Analysis of Conformational Changes during Activation of Protein Kinase Pak2 by Amide Hydrogen/Deuterium Exchange*§

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During apoptotic stress, protein kinase Pak2 is cleaved by caspase 3 to form a heterotetramer that is constitutively activated following autophosphorylation. The active protein kinase migrates slightly slower than the inactive holoenzyme when analyzed by gel filtration, suggesting an expanded conformation. Activation of Pak2 comprises a series of structural changes resulting from caspase cleavage, ATP binding, and autophosphorylation of Pak2. Changes at each step were individually analyzed by amide hydrogen/deuterium exchange coupled with mass spectrometry and compared with inactive Pak2. The autoinhibited form was shown to bind ATP in the active site, with minor changes in the glycine loop and the autoinhibitory domain (AID). Caspase cleavage produced significant changes in solvent accessibility in the AID and upper lobe of the catalytic domain. Cleavage of ATP-bound Pak2 relaxes the allosteric inhibition, as shown by increased solvent accessibility in the upper and lower lobes, including the G-helix, facilitating the autophosphorylation of two sites required for activation, Ser-141 in the regulatory domain and Thr-402 in the catalytic domain. Autophosphorylation increased the amide hydrogen/deuterium exchange solvent accessibility of the contact region between the AID and the G-helix, the E-F loop, and the N terminus. Thus, activation of Pak2 via caspase cleavage is associated with structural relaxation of Pak2 that allows for complete autophosphorylation, resulting in a more comprehensive solvent-exposed and conformationally dynamic enzyme.

The serine/threonine protein kinase Pak2 (also known as γ-Pak) is transiently activated either in response to moderate stress by Cdc42, or during apoptotic stress by caspase cleavage, followed by autophosphorylation (1–3). Cdc42 binds to the Cdc42/Rac interaction and binding sequence (CRIB)2 (residues 74–87) of Pak2, which overlaps with the autoinhibitory domain (AID) (4). Binding of Cdc42 to inactive Pak2 disrupts the inhibition (5). Under apoptotic stress, Pak2 is constitutively activated by caspase 3 cleavage and autophosphorylation (6, 7). Activation of Pak2 in response to stress inhibits transcription and translation of growth-related gene products (8–10). Gene products phosphorylated by Pak2 include histone (4, 7), c-Myc (10), serine/threonine protein kinase Mnk1 (9), tyrosine kinases Syk (11) and c-Abl (12), prolactin (13), and translation initiation factors 3, 4B, and 4G (8, 14). Phosphorylation of these proteins under moderate stress produces a cytostatic state, whereas cleavage of Pak2 by caspase 3 promotes apoptosis.

Pak1, -2, and -3 are ~78% identical, whereas the catalytic domains have 93% identity (3). All three enzymes have an N-terminal regulatory domain and a C-terminal catalytic domain. The regulatory domain includes a conserved AID, which interacts with the active-site cleft, and a switch region (83–132) that interacts with the large lobe of the catalytic domain (5, 15–17). Inactive Pak1 exists in solution as a homodimer, with the AID of each monomer interacting with its partner to inhibit activity (18). Because the dimerization domain (residues 79–86 for Pak2) is homologous in Pak1, Pak2, and Pak3 (15), it is generally assumed that Pak2 also exists as a trans-autoinhibited homodimer as described for Pak1 (18).

Caspase 3 selectively cleaves at Asp-212 in the Pak2 regulatory domain to form two fragments, p27 and p34 (7). The p27 fragment includes most of the regulatory domain, and the p34 fragment includes the entire catalytic domain and a short segment of the regulatory domain. Pak2 has eight autophosphorylation sites, including seven serines in the regulatory domain and one threonine in the catalytic domain (19). Pak2 has a basal activity that autophosphorylates five of the serine residues. Following caspase cleavage, additional autophosphorylation at Ser-141 and Thr-402 is required for full activity (7, 19). Ser-141 regulates the association of Pak2 with Cdc42, and Thr-402 is the conserved threonine in the activation loop of most protein kinases (20).

Although no crystal structure for Pak2 is currently available, a crystal structure of two fragments of a Pak1 mutant (K299R), consisting of the AID (residues 70–149), and the catalytic domain (residues 249–545), mimics the inactive conformation (1F3M.PDB) (15). A crystal structure (1YHV.PDB) of the catalytic domain of the Pak1 mutant (K299R/T423E) mimics the active conformation (21). Because the well studied cAMP-dependent protein kinase (PKA) has a bilobal kinase domain homologous to that of Pak1 and a number of resolved crystal structures of active and inactive conformations, PKA (1CTP, PDB) was used as a model to assist in identifying the function of specific amino acids of Pak2 (22–24). The secondary structure of the Pak1 catalytic domain bound to the AID, and the catalytic
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Experimental Procedures

Materials—Bovine thrombin was obtained from Amersham Pharmacia Biotech. Pepsin immobilized on 6% agarose beads was purchased from Pierce. Deuterium oxide (99.9%) was from Aldrich. ATP, AMP-PCP, and α-cyano-4-hydroxycinnamic acid were obtained from Sigma. The α-cyano-4-hydroxycinnamic acid was re-crystallized and stored in the dark.

Expression of Recombinant Pak2 and Caspase 3—The expression and purification of GST-Pak2 (accession number Q29502) from TN5B1–4 insect cells was described previously (7). The GST was removed by thrombin cleavage. Histagged caspase 3 was overexpressed in BL21(DE3) cells and purified, and the activity was determined (7).

Pak2 Autophosphorylation—To fully phosphorylate Pak2, the protein (20 μl, 12 mg/ml) was cleaved by the addition of 3 units of caspase 3 and incubated at 34 °C for 30 min. The extent of cleavage was analyzed by SDS-PAGE (10%) and Coomassie Brilliant Blue staining. Autophosphorylation of cleaved Pak2 was carried out at a final concentration of 10 mg/ml Pak2 in a 20-μl reaction mixture containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 15 mM MgCl2, 2 mM dithiothreitol, and 10 mM ATP, and incubated at 34 °C for 30 min. Following SDS-PAGE, the level of autophosphorylation with [γ-32P]ATP was determined by quantifying the amount of radiolabel incorporated into intact Pak2 and into the p34 and p27 fragments by scintillation counting.

Gel Filtration—An ÄKTA fast protein liquid chromatograph (Amersham Biosciences) with a Superdex 200 column (1 × 50 cm) was used to determine the molecular weight of Pak2. Intact Pak2, caspase-cleaved inactive Pak2, and autophosphorylated caspase-cleaved Pak2 (50–100 μg in 100 μl of buffer) were analyzed individually at a flow rate of 0.5 ml/min using a mobile phase of 20 mM Tris-HCl and 125 mM NaCl at pH 7.0. The absorbance of the eluate was monitored at 215 nm.

Identification of Pepsin-digested Pak2 Fragments—Pepsin-digested Pak2 (20 μg of Pak2 in 50 μl of 0.1% v/v trifluoroacetic acid) was eluted through a reversed-phase high-performance liquid chromatography C18 column equilibrated with 0.1% trifluoroacetic acid at pH 2.5, with a linear 100-min gradient of 0 to 80% acetonitrile and 0.1% trifluoroacetic acid. Fractions (400 μl) were collected every 2 min. The fractions were dried with a Speed-Vac (3 h) and resuspended in acetonitrile (5 μl) and 0.1% trifluoroacetic acid (5 μl) at pH 2.5. Each fraction was screened for peptides using MALDI-TOF (PerSeptive Biosystems Voyager DE STR, PE Biosystems). All fractions containing peptides were subjected to tandem mass spectrometry (Q-TOF Ultimate-Global and QSTAR XL oMALDI MS/MS, Applied Biosystems) to identify the fragments, based on the primary sequences using Protein Prospector (28).

Measurement of Amide H/D Exchange—H/D exchange was initiated by the addition of 18 μl of buffered D2O (50 mM MOPS, pH 6.98, 125 mM NaCl) to 2 μl of purified intact Pak2 (10 mg/ml) for a final pH of 7.0 at 25 °C. Samples containing nucleotide had a final concentration of 4 mM AMP-PCP or ATP (pH 7.0) and 6 mM MgCl2. H/D exchange was quenched by the addition of 180 μl of ice-cold 0.1% trifluoroacetic acid at pH 2.2, which brought the pH to 2.5 in a final volume of 200 μl. For the
samples containing nucleotide, 180 μl of ice-cold 0.11% trifluoroacetic acid at pH 2.2 was used to maintain the pH at 2.5 in the quenched condition.

The time scale of H/D exchange was 0, 0.5, 1, 3, 5, and 10 min, or 24 h. Six determinations were made at 10 min, and three determinations were made for all other times. An aliquot (100 μl) of each quenched sample was added to 30 μl of activated pepsin-conjugated agarose beads. The digestion was allowed to proceed for 5 min on ice, and the samples were vortexed every 30 s. Digestion was terminated by centrifugation of the sample for 15 s at 12,000 × g at 4 °C to remove pepsin. The samples were rapidly frozen in liquid N2 and stored at −80 °C for no more than 2 days.

The matrix for MALDI-TOF was 5 mg/ml α-cyano-4-hydroxycinnamic acid in a 1:1:1 solution of acetonitrile, ethanol, and 0.1% trifluoroacetic acid at pH 2.5 and held at 0 °C. Each sample was partially thawed quickly, and 1 μl of each sample was mixed with 1 μl of matrix and spotted onto a 4 °C MALDI target plate. A moderate vacuum was applied to the plate to dry the spots in 30 s prior to MALDI-TOF analysis. The time between thawing of the sample and spectrum retrieval was ~3 min.

Data Analysis—The Data Explore 4.0 computer program (Applied Biosystems) performed the baseline correction and noise filtration (medium). Each spectrum was calibrated based on the flight times of two sequenced peaks whose calculated undeuterated m/z were 923.45 and 1697.84. Both peaks had high intensity even after the sample was deuterated. The 24-h deuteron incorporation of the most solvent accessible peptide (m/z = 1105.60) was assumed to represent 100% deuteration. The Centroid Application Computer Program (29) was utilized to calculate the centroids of fragments in the mass spectrum. The incorporated deuteron number was the difference between the centroid of the deuterated and non-deuterated Pak2 peptic peptides. The estimate of deuteron incorporation was corrected for residual deuterium on the side chains (3.3%) and the H/D back exchange (74%). The number of deuterons at time t, D(t), was calculated using Equation 1,

$$D(t) = \frac{(m_t - m(\text{std}))}{(B\%)} - D_s(t) \quad \text{(Eq. 1)}$$

where m(t) is the observed mass at time t, m(stand) is the non-deuterated mass. The back exchange ratio (%B) was calculated by the actual incorporated deuteron number at 24 h of H/D exchange divided by all possible exchangeable sites at peptide m/z 1105.60. The Ds(t) is the amount of residual deuteron on the side chains at time t.

RESULTS

Solution States of Pak2—The apparent molecular weight of inactive Pak2, caspase-cleaved Pak2, and caspase-cleaved autophosphorylated Pak2 was analyzed by fast protein liquid chromatography gel filtration to assess the state of association of Pak2 in solution. A schematic of Pak2 is shown in Fig. 2a. Prior to analysis, the Pak2 proteins were analyzed by SDS-PAGE (Fig. 2b). Inactive Pak2 was a single band of 58 kDa. Caspase cleavage of Pak2 was complete and produced two fragments, p27 and p34. Autophosphorylation of cleaved Pak2 was optimal at 7.6 mol/mol. The migration of autophosphorylated p27 and p34 overlapped on the protein gel due to retarded migration of the fully phosphorylated p27 fragment at seven sites. High-performance liquid chromatography was used to determine the state of association of Pak2, as shown in the elution profiles in Fig. 2c. Intact Pak2 eluted primarily as a single peak with an apparent molecular mass of 115 ± 1 kDa, which was twice the monomeric molecular mass (58 kDa). This was similar to data obtained for Pak1, showing it existed in solution as a homodimer (15). Caspase-cleaved Pak2, consisting of two 27- and 34-kDa fragments, eluted as a single peak at the same volume as intact Pak2 (116 ± 1 kDa), indicating non-phosphorylated caspase-cleaved Pak2 remained as a compact complex. After autophosphorylation, caspase-cleaved Pak2 eluted primarily as a single larger peak (133 ± 3 kDa), indicating autophosphorylation at eight sites left the heterotet-
rameric complex intact, but suggesting alterations in the quaternary structure.

Identification of the Pepsin-digested Fragments—Pepsin-digested peptides were separated by reversed-phase high-performance liquid chromatography on a C18 column; a total of 40 fractions was collected and subjected to tandem mass spectrometry (Q-TOF MS/MS and oMALDI MS/MS) to determine the sequences of the pepsin fragments. The entire mass spectrum of pepsin-digested Pak2 is shown in Fig. 3a. 30 of 42 identified peptides had m/z ratios that were sufficiently different from one another and of sufficient magnitude to use in the amide H/D exchange experiments discussed below. These peptides covered 50% of the primary sequence of Pak2, including all or part of the following regions: the N-terminal region, dimerization domain, AID, glycine-rich loop, C helix, catalytic loop, magnesium-positioning loop, activation loop, and regions without an identified function.

Amide H/D Exchange Analysis—To determine the solvent accessibility of the peptides of Pak2, amide H/D exchange experiments were carried out at specific intervals between 0 and 10 min at 25 °C, and the level of deuterium incorporated into the peptides was quantified. For example, the time course of deuterium incorporation into the peak m/z 1697.85 of intact inactive Pak2 was composed of multiple isotopic peaks as shown by the shift of the mass envelope over time (Fig. 3b). At 10 min post deuterium, four deuterons were incorporated into the 11 available amides, and the level of deuterium incorporation reached a plateau, suggesting saturation of the fast-exchange amide sites (Fig. 3c). The plots of deuterium incorporation for the remainder of the peptides are shown in supplemental Fig. S2. Because 28 of 30 peptides reached the plateau of deuterium incorporation, the 10-min time point was used as a measure of relative amide H/D exchange.

The time course of deuterium incorporation was a combination of multiple-exchange functions. Interestingly, some of the data showed a single class of observed rate constants while others consisted of both a fast and a slow process. These observations were expected, because multiple deuterium atoms were incorporated into
the peptides and the rate of each H/D exchange was different from the others.

To study the structural changes caused by nucleotide binding, caspase cleavage, and autophosphorylation, amide H/D exchange experiments were performed with five samples: intact Pak2 and caspase-cleaved Pak2 in the presence and absence of AMP-PCP, and cleaved autophosphorylated Pak2. The deuterons incorporated into the pepptide fragments of Pak2 after 10 min of amide H/D exchange were measured for all five of the Pak2 samples, and the data for the individual treatments were calculated and compared with that of intact Pak2 (Table 1).

The Pak2 samples were subjected to a two-way analysis of variance analysis followed by a multiple-paired comparison analysis using the Prism 4 computer program. The data showed significant and important (p < 0.0001) variations between the peptides of intact Pak2, and between the individual treatments. Table 2 lists the statistical significance for all paired comparisons between the pepsin-digested fragments under the different conditions. Each treatment produced a unique pattern of changes in the level of deuteron incorporation.

**Table 1**

| Pak2 residues | Structural domains | No. amides | Deuterons exchanged in 10 min* |
|---------------|--------------------|------------|-------------------------------|
|               |                    |            | Pak2 | AMP-PCP | Caspase-cleaved | Caspase-cleaved; AMP-PCP | Cleaved and autophosphorylated |
| m/z           |                    |            |      |         |                |                          |                                 |
| 5–17 (2303.11) | Pro1               | 17         | 9.8±1.7 | 11.1±0.7 | 10.6±1.7 | 13.0±0.5 | 15.3±0.6 |
| 8–17 (1105.60) | Pro1               | 5          | 4.0±0.3 | 4.7±0.4 | 4.8±0.4 | 5.3±0.4 | 5.2±0.3 |
| 58–79 (2414.25) | To CRIB           | 18         | 5.2±1.2 | 6.3±0.9 | 6.2±1.0 | 6.4±0.6 | 5.5±0.1 |
| 80–88 (1086.54) | Di domain/CRIB    | 8          | 2.5±0.4 | 2.8±0.3 | 2.9±0.2 | 3.1±0.1 | 3.4±0.1 |
| 80–89 (1291.86) | Di domain/CRIB    | 9          | 3.1±0.6 | 3.6±0.3 | 4.0±0.1 | 3.8±0.1 | 4.0±0.1 |
| 102–105 (945.32) | AID               | 3          | 1.3±0.1 | 1.5±0.1 | 1.5±0.1 | 1.6±0.1 | 1.6±0.1 |
| 106–124 (2153.23) | AID       | 17         | 7.6±1.0 | 9.2±0.6 | 10.1±0.5 | 11.1±0.2 | 11.9±0.6 |
| 239–252 (1667.95) | β-sheet 1       | 12         | 7.2±0.8 | 7.7±0.5 | 7.4±0.5 | 7.6±0.2 | 7.6±0.4 |
| 239–264 (2842.55) | β1 to Gly loop | 24         | 11.9±1.4 | 13.6±1.1 | 13.6±1.0 | 14.1±0.5 | 14.6±0.6 |
| 242–264 (2543.36) | β1 to Gly loop | 21         | 8.7±1.5 | 10.0±1.2 | 10.8±0.8 | 12.5±0.6 | 12.9±0.4 |
| 283–294 (1467.85) | Loop to C-helix | 10         | 3.2±0.5 | 3.8±0.3 | 3.8±0.6 | 4.2±0.3 | 4.3±0.2 |
| 295–309 (1722.01) | C-helix to loop 13 | 13         | 4.4±0.3 | 4.6±0.4 | 5.6±0.3 | 5.1±0.3 | 4.6±0.2 |
| 297–309 (1545.85) | C-helix to loop 11 | 10         | 2.0±0.3 | 2.5±0.3 | 2.4±0.4 | 2.7±0.2 | 3.3±0.3 |
| 357–375 (2248.18) | Cat. loop 18     | 18         | 1.8±0.4 | 1.9±0.3 | 1.9±0.1 | 2.4±0.1 | 2.3±0.1 |
| 358–375 (2119.14) | Cat. loop 17     | 17         | 1.6±0.3 | 1.7±0.3 | 1.6±0.3 | 2.0±0.1 | 1.7±0.1 |
| 360–375 (1858.99) | Cat. loop 15     | 15         | 1.4±0.4 | 1.3±0.3 | 1.5±0.3 | 1.8±0.1 | 1.6±0.2 |
| 362–375 (1659.80) | Cat. loop 13     | 13         | 1.4±0.2 | 1.5±0.2 | 1.5±0.3 | 1.8±0.1 | 1.6±0.1 |
| 364–375 (1408.79) | Cat. loop 11     | 11         | 0.8±0.2 | 0.9±0.2 | 0.9±0.1 | 1.2±0.1 | 1.2±0.1 |
| 376–389 (1500.74) | Mg loop 13       | 13         | 3.6±0.4 | 3.5±0.8 | 3.2±0.7 | 3.6±0.5 | 3.6±0.4 |
| 381–389 (1013.53) | Mg loop 8        | 8          | 1.4±0.2 | 1.8±0.3 | 1.8±0.4 | 2.5±0.2 | 2.8±0.1 |
| 390–403 (1579.76) | Act. loop 12     | 12         | 6.3±0.9 | 6.9±0.5 | 7.2±0.7 | 7.3±0.7 | 7.4±0.5 |
| 391–403 (1475.85) | Act. loop 11     | 11         | 5.0±0.9 | 5.2±0.8 | 6.1±0.7 | 6.4±0.2 | ND* |
| 417–429 (1532.86) | E-F helix loop 11 | 11         | 3.4±0.9 | 4.1±0.6 | 3.7±0.7 | 3.8±0.4 | 4.7±0.5 |
| 436–451 (1828.89) | F-G helix loop 12 | 12         | 4.7±0.2 | 5.3±0.3 | 5.4±0.3 | 6.1±0.3 | 6.9±0.1 |
| 457–467 (1697.85) | F-G helix loop 11 | 11         | 4.0±0.2 | 4.8±0.1 | 4.5±0.2 | 5.2±0.1 | 5.8±0.1 |
| 455–473 (2095.09) | G-H helix loop 15 | 15         | 7.2±1.4 | 7.9±0.2 | 8.0±0.5 | 8.9±0.2 | 9.5±0.3 |
| 474–480 (923.45) | H-helix 6        | 6          | 0.1±0.1 | 0.0±0.1 | 0.0±0.1 | 0.3±0.2 | 0.3±0.1 |
| 484–500 (1967.07) | Loop to I-helix | 15         | 3.4±0.4 | 3.9±0.3 | 3.7±0.4 | 4.3±0.2 | 4.3±0.2 |
| 485–500 (1852.04) | Loop to I-helix | 14         | 2.2±0.2 | 2.4±0.1 | 2.4±0.1 | 2.4±0.0 | 2.7±0.1 |
| 501–514 (1511.92) | C-terminal 11    | 11         | 1.8±0.4 | 1.9±0.2 | 1.9±0.2 | 1.9±0.1 | 1.9±0.1 |

*The number of deuterons is the average of six replicates with standard deviation.

ND, not detected.

Conformational Changes during Pak2 Activation—To examine the effects of ATP bound to inactive Pak2, AMP-PCP, a non-hydrolysable form of ATP, was used, and amide H/D exchange was measured. Intact Pak2 showed a low level of perturbation in amide H/D exchange of the active site, the AID switch domain, and in the N and C termini (Table 1 and Fig. 5a). The addition of AMP-PCP(Mg) specifically increased deuteron incorporation into five regions of inactive Pak2. These consisted of the 239–264 fragment that included portions of the glycine-rich loop, the 283–294 fragment that included the N-terminal segment of the C helix, the 106–124 fragment that encompassed the AID switch domain, the 5–17 and 8–17 fragments at the N terminus, and the 501–514 fragment at the C terminus. It can be concluded from these results that ATP could bind to autoinhibited Pak2 and that the AID did not prevent nucleotide binding.
Conformational Changes during Pak2 Activation

TABLE 2
Comparisons of H/D exchange data for five activation states of Pak2

| Pak2 residues | Structural domains | Multiple paired comparisons* |
|---------------|--------------------|-----------------------------|
| m/z           | AB     | AC     | AD     | AE     | BC     | BD     | BE     | CD     | CE     | DE     |
| -5–17 (2303.11) /H11002 | Prol | *** NS | *** NS | *** NS | *** NS | *** NS | *** NS | *** NS | *** NS | *** NS |
| 8–17 (1105.60) /H11002 | Prol | NS NS | ** NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS |
| 58–79 (2414.25) /H11002 | To CRIB | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS |
| 80–88 (1086.54) /H11002 | Di domain/CRIB | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS |
| 80–89 (1201.56) /H11002 | Di domain/CRIB | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS |
| 102–105 (545.32) /H11002 | AID | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS |
| 106–124 (2153.23) /H11002 | AID | *** *** | *** *** | *** *** | *** *** | *** *** | *** *** | *** *** | *** *** | *** *** |
| 239–252 (1667.95) /H11002 | β Sheet I | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS |
| 239–264 (2424.55) /H11002 | βI to Gly loop | *** *** | *** *** | *** *** | *** *** | *** *** | *** *** | *** *** | *** *** | *** *** |
| 242–264 (2543.36) /H11002 | βI to Gly loop | *** *** | *** *** | *** *** | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS |
| 283–294 (1467.85) /H11002 | loop to C-helix | NS NS | * NS | NS NS | * NS | NS NS | NS NS | NS NS | NS NS | NS NS |
| 297–309 (1547.96) /H11002 | C-helix to loop | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS |
| 360–375 (2119.14) /H11002 | Cat. loop | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS |
| 358–375 (2185.99) /H11002 | Cat. loop | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS |
| 362–375 (1490.79) /H11002 | Cat. loop | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS |
| 376–389 (1505.74) /H11002 | Mg loop | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS |
| 381–398 (1013.53) /H11002 | Mg loop | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS |
| 390–403 (1579.76) /H11002 | Act. loop | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS |
| 391–403 (1476.75) /H11002 | Act. loop | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS |
| 417–429 (1532.86) /H11002 | Loop to F-helix | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS |
| 436–451 (1828.89) /H11002 | F-G helix loop | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS |
| 437–451 (1697.85) /H11002 | F-G helix loop | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS |
| 455–473 (2069.09) /H11002 | G-H helix loop | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS |
| 474–490 (2293.45) /H11002 | H-helix | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS |
| 494–500 (1967.07) /H11002 | Loop to I helix | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS |
| 485–500 (1852.04) /H11002 | Loop to I helix | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS |
| 501–514 (1511.92) /H11002 | C-terminal | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS | NS NS |

* Results are shown as the indicated statistical significance level: ns, p > 0.05; *, p < 0.05; **, p < 0.01; ***, p < 0.001; -, no detected peptides. Multiple paired comparisons were analyzed by Prism 4.0. (Activation states account for 0.99% of the total variance. F value = 106.0; degree of freedom = 4, p value < 0.0001. Fragments account for 91.12% of the total variance. F value = 1351.0; degree of freedom = 29, p value < 0.0001. Interaction accounts for 6.15% of the total variance. F value = 22.7; degree of freedom = 116, p value < 0.0001.)

Effects of Caspase Cleavage on Solvent Accessibility—Within the primary sequence covered by the MALDI-TOF H/D exchange measurements, the effects of caspase cleavage appeared to be confined to three regions, a fragment close to the N terminus, a portion of the AID switch region, and the active-site cleft. In each region, caspase cleavage increased deuterium incorporation as compared with inactive Pak2 (Table 1).

In the active-site cleft, a net gain of about two deuterons was observed with two overlapping fragments, 239–269 and 242–264, but not with the 239–252 fragment. This suggests the gain in deuterium was confined to residues 253–264 (Table 1 and Fig. 5a). This latter segment included the glycine-rich loop (residues 256–263). In the active site there was a net gain of about one deuteron in the 295–309 fragment following caspase cleavage, but not in the 297–309 or 283–294 fragments, suggesting the change in amide H/D exchange was confined to either residue 295 or 296 adjacent to Glu-294 in the C helix, which is an important link to the glycine-rich loop in ATP binding. The 390–403 fragments, which include the critical Thr-402 in the activation loop (residues 391–406), gained about one deuteron.

Two fragments of the regulatory domain having solvent accessibility are identified above the figures (Fig. 5). Following caspase cleavage, the 58–79 fragment, which included a few residues of the p21-binding domain (73–108), was associated with a net increase of about one deuteron. The 106–124 fragment, part of the AID switch region (83–132), was associated with a net gain of 2–3 deuterons following caspase cleavage.

Thus, caspase cleavage increased the solvent accessibility of the switch region and possibly altered the interaction between the switch region and the catalytic domain.

Perturbation of the AID switch by caspase cleavage could disrupt the autoinhibition and lead to autophosphorylation of Thr-402 in the activation loop and Ser-141 downstream of the AID switch. The combined effects of both nucleotide binding and caspase-cleaved Pak2 are summarized in Table 1 and depicted in Fig. 5c. Nucleotide binding in the absence of autophosphorylation was associated with relatively widespread perturbations of amide H/D exchange. Compared with intact Pak2, changes were observed in the N terminus (−5–17 fragment), the glycine-rich loop (242–264 fragment), and the 390–403 fragment that included a portion of the F helix (423–438).

Effects of Caspase Cleavage plus Autophosphorylation—Compared with the intact Pak2, changes in autophosphorylated Pak2 were observed in the N terminus (−5–17 fragment), the AID region (106–124 fragment), the glycine-rich loop (242–264 fragment), the C helix (297–309 fragment), the magnesium-positioning loop (381–389 fragment), the E-F loop (417–429 fragment), F-G loop (436–451 fragment), and G-H loop (455–473 fragment) (Table 1 and Fig. 5d). There was a significant increase in deuterons in the 106–124 fragment that appeared to link the autoinhibitory switch and its site of contact with the G helix and, subsequently, with the F and H helices. Deuterium incorporation was increased by about four deuterons in the 106–124 fragment associated with the AID switch (Table 1). This switch is shown in the Pak1 crystal structure to
interact with the G helix of the catalytic domain (Fig. 1). Although there was no sequence coverage of the G helix, fragments that include the loop preceding (436–451 and 437–451) and following (455–473) the G helix, but not the H helix (474–480), were each associated with a net gain of 1–2 deuterons. As compared with caspase-cleaved Pak2 alone, the addition of ATP and subsequent complete autophosphorylation of caspase-cleaved Pak2 at eight sites (7.6 mol of phosphate/mol of Pak2) was associated with further perturbations of the AID switch region and the active site (Fig. 5d). In addition, caspase cleavage and autophosphorylation dramatically and specifically increased deuteron incorporation into the N-terminal −5–17 fragment by about five deuterons, bringing the level of incorporation to 90% of the maximum, and indicating a region of high solvent exposure/flexibility (Table 1). The phosphorylated peptides (390–403 and 391–403) in the activation loop were not observed in the MALDI-TOF mass spectrum, because the peptides were not ionizable following phosphorylation under the conditions utilized. Therefore, the segments from the activation loop were detected only in the non-phosphorylated state.

The unique effects identified following autophosphorylation of caspase-cleaved Pak2, as compared with AMP-PCP binding to caspase-cleaved Pak2, showed increased amide H/D exchange in the −5–17 fragment of the N terminus and the E-F loop (417–429 fragment). The N terminus (−5–17 fragment) has an increase of two deuterons, and the E-F loop (417–429 fragment) has an increase of one deuteron. Minor changes of less than one deuteron incorporated were also observed in the AID, the C-helix, the F-G loop, and G-H loop.

**DISCUSSION**

The regulation of Pak2 under apoptotic conditions is different from that during cytostatic and growth conditions. Pak2 is pro-survival and is activated by small G proteins in response to stress-inducing cytostasis, whereas Pak2 is primarily inactive in growing cells (3, 6, 30). In contrast, Pak2 is constitutively activated by caspase 3 during apoptosis in response to apoptotic stresses such as UV radiation and other DNA-damaging agents (3, 31). Analysis of the solution structure of caspase-cleaved Pak2 by gel filtration shows that the caspase cleavage products of Pak2 remain associated as a heterotetramer following both cleavage and autophosphorylation at eight sites. This can be attributed to the fact that the AID binds to the catalytic domain with a high affinity, $K_D \sim 0.9 \mu M$ (32). Strong Pak2 trans-dimerization is established through two anti-parallel dimerization domains and hydrophobic interactions between the G-helix and the AID of the autoinhibited structure (15). Consequently, the results support the concept of a fully catalytically active heterotetrameric Pak2 complex.
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of course, does not preclude the existence of proteins that mediate the selective translocation of the p34 catalytic domain to one or more subcellular compartments or the dissociation of p34 from the AID at low Pak2 concentration (33). Pirruccello et al. (32) have reported the dimerization of the phosphorylated catalytic domains of Pak2, but not the non-phosphorylated domains, suggesting a possible conformational change of the Pak2 complex. In vivo, regulation of p27 and the local concentration of Pak2 could play an important role in its activation.

Autoinhibited Pak2, as illustrated in Fig. 4, shows low solvent accessibility in the catalytic loop and the magnesium positioning loop, as evidenced by a slow amide H/D exchange. The catalytic loop has low solvent accessibility in both active and autoinhibited Pak2, which suggests that Pak2 activation does not involve an opening up of the catalytic loop and exposure of the catalytic serine to the solvent. The protein substrates may interact with the solvent-exposed binding sites to dock the consensus sequence properly. The basal serine autophosphorylation sites do not contain the substrate consensus sequence but are tethered within distance for phosphorylation by the active site Asp-368. The activation loop, which includes the autophosphorylated activation loop (Fig. 5a and Table 1). The activation loop has been shown to be stabilized and docked on the E-F loop in the crystal structure of the catalytic domain (1YHV.PDB) (21). However, there was no signal for the phosphorylated activation loop in our studies, as it was not detected by mass spectrometry due to a change in the ionization properties following autophosphorylation of Thr-402.

The binding of AMP-PCP alone produces changes of 10–25% in amide H/D exchange in the glycine-rich loop and minor changes on the C-helix (Fig. 5a and Table 1). The binding of AMP-PCP after caspase cleavage also shows a similar trend (Fig. 5c). The glycine loop and C-helix are two important regions for the orientation of ATP binding (24). Lys-72 interacts with Glu-91 in C-helix for docking of ATP in PKA. Our laboratory has shown the K278R mutation on Pak2 completely knocks out Pak2 activity (7). This lysine corresponds to Lys-72 in PKA, which has been shown to disrupt the interaction with Glu-91 in PKA; Glu-91 corresponds to Glu-294 in Pak2 (7, 24). The AID switch domain (83–132) and the N-terminal (1–17) and the C-terminal J-helix (501–514) of autoinhibited Pak2 show that AID occupation of the active site does not prevent ligand binding to the nucleotide pocket located deep in the active-site cleft. This reflects a significant level of conformational flexibility of the AID segment occupying the active-site cleft.

Caspase cleavage at Asp-212 is associated with increased amide H/D exchange in the active site (glycine-rich loop, C helix, and activation loop), the AID, and the N terminus. The increased amide H/D exchange appears to be the result of breaking covalent bonds, Asp-212 and Ser-213, in the trans-dimerized conformation. The Asp-212 cleavage site is 15 residues upstream of the small lobe, and the AID binds to the large lobe of the partner Pak2. The observed increase in the solvent accessibility of the N terminus could be from the disturbance or release of a docking site for the N terminus as a result of caspase cleavage. Autophosphorylation at the two critical sites required for activation, Ser-141 in the autoinhibitory domain and Thr-402 in the activation loop, is the major difference between cleaved and intact autoinhibited Pak2 (19, 20). It has been suggested that the seven serine residues in the regulatory domain are phosphorylated by an intramolecular mechanism, and Thr-402 in the kinase domain is phosphorylated by an intermolecular mechanism (35). We showed that mutation of the conserved threonine to T402A dramatically decreased the protein kinase activity to 2%, whereas T402E was highly active (20). In addition, the S141A mutant decreased the level of autophosphorylation to 65%. Our study shows that caspase cleavage causes a rearrangement of the AID that allows intramolecular autophosphorylation at the critical Ser-141 site in the AID and exposes Thr-402 in the activation loop to facilitate intermolecular autophosphorylation.

Two x-ray crystal structures of Pak1 (1F3M.PDB and 1YHV.PDB) show the active conformation uses the hinge region as an axis to make a 15° movement between the two lobes of the catalytic domain toward a more closed conformation. This movement changes the position of glycine-loop, C-helix, and G-helix. Lei et al. (21) show the activation loop docks on the E-F loop without interference of the AID in the active conformation of Pak1. Within the regions of the primary sequence...
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covered by MALDI-TOF H/D exchange analysis, the effects of autophosphorylation are similar to those observed with nucleotide-bound caspase-cleaved Pak2, with an increased level of deuterium in the C-helix, the AID switch region, and the G-helix region. We have also observed a perturbation of the E-F loop upon autophosphorylation of cleaved Pak2. This suggests Pak2 has a conformational change in the E-F loop, which is related to docking of the activation loop. The increase of deuterium in the F-G loop and the G-H loop indicates activation of Pak2 reduces the contact between the AID and the G-helix, suggesting the G-helix may be an allosteric control region for the transition between the autoinhibited and the active conformations. Mutation of L455Q in the G-helix disrupts the dimerization of the two individual kinase domains (31), which would inhibit the intermolecular autophosphorylation of Thr-402 in the activation loop.

The effects of autophosphorylation are the result of both independent and overlapping effects of nucleotide binding and caspase cleavage. The resulting autophosphorylation produces a more solvent-exposed and conformationally dynamic enzyme, which includes the active site, AID, dimerization, and N-terminal domains, as well as novel sites that link the AID switch at the G helix to the F and H helices.

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