsewhere (5). To terminate autophosphorylation, 10 μl of SDS sample buffer was added. Autophosphorylation of Pak2 was analyzed by SDS-PAGE followed by staining with Coomassie blue and autoradiography.

**Protein Kinase Assays**—To measure the protein kinase activity of Pak2, phosphorylation of the peptide substrate S3 (AKRESAA) was carried out in a 25-μl reaction mixture containing pre-autophosphorylated GST-Pak2 (0.22 μg) as described above, with the addition of 1 mM S3 and 10 μg of bovine serum albumin (24). The reaction was incubated for 10 min at 30°C and terminated on ice with 6 μl of 100 mM non-radio-labeled ATP. The peptide was precipitated on P81 phosphocellulose paper with 75 mM H3PO4. The paper was washed with 75 mM H3PO4 three times, washed with water twice, and dried. S3 phosphorylation was quantified by scintillation counting.

**Tryptic Phosphopeptide Mapping**—To perform tryptic phosphopeptide mapping, autophosphorylated GST-Pak2 was excised from the polyacrylamide gel and extensively digested with trypsin as described by Tuazon et al. (25). Peptides were separated in the first dimension by electrophoresis and in the second dimension by thin layer chromatography. 32P-labeled peptides were detected using the PhosphorImager SI (Amersham Biosciences).

**Culture and Transfection of 293T Cells**—Cells (5 × 105) were plated in 6-well plates and transfected with HA-Pak2 in the pcDNA 3.1+ vector alone (0.4 μg) or with HA-Cdc42 L61 or N17 in pcDNA 3.1+ (0.2 μg) using the FuGENE6 reagent as described by the manufacturer. The transfected DNA was normalized to 0.6 μg by addition of the empty vector. At 24 h post-transfection the cells were collected, washed twice with chilled phosphate-buffered saline, and frozen at −80°C prior to further analyses.

**Immunoprecipitation and Western Blotting**—To prepare the cell lysate for immunoprecipitation of HA-Pak2, frozen cell pellets were resuspended in 200 μl of lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 5 mM dithiothreitol, 1% Nonidet P-40, phosphatase inhibitors (50 mM NaF, 5 mM Na3VO4, 10 mM okadaic acid, and 1 mM Na2VO4), and protease inhibitors (4 μg/ml aprotinin, 4 μg/ml leupeptin, 10 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride). Resuspended cells were kept on ice for 20 min, and lysates were cleared by centrifugation at 14,000 × g for 10 min at 4°C. Total protein was quantified using Bradford assay with bovine serum albumin as a standard protein.

To analyze the interaction between Pak2 and Cdc42, 200 μg of lysate were incubated with 0.4 μg of Pak2 antibody N-19 for 1 h at 4°C. Additionally, 20 μl of protein A/G agarose beads (1:1 slurry solution) were added, and the sample was incubated for 1 h. Immunoprecipitates were collected by centrifugation at 1,000 × g for 1 min and washed three times with lysis buffer. The samples were separated by SDS-PAGE on a 15% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. HA-Pak2 and HA-Cdc42 were detected by Western blotting with anti-HA antibody as described (20).

**GST Pull-down Assay**—To study the effect of autophosphorylation on the interaction of Pak2 with Cdc42, 0.2 μg of Pak2 was incubated
with 1 μg of GST-Cdc42 pre-loaded with GTPγS in the absence and presence of ATP (0.2 mM) for 30 min at 30 °C in a 40-μl reaction containing 20 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, and 30 mM β-mercaptoethanol. Glutathione beads (20 μl) were added, and the samples were incubated for 30 min at room temperature. The beads were washed three times with 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.5% Triton X-100. Pak2 bound to Cdc42 was analyzed by SDS-PAGE and Western blotting with the Pak2 antibody N-19. GST-Cdc42 was detected by Coomassie staining of the membrane.

GST-Cdc42(GTPγS) was incubated with 293T cell lysate (200 μg) containing WT or mutants of HA-Pak2 at 4 °C for 30 min and then incubated with 30 μl of glutathione beads at 4 °C for 1 h. The beads were spun down and washed three times with lysis buffer. Bound Pak2 was analyzed by SDS-PAGE and Western blotting with anti-HA antibody and quantified using NIH ImageJ.

Data Analysis—All of the observations were confirmed in at least three independent experiments. The statistical analyses were performed using the Student’s t test. A p value of 0.05 was considered to be statistically significant.

RESULTS

Analysis of Autophosphorylation of Wild-type and Mutants of Pak2—Autophosphorylation of Pak2 at serines 141 and 165 required Cdc42(GTP). Ser-141 is situated at the C terminus of the AID domain, whereas Ser-165 is located in a region of undefined function (Fig. 1A). Ser-141 is conserved in Pak1 (Ser-144) and Pak3 (Ser-139); Pak1 also contains a second autophosphorylation site at Ser-149. Ser-165 is present only in Pak2 from rabbit and rat; all other isoforms lack an autophosphorylation site at this position. Ser-141 and Ser-165 are conserved in all forms of Pak. S165 is present only in rabbit and rat Pak2 and is replaced by proline in human Pak1/2/3.

To examine the effects of autophosphorylation of Ser-141 and Ser-165 on Cdc42-mediated activation of Pak2, the two sites were mutated to either alanine or aspartate mimicking a non-phosphorylated or a phosphorylated residue, respectively. WT Pak2, the mutants S141A, S165A, A141D, S165D, and T402A were expressed and purified from insect cells as GST fusion proteins and autophosphorylated in the absence and presence of Cdc42(GTPγS). The extent of autophosphorylation of WT Pak2 with Cdc42 was maximal at 20 min. WT Pak2 and the mutants, autophosphorylated in the absence of Cdc42, migrated as a single band. Slower migrating bands were observed on SDS-PAGE when Pak2 was incubated with Cdc42(GTPγS) because of increased autophosphorylation (Fig. 2A, top section). With Cdc42, the two major bands of WT Pak2 were shifted upward on SDS-PAGE. Similar shifts were detected with serine 141 and serine 165 mutants. One shifted band was observed for T402A with Cdc42, which matched the faster migrating band of the two bands for WT with Cdc42.

As shown by autoradiography (Fig. 2A, bottom section), there was a significant increase in the autophosphorylation of WT, S141A and S141D, and S165A and S165D upon the addition of Cdc42. Essentially no phosphorylation of T402A was observed in the absence of Cdc42, and T402A had a low level of autophosphorylation with Cdc42. To compare the extent of autophosphorylation between WT and mutant forms of GST-Pak2, the level of autophosphorylation was normalized to the amount of Pak2 protein, and autophosphorylation of WT Pak2 in the presence of Cdc42(GTPγS) was set at 1.00 (Fig. 2A). With Cdc42, the two major bands of WT Pak2 were shifted upward on SDS-PAGE. Similar shifts were detected with serine 141 and serine 165 mutants. One shifted band was observed for T402A with Cdc42, which matched the faster migrating band of the two bands for WT with Cdc42.

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To further evaluate the mutants of Pak2 in terms of autophosphorylation, WT Pak2 and the mutants phosphorylated in the presence of Cdc42(GTP[S]) were analyzed by tryptic phosphopeptide mapping. The two-dimensional map of WT Pak2 had 10 identified phosphopeptides as shown in the schematic (Fig. 3, upper left and middle sections). Previously, Gatti et al. (7) sequenced the tryptic phosphopeptides of Pak2 following two-dimensional peptide gel electrophoresis. The identified phosphopeptides were individually eluted from the peptide gel and analyzed in the two-dimensional system used in these experiments.3 The phosphopeptides are summarized in Fig. 3, lower left section.

To examine the result of autophosphorylation, the three mutants were compared with WT Pak2. Two phosphopeptides, 7 and 8, previously identified as Thr-402, were missing in the map of T402A (Fig. 3, upper right section). Spot 3 corresponded to Ser-141, and phosphopeptide 3 was missing in the map of S141A (Fig. 3, lower middle panel). In the map of S165A, all of the spots were detected (Fig. 3, lower right panel). This was due to the fact that spots 6 and 9 also contained peptides with autophosphorylated Ser-197 (7). Although the extent of autophosphorylation of S141A and T402A was reduced as compared with the WT level, all of the phosphopeptides except those containing the mutation site were detected in the two-dimensional maps. Thus, phosphorylation of Ser-141 and Thr-402 could alter the conformation of Pak2 rather than directly regulate autophosphorylation at other sites.

The Protein Kinase Activity of WT Pak2 and Pak2 Mutants—To determine the effects of the mutations of the autophosphorylation site mutants, WT and mutant forms of Pak2 were assayed with the peptide substrate S3. Pak2 was pre-autophosphorylated in the absence and presence of Cdc42(GTP[S]), and the substrate was added and phosphorylated under kinetically valid conditions where <5% of S3 was phosphorylated. The rate of phosphorylation of S3 with WT Pak2 was increased 6-fold with Cdc42(GTP[S]); the level of phosphorylation of S3 by Cdc42-activated Pak2 was set as 1.00 (Fig. 4). The rate of phosphorylation with S141A was stimulated 5-fold by Cdc42(GTP[S]), but the level was reduced to 0.56 as compared with that of WT, although S141D was similar to WT (0.95). Phosphorylation of S3 with S165A was increased 10-fold by Cdc42 to 1.21, whereas the activity of S165D was 0.88. There was little or no substrate phosphorylation by T402A with and without Cdc42. Taken together, the data indicated that autophosphorylation of Ser-141 was required for full protein kinase activity. In contrast, the substitution of Ser-165 to alanine or aspartate had essentially no inhibitory effect on Pak2 activity. Similar effects were observed with histone 4 and myelin basic protein (data not shown).

Interaction of Pak2 with Cdc42—Because Pak2 was activated by association with Cdc42 and autophosphorylation, we examined whether autophosphorylation of Pak2 altered the interaction between Pak2 and Cdc42. Purified Pak2 was incubated with GST-Cdc42(GTP[S]) in the absence and presence of ATP, and Pak2 bound to Cdc42 was analyzed by SDS-PAGE and Western blotting (WB) with anti-Pak2 antibody (top section, GST pull-down). The total amount of Pak2 is shown (input). The amount of GST and GST-Cdc42 proteins are shown by Coomassie staining (bottom section). The result is a representative of four independent experiments.

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3 P. T. Tuazon and J. A. Traugh, unpublished data.
expression of S141A was 60% lower than that of WT, whereas the expression S165A was slightly higher than that of WT (Fig. 6A, upper section). In contrast, other mutants such as kinase-negative K278R and T402A, kinase-active T402E, and the phosphomimics S141D and S165D were expressed at a 3- to 5-fold higher level than WT, as shown by SDS-PAGE and Western blotting. This result is consistent with previous data showing that the expression of Pak2 is tightly regulated by ubiquitination and degradation through the proteosome pathway because of the cystostatic nature of the protein kinase (20, 26). In contrast, mutants that lack the ability to induce cytostasis are expressed at significantly higher levels. The reduced expression of S141A suggests that it has similar cystostatic properties.

Cell lysates containing expressed HA-WT or mutants of Pak2 were incubated with GST-Cdc42 preloaded with GTPγS, and the Pak2 associated with GST-Cdc42 was analyzed by SDS-PAGE and Western blotting. WT Pak2 and the mutants S141D and S141A bound to GST-Cdc42 (Fig. 6A, lower sections). The other mutants, K278R, T402A, T402E, S165D, and S165D, were not detected by Western blotting and thus did not bind to Cdc42(GTPγS). When the level of Pak2 bound to Cdc42 was normalized to the amount of expressed protein, the association of S141A with Cdc42 was similar to that of WT and 6-fold higher than that of S141D (Fig. 6B). The amount of Cdc42 was not a limiting factor in the assay, as the amount of GST-Cdc42 used in these experiments was 5-fold higher than that of HA-Pak2 in the cell lysate. Thus the phosphomimic S141D bound Cdc42(GTP) to a lesser extent than S141A or WT.

The interaction between Pak2 and Cdc42 was also analyzed in vivo. 293T cells were cotransfected with HA-Pak2 and the HA-Cdc42 dominant negative N17 or constitutively active L61. Pak2 was immunoprecipitated (IP) as described under "Experimental Procedures." The immunoprecipitates were analyzed by SDS-PAGE and Western blotting (WB) with an anti-HA antibody (middle section). The GST-Cdc42 protein was detected by Coomassie staining (bottom section). & the extent of binding of Pak2 WT, S141D, and S141A to Cdc42 was quantified by densitometric analysis. The histogram shows the mean and S.D. of three independent experiments.

The expression levels of the Pak2 proteins were similar to the data shown in Fig. 6. K278R, T402A, T402E, S141D, and S165D were expressed at a 3- to 5-fold higher level than WT Pak2. WT Pak2, S141A, and S165A were significantly reduced. Cdc42 L61 and N17 were coexpressed at a similar level throughout (Fig. 7, lower sections). When the data were quantified and the ratio of Cdc42 bound to Pak2 WT set as 1.0, the ratio for S141D was 1.1 whereas that of S141A was 3.6. Thus, the interaction of Cdc42 L61 with S141A was 3-fold higher than that with S141D, which was consistent with the result of the GST pull-down assay. The data suggest that the phosphorylation status at Ser-141 can alter the interaction of Pak2 and Cdc42.

**DISCUSSION**

Pak2 is activated through dissociation of the AID from the catalytic site by the binding of Cdc42(GTP) or following caspase 3 cleavage and subsequent autophosphorylation at multiple sites. Autophosphorylation of Ser-141, Ser-165, and Thr-402 is correlated with Cdc42-dependent activation of Pak2 (7). Phosphorylation of Thr-402 is essential for the full level of autophosphorylation and activation of Pak2 following binding of active Cdc42 or cleavage with caspase 3 (8, 10). In this study we showed that autophosphorylation of Ser-141 in the regulatory domain is necessary for activation of Pak2 by Cdc42 and also has a second function, modulating the interaction with Cdc42. As summa-
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| Pak2   | Activity* | Expression in 293T cells* | Stable association with active Cdc42* |
|--------|-----------|---------------------------|-------------------------------------|
| WT     | 2         | 1                         | 2                                   |
| S141A  | 1         | 1                         | 3                                   |
| S141D  | 2         | 3                         | 1                                   |
| S165A  | 2         | 2                         | 0                                   |
| S165D  | 2         | 3                         | 0                                   |
| T402A  | 0         | 2                         | 0                                   |
| T402E  | 2         | 3                         | 0                                   |
| K278R  | 0         | 3                         | 0                                   |

* The relative activity of Pak2 was measured by S3 phosphorylation in vitro.
* The relative expression of Pak2 was determined by Western blotting following transfection of HA-Pak2 in 293T cells.
* Interaction with active Cdc42 was determined by pull-down assay with GST-Cdc42(GTPγS) and 293T cell lysates containing HA-PAK2.
* Data not shown.

Autophosphorylation of Pak2, induced by binding of Cdc42(GTP), also negatively regulates the interaction between Pak2 and Cdc42 as well as activating the protein kinase. The interaction of brain-specific Pak3 with Cdc42 and Rac1 was significantly decreased by p21-induced autophosphorylation (3). It was suggested that autophosphorylation of Pak could release the GTPases from Pak as Pak became activated. Our studies take this further. WT Pak2, S141D, and S141A binds to Cdc42(GTPγS) and constitutively active Cdc42 L61, as shown by GST pull-down assays and coinmunoprecipitation. This association is stable as compared with that of other kinase mutants that are activated by Cdc42 such as S165A, S165D, and T402A. Although the autophosphorylation and activity of these enzymes are stimulated by Cdc42, they do not form a stable complex with Cdc42. Following autophosphorylation, the continued association of Pak with Cdc42 is not required for Pak activity (2). Binding of S141A to Cdc42 is 6-fold higher than the association of S141D with Cdc42. The stable association with Cdc42 could be involved in maintaining the subcellular localization of Pak2 in the ER, which is modulated by the phosphorylation/dephosphorylation of Ser-141.

Subcellular localization as well as protein kinase activity is critical for the physiological functioning of Pak. Cdc42/Rac1-activated Pak1 has been shown to localize to focal adhesions, modulate cytoskeleton reorganizations, and stimulate cell migration (14, 28). In contrast Cdc42-activated Pak2 is localized to the ER. In exponentially growing 3T3-L1 cells, Pak2 is primarily inactive and only a small amount of total Pak2 (~5%) is associated with the ER (17, 20). In response to stress such as hyperosmolarity, Cdc42 translocates Pak2 to the ER, which results in autophosphorylation and activation (17). The association of Pak with the ER inhibits cell division, indicating a cytoplastic state (20). The Pak2 mutant S490D and the C-terminal deletion mutant ΔA488 do not localize to the ER and do not inhibit cell division even though these mutants have high levels of protein kinase activity, whereas S490A has cytoplastic properties similar to those of WT Pak2 (20).

As shown in the model in Fig 8, under cell growth conditions Pak2 is primarily inactive through the interaction of AID with the active site of the catalytic domain. Pak2 can be partially autophosphorylated without being activated. This basal level of autophosphorylation is at five serine sites (Ser-19, Ser-20, Ser-55, Ser-192, and Ser-197) in the regulatory domain (soluble inactive Pak2). When Cdc42(GTP) binds Pak2, disrupting the autoinhibitory conformation of Pak2, the Cdc42-associated Pak2 is translocated to the ER and activated by autophosphorylation at Ser-165 and Thr-402 (ER-associated, partially active Pak2). With additional phosphorylation at Ser-141, Pak2 is fully activated, leading to phosphorylation of downstream target protein(s) and cell growth arrest. At the same time, phosphorylation of Ser-141 lowers the affinity of the

TABLE ONE
Summary of the activity and the levels of expression and association with Cdc42 of WT Pak2 and the mutants

![Diagram](image-url)

**FIGURE 8.** Model for the activation of Pak2 and the association of Pak2 with Cdc42. Inactive Pak2 is stabilized by the autoinhibitory interaction between AID and the active site under growing conditions. Autophosphorylation is in the regulatory domain at the basal level. When GTP-bound Cdc42 interacts with Pak2 at the p21-binding domain (PBD), it translocates Pak2 to the membrane (ER) where it becomes autophosphorylated and activated because of a conformational change induced by Cdc42 binding, which disrupts the inhibitory interaction and enhances autophosphorylation at Thr-402 (T^PP) and Ser-165 (S^P). The partially active Pak2 is further activated by phosphorylation on Ser-141 (S^P), which leads to dissociation of Cdc42(GTP). GTP bound to Cdc42 is hydrolyzed to form Cdc42(GDP). More details of this model are described in the text.
interaction between Pak2 and Cdc42. This could alter the association of Pak2, which could promote Cdc42 to hydrolyze GTP to GDP, resulting in dissociation of Cdc42(GDP) from Pak2 (ER-associated fully active Pak2).

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