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PASK-mediated Regulation of NKCC1

alnine-rich kinase (11)) is a 55-kDa serine/threonine kinase within the germinal center kinase VI subfamily of STE20 group kinases (12). First isolated from rat brain, PASK was found to be heavily expressed in epithelial cells active in ion transport and to associate with the cytoskeleton (7). The only demonstrated biological function for this kinase was reported for Fray, the Drosophila PASK homologue, which was shown to be expressed in glial cells and required for the proper ensheathing of axons (13).

In this report, we describe the regulation of NKCC1 by PASK in transfected HEK cells. We show that overexpression of PASK causes a small but significant increase in NKCC1 activity. We also show that a dominant-negative PASK mutant drastically inhibits shark and human NKCC1 activation. This inhibition is via a decrease in NKCC1 regulatory phosphorylation, not a decrease in number of functional cotransporter molecules or a down-regulation in expression. We also show that PASK phosphorylation parallels that of NKCC1 in low [Cl], and that binding of the kinase does not appear to be regulated by or dependent upon the activation state of PASK or NKCC1.

EXPERIMENTAL PROCEDURES

cDNA Constructs and the Production of Stable Cell Lines—The rat PASK clone was tagged on the N terminus and subcloned into the pIREs puro3 vector (Invitrogen) using PCR. Briefly, a forward primer (p1) containing the HA antigen and EcoRI site (gggattcaatgatcagtccagtcagtccagtcag) and its reverse complement (p2) containing a stop codon and BamHI site (gaggctctcagcactccactcacacacgacgacag) were used in a PCR reaction with pGST-PASK as a template (a kind gift of William Leiserson) (7). The PCR product was then subcloned into the pIREs puro3 vector using EcoRI and BamHI.

The dominant negative, kinase inactive K101R PASK was created using a two-step PCR approach. First, an internal primer (p3) (CGCG-TAGCCATAAGCGGATCAACTTG) and its reverse complement (p4) (TGGCGGAGCCGAGCGGCTCG) were created containing a single nucleotide substitution to produce the K101R mutation (mutation in lowercase). PCR reactions were completed using p1/p4 and p2/p3 primer combinations. After gel purification of the two products, equal amounts were subjected to five cycles of PCR with Pfu polymerase but without primers (for extension). The p1 and p2 primers were then added to amplify the full-length mutant. Subcloning into pIREs puro3 (Clontech) was completed as described above. Sequence verification was completed by the Mount Desert Island Biological Laboratory DNA Sequencing Center.

For most experiments a shark protein kinase (sMST2/2) with significant homology to mammalian MST1 (accession number AAA83254) and MST2 (accession number 2204254A) was used as a control in experiments. Cells were selected and maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 1 µM puromycin (Clontech), and 0.8 µg/ml Geneticin (Invitrogen). With the use of the IRES vector, clonal selection was not needed in isolation of stable lines. The IRES vector is herein referred to as “vector 2,” whereas the pJB20 vector used for sNKCC1 and hNKCC1 expression is referred to as “vector 1.”

32P Incorporation and Immunoprecipitation Experiments—Near confluent 10-cm dishes were rinsed with phosphate-free isosmotic media (135 mM NaCl, 5 mM RbCl, 1 mM CaCl2/MgCl2, and 15 mM NaHEPES, pH 7.4) and incubated with 250 µCi of 32P-P for 20 min. Cells were then rinsed 2× with either phosphate-free isosmotic or phosphate-free 3 mM CI- with gluconate replacement (135 mM sodium gluconate, 5 mM potassium gluconate, 1 mM CaCl2, 1 mM MgCl2, 1 mM Na2HPO4, 1 mM NaSO4) for 1 h at room temperature. Cells were lysed on ice in 1 ml of lysis buffer (300 mM NaCl, 50 mM HEPES, 2.5 mM β-glycerophosphate, 1% Nonidet P-40, 1 mM sodium vanadate, 5 mM EDTA, 0.5 µM calyculin A, protease inhibitor mixture (Roche Applied Science)) and centrifuged to remove debris. Bovine serum albumin (0.25% final concentration) and 5 µg of antibody were added before a 2-h incubation at 4 °C. Protein G beads (Fischer) were blocked in 5% milk/phosphate-buffered saline, 0.1% Tween 20, washed in lysis buffer, added to lystate, and incubated at 4 °C for an additional hour. Immunocomplexed beads were washed 3× in ice-cold wash buffer (lysis buffer without calyculin A and protease inhibitors) and 1× in ice-cold phosphate-buffered saline. Sample buffer with 2-mercaptoethanol was added before boiling. Samples were then subjected to SDS-PAGE using a 10% acrylamide gel. Western blotting was completed as previously described (4). For protein determinations of 96-well flux plates, cells were solubilized with 1% SDS after being exposed to phosphorimaging plates, and the DC-BCA kit (Pierce) was used according to the manufacturer’s instructions.

In the post-lysis binding experiment, immunoprecipitation was conducted as described above except for the following changes; after lysis and centrifugation, 200 µl of HEK cell lystate was added to 200 µl of vector 1/PASK, sNKCC1/vector 2, or sNKCC1/PASK. In a fourth tube, 200 µl of lystate from vector 1/PASK and sNKCC1/vector 2 were combined before the immunoprecipitation step.

Regulatory Phosphorylation Time Course—Transfected HEK cells were grown to confluence in a 96-well polystyrene-treated plate. Wells were rinsed with incubation media for specific time points, and the reaction was terminated with the addition of 1 mM H3PO4, 1% SDS. Samples were neutralized with 3× Tris, 1× NaOH and loaded in duplicate onto two separate gels. SDS-PAGE with Western analysis using the phosphospecific R5 and J3 antibodies was completed in parallel. Comparison of parallel blots was necessary due to selective loss of signal on stripping.

Antibodies—The sNKCC1-specific J3 antibody and R5 phospho-specific NKCC1 have been previously described (4, 14). Polyclonal (MBL) and monoclonal (Roche Applied Science) anti-HA antibodies were purchased.

General—All incubations were carried out at room temperature (21–24 °C) unless otherwise noted. Data are presented as the means ± S.E. from replicate experiments except as noted (Fig. 1). Statistical significance was determined for summary data by the paired t test (data related to Figs. 1 and 2) or Wilcoxon paired rank test (data related to Fig. 4).

RESULTS AND DISCUSSION

To address the effect of PASK activity upon NKCC1 activation, HEK clonal cell lines stably expressing sNKCC1 or hNKCC1 were transfected with PASK or a kinase inactive, DN-PASK. This previously described PASK mutation (7, 11), consisting of a single amino acid substitution (K101R), involves an essential lysine residue found within the catalytic domain of most kinases and has been shown to abolish the positioning of the terminal phosphate of ATP (17). sNKCC1 cells were also transfected with the shark homologue of MST1/2 or with a MST1/2 kinase inactive mutant created via a Thr→Ala mutation in the activation loop of its catalytic domain. This mu-
PASK-mediated Regulation of NKCC1

The expression of DNPASK results in a dramatic inhibition of NKCC1 activation in HEK cells. Fig. 1 illustrates the results of several experiments in which $^{86}$Rb influx activity was exam-

![Figure 1](image1.png)

**FIG. 1.** The effect of PASK and DNPASK on the activation of NKCC1 cotransport. A, phosphorimage of $^{86}$Rb influx assays using HEK-hNKCC1 clonal cell line transfected with vector 2 DNPASK, or PASK. Confluent cells in a 96-well plate were assayed in a 2-min $^{86}$Rb influx after a 60-min preincubation in media of various Cl$^-$/H$^+$ concentrations and tonicity, as noted. [Cl$^-$/H$^+$] was altered isotonically with gluco-

nate replacement. Hypo/1.5 Cl$^-$/H$^+$ is a 3 mm Cl$^-$ solution diluted 1:1 with H$_2$O. Furosemide (Furo, 50 μM) was added to stated preincubation solutions to inhibit NKCC1-mediated restoration of [Cl$^-$/H$^+$] and was removed by rinsing immediately before the flux. B and C, graphical representation of similar experiments with hNKCC1 (B) or sNKCC1 (C) (vector 2 (○) PASK (■) DNPASK (▲)). Data shown are the means of 12 replicate wells ± S.D. from a single representative experiment; similar data, summarized in the text, were obtained in 13 other experiments with hNKCC1 and 5 other experiments with sNKCC1.

![Figure 2](image2.png)

**FIG. 2.** The effect of PASK and DNPASK on NKCC1 regulatory phosphorylation. A, the time course of NKCC1 activation was mea-

ured using the anti-phospho-NKCC1 antibody (R5; top four rows). Before lysis, cells were incubated in hypotonic (150 mosmol) 1.5 mM Cl$^-$ for the time noted. The lower band on the R5 blots is believed to represent endogenous hNKCC1 (see Flemmer et al. (4)). The sNKCC1 immuno-

blot (using the J3 antibody) was utilized as a measure of total NKCC1; Coomassie Blue staining illustrates that there are similar protein levels in the control cells. B, regulatory NKCC1 phosphorylation at three time points, analyzed as the R5/J3 ratio normalized to the vector 2 control within each of four experiments. Differences are significant ($p < 0.01$) for DNPASK as well as ($p < 0.05$) for PASK at the 10-min time point and sMST1/2 at the 3-min time point.

The expression of DNPASK results in a dramatic inhibition of NKCC1 activation in HEK cells. Fig. 1 illustrates the results of several experiments in which $^{86}$Rb influx activity was exam-
ined in NKCC1-expressing HEK cells transfected with DNAPASK, PASK, or vector 2 alone. In these experiments, cotransport activity was activated by preincubation in media of various Cl\(^-\) concentrations, as previously described (5). As shown here, DNAPASK expression produced a dramatic decrease in the level of NKCC1 activity for both human (Fig. 1A and B) and shark (Fig. 1C) cotransporters. Under conditions of maximal activation by preincubation in 3 mM Cl\(^-\)/H\(^+\) conditions, DNAPASK decreased the activity of hNKCC1 by 80 ± 3% in 14 experiments including those of Fig. 1, and it decreased sNKCC1 activity by 60 ± 5% in 6 experiments. This high level of inhibition mediated by the DN mutant strongly suggests that PASK activity is essential for the activation of NKCC1 in HEK cells.

As seen in Fig. 1, overexpression of PASK causes an increase in the activity of NKCC1. Under conditions of maximal activation, the differences were not statistically significant when all of the experiments were analyzed, but at intermediate activation levels the differences were larger. For instance, in the 14 experiments with hNKCC1 and 6 with sNKCC1, after preincubation in 30 mM Cl\(^-\), the PASK-induced increase in NKCC1 activity was 12 ± 3% (p < 0.01) and 22 ± 8% (p < 0.05), respectively, after each curve was normalized to the maximal activity in 1.5 mM Cl\(^-\)/hypotonic conditions. These results are consistent with a situation in which there is sufficient endogenous PASK to fully regulate NKCC1 but in which overexpression of PASK creates a shift toward higher activation at intermediate levels of stimulation. The present data are the first to directly link PASK with the regulation of NKCC1 activity, complementing an earlier study in which PASK binding to NKCC was demonstrated (6).

Previous work in our laboratory has identified an N-terminal regulatory domain in which the phosphorylation state of two threonines (Thr\(^{184}/\text{Thr}^{189}\) in shark) correlates with NKCC1 activation (3). To determine whether DNAPASK-mediated NKCC1 inhibition also causes a reduction in regulatory phosphorylation, we used the previously described, phosphospecific antibody (R5) raised against two of these phosphorylated threonines (4). As shown in Fig. 2A, overexpression of DNAPASK causes a dramatic reduction in the Thr\(^{184}/\text{Thr}^{189}\) phosphorylation of sNKCC1 throughout the activation time course; in the four experiments summarized in Fig. 2B, the reduction is 75 ± 8% at the 10-min time point. This result is consistent with the results of \(^{86}\)Rb\(^+\) influx assays and it links PASK to both the activation and phosphorylation of NKCC1. As in the \(^{86}\)Rb influx assays, any effect of PASK under conditions of maximal stimulation is small, and statistical significance is achieved in these four experiments only at the 10-min time point (14% increase).
The experiments of Fig. 2 rule out nonspecific interactions like that of the myosin light chain kinase inhibitor ML-7, where inhibition of NKCC1 did not alter the level of NKCC1 phosphorylation (8). Importantly, DNPASK-mediated inhibition of NKCC1 is not due to a decrease in cotransporter expression, as shown using the shark-specific sNKCC1 antibody, J3. These data are also consistent with the decrease of sNKCC1 32P incorporation under low [Cl] e conditions (see Fig. 4) and indicate that the action of DNPASK is to prevent phosphorylation of NKCC1.

To further test the action of DNPASK in affecting the NKCC1 phosphorylation state, we measured 86Rb influx in the presence of the protein phosphatase type 1 inhibitor calyculin A. We have previously shown that this compound causes a rapid and dramatic increase in NKCC1 activity via the inhibition of the regulatory phosphatase (5). In the experiments depicted in Fig. 3, b–h, transfected cells are preincubated in various [Cl] i or hypo- or hypertonic media before the addition of calyculin A. Independent of the preincubation condition, NKCC1 activity was initially at a very low level in DNPASK cells (triangles) before calyculin A, similar to the result in Fig. 1. Upon the addition of the phosphatase inhibitor, activity is seen to rise to a level similar to that in vector 2 (closed circles) and PASK (open circles) cells. Expressed as the ratio of DNPASK/vector 2 activity at maximal activation (1.5 mM Cl−, hypotonic preincubation) in 12 experiments, the restoration of activity was 0.97 ± 0.1% after 16 min in calyculin A. These data clearly confirm that the effect of DNPASK is due to an alteration in phosphorylation/dephosphorylation and not to a nonspecific effect such as a reduction in functional NKCC1 protein levels.

The preincubation condition for Fig. 3a is 150 mM Cl−, 10 mM K+ media; this has previously been used to fully inactivate NKCC1, presumably by raising [Cl] i (1, 5). In each cell line, NKCC1 activity is seen to increase during the incubation with calyculin A; interestingly, the rate of activation is similar for cells transfected with PASK, DNPASK, or vector 2. This result can be explained if PASK activity is completely inhibited in this preincubation condition, implying that the low rate of NKCC1 phosphorylation seen on the addition of calyculin A must be due to another kinase whose identity is yet unknown.

The rates of calyculin A-induced NKCC1 activation were
calculated from the data in Fig. 3 for conditions when NKCC1 is inactive (Fig. 3a) and when it is partially activated by pre-incubation in 30 mM Cl− medium (Fig. 3d) or cell shrinkage (Fig. 3g); the derived rate constants are presented in the figure legend. Although there was a greater than 15-fold increase in the rate constant of PASK- and vector 2-transfected cells during incubation in either of the two activation conditions, the DNPASK-transfected cells displayed less than a 2-fold increase in the rate constant of calyculin A-induced NKCC1 activation. This result is consistent with the idea that most of the residual kinase activity in the DNPASK-transfected cells is due to a kinase other than endogenous PASK.

To investigate PASK association with the cotransporter, reciprocal communoprecipitation experiments were carried out in double-transfected HEK cell lines (Fig. 4). sNKCC1 was immunoprecipitated from cell lysate using a shark-specific anti-sNKCC1 antibody (Fig. 4A), or the tagged kinases were immunoprecipitated with an anti-HA antibody (Fig. 4C). Western blotting shows a much higher level of PASK and DNPASK compared with sMST1/2 as control in immunoprecipitates using anti-sNKCC1 (Fig. 4A). Cells not expressing sNKCC1 (vector 1) are also shown as a further control for specificity. These data are consistent with previous GST pull-down experiments (6) and directly demonstrate the co-association of PASK and DNPASK with NKCC1 in a mammalian cell system.

In this experiment we also address the question as to whether PASK activity is necessary for association with sNKCC1. In Fig. 4A, middle panel, we find that both HA-tagged DNPASK and PASK are present in the immunocomplex when precipitated with the anti-sNKCC1 antibody (J3). Although DNPASK levels are significantly less than PASK in the J3 immunoprecipitate, this coincides with a lower expression level of DNPASK (not shown). The reason for this lower level of expression is unclear but possibly represents clonal selection resulting from a DNPASK-induced, negative-selective pressure. The amounts of sNKCC1 that are co-immunoprecipitated with HA-tagged DNPASK and PASK (Fig. 4C, lower panel) are similar in 5 experiments (PASK/DNPASK ratio 1.7 ± 0.4 for stimulated and 1.1 ± 0.3 for unstimulated cells) and appear to reflect the amounts of immunoprecipitated kinase shown in the HA blot (Fig. 4C, middle panel). These results suggest that PASK activity may not be required for PASK binding to NKCC1.

Additionally in this experiment we address whether PASK binding is dependent upon the activation state of NKCC1. Figs. 4, A, middle panel, and C, bottom panel, show that cells preincubated for 60 min in either a low (+) or normal (-) [Cl-] solution before lysis display similar levels of the HA-tagged PASK or DNPASK associated with sNKCC1. The slight increase shown in this figure was not reproducible in the four replicate experiments; the ratio of stimulated to non-stimulated binding was 1.2 ± 0.3 for PASK and 0.8 ± 0.2 for DNPASK. Thus, it appears that the activated state of sNKCC1 is not a prerequisite for, or consequence of, PASK binding, and the possibility of NKCC1 regulation via a binding competition between protein phosphatase type 1 and PASK seems unlikely.

The cells used in this communoprecipitation experiment were preloaded with 32P to measure phosphate incorporation into both the kinase and the cotransporter. Stimulation of NKCC1 under these conditions is confirmed in Figs. 4, A, top panel, i, and B by the increase in phosphorylation in J3 immunoprecipitates. In eight experiments, we found a 64 ± 3% DNPASK-mediated reduction in NKCC1 phosphorylation under stimulatory conditions. The reduction in phosphorylation mirrors the 68% inhibition of transport activity of sNKCC1 activity reported above (e.g. Fig. 1C), and it is consistent with the −75% inhibition of regulatory phosphorylation seen with the phosphospecific R5 antibody (Fig. 2B). No significant changes in NKCC1 phosphorylation were seen in PASK (1.01 ± 0.20-fold, n = 8) or sMST1/2 (1.01 ± 0.14-fold, n = 8) -transfected cells, consistent with the above-noted lack of significant differences in flux activity under maximally activating conditions.

Also in this experiment we investigated whether PASK phosphorylation coincides with NKCC1 activation. The activation requirements for PASK have not been previously reported. In Fig. 4C, upper panel, middle band, PASK immunoprecipitated with the anti-HA antibody displays a dramatic increase of 32P incorporation in cells incubated for 60 min in low [Cl], before lysis; this change was 5.5 ± 0.4-fold in 5 experiments (Fig. 4D). The change in the phosphorylation of immunoprecipitated DNPASK (1.9 ± 0.6-fold) was not significant. These data are mirrored in the level of phosphorylation of the kinase that is co-immunoprecipitated with sNKCC1 (Fig. 4A, upper panel, ii, 4.5 ± 0.5 for PASK; 1.8 ± 0.3 for DNPASK). Our interpretation of this result is that the activity of PASK is necessary for its own phosphorylation.

The above finding suggests that either the kinase undergoes intramolecular autophosphorylation when preincubated in [Cl], or that PASK activity initiates a positive feedback loop that results in its own phosphorylation by another downstream kinase. Although the former ties the activation of PASK to its phosphorylation, the latter does not. Although an increase in kinase phosphorylation commonly corresponds with an increase in its activity, there are known exceptions; therefore, at this time we cannot link the phosphorylation of PASK with its activation. In Fig. 4A, upper panel, ii, we failed to detect a 32P-labeled band that corresponds with the endogenous PASK in sNKCC1/vector 2 immunoprecipitates. Given the low level of phosphorylation detected even within the overexpressed cell lines, we believe that the level of phosphorylated, endogenous PASK is below the limits of detection in this experiment.

We wondered if post-lysis binding during immunoprecipita-
tion might contribute to the result shown in Fig. 4. To test this, we lysed four separate cell lines, (a) HEK cells exogenously expressing HA-tagged PASK alone, (b) HEK cells exogenously expressing sNKCC1 alone, (c) HEK cells exogenously expressing both sNKCC1 and PASK, and (d) non-transfected HEK cells. Cell lysates of (a), (b), and (c) were then each combined 1:1 with (d) as controls. In a fourth tube, lysate from (a) + (b) was combined before the sNKCC1 immunoprecipitation step. Although binding occurs in the cell line exogenously expressing both PASK and sNKCC1 (c), little binding occurs when lysate from cells singly expressing either sNKCC1 or PASK ((a) + (b)) are combined for the immunoprecipitation (Fig. 5). These data strongly suggest that the observed PASK binding does indeed occur pre-lysis.

We have previously reported large regulatory differences between exogenously expressed hNKCC1 and endogenous NKCCs bumetanide-sensitive 86Rb influx in HEK cells. The regulation of this endogenous NKCC1 in HEK cells differs greatly when compared with other mammalian and shark NKCCs investigated, both in native tissue and transfected cell systems (characterization of these phenomena are discussed in detail in Flemmer et al. (4) and Isenring et al. (19)). To address whether PASK regulates endogenous NKCC activity, we transfected a stable clonal cell line of HEK pcDNA3.1 empty vector whether PASK regulates endogenous NKCC activity, we transfected a stable clonal cell line of HEK pcDNA3.1 empty vector

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The regulatory pathways of NKCCs and KCCs have been proposed to be linked due to their converse activation requirements (for review, see Ref. 24). The activity of the cotransporter kinase (adapted from Jennings and Al-Rohil v-kinase (25)) would, therefore, determine the set point for cell volume via reciprocal regulation of both NKCC and KCC. This set point has repeatedly been reported to be modified by [Cl], (23), making PASK-mediated regulation of both NKCC1 and KCC an attractive hypothesis.

As shown in Fig. 2 and described above, PASK mediates the phosphorylation of two threonines found within an N-terminal, regulatory domain. This domain is present and highly conserved in two proteins found in the distal tubule of the mammalian kidney, NKCC2 and the Na-Cl cotransporter (NCC); NKCC2 has also been reported to contain a PASK binding site (6). We propose that PASK may regulate the activity of NKCC2 and NCC and, therefore, play an important regulatory role in salt reabsorption and plasma fluid volume.

The data presented in this report strongly link PASK with the regulation of the secretory or housekeeping isoform of the Na-K-Cl cotransporter (NKCC1). We show that disruption of PASK activity, although not affecting the ability of kinase to bind NKCC1, results in up to an 80 ± 3% decrease in cotransport activity, as measured by 86Rb influx. Inhibition of PASK activity decreases total NKCC1 phosphorylation (32P incorporation) as well as the phosphorylation of the regulatory Thr184 residues found within the N terminus (R5 binding). These data combined with the previously reported high expression level of PASK in solute transporting epithelial cells strongly suggest that this kinase plays an important role in the regulation of NKCC1 and, consequently, fluid secretion.

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PASK (Proline-Alanine-rich STE20-related Kinase), a Regulatory Kinase of the Na-K-Cl Cotransporter (NKCC1)
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