Characterization of the Interaction of the Stress Kinase SPAK with the Na\(^+\)-K\(^+\)-2Cl\(^-\) Cotransporter in the Nervous System

EVIDENCE FOR A SCAFFOLDING ROLE OF THE KINASE

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Activity of heterologously expressed NKCC1 was analyzed under basal and activated conditions in the presence and absence of binding of Ste20-related proline-alanine-rich kinase (SPAK). Mutant NKCC1 that lacks the ability to bind to this kinase showed K\(^+\) transport function identical to wild-type NKCC1. Thus, preventing the binding of the kinase to the cotransporter does not affect cotransporter function. In contrast, several experiments suggest a possible role for SPAK as a scaffolding protein. First, Western blot analysis revealed the presence, and in some tissues abundance, of truncated forms of SPAK and OSR1 in which the kinase domains are affected and thus lack kinase activity. Second, a yeast two-hybrid screen of proteins that interact with the regulatory (binding) domain of SPAK identified several proteins all involved in cellular stress pathways. Third, p38, one of the three major MAPKs, can be coimmunoprecipitated with SPAK and with NKCC1 in an activity-dependent manner. The amount of p38 coimmunoprecipitated with the kinase and the cotransporter significantly decreases upon cellular stress, whereas the interaction of the kinase with NKCC1 remains unchanged. These findings suggest that cation-chloride cotransporters might act as “sensors” for cellular stress, and SPAK, by interacting with the cotransporter, serves as an intermediate in the response to cellular stress.

Cation-chloride cotransporters, which mediate the tightly coupled, electroneutral movement of cations (Na\(^+\) and K\(^+\)) together with Cl\(^-\), can be divided into Na\(^+\)-dependent transporters, such as Na-K-2Cl cotransporters (NKCC1–2) and the Na-Cl cotransporter (NCC), and Na\(^+\)-independent K-Cl cotransporters (KCC1–4). All of these transporters have a well conserved topology with large intracellular amino-terminal and carboxyl-terminal tails and 12 transmembrane spanning domains. The core protein shares some homology to amino acid permeases (for reviews see Refs. 1 and 2). A variety of stimuli regulate these transporters, including hormonal (3), cytokines (4, 5), cell volume (6, 7), oxidative stress (8), etc. There is also accumulating evidence that cation-chloride cotransporters participate in pathways leading to cell differentiation, growth and proliferation (9, 10), and apoptosis (11, 12). At the molecular level, the activation-deactivation of these cotransporters mostly involves phosphorylation/dephosphorylation mechanisms, the details of which are still the subject of intense investigation.

To identify proteins that directly interact and regulate cation-chloride cotransporters, we recently performed a yeast two-hybrid screen using the cytosolic amino-terminal tail of KCC3. We identified two closely related kinases that bind to KCC3, NKCC1 and NKCC2 (13). These kinases belong to the group of Ste20 kinases that function as regulators of MAPK cascade (14). The first kinase, SPAK (Ste-20 related proline-glutamine-rich kinase, or PASK, as the rat homologue), is highly expressed in epithelia and neurons (15). Its gene is located on human chromosome 2q31.1. The second kinase, OSR1, which is named based on a 30% homology to an oxidative stress response kinase, has much higher homology to SPAK (67%). The OSR1 gene is located on human chromosome 3p22-p21.3 (16). There are orthologues of OSR1 in Caenorhabditis elegans and Drosophila. Inactivation of the Drosophila ortholog, fray, results in a phenotype with axonal ensheathment deficits (17).

The catalytic (kinase) domains of SPAK and OSR1 are located at the amino terminus, and the regulatory domains are at the carboxyl terminus (14). We demonstrated that the binding with cation-chloride cotransporters takes place at the extreme carboxyl termini of SPAK and OSR1 (13). The two kinases bind to peptides with a minimum of 9 residues with the following initial consensus sequence: (R/K)F(V/I). This motif was found twice in the amino terminus of NKCC1, and each sequence was shown to interact with the kinases. Recently, Dowd and Forsbus (18) proposed a regulatory role of SPAK (PASK) on the activity of NKCC1. They showed that overexpression of a dominant negative PASK (inactive kinase) resulted in a lack of activation of NKCC1 upon lowering the intracellular Cl\(^-\). They also demonstrated that inactive SPAK failed to phosphorylate NKCC1 upon activation. Whether or not the kinase directly phosphorylated and activated the cotransporter still remains unclear.

In the present study, we show that preventing the interaction of SPAK and OSR1 with NKCC1 fails to influence the activity of the cotransporter under basal or activated conditions. Our results therefore suggest that binding of the kinase to the two SPAK binding domain is not required for NKCC1 activation. We demonstrate that SPAK, besides colocalizing with cation-chloride cotransporters, interacts with additional proteins that are involved in the response of the cell to stress.

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‡ The abbreviations used are: MAPK, mitogen-activated protein kinase; MAP2, microtubule-associated protein 2; PVDF, polyvinylidene difluoride; PBS, phosphate-buffered saline; GFAP, glial fibrillary acidic protein; AATYK, apoptosis-associated tyrosine kinase.
Furthermore, we show that the ability of SPAK to communoprecipitate p38 changes upon extracellular stress. Thus, we propose that the kinase serves as a scaffolding protein, gathering stress-response proteins in the vicinity of the cotransporters.

**EXPERIMENTAL PROCEDURES**

**NKCC1 Function in Xenopus laevis Oocytes**—The open reading frame of the mouse NKCC1 (3.7 kb) was ligated into pBF, a vector suitable for expression in amphibian oocytes. The cDNA was linearized with MluI and transcribed into cRNA using the mMESSAGE mACHINE SP6 transcription system from Ambion. cRNA was quantitated by measurement of its absorbance at 260 nm, and its quality was verified by agarose gel electrophoresis (1% agarose). A small Narl-NcoI fragment was subcloned into the vector pBluescript (pBSK+) for site-directed mutagenesis. Complementary sense and antisense oligonucleotides containing specific mutations were synthesized and used to amplify the subclone inserted in pBSK+ (QuickChange, Stratagene). The parental DNA was digested with DpnI, a restriction enzyme that recognizes and cleaves methylated GATC sequence. After DpnI treatment, a 1-μl aliquot of the PCR was transferred into *Escherichia coli*. Several clones were isolated and sequenced between Narl and NcoI to verify proper sequence and mutation. The fragment was then reinserted into the original NKCC1 clone in pBF and transcribed.

Stages V–VI X. laevis oocytes were isolated as described previously (10), and incubated for 18 h at 20°C in modified L15 solution diluted with water to a final osmolarity of 195–200 mosmol. A small aliquot of the supernatant was saved for protein assay (Bradford, Bio-Rad). Once with 3 ml of isosmotic saline (96 mM NaCl, 4 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 5 mM HEPES [pH 7.4]) and preincubated for 20 min in 1 ml of isosmotic saline containing 1 mM ouabain. The solution was then aspirated and replaced with isosmotic or hypertonic solutions complemented with 2–5 μCi of 82Rb. The composition of the hyperosmotic solution was identical to the isosmotic solution but with an additional 50 mM sucrose. Two aliquots of 5 μl of flux solution were sampled at the beginning of the incubation and used as controls. After 1 h of uptake, the radioactive solution was aspirated, and the oocytes were washed 4 times with 3 ml of ice-cold isosmotic or hypertonic solution. Preliminary experiments have shown that the flux is linear over a 3-h period. NKCC1 flux is expressed in picomoles of K+/oocyte-h. Statistical analysis was performed using Student’s t-test and analysis of variance.

**Surface Expression of NKCC1 in X. laevis Oocytes**—Groups of 20 oocytes were injected with 27.5 ng of wild-type NKCC1 or deletion mutant NKCC1 cRNA and kept at 16°C for 4 days in modified L15 medium (Leibovitz L15) connected to a G4 Apple computer. The first half was homogenized promptly in 5 ml of lysis buffer (100 mM NaCl, 50 mM NaF, 2 mM EDTA, 150 mM Na3VO4, 0.5% Triton X-100, 10 mM HEPES, 0.5 mM DTT, and protease inhibitors) and incubated for 10 min on ice in the lysis buffer, the immunoprecipitate-agarose beads complexes were resuspended into 65 μl of sample buffer containing 4% β-mercaptoethanol, heated at 50°C for 20 min, and subjected to Western blot analysis.

**Preparation of an OSR1-specific Antibody**—Alignment of SPAK and OSR1 proteins reveals a small region within the carboxyl terminus with very low amino acid conservation (9% identity, see Fig. 1A of our previous publication (13)). Thus, we selected a small fragment within that particular region to create an OSR1-specific rabbit polyclonal antibody. A 74-aminoc acid peptide (residues 363–436) of the mouse OSR1 was PCR-amplified from IMAGE clone 5341146 and fused to glutathione-coupled Sepharose beads. Polyclonal antibodies were produced by subcutaneous injections of the entire fusion protein in complete Freund’s adjuvant. White rabbits were immunized with glutathione-coupled Sepharose beads from the last production bleed was affinity-purified as described previously (20).

**Yeast Two-hybrid Screen**—The binding domain of SPAK to cation-chloride cotransporters (amino acids 461–556) was amplified by PCR from mouse IMAGE clone 2135848 using a sense primer containing an EcoRI site and an antisense primer containing a stop codon following a BamHI site. The fragment was then ligated downstream of the Gal4 binding domain in pGBD-U2 (yeast two-hybrid vector) and transformed into PJ69-4A cells (21). SPAK-containing yeast cells were subsequently transformed with a mouse brain library in pACTII (Clontech), and a total number of 18.9 × 10^9 clones were screened as described previously (22). To test for interaction between SPAK and the amino terminus of NKCC1, the entire amino terminus of the cotransporter was PCR-amplified and ligated into pGBD-U2. For the mutants, Narl-Sacl fragments were isolated from full-length NKCC1 mutants in pBF and exchanged with the wild-type Narl-Sacl fragment in pGBD-U2.

**Yeast Two-hybrid Screen—**To test for interaction between SPAK and the amnioterminal fragment of NKCC1, the entire amino terminus of the cotransporter was PCR-amplified and ligated into pGBD-U2. For the mutants, Narl-Sacl fragments were isolated from full-length NKCC1 mutants in pBF and exchanged with the wild-type Narl-Sacl fragment in pGBD-U2.
RESULTS

NKCC1 Mutants—As indicated in Fig. 1, two SPAK binding domains can be found in the amino terminus of NKCC1. The first domain, RFQVDFPESV, is located 76 residues downstream of the start methionine. The second motif, RFRVNDPDA, is located 48 residues downstream of the first motif and overlaps with a putative PP1-binding motif (24). By using the yeast two-hybrid method, we demonstrated that these two minimal motifs constitute sites of interaction with the kinases SPAK and OSR1 (13). We also demonstrated previously that mutations of the phenylalanine residue at position 2 or the valine residue at position 4 to alanine completely abolished the interaction. Note that the SPAK/OSR1-binding sites are located far upstream of the threonine residue (Thr-211 for mouse NKCC1) involved in phosphorylation. In contrast to the double mutant, a deletion of a large fragment containing both SPAK/OSR1 binding domains as well as a long polyalanine stretch inbetween significantly reduced NKCC1 activity (60% decrease) under isotonic, hypertonic, and low Cl− conditions. However, the percent activation by hypertonicity and low Cl− compared with wild type was unchanged (Fig. 2D). To assess whether the observed overall decrease in NKCC1 activity results from a decrease in NKCC1 trafficking, we injected groups of 20 oocytes with wild-type NKCC1 and deletion mutant NKCC1. Three days post-transfection, the oocytes were biotinylated, washed, and lysed. Western blot analysis of biotinylated protein revealed a higher number of cotransporter in the membrane for the mutant cotransporter, compared with wild-type NKCC1 (see Fig. 4). Effects of the single and double mutations were also tested while coexpressing mouse SPAK cDNA in X. laevis oocytes to ensure that the level of endogenous OSR1 is not a limiting factor. As with the uptake curves shown in Fig. 2D, there were no significant differences between the activity of mutants and wild-type NKCC1 proteins in SPAK coexpressing oocytes (data not shown). These data indicate that preventing SPAK/OSR1 interaction with NKCC1 by preventing the binding has no

![Image](58x207)
Expression of SPAK in the Brain, Spinal Cord, and Sciatic Nerve—SPAK was localized in brain, spinal cord, and sciatic nerve using the immunopurified anti-SPAK antibody, which we characterized previously (13). As negative controls, the primary antibody (SPAK) was omitted from the immunostaining protocol on a series of brain sections adjacent to the ones probed with one antibody. As seen in Fig. 6A, the sizes of the large molecular bands and low molecular bands are unambiguously different for SPAK and OSR1. This clearly suggests that there are two distinct SPAK and two distinct OSR1 proteins. Analysis of the SPAK and OSR1 sequences revealed the presence of downstream second Kozak consensus sequences in both proteins (see Fig. 6C). Initiations at the first methionine give full-length proteins of 60.3 and 58.3 kDa for SPAK and OSR1, respectively. In contrast, translation initiation at the second methionine results in truncated proteins of 49.2 and 52.1 kDa, respectively. Our results of Figs. 5 and 6A are consistent with the hypothesis of two translation initiation sites since the Western analysis clearly shows that the size difference between large and small bands is larger for SPAK than for OSR1. Furthermore, the second translation initiation site for OSR1 is a slightly better Kozak consensus than the first (Fig. 6B), consistent with the higher abundance of the lower protein band. These results suggest that truncated SPAK and OSR1 kinase-inactive proteins are expressed in tissues.

Effect of NKCC1 mutations on SPAK binding, as revealed by yeast two-hybrid analysis. A, the positive interaction between SPAK and the amino terminus of NKCC1 promotes growth of the yeast in plates lacking histidine, leucine, and uracil and containing 3-amino-1,2,4-triazole. B and C, similar interaction between SPAK and each single NKCC1 mutant. D, absence of growth (interaction) in yeast transfected with SPAK and double NKCC1 mutant.

Western blot analysis of biotinylated (streptavidin bead-bound) NKCC1 protein isolated from X. laevis oocytes. Three groups of 20 oocytes expressing wild-type and deletion mutant NKCC1 were treated with EZ-Link sulfo-NHS biotin, washed, and lysed. Biotinylated (surface) proteins were isolated using streptavidin beads and separated on a 7.5% SDS-polyacrylamide gel. Experiment was repeated once with identical results.

In the brain, brain stem, and cerebellum, the strongest SPAK immunolabeling was found on the apical membrane of epithelial choroid plexus cells (Fig. 7A), as described previously (13). We also found prominent staining for SPAK at the nodes of Ranvier in teased sciatic nerve (Fig. 7B), corresponding to the nodal expression of NKCC1 (26). In the spinal cord, cells in both the white matter and the gray matter were labeled (Fig. 7, C–E). Double staining of spinal cord sections with anti-SPA and anti-MAP2 antibodies shows that neurons as well as glia cells are positive for SPAK; adjacent to motor neurons in the ventral horn in the gray matter we detected smaller cells with the solely red fluorescence caused by the anti-SPA-Cy3 conjugate (Fig. 7F). We tested the entire brain for SPAK expression and found significant labeling only in the brain stem. The schematic drawing in Fig. 7G indicates the location of nuclei shown in Fig. 7, H and I, and the position of the section on a rostro-caudal axis (Fig. 7G, inset). A moderate signal was observed in the nucleus of the hypoglossus (Fig. 7H).
and in the inferior cerebellar peduncle (Fig. 7I, icp). The strongest labeling in the brain stem was found in the spinal tract of the trigeminal nerve (Fig. 7I, sp5) along its entire extension (Fig. 7, I and L, and Fig. 8B). In sections further rostral (Fig. 7J), we found moderate immunolabeling in the motor neurons of the facial nerve (Fig. 7K), in cells of the dorsal (Fig. 7M, DCN) as well as ventral cochlear nuclei (Fig. 7N, VCN), and in giant reticular neurons (Fig. 7O). A faint signal was observed in the vestibulocochlear nerve (Fig. 7L). The most rostral sections containing significantly SPAK-immunolabeled structures still derived from the brain stem (Fig. 8A). Within this area, the strongest signal was detected in the aforementioned sp5 (Fig. 8B). The adjacent brain section was not exposed to the primary antibody (negative control) and was devoid of labeling (Fig. 8C). Cells in the medial nucleus of the trapezoid body (MNTB) were positive for SPAK (Fig. 8D) and were identified as neurons by double-labeling with MAP2 antibody (Fig. 8, E and F). Other auditory structures that were moderately labeled were the lateral nucleus of the trapezoid body (Fig. 8G, LNTB), the anterior ventral cochlear nucleus (Fig. 8H, AVCN), as well as the posterior ventral cochlear nucleus (not shown). SPAK labeling was also found in motor neurons of the trigeminal nerve (Fig. 8J). In contrast to the 8th cranial nerve, which was SPAK-immunolabeled (Fig. 8J), no staining was found in the 7th nerve as shown by the lack of staining in Fig. 8K and by the double labeling of the same section with SPAK and GFAP in Fig. 8L. These results suggest that SPAK is expressed in the gray and white matter of the spinal cord, the nodes of Ranvier in the sciatic nerve, and in several brain stem structures. More rostral areas were devoid of signal except for the choroid plexus. Furthermore, SPAK expression was shown in both neuronal and glial cells.

Yeast Two-hybrid Screen of a Mouse Brain Library with the Carboxyl-terminal Binding Domain of SPAK—To better delineate the functional pathway linking cation-chloride cotransporters to SPAK, we sought to identify additional binding partners of the kinase. We screened a yeast two-hybrid mouse brain library (cDNAs inserted in pACT2) by using the binding domain of SPAK as a bait. From 18.9 × 10⁶ clones plated on growth restrictive substrate (−uracil, −leucine, and −histidine), we isolated 139 clones that survived several rounds of selection. From these surviving clones, we identified several SPAK-binding partners, some of which were identified multiple times through independent cDNA clones (see Table I). For instance, apoptosis-associated tyrosine kinase (AATYK) was identified 7 times through 4 distinct cDNA clones, one of which was isolated 4 times. One interactor isolated several times was identified through BLAST searches as 5E5 antigen using the NCBI data base and as a tyrosine kinase using the mouse Celera data base. This novel Celera protein contains a typical tyrosine kinase domain. Its alignment with AATYK revealed an overall 29% homology with several regions of high conservation, indicating a relationship between the two proteins. Analysis of the amino acid sequence of these clones revealed the presence of SPAK-binding motifs. Again for the apoptosis-associated tyrosine kinase, we identified three sequences within the carboxyl terminus that resemble the SPAK-binding motif. These sequences were RFEDWDGFDP, RFTVSPTPA, and RFSITHISD. We tested all three sequences for interaction with SPAK showing the two putative start sites. The shaded box represents the kinase domain. Note that the second methionine truncates the kinase domain.

FIG. 6. Evidence for two translational start sites in mouse SPAK and OSR1. A, Western analysis of 60 μg of kidney protein with SPAK and OSR1 antibody. After transfer to PVD membranes, the lane was cut in the middle to precisely size the SPAK and OSR1 bands. Note that the SPAK and OSR1 bands do not correspond and that the lane was cut in the middle to precisely size the SPAK and OSR1 with SPAK and OSR1 antibody. After transfer to PVDF membranes, kinase domain. Note that the second methionine truncates the kinase domain. 

Coimmunoprecipitation of SPAK, NKCC1, and p38—To examine further the relationship between NKCC1, SPAK, and cellular stress, we isolated proteins from control brain and ischemic brain tissue, which was exposed to non-carboxygenated aCSF, and performed coimmunoprecipitation experiments. Evidence for ischemic stress was verified by the marked activation of phospho-p38 (Fig. 9A, pp38). The figure panel also shows that the amount of NKCC1 and SPAK coprecipitated with pp38 decreases with cellular stress. In contrast, the amount of SPAK coprecipitated with NKCC1 does not change significantly with stress. Of interest is the observation that only the larger molecular form of NKCC1, which represents phosphorylated NKCC1, interacts with pp38. Indeed, Fig. 9B shows two distinct NKCC1 bands in the NKCC1 immunoprecipitate, with the lower molecular size much more abundant than the higher molecular size. Only the larger band is observed in a pp38 immunoprecipitate run in the same gel in an adjacent lane. These data suggest that NKCC1, SPAK, and p38 form a complex and that p38 or pp38 but not SPAK is released upon cellular stress.

DISCUSSION

In an effort to characterize the SPAK/OSR1-binding site, we have shown previously by alanine-scanning mutagenesis on KC3 and NKCC2 motifs that a mutation of the phenylalanine residue at position 2 or the valine residue at position 4 into alanine completely disrupted SPAK interaction with these cotransporters (13). The absence of interaction was also verified by glutathione S-transferase pull-down assays. In the present study, we created several NKCC1 mutants disrupting one or both binding motifs that we had identified previously at the amino terminus of NKCC1, and we examined their transport activity in X. laevis oocytes. Our rationale was as follows: if the kinase interacts and phosphorylates NKCC1, thus leading to a change in its activity, preventing the kinase from interacting with the cotransporter should prevent activation of transport. Fig. 3 demonstrates that the two SPAK-binding motifs are functional binding sites; mutation of either one alone does not preclude interaction, whereas the double mutant shows a complete absence of interaction. Our flux experiments demonstrate that a double mutant cotransporter that is unable main of SPAK as a bait. From 18.9 × 10⁶ clones plated on growth restrictive substrate (−uracil, −leucine, and −histidine), we isolated 139 clones that survived several rounds of selection. From these surviving clones, we identified several SPAK-binding partners, some of which were identified multiple times through independent cDNA clones (see Table I). For instance, apoptosis-associated tyrosine kinase (AATYK) was identified 7 times through 4 distinct cDNA clones, one of which was isolated 4 times. One interactor isolated several times was identified through BLAST searches as 5E5 antigen using the NCBI data base and as a tyrosine kinase using the mouse Celera data base. This novel Celera protein contains a typical tyrosine kinase domain. Its alignment with AATYK revealed an overall 29% homology with several regions of high conservation, indicating a relationship between the two proteins. Analysis of the amino acid sequence of these clones revealed the presence of SPAK-binding motifs. Again for the apoptosis-associated tyrosine kinase, we identified three sequences within the carboxyl terminus that resemble the SPAK-binding motif. These sequences were RFEDWDGFDP, RFTVSPTPA, and RFSITHISD. We tested all three sequences for interaction with SPAK showing the two putative start sites. The shaded box represents the kinase domain. Note that the second methionine truncates the kinase domain.

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to interact with the kinase is fully functional and activable to
the same degree by hyperosmotic and low internal Cl
stimuli as wild-type NKCC1. Our approach is independent of a distinc-
tion between endogenously expressed SPAK in oocytes versus
heterologously coexpressed SPAK, since the binding prevent-
ing mutation is on the cotransporter. In addition, the activity of
NKCC1 and its correct expression can be directly monitored via
the flux studies. In our assay system, which differs from Dowd

Fig. 7. A, immunohistochemical labeling of brain slices with anti-SPAK antibody was strongest on the apical membrane of epithelial choroid
plexus cells. B, SPAK immunolabeling at the node of Ranvier of teased sciatic nerve. C–E, in the spinal cord, cells in both the white matter (WM)
and gray matter (GM) were immunolabeled. F, double staining of motor neurons in the ventral horn of the spinal cord with anti-SPAK and
anti-MAP2. Note the exclusively SPAK-stained glial cells adjacent to the motor neurons. G and J, schematic drawing of brainstem slices indicating
the positions of SPAK-positive nuclei shown in the following pictures. Insets in G and J, the vertical line indicates the plane of the slice on a
rostro-caudal axis. H–O, SPAK immunolabeling was detected as follows: H, motor neurons of the hypoglossus; I, the spinal tract of the trigeminal
nerve (sp5); K, motor neurons of the facial nerve; L, the vestibulocochlear nerve; M, the dorsal cochlear nucleus (DCN); N, the ventral cochlear
nucleus (VCN); O, giant reticular neurons. Scale bars: 30 μm in A and B, D–F, H, K, and M–O; and 100 μm in C, I, and L.

SPAK Interaction with NKCC1
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and Forbush (18), we were thus unable to demonstrate functional changes. These authors had recently reported a reduction of human and shark NKCC1 activity by overexpressing the kinase negative, dominant negative PASK mutant. Differences between the two sets of data could originate from differences between HEK293 cells and *X. laevis* oocytes. However, this would suggest that different regulatory proteins (e.g., kinases) and separate regulatory pathways would produce identical activation from identical stimuli in different cell types. One could also conceive that the binding and the phosphorylation are completely independent events, in which case the kinase could phosphorylate NKCC1 in the absence of binding. If true, one has to conclude that the binding of the kinase to the cotransporter serves a function that is completely independent of phosphorylation. It is noteworthy that in PASK overexpression studies, Dowd and Forbush (18) detected a significant, small difference only at an intermediate activation level (preincubation in 30 mM extracellular Cl⁻) but not at lower or maximal levels of activation. Thus, at maximal activation levels, the study of Dowd and Forbush (18) is in line with our finding of no significant change of transport activity. Finally, the overexpression of the dominant negative SPAK mutant in HEK293 cells in the study by Dowd and Forbush (18) might have produced indirect effects on NKCC1 activation. Their data using immunoprecipitated HA-SPAK on cotransporters are more difficult to reconcile with our experiments. It is possible that the coimmunoprecipitated HA-SPAK interact with SPAK (see below). However, if these kinases

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**Fig. 8.** A, schematic drawing of brainstem slice locating SPAK positive structures shown in the following pictures. The vertical line in the inset indicates the plane of the slice on a rostro-caudal axis. B, in the brainstem, SPAK immunolabeling was most intense in the spinal tract of the trigeminal nerve (*sp5*). C, the adjacent control section, shows no staining above background. D, moderate SPAK labeling was detected in the medial nucleus of the trapezoid body. E, staining for MAP2 of the same slice shown in D, F, superimposing pictures from D and E, identifies SPAK-positive cells as neurons. SPAK labeling was also found as follows: G, in the lateral nucleus of the trapezoid body (*LNTB*); H, anterior ventral cochlear nucleus (*AVCN*); I, motor neurons of the trigeminal nerve; and J, the auditory nerve (*VIII nerve*). K, the trigeminal nerve (*VII nerve*) is devoid of SPAK immunolabeling. L, same section as in K. Double staining of the VIIth nerve with anti-SPAK and anti-GFAP antibodies. Scale bar: 30 μm in B (for B–I).
are brought into contact with the cotransporter via SPAK binding, disrupting the interaction of the scaffolding protein should disrupt the availability of the associated kinase and thus affect the activity of the flux. Further work is needed to address this issue and reconcile these data.

Immunofluorescence studies revealed that SPAK expression in the nervous system is far more abundant than OSR1 expression. OSR1 is generally found in structures similar to those expressing SPAK, but at much lower levels (data not shown). As shown in our previous study, SPAK expression is highest in choroid plexus, solely localized on the apical membrane (Fig. 8A). Apical expression coincides with NKCC1 (13, 27, 28). In spinal cord, the kinase is found in both white and gray matter, a pattern that correlates well with KCC3 staining (20). In the nerve fiber, abundant SPAK expression is found in the node of Ranvier, where NKCC1 is abundantly expressed (26). Thus, SPAK expression seems to correlate tightly with cation-chloride cotransporter expression. Outside the choroid plexus, SPAK is found in the brain and mostly in brain stem structures, with the most prominent labeling seen in the spinal tract of the 5th nerve and in the auditory nuclei of the medial nucleus of the trapezoid and the cochlear nucleus. Our immunofluorescence data therefore agree with the conclusions of Ushiro et al. (15) that SPAK is rich in epithelia and neurons and add more detailed information about SPAK-positive nuclei. The question of why SPAK expression is high specifically in the aforementioned nuclei remains to be solved.

By using the extreme carboxyl terminus of SPAK as a bait in a yeast two-hybrid screen, we identified several interesting proteins as interacting candidates of SPAK/OSR1 as follows: HSP105, a heat shock protein that was originally cloned based on its marked up-regulation by dehydration in the renal medulla (29, 30); gelsolin, an actin-binding protein that protects against stress when overexpressed (31) and that is depleted in cells after oxidative injury (32); otoferlin, a protein originally cloned from inner ear (33) with alternatively spliced isoforms in brain, believed to be involved in membrane trafficking in a Ca2+-dependent manner (such as synaptotagmin) (34); a tyrosine kinase (AATYK) that is related to the apoptotic pathway (35); a novel tyrosine kinase with unidentified function but related to AATYK; and WNK4 a kinase that has been implicated in hypertension (36) and that is involved in regulating trafficking of the Na-Cl cotransporter (37). Together with the study by Johnston et al. (38) and our demonstration by co-immunoprecipitation that SPAK interacts with p38, these

| INTERACTING PROTEINS | LENGTH | # | LAC Z | CLONE STARTS | SPAK MOTIFS |
|----------------------|--------|---|-------|--------------|-------------|
| Apoptosis-associated TK (AATYK) | 1317 | 7 | 3.6–36.1 | EDSEE... 1127 (4) | RFTVs [1279]* |
| Glial fibrillary acidic protein (GFAP) | 731 | 6 | 5.0–16.8 | LFQVR... 514 (1) | RFVle [627] |
| Otoferlin | 1997 | 2 | 8.4–41.3 | NQSPGL... 1386 (2) |
| WNK4 | 954 | 2 | 6.1–19.9 | PSILLP... 579 (2) | RFQy [738] |
| Heat shock protein 105 (HSPA8) | 859 | 1 | 4.1 | PYPEA... 473 (1) | RFVg [461] |
| Novel tyrosine kinase (similar to AATYK) | 1307 | 7 | 3.6–16.8 | REAPV... 1108 (1) | RFSVs [1234] |

Table I: Summary of yeast two-hybrid screening with the binding domain (C terminus) of SPAK

The 1st column lists the mouse clones identified as positive interactors of SPAK. The 2nd column indicates the length of the mouse protein (in number of residues). The 3rd column reports the number of times each clone was isolated. The 4th column includes the range of the LacZ values (out of 139 clones measured, the average LacZ was 5 ± 6). The 5th column lists the independent clones isolated for each interacting protein: the beginning of the library clone amino acid sequence, its position within the protein, and in parentheses the number of times the clone was isolated. In the last column, we list the SPAK motifs identified within the interacting protein. The number in brackets indicates the position of the first residue within the protein. Asterisk, the motif was tested for interaction with SPAK using yeast two-hybrid assay, and the interaction was positive.
newly identified interactor candidates of SPAK strongly suggest a role for this Ste20 kinase in cellular stress.

Consistent with our hypothesis that SPAK and OSR1 function as scaffolding proteins is the observation that the two stress-related proteins exist in two forms in cells: a large molecular weight form that conserves the catalytic domain of the kinase, and a lower molecular weight form with a truncated catalytic domain. For SPAK, it is only in brain tissue that the large molecular weight form is far more abundant than the lower molecular form. For OSR1, which is phylogenetically older than SPAK, the truncated form is more abundant than the full-length form. This suggests either an important regulatory role as a native kinase or a function of the protein that is completely independent of kinase activity. It is of interest to note that the second methionine is conserved in amphibians and in Drosophila but is substituted by leucine, isoleucine, or valine residues in C. elegans and plants. The prevalence of the shorter version is therefore likely to represent an adaptation process of OSR1 in higher organisms such as amphibians, insects, and mammals.

Strong support for the notion that SPAK and OSR1 might function as scaffolding proteins stems from our demonstration that SPAK interaction with NKCC1 (amount of SPAK communoprecipitated with NKCC1) is not affected by cellular stress, such as ischemic stress, whereas the interaction of p38 with SPAK (p38 communoprecipitated with SPAK) and with NKCC1 (p38 communoprecipitated with NKCC1) is significantly reduced upon stress.

Together with the fact that SPAK expression was shown to highly correlate with NKCC1 (on the apical membrane of cholinergic plexus, on the basolateral membrane of salivary epithelial cells, at the node of Ranvier) and NKCC2 (apical membrane of thick ascending limb of Henle), data shown under normal non-stress conditions, our data suggest that SPAK might play a scaffolding role. We suggest that SPAK/OSR1 gather stress-related proteins in the proximity of cation-chloride cotransporters and, as we have shown here for phosphorylated p38 and SPAK, release proteins upon stress stimuli. It is therefore tempting to speculate that the cotransporters might play a sensing and/or transducing role in the response of cells to environmental stress.

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