

# MAPKAPK-2 Is a Critical Signaling Intermediate in NHE3 Activation Following Na<sup>+</sup>-Glucose Cotransport\*

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Villus enterocyte nutrient absorption occurs via precisely orchestrated interactions among multiple transporters. For example, transport by the apical Na<sup>+</sup>-glucose cotransporter, SGLT1, triggers translocation of NHE3, Na<sup>+</sup>-H<sup>+</sup> antiporter isoform 3, to the plasma membrane. This translocation requires activation of p38 mitogen-activated protein kinase (MAPK), Akt2, and ezrin. Akt2 directly phosphorylates ezrin, but the precise role of p38 MAPK in this process remains to be defined. Sequence analysis suggested that p38 MAPK could not directly phosphorylate Akt2. We hypothesized that MAPKAPK-2 might link p38 MAPK and Akt2 activation. MAPKAPK-2 was phosphorylated after initiation of Na<sup>+</sup>-glucose cotransport with kinetics that paralleled activation of p38 MAPK, Akt2, and ezrin. MAPKAPK-2, Akt2, and ezrin phosphorylation were all attenuated by p38 MAPK inhibition but were unaffected by dominant negative ezrin expression. Akt2 inhibition blocked ezrin but not p38 MAPK or MAPKAPK-2 phosphorylation, suggesting that MAPKAPK-2 could be an intermediate in p38 MAPK-dependent Akt2 activation. Consistent with this, MAPKAPK-2 could phosphorylate an Akt2-derived peptide *in vitro*. siRNA-mediated MAPKAPK-2 knockdown inhibited phosphorylation of Akt2 and ezrin but not p38 MAPK. MAPKAPK-2 knockdown also blocked NHE3 translocation. Thus, MAPKAPK-2 controls Akt2 phosphorylation. In so doing, MAPKAPK-2 links p38 MAPK to Akt2, ezrin, and NHE3 activation after SGLT1-mediated transport.

Intestinal nutrient absorption occurs via precisely orchestrated regulation of multiple transporters. One critical example involves coordinated Na<sup>+</sup> and glucose absorption by villus enterocytes (1, 2). This process may, in part, explain why inclusion of Na<sup>+</sup> and glucose in oral rehydration solutions used to treat life-threatening diarrheal disease augments therapeutic efficacy (3, 4). In mechanistic terms, Na<sup>+</sup>-glucose cotransport mediated by SGLT1 leads to activation of the apical Na<sup>+</sup>-H<sup>+</sup> antiporter NHE3. This generates a favorable osmotic gradient that drives transepithelial water absorption (5, 6). Coordination of SGLT1 and NHE3 involves a complex signal transduction

pathway where activation of p38 mitogen-activated protein kinase (MAPK)<sup>2</sup> leads to Akt2 activation, ezrin phosphorylation, and NHE3 translocation to the brush border (1, 7, 8).

Although it is clear that p38 MAPK is critical to this process (1), the mechanisms by which p38 MAPK activates Akt2 are unknown. Available data suggest that p38 MAPK cannot activate Akt2 directly. We therefore considered p38 MAPK effectors as potential signaling intermediates in this process.

A large number of kinases have been reported to be p38 MAPK effectors, including Mnk1, Mnk2, PRAK, MEF2, ATF-2, Stat1, and MAPK-activated protein kinase-2 (MAPKAPK-2) (9, 10). These, in turn, act on additional substrates, including CREB and HSP27 (11–17). Among these effectors, two recent reports suggest that MAPKAPK-2, a serine/threonine protein kinase, may link p38 MAPK and Akt. These studies showed that recombinant MAPKAPK-2 can phosphorylate Akt *in vitro* (18) and that pharmacological MAPKAPK-2 inhibition prevented Akt1 phosphorylation in smooth muscle myocytes (19). In contrast, most studies have suggested that phosphoinositide 3-kinase, and not p38 MAPK, is responsible for activation of Akt isoforms. Other data suggest that MAPK activation may inhibit Akt activation (20–22), particularly in intestinal epithelia, or that Akt activation can lead to p38 MAPK activation (23, 24). Thus, while existing data suggest that a relationship between p38 MAPK and Akt exists in many systems, the intermediates involved in this process, and even the direction of the effect, are controversial.

To define the signaling mechanisms that link p38 MAPK to NHE3 translocation after SGLT1-mediated Na<sup>+</sup>-glucose cotransport and to better understand the relationship between p38 MAPK and Akt in general, we sought to identify the p38 MAPK effector involved in this process. The data show that p38 MAPK-dependent MAPKAPK-2 activation triggers Akt2 phosphorylation on serine 473. This results in Akt2-dependent ezrin phosphorylation and subsequent NHE3 translocation. We therefore conclude that MAPKAPK-2 is a critical intermediate in the events that cause NHE3 translocation after SGLT1-mediated transport.

## EXPERIMENTAL PROCEDURES

**Cell Culture and Stimulation of SGLT1-mediated Na<sup>+</sup>-Glucose Cotransport**—SGLT1-expressing Caco-2BBE cells were maintained as described previously (1, 7). For experimental use, cells were grown as confluent monolayers (7, 8). For studies

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<sup>2</sup> The abbreviations used are: MAPK, mitogen-activated protein kinase; MAPKAPK-2, MAPK-activated protein kinase-2; MOPS, 4-morpholinepropane-sulfonic acid; siRNA, small interfering RNA.

## MAPKAPK-2 Links p38 MAPK to Akt2

using dominant negative ezrin, Caco-2 cells stably transfected with VSVG tagged NH<sub>2</sub>-terminal (1–309) ezrin were used (7, 8) and Caco-2 cells stably transfected with wild type ezrin were used as controls.

Before initiation of SGLT1-mediated Na<sup>+</sup>-glucose cotransport, monolayers were preincubated for 20 min at 37 °C in nominally HCO<sub>3</sub><sup>-</sup>-free HBSS with 25 mM mannose and 0.5 mM phloridzin, to inhibit Na<sup>+</sup>-glucose cotransport. When used, 10 μM PD169316 or 5 μM Akt1/2 (Calbiochem) were included during this preincubation as well as all subsequent media before cell lysis. Na<sup>+</sup>-Glucose cotransport was initiated by iso-osmotic transfer to nominally HCO<sub>3</sub><sup>-</sup>-free Hanks' balanced salt solution containing 25 mM glucose. Control monolayers were transferred to nominally HCO<sub>3</sub><sup>-</sup>-free HBSS with 25 mM mannose. At the times indicated, monolayers were scraped directly into SDS-PAGE sample buffer at 4 °C and lysates immediately boiled for 5 min before immunoblot analysis.

**Immunoblot Analysis**—Cell lysates were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Phosphorylation of p38 MAPK, MAPKAPK-2, Akt2, and ezrin were detected using phospho-specific polyclonal antisera recognizing phosphorylation at threonine 180/tyrosine 182, threonine 334, serine 473, and threonine 567, respectively (Cell Signaling Technology, Beverly, MA). Monoclonal 3C12 anti-ezrin (Sigma) and polyclonal antisera recognizing p38, MAPKAPK-2, and Akt2 (Cell Signaling Technology) were used to probe total levels of these proteins. Antibody binding was detected with goat anti-mouse or anti-rabbit IgG peroxidase-conjugated antibodies (Cell Signaling Technology) and chemiluminescence (Pierce) on Kodak BioMax MR film. Signal intensity, corrected for background, was analyzed by densitometry using MetaMorph 6 (Universal Imaging, Downingtown, PA). For quantitative analysis of protein phosphorylation, paired aliquots of each sample were immunoblotted for phosphorylated or total protein and the ratio of these values was considered the phosphorylation state of that sample in arbitrary units. Experiments were performed in triplicate or greater. These values were grouped for statistical analysis.

**In Vitro Kinase Reaction**—Active recombinant MAPKAPK-2 (EMD Biosciences, Madison, WI) was diluted with kinase assay buffer (20 mM MOPS, 150 mM MgCl<sub>2</sub>, 12.5 mM β-glycerophosphate, 5 mM EGTA, 2 mM EDTA, 250 μM dithiothreitol, pH 7.2) so that each reaction mixture included 100 ng of kinase. One μg of synthetic Akt peptide containing the core PQFSYSAS sequence surrounding human Akt2 serine 474 (GeneTex, Inc., San Antonio, TX) and 1 μCi of [γ-<sup>32</sup>P]ATP (2 μM, MP Biomedicals, Irvine, CA) was combined in a final reaction volume of 12 μl. To confirm specificity, excess cold ATP (50 μM) was included in some reaction mixtures. After incubation for 15 min at 30 °C, reactions were terminated by adding 20 μl of 50% trichloroacetic acid. For analysis of phosphorylation, 20 μl of each reaction was spotted onto P81 paper, allowed to dry, and washed three times in 1% phosphoric acid. The washed paper discs were then used for scintillation counting. Specific <sup>32</sup>P incorporation was normalized using two-point calibration where reactions lacking enzyme and substrate peptide were set to 0 and a complete reaction mixture with enzyme, peptide, and [<sup>32</sup>P]ATP (without cold ATP) was set to 100. For autora-

diographic analysis 10 μl of terminated reaction was blotted onto nitrocellulose membranes.

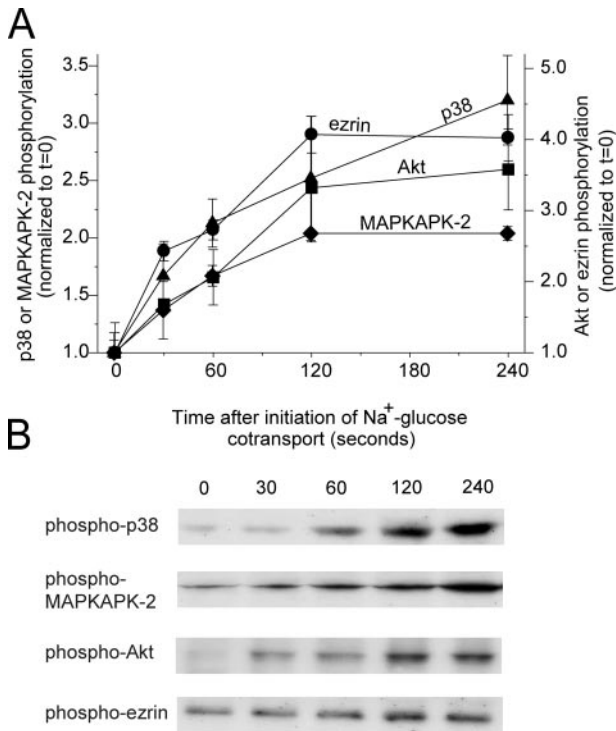
**siRNA Knockdown of MAPKAPK-2 Expression**—Caco-2 monolayers were transfected with SignalSilence MAPKAPK-2 siRNA duplexes targeting either exon 2 (catalog number 1598) or exons 3 and 4 (catalog number 1688) (Ambion, Austin, TX) or nonspecific control GC content-matched siRNA duplexes. Preliminary optimization studies showed that optimal MAPKAPK-2 protein knockdown occurred 4 days after transfection using 200 nM MAPKAPK-2 siRNA and siLentFect lipid reagent (Bio-Rad) and that the exon 2 targeted siRNA was most effective. Immunoblot analyses showed that expression of p38, Akt2, ezrin, and actin were unaffected by MAPKAPK-2 siRNA treatment. Similar immunoblots were routinely used to verify specific MAPKAPK-2 knockdown in all experiments.

**Immunofluorescent Detection of Surface NHE3**—Surface NHE3 expression was assessed as described previously (7, 8). In brief, monolayers were fixed in 1% paraformaldehyde in phosphate-buffered saline for 15 min, washed, blocked, and incubated with affinity-purified rabbit polyclonal antisera at 4 °C. After washing, bound anti-NHE3 antibodies were detected using Alexa-594 conjugated goat anti-rabbit antisera (Molecular Probes, Eugene, OR). Total NHE3 detection was performed identically except for the inclusion of 0.05% saponin in wash and antibody incubation buffers. Stained monolayers were mounted in Slowfade (Molecular Probes), and images were collected using a 100× Plan Apo objective and DM4000 epifluorescence microscope (Leica Microsystems Inc., Bannockburn, IL) equipped with a 41004, shift filter cube (Chroma Technology, Rockingham, VT) and a 12-bit Coolsnap HQ camera (Roper Scientific, Tucson, AZ) controlled by MetaMorph 6. Post-acquisition analyses of mean pixel intensity were performed using MetaMorph 6. In all image acquisition and analysis, identically timed exposures within a single experiment were used for quantitative comparisons.

## RESULTS

**MAPKAPK-2 Activation Follows Initiation of Na<sup>+</sup>-Glucose Cotransport**—We have previously shown that both p38 MAPK and Akt2 phosphorylation are required for downstream ezrin and NHE3 activation after initiation of Na<sup>+</sup>-glucose cotransport (8). While it is clear that the role of Akt2 is to phosphorylate ezrin (8), the kinase that activates Akt2 has not been identified. Because p38 MAPK inhibition blocks both Akt2 and ezrin phosphorylation, we considered the possibility that p38 MAPK might directly phosphorylate Akt2. However, the critical serine 473 residue of Akt2 that is phosphorylated following Na<sup>+</sup>-glucose cotransport lacks the trailing proline residue that characterizes p38 MAPK targets. We therefore considered the possibility that an intermediate kinase might link p38 MAPK to Akt2 activation.

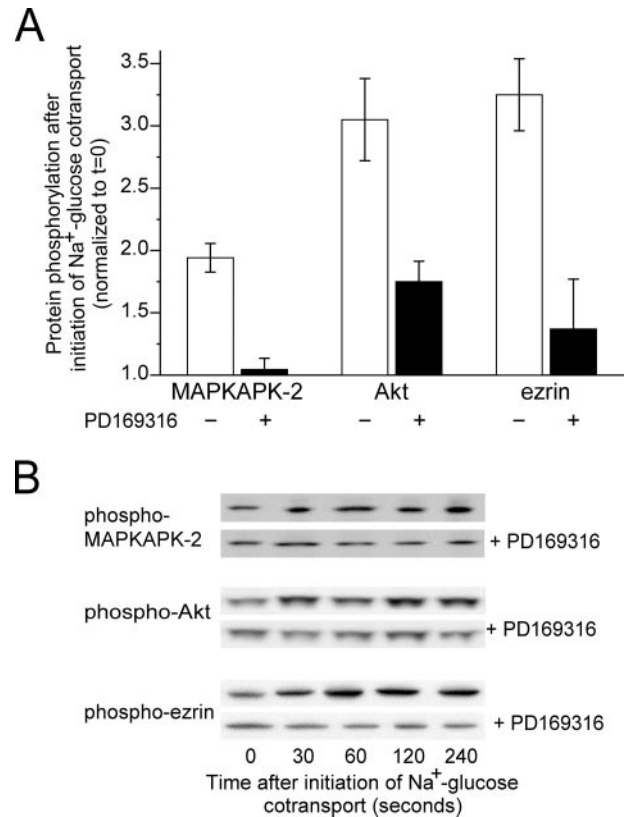
MAPKAPK-2 is a major p38 MAPK effector kinase that has been reported to phosphorylate Akt serine 473 *in vitro* (18, 19, 25). We therefore considered the hypothesis that p38 MAPK-dependent MAPKAPK-2 activation leads directly to Akt2 phosphorylation. To determine whether MAPKAPK-2 is activated after initiation of Na<sup>+</sup>-glucose cotransport, MAPKAPK-2 phosphorylation at threonine 334 was assessed by SDS-PAGE



**FIGURE 1. Initiation of Na<sup>+</sup>-glucose cotransport results in increased MAPKAPK-2 phosphorylation at threonine 334 that is accompanied by increased phosphorylation of p38 MAPK, Akt2, and ezrin.** Monolayers were lysed at indicated times after initiation of Na<sup>+</sup>-glucose cotransport, and lysates were immunoblotted for phosphorylated and total p38 MAPK, threonine 334 phosphorylated and total MAPKAPK-2, serine 473 phosphorylated and total Akt2, and threonine 567 phosphorylated and total ezrin. Densitometric analysis of triplicate samples from a single experiment are shown and represent the mean and S.D. of the ratios of phosphorylated to total protein at indicated times after initiation of Na<sup>+</sup>-glucose cotransport. *A*, p38 MAPK (triangles), MAPKAPK-2 (diamonds), Akt2 (squares), and ezrin (circles) phosphorylation all increased with comparable kinetics. *B*, immunoblots show representative data for each protein. Results are typical of at least five independent experiments, each with triplicate samples.

immunoblot. Phosphorylation was detected within 30 s and reached a maximum at 120 s after initiation of Na<sup>+</sup>-glucose cotransport (Fig. 1). The kinetics of MAPKAPK-2 activation were similar to those of p38 MAPK as well as Akt2 and ezrin (Fig. 1). Thus, MAPKAPK-2 is activated in intestinal epithelia after initiation of Na<sup>+</sup>-glucose cotransport and this activation occurs with kinetics that are consistent with a functional role linking p38 MAPK and Akt2 activation.

**MAPKAPK-2 Activation Is p38 MAPK-dependent**—To determine whether MAPKAPK-2 activation after initiation of Na<sup>+</sup>-glucose cotransport was fully dependent on p38 MAPK activity, monolayers were treated with the specific p38 MAPK inhibitor PD169316. Although transient MAPKAPK-2 activation was observed in the presence of PD169316, sustained MAPKAPK-2 activation was prevented (Fig. 2). Thus, Na<sup>+</sup>-glucose cotransport-dependent p38 MAPK activation is required for downstream activation of MAPKAPK-2. Moreover, p38 MAPK inhibition completely blocked Na<sup>+</sup>-glucose cotransport-dependent downstream phosphorylation of Akt2 and ezrin. While these data do not demonstrate a required role for MAPKAPK-2 in Akt2 and ezrin phosphorylation, they are consistent with the hypothesis that p38 MAPK-dependent MAPKAPK-2 activation leads directly to Akt2 phosphorylation.

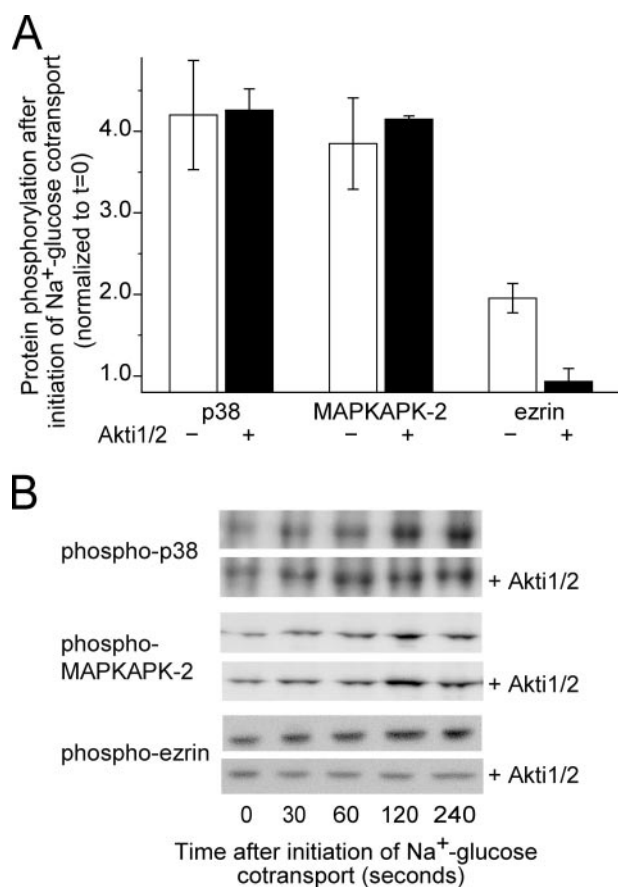


**FIGURE 2. Inhibition of p38 MAPK prevents MAPKAPK-2, Akt2, and ezrin activation.** Monolayers were pretreated with 10 μM PD169316, a specific p38 MAPK inhibitor, and lysed at indicated times after initiation of Na<sup>+</sup>-glucose cotransport. Lysates were immunoblotted for phosphorylated and total MAPKAPK-2, Akt2, and ezrin. *A*, densitometric analysis of lysates harvested 240 s after initiation of Na<sup>+</sup>-glucose cotransport shows that p38 MAPK inhibition markedly attenuated phosphorylation increases. *B*, representative immunoblots for times from 0 to 240 s after initiation of Na<sup>+</sup>-glucose cotransport are shown. Results are typical of more than three independent experiments, each with triplicate samples.

**MAPKAPK-2 Activation Does Not Require Akt2 or Ezrin Function**—Although the data above show that Akt2 and ezrin are downstream of p38 MAPK signaling, they do not demonstrate that MAPKAPK-2 activation is necessary for Akt2 and ezrin activation. Moreover, the data do not exclude the possibility that Akt2- or ezrin-dependent events feed back to trigger MAPKAPK-2 phosphorylation. Thus, to determine whether MAPKAPK-2 phosphorylation requires Akt2 or ezrin activation, monolayers treated with a specific Akt inhibitor (26) or expressing dominant negative ezrin (7) were studied. Although Akt2 inhibition prevented ezrin phosphorylation, it did not prevent p38 MAPK or MAPKAPK-2 activation after initiation of Na<sup>+</sup>-glucose cotransport (Fig. 3). Moreover, expression of dominant negative ezrin had no effect on Na<sup>+</sup>-glucose cotransport-dependent MAPKAPK-2 activation (Fig. 4). These data therefore show that MAPKAPK-2 activation does not require Akt2 or ezrin function.

**MAPKAPK-2 Phosphorylates Akt2 in Vitro**—The data above support the hypothesis that p38 MAPK-dependent MAPKAPK-2 activation leads directly to Akt2 phosphorylation. However, data such as these cannot exclude the possibility that MAPKAPK-2 activation leads to Akt2 phosphorylation by indirect means, *i.e.* through another kinase. To determine whether

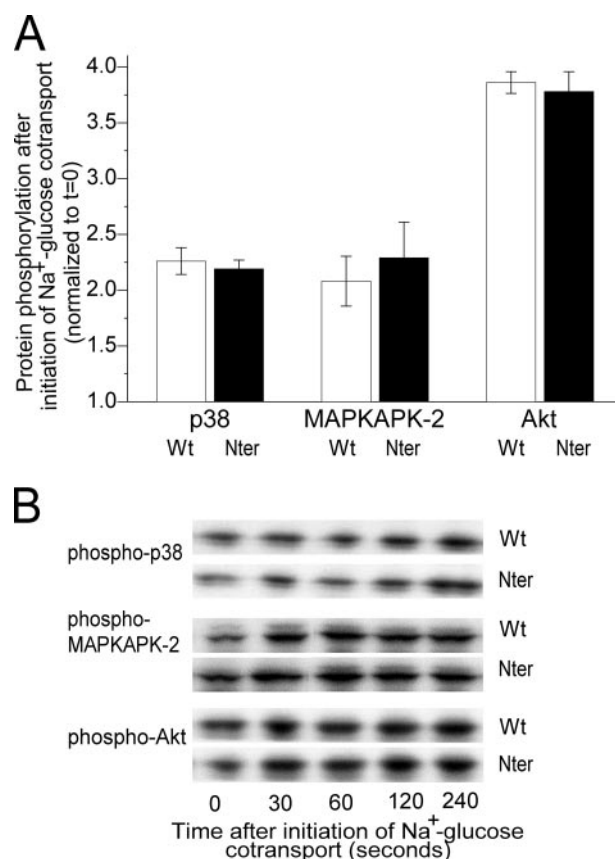




**FIGURE 3. Akt2 inhibition does not prevent p38 MAPK or MAPKAPK-2 phosphorylation.** Monolayers were pretreated with the Akt inhibitor Akt1/2 (5  $\mu$ M) and lysed at indicated times after initiation of Na<sup>+</sup>-glucose cotransport. Lysates were immunoblotted for phosphorylated and total p38 MAPK, MAPKAPK-2, and ezrin. *A*, densitometric analysis of lysates harvested 240 s after initiation of Na<sup>+</sup>-glucose cotransport shows that Akt2 inhibition markedly attenuated ezrin phosphorylation. *B*, representative immunoblots for times from 0 to 240 s after initiation of Na<sup>+</sup>-glucose cotransport are shown. Results are typical of more than three independent experiments, each with triplicate samples.

MAPKAPK-2 could directly phosphorylate Akt2, purified recombinant active MAPKAPK-2 was incubated in a reaction including only [<sup>32</sup>P]ATP and a synthetic peptide including serine 473, the phosphorylation site necessary for Akt2 activation and detected by the phosphospecific anti-Akt antibodies used in the studies above. Full-length Akt2 was not used, as it may be phosphorylated at multiple sites and is also capable of autophosphorylation. Significant <sup>32</sup>P incorporation was observed when recombinant active MAPKAPK-2 was incubated with Akt2 peptide substrate and [<sup>32</sup>P]ATP (Fig. 5). This was specific, as it could be competed by a 25-fold excess of unlabeled cold ATP and no <sup>32</sup>P incorporation occurred when recombinant active MAPKAPK-2 was incubated with [<sup>32</sup>P]ATP but without the Akt2 peptide substrate. These data therefore show that active MAPKAPK-2 can directly phosphorylate Akt2.

**MAPKAPK-2 Is Required for Akt2 and Ezrin Phosphorylation After Initiation of Na<sup>+</sup>-Glucose Cotransport**—The data above show that p38 MAPK activation is required for phosphorylation of MAPKAPK-2, Akt2, and ezrin after initiation of Na<sup>+</sup>-glucose cotransport and that neither Akt2 nor ezrin function are required for MAPKAPK-2 activation. However, the data do



**FIGURE 4. Dominant negative ezrin expression does not prevent activation of p38 MAPK, MAPKAPK-2, or Akt2.** Monolayers stably transfected with wild type or truncated amino-terminal dominant negative ezrin constructs (7) were lysed at the indicated times after initiation of Na<sup>+</sup>-glucose cotransport. Lysates were immunoblotted for phosphorylated and total p38 MAPK, MAPKAPK-2, and Akt2. *A*, densitometric analysis of lysates harvested 240 s after initiation of Na<sup>+</sup>-glucose cotransport shows that dominant negative ezrin expression does not interfere with phosphorylation of p38 MAPK, MAPKAPK-2, or Akt2. *B*, representative immunoblots for times from 0 to 240 s after initiation of Na<sup>+</sup>-glucose cotransport are shown. Results are typical of more than three independent experiments, each with triplicate samples.

not provide evidence for a relationship between MAPKAPK-2 activation and Akt2 and ezrin phosphorylation. As well characterized MAPKAPK-2 inhibitors are not available, we used MAPKAPK-2-specific siRNA to knockdown expression 52.0  $\pm$  6.0% (Fig. 6). MAPKAPK-2 expression was not affected by control scrambled siRNA. Moreover, expression of  $\beta$ -actin, p38 MAPK, Akt2, and ezrin were unaffected in monolayers treated with MAPKAPK-2-specific siRNA or control siRNA (Fig. 6). Thus, the specific siRNA knocked down MAPKAPK-2 expression without affecting expression of other potential signaling pathway components.

MAPKAPK-2 knockdown severely attenuated Akt2 and ezrin phosphorylation after initiation of Na<sup>+</sup>-glucose cotransport (Fig. 7). This effect of MAPKAPK-2 knockdown was most obvious within 2 min of Na<sup>+</sup>-glucose cotransport initiation, when MAPKAPK-2 knockdown completely blocked Akt2 and ezrin phosphorylation (Fig. 7). At later times some Akt2 and ezrin phosphorylation was apparent, potentially reflecting activity of the 48.0  $\pm$  6.0% of cellular MAPKAPK-2 that could not be knocked down by siRNA. MAPKAPK-2 knockdown did not interfere with p38 MAPK phosphorylation after initiation

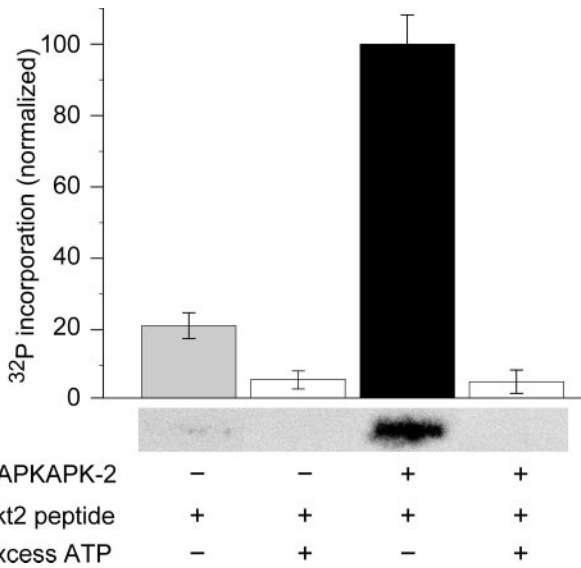


FIGURE 5. **MAPKAPK-2 specifically phosphorylates Akt2.** Reactions contained active recombinant human MAPKAPK-2, a synthetic Akt2 peptide substrate, or excess cold ATP, as indicated. All reactions contained [<sup>32</sup>P]ATP. Specific peptide phosphorylation that could be competed with excess cold ATP was only seen when both active MAPKAPK-2 and peptide substrate were included in the reaction. Results are typical of three independent experiments, each with duplicate samples.

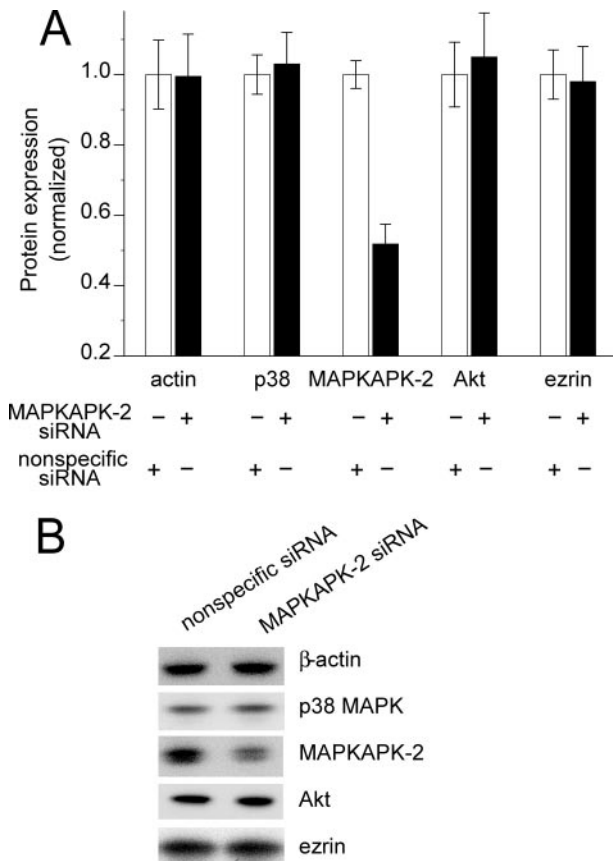


FIGURE 6. **MAPKAPK-2 siRNA specifically knocks down MAPKAPK-2 expression.** A, treatment of monolayers with exon 2 MAPKAPK-2-specific siRNA resulted in knockdown of protein levels by  $52.0 \pm 6.0\%$  relative to control monolayers transfected with nonspecific siRNA ( $p < 0.05$ ). Expression of p38 MAPK, Akt2, ezrin, and  $\beta$ -actin was not changed by either MAPKAPK-2-specific or nonspecific siRNA. B, representative immunoblots demonstrating protein expression levels. Results are typical of more than four independent experiments, each with triplicate samples.

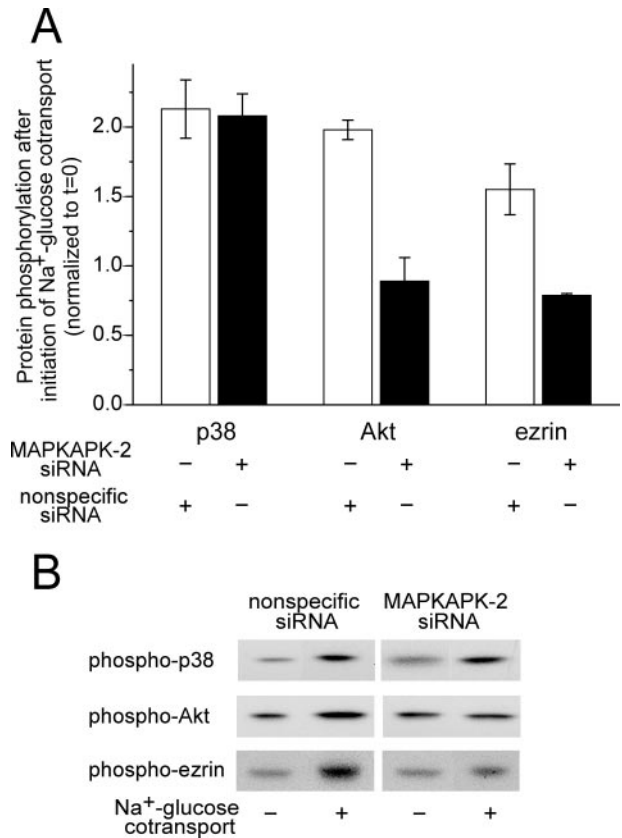
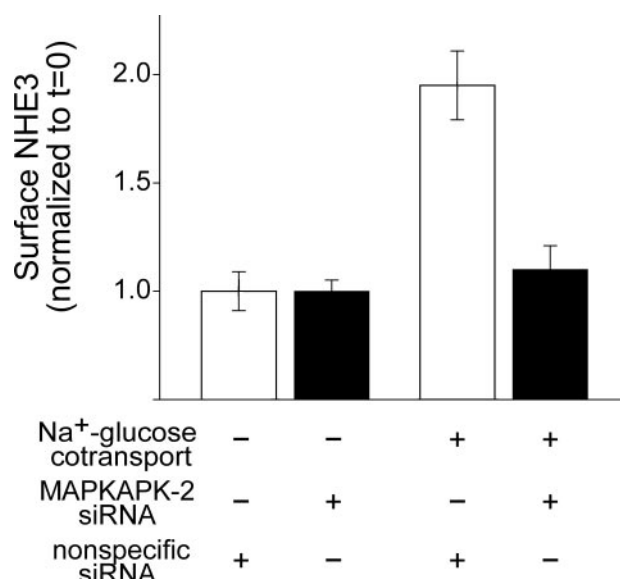


FIGURE 7. **Akt2 and ezrin activation after initiation of Na<sup>+</sup>-glucose cotransport require MAPKAPK-2 expression.** Monolayers transfected with exon 2 MAPKAPK-2-specific or control nonspecific siRNA were lysed 60 s after initiation of Na<sup>+</sup>-glucose cotransport. Lysates were immunoblotted for phosphorylated and total p38 MAPK, Akt2, and total MAPKAPK-2. MAPKAPK-2 expression was knocked down as shown in Fig. 5. A, densitometric analysis shows Akt2 and ezrin phosphorylation after initiation of Na<sup>+</sup>-glucose cotransport are severely attenuated by MAPKAPK-2 knockdown. In contrast, p38 MAPK phosphorylation is not affected by MAPKAPK-2 knockdown. B, representative immunoblots are shown. Results are typical of three independent experiments, each with triplicate samples.

of Na<sup>+</sup>-glucose cotransport (Fig. 7). The data thus show that Akt2 and ezrin phosphorylation, but not p38 MAPK phosphorylation, depend on MAPKAPK-2 activity after initiation of Na<sup>+</sup>-glucose cotransport. Together with our data showing that MAPKAPK-2 can phosphorylate Akt *in vitro*, these data suggest that Akt2 activation after initiation of Na<sup>+</sup>-glucose cotransport is directly mediated by MAPKAPK-2.

**MAPKAPK-2 Is Required for Na<sup>+</sup>-Glucose Cotransport-induced NHE3 Translocation**—Initiation of Na<sup>+</sup>-glucose cotransport triggers NHE3 translocation to the plasma membrane, thereby activating Na<sup>+</sup>-H<sup>+</sup> exchange and resulting in net intestinal Na<sup>+</sup> absorption (6, 7, 27, 28). Moreover, the net cytosolic alkalinization that results from this NHE3 activation (1) provides the driving force for intestinal H<sup>+</sup>-dipeptide transport (29). We have previously shown that this NHE3 translocation requires ezrin function. To determine whether inhibition of ezrin phosphorylation by MAPKAPK-2 knockdown prevented NHE3 translocation, surface NHE3 content was assessed before and after Na<sup>+</sup>-glucose cotransport initiation. Although MAPKAPK-2 knockdown did not alter surface NHE3 expression in resting monolayers, it nearly completely prevented increases in surface NHE3 induced by Na<sup>+</sup>-glucose



**FIGURE 8. MAPKAPK-2 knockdown prevents Na<sup>+</sup>-glucose cotransport-induced NHE3 translocation.** Surface NHE3 expression was measured in monolayers transfected with MAPKAPK-2-specific or control nonspecific siRNA 240 s after initiation of Na<sup>+</sup>-glucose cotransport. MAPKAPK-2 knockdown prevented 84.0 ± 11.0% of the increase in surface NHE3 expression detected in monolayers transfected with nonspecific siRNA (*p* < 0.01). Total NHE3 expression was unchanged. Results are typical of three independent experiments, each with triplicate samples.

cotransport (Fig. 8). Thus, MAPKAPK-2 expression is required for acute NHE3 translocation. Taken as a whole, these data demonstrate that MAPKAPK-2 is the critical intermediate that links upstream Na<sup>+</sup>-glucose cotransport-dependent p38 MAPK activation to downstream Akt2 and ezrin phosphorylation and NHE3 translocation.

### DISCUSSION

We have characterized activation of NHE3 following initiation of SGLT1-mediated Na<sup>+</sup>-glucose cotransport as an example of the finely regulated coordination that results in intestinal nutrient absorption (1, 7, 8). This results in increased Na<sup>+</sup> absorption as well as cytosolic alkalization that drives H<sup>+</sup>-dipeptide transport (1, 29). The mechanism of this NHE3 activation is incompletely understood but clearly involves a signaling cascade that includes activation of multiple kinases, the cytoskeletal linker protein ezrin, and NHE3 translocation from intracellular stores to the microvillus brush border (1, 7, 8). Although the distal arm of this signaling cascade has been shown to directly follow Akt2 activation (8), the mechanism of Akt2 activation is unclear. One clue is that p38 MAPK activity is required for Akt2, ezrin, and NHE3 activation (1, 7, 8). To identify the mechanism of Akt2 activation, we considered the possibility that p38 MAPK might directly phosphorylate and activate Akt2. However, analysis of the sequence surrounding serine 473 of Akt2 suggested that this was not a viable hypothesis. We therefore asked if one of the many described p38 MAPK effectors might activate Akt2. Two recent studies suggest that the p38 MAPK effector MAPKAPK-2 may phosphorylate Akt under some conditions. First, *in vitro* kinase assays using purified recombinant proteins showed that active MAPKAPK-2 could phosphorylate Akt *in vitro* (18). Moreover,

intracellular delivery of a MAPKAPK-2 peptide inhibitor blocked angiotensin II-induced Akt1 phosphorylation on serine 473 in smooth muscle myocytes (19). However, in human intestinal epithelia, p38 MAPK activation has been linked to decreased Akt activation (20, 22). Thus, the relationship between p38 MAPK and Akt isoforms appears to be complex and, in part, dependent on the stimulus and cell type studied.

To examine the potential role of MAPKAPK-2 in mediating p38 MAPK-dependent Akt2 activation following initiation of Na<sup>+</sup>-glucose cotransport in intestinal epithelia we first asked if MAPKAPK-2 was activated after initiation of Na<sup>+</sup>-glucose cotransport. We found that MAPKAPK-2 is activated after initiation of Na<sup>+</sup>-glucose cotransport with kinetics that paralleled those of Akt2 and ezrin phosphorylation. This MAPKAPK-2 activation required p38 MAPK but not Akt2 or ezrin activation, consistent with a model where MAPKAPK-2 links p38 MAPK to Akt2. Consistent with this, *in vitro* kinase reactions confirmed that MAPKAPK-2 could directly phosphorylate Akt2 at serine 473. Furthermore, specific siRNA-mediated MAPKAPK-2 knockdown prevented activation of Akt2 and ezrin as well as NHE3 translocation but had no effect on p38 MAPK activation. Thus, the data show that MAPKAPK-2 is a required intermediate that links p38 MAPK to Akt2 activation in intestinal epithelia. We conclude that MAPKAPK-2 is an Akt2 3-phosphoinositide-dependent kinase-2 in human enterocytes.

These studies clearly show that MAPKAPK-2 is responsible for the downstream Akt2 phosphorylation that links NHE3-mediated Na<sup>+</sup> absorption to Na<sup>+</sup>-glucose cotransport, thereby defining a new signaling pathway for p38 MAPK-dependent regulation of membrane traffic and cytoskeletal structure. While our data focus on the role of these signaling events in NHE3 translocation downstream of SGLT1-mediated transport, they may also be significant in explaining the critical roles of MAP kinases and ezrin in epithelial organization and differentiated function (30–35).

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