Proline- and Alanine-rich Ste20-related Kinase Associates with F-actin and Translocates from the Cytosol to Cytoskeleton upon Cellular Stresses*

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Proline- and alanine-rich Ste20-related kinase (PASK) is a Ste20-related protein kinase isolated from rat brain. Cell fractionation studies showed that PASK was present both in the cytosol and in Triton X-100-insoluble cytoskeletal fraction in rat tissues. In brain, PASK associated with protein complexes that contained actin and tubulin, confirming the association of PASK with the cytoskeleton in vivo. Glutathione S-transferase-PASK fusion protein cosedimented with F-actin, indicating that PASK binds to F-actin. In contrast to rat tissues, PASK was detected only in the Triton X-100-soluble cytosolic fraction in cultured PC12 and NIH 3T3 cells. Cytosolic PASK translocated to the cytoskeleton when these cells were stimulated with severe cellular stresses such as hypertonic sodium chloride, hydrogen peroxide, and heat shock at 45 °C. Our results suggest that PASK may be involved in the regulation of the cytoskeleton in response to cellular stresses such as hyperosmotic shock.

In addition to their roles as upstream activators of mitogen-activated protein kinase pathways, members of the Ste20 family have been implicated in the regulation of cytoskeletal reorganization. The small GTPase Rac1 and Cdc42, upstream activators of PAK/Ste20 subfamily members, are considered key regulatory molecules that link surface receptors to the organization of the actin cytoskeleton (10). A Drosophila homolog of PAK, DPAPK, has been reported to colocalize with focal adhesion and focal complexes (11). PAK1 in fibroblast cell lines has been shown to translocate from the cytosol to Rac1 and Cdc42-dependent actin structures when cells were stimulated with platelet-derived growth factor (12). It has been reported that expression of activated mutants of PAK1 in mammalian cells induces actin reorganization (13, 14). For Sps1/germinal center kinase subfamily members, there are no reports concerning their roles in the regulation of cytoskeletal proteins except that Dictyostelium Severin kinase has been shown to phosphorylate actin binding protein severin (15).

We have recently cloned a rat Ste20-related protein kinase of the Sps1/germinal center kinase subfamily and named it PASK (proline- and alanine-rich Ste20-related kinase; Ref. 16). PASK was present in both the cytosol and particulate fraction in the rat brain. The particulate PASK was not solubilized by extraction with Triton X-100, suggesting that it was associated with complexes of high density such as cytoskeleton. In this report, we identified actin and tubulin as the major constituents of PASK-associated proteins in the brain, confirming the association of PASK with the cytoskeleton in vivo. In addition, we examined changes in the subcellular distribution of PASK in cultured PC12 and NIH 3T3 cells in response to extracellular stimuli. We demonstrated that PASK translocates from the cytosol to the cytoskeleton upon stimulation of the cells with severe cellular stresses. PASK is the first example of Sps1/germinal center kinase subfamily kinase that is shown to associate with the cytoskeleton.

EXPERIMENTAL PROCEDURES

Fractionation of Rat Tissues—All procedures were carried out at 0–4 °C. The experimental protocol was approved by the Committee for Animal Research of Mie University. Tissues from adult Harlan Sprague Dawley rats (13 weeks old) were homogenized with a Polytron in 10 volumes of extraction buffer containing 20 mM Tris-HCl, pH 7.5, 10 mM EDTA, 10 mM 2-mercaptoethanol, and 1 mM p-phenylmethylsulfonyl fluoride (buffer A). To the homogenates, we added 1 volume of buffer A containing 4% Triton X-100. After incubation on ice for 10 min, the homogenates were centrifuged at 10,000 × g for 10 min. The supernatants (Triton X-100-soluble fraction) were saved, and the pellets were washed with buffer A containing 1% Triton X-100 to obtain the Triton X-100-insoluble cytoskeletal fractions.

Preparation of Anti-PASK Antibody—Antibody against the carboxyl-terminal region of PASK (amino acid residues 424–553) was prepared as described previously (16). This antibody recognized a protein of 66 kDa on Western blot in COS-7 cells transfected with a full-length PASK cDNA but not in cells transfected with an empty vector. The antibody
also detected a single protein of 66 kDa in many rat tissues, indicating that the antibody specifically recognizes PASK. In addition, the antibodies immunoprecipitated PASK from the lysate of the transfected cells.

**Immunoaffinity Purification of PASK from Rat Brain**—Anti-PASK antibody (0.3 mg) was covalently bound to 1 ml of N-hydroxysuccinimide-activated Sepharose (Amersham Pharmacia Biotech) to prepare an anti-PASK immunoaffinity column. Frozen rat brains (4 g) were homogenized with Polytron in 20 ml of extraction buffer containing 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10 mM 2-mercaptoethanol, and 1 mM p-phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 30,000 × g for 15 min, and the resulting supernatant (10 mg of protein) was applied on the anti-PASK immunoaffinity column. After washing the column with phosphate-buffered saline and 10 mM Tris-HCl, pH 7.5, the bound proteins were eluted with 0.1 M glycine-HCl, pH 2.5.

**Immunoprecipitation of PASK from Rat Brain Extract**—Rat brain extract (14 mg of protein) prepared as described above was incubated with 10 μg of anti-PASK antibody for 1 h at 4 °C, and the immune complex was precipitated with 10 μl of protein A-Sepharose CL-4B (Amersham Pharmacia Biotech). After washing the gel with phosphate-buffered saline containing 0.1% Nonidet P-40, 10 mM Tris-HCl, pH 7.5, containing 0.5 mM NaCl, and 10 mM Tris-HCl, pH 7.5, bound proteins were eluted with 0.1 M glycine-HCl, pH 2.5.

**Immunoblot Analyses**—After electrophoresis on 10% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membranes, proteins were separated on a 10% SDS-polyacrylamide gel and stained with Coomassie brilliant blue.

**Subcellular Distribution of PASK**—We performed fractionation studies to determine whether PASK associates with the cytoskeleton in rat tissues and cultured PC12 and NIH 3T3 cells. Homogenates of rat brain, adrenal gland, heart, stomach, testis, epididymis, PC12 cells, and NIH 3T3 cells prepared in 1% Triton X-100 were centrifuged at 30,000 × g for 30 min. The supernatant was subjected to immunoaffinity chromatography and immunoprecipitation analysis.

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with anti-PASK antibody. Brain extracts were applied to the anti-PASK immunoaffinity column. After washing and elution of bound proteins, the adsorbed proteins were analyzed by SDS-PAGE. Fig. 2A, lane 2, shows silver staining of the proteins eluted from the anti-PASK antibody affinity column. A band at 45 kDa and a doublet at 50 kDa were the major proteins copurified with PASK. As shown in Fig. 2B, left panel, these proteins were identified as actin and tubulin by immunoblotting with anti-actin and anti-α-tubulin antibodies, respectively. Actin and tubulin were also coprecipitated with PASK by anti-PASK antibody and protein A-Sepharose, whereas none of these proteins was precipitated by nonimmune IgG (Fig. 2B, right panel). These results indicate that PASK forms molecular complexes with actin and tubulin.

**PASK Associates with F-actin in Vitro**—To assess the actin-binding properties of PASK and to determine which region of PASK binds actin, the behavior of various GST-PASK and truncated PASK fusion proteins (Fig. 3A) in an actin sedimentation assay was tested. Rabbit muscle actin was incubated with GST-PASK fusion proteins for 1 h in the presence or absence of KCl and MgCl₂. Subsequently, the actin filaments were sedimented by centrifugation. Both pellet and supernatant were analyzed by SDS-PAGE. As shown in Fig. 3B, when actin was polymerized by the addition of KCl and MgCl₂, GST-PASK (full-length), GST-PASK (66–553), and GST-PASK (245–424) cosedimented with F-actin and were found almost entirely in the pellet. GST-PASK (1–360) also cosedimented with F-actin, although a portion of it remained in the supernatant. GST-PASK (1–72) and GST-PASK (424–553) did not cosediment with F-actin and were found entirely in the supernatant. When actin was left unpolymerized (Fig. 3B, –(KCl, MgCl₂)), all the fusion proteins remained in the supernatant. To determine the specificity of the association between PASK and F-actin, control experiments were performed with GST-JNK2 and GST-xylanase (33–547), which are similar in size to GST-PASK. These proteins did not cosediment with F-actin under the assay conditions (Fig. 3B). These results indicated that PASK associates with F-actin in vitro and that PASK interaction with F-actin is mediated by the region within amino acid residues 245–424.

To determine whether PASK could bind to unpolymerized G-actin, GST-PASK and GST bound to glutathione-Sepharose were incubated with G-actin. Sepharose beads were then washed with incubation buffer containing 1% Triton X-100. Sepharose-bound proteins were analyzed by SDS-PAGE. As shown in Fig. 3C, no actin was detected in association with GST-PASK or GST, indicating that PASK did not bind to G-actin under the assay conditions.

**Translocation of PASK from the Cytosol to Cytoskeleton Is Induced by Cellular Stresses in PC12 and NIH 3T3 Cells**—It is known that certain protein kinases, including PKCs and p160ROCK, interact with cytoskeletal proteins and translocate from the cytosol to cytoskeleton in response to extracellular stimuli (20–22). We therefore tested whether translocation of PASK from the cytosol to the cytoskeleton was induced upon stimulation of PC12 and NIH 3T3 cells. For this purpose, PASK distribution was analyzed by cell fractionation after treatment of these cells with various stimuli. As shown in Fig. 4, A and B,
upper two panels, PASK was detected in the Triton X-100-insoluble cytoskeletal fraction when PC12 and NIH 3T3 cells were osmotically stressed with NaCl or sorbitol, oxidatively stressed with H$_2$O$_2$, or heat-shocked at 45 °C. In these cells, the amount of PASK in the Triton X-100-insoluble fraction increased in a time-dependent manner (Fig. 4, C and D). To quantify the changes in the distribution of PASK, dilution series of the Triton X-100-soluble and -insoluble fractions were resolved in the same gel and subjected to densitometric analysis. In cells stimulated with +0.5 M NaCl for 1 h and in cells treated at 45 °C for 1 h, ~30 and 80% of total PASK for PC12 cells and 20 and 80% of it for NIH 3T3 cells, respectively, were found in the Triton X-100-insoluble fraction. There was a concomitant stoichiometric decrease in the amount of soluble PASK. In Fig. 4, the reduction of soluble PASK is less obvious than the increase of insoluble PASK, because 5-fold excess amounts of the insoluble fractions were subjected to the immunoblot analysis. These results indicated that PASK translocated from the cytosol to the cytoskeleton in response to cellular stresses. The translocation was unique to PASK and not attributable to an artifact of cell fractionation, because the distribution of JNK1 and JNK2 in these stimulated cells was considerably different from that of PASK (Fig. 4, A and B, lower two panels); there was no significant increase in JNK2 in the insoluble fraction by any of these stresses; JNK1 did not translocate by hypertonic stress in NIH 3T3 cells, although its amount in the insoluble fraction increased by the other stresses.

In contrast to the translocation by these stresses, PASK in PC12 cells did not translocate by hypotonic stress, stimulation
of acetylcholine receptor with 1 mM carbachol, inhibition of protein synthesis with 50 µg/ml anisomycin, or exposure of the cells to 50 ng/ml nerve growth factor for 1 h (Fig. 4E and data not shown). Relatively mild stresses such as +0.2 mM NaCl, 0.5 mM H_2O_2, and heat shock at 42 °C also failed to induce PASK translocation (Fig. 4F and data not shown). These results suggested that PASK translocation was specifically induced by severe cellular stresses. As shown in Fig. 4G, hypertonic NaCl-induced PASK translocation was reversible: the amount of PASK in the Triton X-100-insoluble fraction returned to a control level when cells were incubated in an isotonic medium for 4 h after hypertonic stress.

To investigate the mechanism of hypertonic NaCl-induced PASK translocation, we examined whether such a process involved phosphorylation of PASK. PC12 cells were labeled with [32P]Pi and stimulated with a hypertonic medium (+0.5 mM NaCl) for 1 h. The cells were extracted to obtain Triton X-100-soluble and -insoluble fractions. PASK was immunoprecipitated with anti-PASK antibody directly from the Triton X-100-soluble fraction and after extraction in 0.5% SDS from the Triton X-100-insoluble fraction of the stimulated cells. This band was not counted, because it was undetectable by immunoblot with anti-PASK antibody. Our results showed that translocation of PASK from the cytosol to cytoskeletal fraction was undetectable in the cytoskeletal fraction of PC12 and NIH 3T3 cells. Although the molecular mechanisms that regulate the association of PASK with the cytoskeleton remain to be elucidated, these differences in the distribution of PASK suggest that the interaction between PASK and the cytoskeleton may be regulated by some factor and may reflect different activities of such factors in different tissues.

Our results showed that translocation of PASK from the cytosol to cytoskeleton occurred in PC12 and NIH 3T3 cells when these cells were stimulated with severe cellular stresses. The observation that hypertonic stress-induced PASK translocation was unaffected by cytochalasin B or colchicine treatment suggests that PASK translocation is not induced by rearrangement of the cytoskeleton. Cytosolic PASK probably does not associate with actin or tubulin monomers because none of these proteins was coimmunoprecipitated with PASK from a 100,000 x g supernatant of brain extract by anti-PASK antibody (data not shown). This is also supported by the observation that GST-PASK did not bind to unpolymerized G-actin. Therefore, we do not believe that cytosolic PASK associated with actin or tubulin monomers translocates to the cytoskeleton.
toskeleton by polymerization of these cytoskeletal proteins. PASK translocation was also not affected by inhibition of protein synthesis with cycloheximide or anisomycin (data not shown), suggesting that PASK translocation is not induced by de novo synthesis of adapter proteins, which bind PASK to the cytoskeleton. In vivo 32P labeling of PC12 cells showed that the phosphorylation level of PASK translocated to the cytoskeleton decreased to ~40% of that in the cytosol. Thus, it is conceivable that the level of PASK phosphorylation may modulate the affinity of PASK for the target molecule of the cytoskeleton. At this point, it is unclear whether PASK translocation is induced by PASK dephosphorylation. Further experiments using mutants lacking the phosphorylation site(s) will be needed to establish a causal relationship.

It has been reported that Ste20-like oxidant stress response kinase-1 and mammalian sterile twenty-like/kinase regulated by stress, Ste20 family members to which PASK is most closely related, are activated by cellular stresses (24, 25). To investigate whether PASK is similarly activated by cellular stresses, PASK was immunoprecipitated from Triton X-100-soluble extracts of hypertonic or oxidant stress-stimulated PC12 cells, and its activity was determined in vitro with myelin basic protein as an exogenous substrate. However, we could not detect any significant changes in PASK activity, because it was very weak under the assay conditions used (data not shown). Taken together with the observation that cytosolic PASK was decreased to ~40% of that in the cytosol, Thus, it is conceivable that PASK may be involved in the regulation of cytoskeleton when these cells respond to changes in osmolarity of body fluids.

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