Serum and glucocorticoid-regulated kinase Sgk1 inhibits insulin-dependent activation of phosphomannomutase 2 in transfected COS-7 cells

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Menniti, Miranda, Rodolfo Iuliano, Rosario Amato, Rosalia Boito, Monica Corea, Ilaria Le Pera, Elio Gulletta, Giorgio Fuiano, and Nicola Perrotti. Serum- and glucocorticoid-regulated kinase Sgk1 inhibits insulin-dependent activation of phosphomannomutase 2 in transfected COS-7 cells. Am J Physiol Cell Physiol 288: C148–C155, 2005. First published September 1, 2004; doi:10.1152/ajpcell.00284.2004.—Serum- and glucocorticoid-regulated kinase (Sgk1) is considered to be an essential convergence point for peptide and steroid regulation of ENaC-mediated sodium transport. We tried to identify molecular partners of Sgk1 by yeast two-hybrid screening. Yeast two-hybrid screening showed a specific interaction between Sgk1 and phosphomannomutase (PMM)2, the latter of which is an enzyme involved in the regulation of glycoprotein biosynthesis. The interaction was confirmed in intact cells by coimmunoprecipitation and colocalization detected using confocal microscopy. We were then able to demonstrate that Sgk1 phosphorylated PMM2 in an in vitro assay. In addition, we found that the enzymatic activity of PMM2 is upregulated by insulin treatment and that Sgk1 completely inhibits PMM2 activity both in the absence and in the presence of insulin stimulation. These data provide evidence suggesting that Sgk1 may modulate insulin action on the cotranslational glycosylation of glycoproteins.

Sgk, protein glycosylation; CDGla

SGK1 IS A SERINE THERONINE KINASE (22) that regulates sodium absorption by the amiloride-sensitive sodium channel in kidney principal cells (1, 11). The kinase is activated by serum, steroids, insulin, and cAMP (6, 14). Recent evidence suggests that serum- and glucocorticoid-regulated kinase (Sgk1) is an important molecular target that integrates the multiple endocrine inputs regulating sodium transport (2, 3). Studies with stably transfected A6 cell lines, a well-characterized model of the principal cells of the distal nephron, have demonstrated that the activation of the kinase is required for basal as well as hormone-stimulated sodium transport. The results are compatible with a model in which Sgk1, once activated by hormonal stimulation, interacts with molecules that mediate Sgk1 action on the epithelial sodium channel (ENaC). One of the classic tools with which to search for an interaction between molecules is based on the possibility of studying the expression of reporter genes in yeast two-hybrid systems (4). This method was recently used by Maiyar et al. (8) and allowed the identification of importin-α as an Sgk1-interacting protein, leading nuclear localization of Sgk1.

Because our main interest was focused on Sgk1 regulation of sodium absorption in kidney cells, we decided to perform yeast two-hybrid screening using a kidney library as a source of prey cDNA.

We found several clones coding for putative Sgk1-interacting molecules. One of them contained the full sequence of phosphomannomutase (PMM)2, a key enzyme regulating the early steps of protein glycosylation and responsible for autosomal recessive congenital disorders of glycosylation type Ia (CDGia) (19).

We present evidence based on colocalization and coimmunoprecipitation experiments that the interaction is specific and occurs in yeast as well as in eukaryotic cells. Moreover, we demonstrate that insulin enhances PMM2 activity in intact cells and that Sgk1 phosphorylates enzyme-inhibiting basal and insulin-stimulated PMM2 activity.

The present study represents the first demonstration of the hormonal regulation of PMM2 and opens new perspectives on the pathophysiology of glycoprotein metabolism.

MATERIALS AND METHODS

Constructs

pBridge-Sgk1. Polymerase chain reaction (PCR) (Klen Taq; BD Biosciences/Clontech, Palo Alto, CA) was used to amplify wild-type Sgk1 from pCINeo-Sgk1 (14). Primers introducing the EcoRI restriction site (ATGCGGAATTCATGACGGTGAAAACTGAGGCTGCT- AAGGGG) and the BamHI restriction site (GTATGGGATCCTCA- GAGGAAAGAGTCCGTGG) were used.

The amplified fragment, flanked by an EcoRI site upstream and a BamHI site downstream, was ligated into the TA cloning PCR 2.1 plasmid (Invitrogen, Carlsbad, CA) and subcloned into the multiple cloning sites of pBridge (BD Biosciences/Clontech, Palo Alto, CA). pcDNA4TO Myc-Sgk1. The coding sequence of Sgk1, including the Myc epitope within pCINeo (14), was subcloned into the EcoRI and Not I sites of expression vector pcDNA4TO (Invitrogen), which demonstrated much more efficient expression in COS-7 cells (data not shown).

pCELFHA-PMM2. The full-length human PMM2 cDNA was subcloned into pCELFHα. The coding sequence of PMM2 was amplified by Klen Taq polymerase using pACT2-PMM2, from the screening, as a template. Primers introducing EcoRV (AGC ATG TCC CAT GCC CCC ACC ACC TGG T) and XbaI (ACT CTA GAT TAG GAG AAC AGC AGT TCA CA) restriction sites (underlined) were used.

pGEX4T3-PMM2. The full-length human PMM2 cDNA was subcloned into pGEX4T3 (Amersham Biosciences, Freiburg, Germany) in BamHI XhoI sites within the glutathione-S-transferase (GST).

The coding sequence of PMM2 was amplified by Klen Taq polymerase using pACT2-PMM2 from the screening as a template. Primers introducing the BamH I (GAC GGA TCC ATG CAG CGC TG)
and XhoI (CGA CTG GAC CCC ACG TTA GGA GAA CAG) restriction sites (underlined) were used.
pACT2-(Δ1–125)/PMM2. Human cDNA PMM2 coding for the COOH-terminal 101 amino acids (from M126 to the stop codon) was subcloned into pACT2 by means of a PCR-based method (Klen Taq polymerase) using pACT2-PMM2 from the screening as a template. Primers introducing EcoRI (CGGAATTCTGAATGTGTA- AACGTTGCTCCCT) and XhoI (CGAATTCGAGCCCAGCTTGA-GAGAACG) restriction sites (underlined) were used.

PDK1-activated (Δ1–59)S422D Sgk1 mutant (active Sgk1) was purchased from Upstate Biotechnology (catalog no. 14-331; Charlottesville, VA).

**Yeast Two-Hybrid Screening**

To identify Sgk1 binding proteins, a yeast two-hybrid system was used (MATCHMAKER Gal4 Two-Hybrid; BD Biosciences/Clontech, Palo Alto, CA). The bait plasmid pBridge-Sgk1, with full-length human Sgk1 expressed as a fusion protein with the DNA binding domain (BD) of the yeast transcription factor GAL4, was used to transform a suitable yeast strain (Saccharomyces cerevisiae AH109). The yeasts transformed by pBridge-Sgk1 were used for mating for 20 h with a Saccharomyces cerevisiae host strain Y187 (BD Biosciences/Clontech Laboratories) pretransformed using a MATCHMAKER human kidney cDNA pACT2-derived library cloned into a sciences/Clontech Laboratories) pretransformed using a MATCHMAKER Gal4 Two-Hybrid; BD Biosciences/Clontech, Palo Alto, CA). The bait plasmid pBridge-Sgk1, with full-length human Sgk1 expressed as a fusion protein with the DNA binding domain (BD) of the yeast transcription factor GAL4, was used to transform a suitable yeast strain (Saccharomyces cerevisiae AH109). The yeasts transformed by pBridge-Sgk1 were used for mating for 20 h with a Saccharomyces cerevisiae host strain Y187 (BD Biosciences/Clontech Laboratories) pretransformed using a MATCHMAKER human kidney cDNA pACT2-derived library cloned into a yeast GAL4 activation domain (AD) expressing proteins containing a hemagglutinin (HA) tag at the NH2 terminal. After 20 h, we spread the yeast-Y187 mating mixture on SD/-Ade/-His/-Leu/-Trp plates.

Yeast colonies that demonstrated activation of both reporters, galactose-dependent blue staining in the presence of X-Gal (5-bromo-4-chloro-3-indolyl-galactose-dependent blue staining in the presence of X-Gal (5-bromo-4-chloro-3-indolyl-galactose)-dependent blue staining in the presence of X-Gal (5-bromo-4-chloro-3-indolyl-galactoside) (4-chloro-3-indolyl-galactoside) (4-chloro-3-indolyl-β-D-galactopyranoside) (4-chloro-3-indolyl-β-D-galactopyranosidase), and adenine- and histidine-independent growth were selected and considered for further evaluation to screen for putative Sgk1-interacting proteins.

Library plasmid DNA was isolated from this selection of clones with lyticase solution and rescued into HB101 Escherichia coli strain using the CaCl2 method (Invitrogen). The transformants were recovered on minimal M9 selective medium lacking leucine for nutritional selection.

The specificity of the interaction was tested for several clones by retransforming the interactor plasmid into yeast expressing pBridge-Sgk1 bait, as well as yeast strain containing the empty vector pBridge. To check the specificity of the interaction, the plasmid was expressed as a fusion protein with the DNA binding domain (BD) of the yeast transcription factor GAL4.

**Preparation of GST-PMM2**

The GST-PMM2 fusion protein was isolated from the bacterial strain BL21. Cells were transformed with pGEX4T3-PMM2 construct. Bacteria were exponentially grown at 37°C for 2 h and subsequently induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 30°C. Cells were centrifuged and then lysed with lysis buffer: PBS, pH 7.4, with 1% Triton X-100, 0.1% glycerol, and 1 mM dithiothreitol (DTT) in the presence of protease inhibitors (10 mM lysis buffer; Complete Roche Molecular Biochemicals, Mannheim, Germany). The GST-PMM2 fusion protein was purified on glutathione sepharose beads (Pharmacia/Pfizer) and then dialyzed overnight at 4°C against a buffer containing (in mM) 50 Tris, pH 7.5, 1 EDTA; and 50 NaCl. The concentration of GST-PMM2 was calculated using the Bradford method and by performing in SDS-PAGE with Coomassie blue stain.

**In Vitro Phosphorylation of PMM2**

Thrombin cleavage (25 μM/ml, 16 h, 30°C) of GST-PMM2 released a 28-kDa band corresponding to PMM2 and a 24-kDa band corresponding to the GST molecule lacking the 16-amino acid NH2 terminal to the thrombin site. For the in vitro phosphorylation assay, equimolar amounts of GST-PMM2 and GST were bound to 30 μl of glutathione sephadex. Thrombin was added in all the reactions to cleave PMM2 from GST using the thrombin site within the fusion protein. The resin was separated with the use of supernatants by performing centrifugation and the pellets (~30 μl) in which the thrombin-cleaved PMM2 was recovered were used in a phosphorylation reaction.

PDK1-activated (Δ1–59)S422D Sgk1 (active Sgk1) was then used to phosphorylate thrombin-cleaved GST-PMM2 in kinase buffer (in mM: 20 Tris, pH 7.4, and 10 MgCl2) in the presence of 5 μM ATP, 1 mM DTT, and γ-32P-ATP (0.02 mCi/sample). The reaction was allowed to occur for 30 min at room temperature and was stopped by boiling in Laemmli sample buffer. Phosphoproteins were separated using 12% SDS-PAGE and detected by performing autoradiography.

**Expression in COS-7 Cells**

COS-7 cells were plated at a density of 3.5 × 105 cells/ml in six-well 35-mm plates. For the immunofluorescence studies, cells were plated at a density of 2.0 × 105 cells/ml on glass coverslips in individual 35-mm plates. Cells were cultured overnight in DMEM (Invitrogen) containing 10% fetal bovine serum (Invitrogen). The next day, the cells were transfected with expression plasmids using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer’s instructions. We used pCINeoMyc-Sgk1 (600 ng/well) (14) for the confocal microscopy experiments and pCDNA4TO Myc-Sgk1 (600 ng/well) to express Sgk1 for the coimmunoprecipitation experiments and for the evaluation of the Sgk1 effect on PMM2 activity in intact cells. pCELFHA-PMM2 (600 ng/ml) was used in the studies of coimmunoprecipitation and regulation of PMM2 activity in intact cells. Empty vectors were transfected in the sham-transfected cells.

Five hours after transfecting the cells, the transfection medium was exchanged with DMEM containing 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). Twenty-four hours after transfection, the medium was replaced with serum-free medium (DMEM containing 0.1% insulin-free bovine serum albumin plus antibiotics); the next day, the cells were used to study the regulation of PMM2 activity as well as coimmunoprecipitation and in the colocalization experiments. Confocal microscopy experiments showed that the efficiency of transfection reached ~30%.

**Immunofluorescence**

Thirty-six hours after transfection, cells were washed twice with phosphate-buffered saline (PBS) and fixed for 15 min in 4% paraformaldehyde in PBS. Fixed cells were washed twice with PBS and then permeabilized for 5 min with 0.5% Triton X-100 in PBS. To visualize Sgk1, the permeabilized monolayers were incubated for 1 h with a rabbit anti-Myc (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:200 in PBS. To visualize PMM2, the permeabilized monolayers were incubated for 1 h with a mouse anti-HA antibody (Roche Diagnostics, Indianapolis, IN) diluted 1:200 in PBS. To detect the staining for Sgk1, the coverslips were washed twice with PBS and incubated for 45 min at room temperature with a 1:800 dilution of Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes, Eugene, OR) in a humidified chamber. To detect staining for PMM2, the coverslips were washed twice with PBS and incubated for 45 min at room temperature with a 1:800 dilution of Alexa Fluor 568 donkey anti-mouse IgG (Molecular Probes, Eugene, OR) in a humidified chamber.

After being washed with PBS, cells were mounted in Prolong antifade reagent (Molecular Probes) and visualized using a confocal microscope (Leica Microsystems, Wetzlar, Germany).
Coimmunoprecipitation Experiments

The transfected cells were solubilized for 20 min at 4°C in a solubilization buffer (250 μl/well) containing 50 mM Tris, pH 7.8, 300 mM NaCl, and 0.5% Triton X-100, with the protease inhibitor complete TM, phosphatase inhibitors (in mM: 100 NaF, 5 sodium pyrophosphate, and 2 sodium orthovanadate and 5 EDTA). Protein extracts were quantified by means of a Bradford-based assay (Bio-Rad, Hercules, CA), and an aliquot (20 μg) of protein extracts was analyzed by performing immunoblotting with rabbit anti-HA (Roche Diagnostics) and rabbit anti-Myc (Santa Cruz Biotechnology) to assess the expression of HA-PMM2 and Myc-Sgk1. Proteins (400 μg) were immunoprecipitated with mouse anti-HA antibodies (8 μl; Roche Diagnostics) at 4°C overnight. The antibody was bound to protein G-Ultralink (15 μl; Pierce, Rockford, IL) at 4°C for 60 min. The immune complexes were sedimented and then washed three times with a washing buffer containing 25 mM HEPES, 100 mM KCl, 12.5 mM MgCl2, 0.1 mM EDTA, 10% glycerol, and 0.1% Nonidet P-40, pH 7.9 (3). The pellets were then resuspended in Laemmli sample buffer containing DTT (1 mM), boiled for 5 min, and separated by performing SDS-PAGE using a 12% gel. The proteins were transferred to nitrocellulose, blocked in 5% nonfat dry milk in Tris-buffered saline plus Tween 20 (TTBS), and incubated with mouse anti-HA antibody at a dilution of 1:1,000 in 5% nonfat dry milk in TTBS to detect immunoprecipitated PMM2, rabbit anti-peptide 267 Sgk1 antibody (2) (Zymed Laboratories, South San Francisco, CA), or preimmune rabbit serum at a dilution of 1:1,000 in 5% nonfat dry milk in TTBS for the detection of Sgk1.

PMM2 Activity in HA Immunoprecipitates

After 16 h of serum starvation (see Expression in COS-7 Cells), the cells were incubated in the presence or in the absence of human recombinant insulin (1 μM for 45 min; Sigma-Aldrich). The medium was aspirated, and the cells were solubilized. The extracts were treated as described above for the immunoprecipitation experiments; however, after the pellets were washed, instead of resuspending in Laemmli sample buffer, the proteins immunoprecipitated by the anti-HA antibody were washed once and resuspended in the mammomutase buffer containing 50 mM HEPES, pH 7.1, 5 mM MgCl2, 5 μg/ml glucose-6-phosphate dehydrogenase, 10 μg/ml phosphoglucone isomerase, 3.5 μg/ml phosphomannose isomerase, and 0.2 μM glucose 1,6-biphosphatase (Sigma-Aldrich, Milan, Italy), according to the method described by Van Schaftingen and Jaeken (10). The reaction was started by the addition of 0.25 mM NADP and 0.1 mM mannose-1-phosphate. The reaction was allowed to occur for 30 min at 30°C on a rotating wheel and was stopped by heating at 80°C for 5 min. The immunoglobulins bound to protein G-Sepharose were sedimented by centrifugation (microfuged at 14,000 rpm for 2 min). The absorbance at 340 nm was read in spectrophotometers to measure the amount of NADPH produced in the reaction.

RESULTS

Yeast Two-Hybrid Screening Reveals Specific Sgk1/PMM2 Interaction

Two-hybrid screening allowed us to identify different independent clones interacting specifically with Sgk1. Approximately 15 million yeast transformants were screened. Among several putative interacting clones, four isolated clones contained cDNA inserts of similar size. BLAST analysis revealed that all four library clones contained the coding sequence for full-length PMM2 (gi:14249867). The interaction was specific because the growth on plates SD/-Ade/-His/-Leu/-Trp was observed only when the interacter plasmid pACT2-PMM2 was retransformed into yeast expressing pBridge-Sgk1 bait. No growth was observed when the interacter plasmid pACT2-PMM2 was retransformed into yeast containing the empty vector pBridge or two unrelated bait plasmids: pBridge-HMG(Y) and pBridge-PTP7 (Fig. 1A). The interaction between PMM2 and Sgk1 required the integrity of the PMM2 molecule. In fact, no growth was observed when a pACT2 plasmid expressing a PMM2 deletion mutant [pACT2-(Δ1–Δ25)PMM2] was retransformed into yeast containing either pBridge-Sgk1 or the empty vector pBridge (Fig. 1B). We found other clones containing genes coding for putative Sgk1-interactive proteins. One of them, transportin-β, is related to importin-α, previously described as an Sgk1-interacting protein (8). Other clones are currently under investigation.

PMM is the enzyme that catalyzes the interconversion between mannose 6-phosphate and mannose 1-phosphate. Mannose 1-phosphate is required for the synthesis of GDP-mannose, the activated intermediate essential for the synthesis of the dolichol-linked oligosaccharide early in the N-glycosylation process.

In Vitro Phosphorylation of PMM2

To verify whether Sgk and PMM2 were related in an enzyme/substrate molecular interaction, we used PDK1-activated Sgk to phosphorylate PMM2 in vitro. Active Sgk1 appeared to be heavily phosphorylated in the conditions used for the assay (Fig. 2B, top, lanes 2 and 4). Because GST-PMM2 had electrophoretic mobility similar to that of phosphorylated Sgk1 in SDS-PAGE, it was impossible to detect Sgk1-dependent phosphorylation of GST-PMM2 in the presence of phosphorylated Sgk1. We used a thrombin-cleaved GST-Sgk1 as a substrate for a kinase reaction in the presence of active Sgk1. The thrombin-digested proteins were loaded onto an SDS-PAGE gel and detected using Coomassie blue staining. A 48-kDa band corresponding to undigested GST-PMM2 (Fig. 2A, top, lanes 1 and 2), comigrating with active Sgk1, was detected (Fig. 2, A and B, top, lanes 2 and 4). A 28-kDa band just above the 25-kDa marker corresponded to PMM2, and a 24-kDa band just underneath the 25-kDa marker corresponded to the GST molecule lacking the 16-amino acid NH2 terminal to the thrombin site (Fig. 2A, bottom, lanes 1 and 2). In the lanes in which GST alone was loaded, Coomassie blue staining allowed the detection of a 25-kDa band corresponding to undigested GST and the 24-kDa band corresponding to the GST molecule lacking 16-amino acid NH2 terminal to the thrombin site (Fig. 2A, bottom, lanes 3 and 4). Thrombin-cleaved GST-PMM2 and GST were used as substrates for a phosphorylation reaction in the absence (Fig. 2, A and B, lanes 1 and 3) and in the presence (Fig. 2, A and B, lanes 2 and 4) of active Sgk1. In the presence of active Sgk1, thrombin-digested PMM2 was phosphorylated (Fig. 2B, bottom, lane 2), whereas no phosphorylation was observed when thrombin-digested GST was used as a substrate for active Sgk1 (Fig. 2B, bottom, lane 4).

Coimmunoprecipitation

To verify whether the interaction between Sgk1 and PMM2 also occurred in eukaryotic cells, we performed coimmunoprecipitation experiments in transfected COS-7 cells. Rabbit anti-Myc and anti-HA immunoglobulins allowed the detection of Myc-Sgk1 (Fig. 3A, lanes 2 and 4) and HA-PMM2 (Fig. 3A, lanes 3 and 4) in cell extracts. Mouse anti-HA was
used to immunoprecipitate HA-PMM2, which was detected by performing blotting with rabbit anti-HA antibodies as expected (Fig. 3B, lanes 3 and 4). Weak and nonspecific bands were detected in extracts from cells that had not been transfected with HA-PMM2 (Fig. 3B, lanes 1 and 2). These weak bands are likely to correspond to the light chains of the mouse immunoglobulins used to immunoprecipitate HA-PMM2, cross-reacting with the HRP anti-rabbit immunoglobulins used to detect the immunoprecipitated proteins in the blot. Sgk1 was specifically detected by blotting with a rabbit anti-Sgk1 antibody (peptide 267) (6) (Fig. 3C, lane 4) but not by blotting with the preimmune serum from the same rabbit (Fig. 3D, lane 4). Nonspecific bands were detected with both the preimmune and the immune anti-Sgk antibodies. These bands have an apparent molecular weight corresponding to the heavy chains of the mouse immunoglobulins used for the HA immunoprecipitation. Because Sgk1 was detected only in HA immunoprecipitates from cells transfected with vectors coding for PMM2 and Sgk1, we conclude that the interaction between Sgk1 and PMM2 occurred in eukaryotic cells.

**Colocalization**

The localization of Sgk1 and PMM2 was studied in COS-7 cells using confocal microscopy (Fig. 4) to verify whether the interaction of the two molecules was possible in eukaryotic cells.

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**Fig. 1.** Identification of phosphomannomutase (PMM2) as an serum- and glucocorticoid-regulated kinase (Sgk1)-interacting protein using yeast two-hybrid assay. AH109 yeast strain cells cotransformed with pBridge-Sgk1 and pACT2-PMM2 were able to grow in selective medium lacking histidine, tryptophan, leucine, and adenine. The interaction was specific because yeasts cotransformed by vectors expressing different molecules, pACT2-PMM2 with pBridge HMG(Y), pBridge rPTPγ, and pBridge empty vector, were unable to grow in selective medium (A). AH109 yeast strain cells transformed with pBridge-Sgk1 or with pBridge empty vector were unable to grow in selective medium lacking histidine, tryptophan, leucine, and adenine when retransformed with pACT2-(Δ1–125)PMM2 (B).

**Fig. 2.** In vitro phosphorylation of PMM2 by active Sgk1. Active Sgk1 was used to phosphorylate thrombin-cleaved glutathione-S-transferase (GST)-PMM2 and GST as indicated in MATERIALS AND METHODS. The kinase reaction was stopped by boiling in Laemmli sample buffer. The proteins were separated by performing SDS-PAGE, stained with Coomassie blue (A), and detected by performing autoradiography (B). Thrombin-cleaved PMM2 (A and B, lanes 1 and 2, bottom) and thrombin-cleaved GST (A and B, lanes 3 and 4, bottom) were used as substrates of a kinase reaction in the presence (A and B, lanes 2 and 4) and in the absence (A and B, lanes 1 and 3) of active Sgk1.
COS-7 cells were transiently transfected with pCINeo Myc-
Sgk1 and pCELFHA-PMM2. The Myc epitope of Sgk1, rec-
ognized by rabbit anti-Myc antibodies, was detected by means of a secondary fluorescein-conjugated anti-rabbit antibody (Fig. 4A). The HA epitope of PMM2, recognized by mouse anti-HA antibodies, was detected using a secondary rhoda-
mine-conjugated anti-mouse antibody (Fig. 4B).

Under the conditions studied, both the proteins localized in the cytoplasm. The localization of Sgk was very similar to that reported in a recent study (12). Colocalization of Sgk1 and PMM2 was strongly suggested by the appearance of the yellow color in the Sgk1-PMM2 overlay in the vast majority of cells in which both the vectors were expressed (Fig. 4C).

**Sgk1 Inhibits Basal and Insulin-Stimulated PMM2 Activity in Intact Cells**

PMM2 is a key enzyme in the posttranslational glycosyla-
tion of proteins. Given the interaction between Sgk and PMM2, we hypothesized that Sgk could regulate the activity of PMM2 in basal conditions or after insulin stimulation.

HA immunoprecipitates from Sgk1- and PMM2-transfected COS-7 cells were assayed in a PMM2 reaction as described in MATERIALS AND METHODS. PMM2 activity was calculated by subtracting the background in HA immunoprecipitates from untransfected cells in the absence and in the presence of insulin. The raw data are reported in Table 1. To average data from multiple experiments, the data were expressed as frac-
tions of the mean PMM2 activity immunoprecipitated from cells incubated in the presence of insulin. Data are means ± SE from three independent experiments. The PMM2 activity recovered from cells transfected with Sgk1 alone was similar to the background and was not significantly modified by insulin (Fig. 5A, columns 1 and 2).

On the other hand, clearly measurable PMM2 activity was associated with the HA immunoprecipitates of cells transfected with HA-PMM2. Interestingly, incubation with insulin for 45 min led to a more than twofold activation of the enzyme (Fig. 5A, columns 3 and 4).

In cells cotransfected with both wild-type Sgk1 and PMM2, the PMM2 activity associated with the HA immunoprecipitates was again similar to the background, both in the absence and in the presence of insulin (Fig. 5A, columns 5 and 6).

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**Fig. 3. Coimmunoprecipitation of Sgk1 and PMM2.** COS-7 cells were trans-
fected with empty vector (A, B, C, and D, lane 1), pCDNA4TO-Myc Sgk1 (A, B, C, and D, lane 2), pCELF-HA PMM2 (A, B, C, and D, lane 3), and both pCDNA4TO-Myc Sgk1 and pCELF-HA PMM2 (A, B, C, and D, lane 4). A: solubilized extracts were diluted with Laemmli sample buffer and analyzed by performing SDS-PAGE followed by immunoblotting with rabbit anti-Myc and mouse anti-hemagglutinin (anti-HA) immunoglobulins. HA-PMM2 was de-
tected in lanes 3 and 4, and Myc-Sgk1 was detected in lanes 2 and 4. B: solubilized extracts were immunoprecipitated using mouse anti-HA immuno-
globulins and blotted with rabbit anti-HA antibodies. HA-PMM2 was detected in lanes 3 and 4. In lanes 1 and 2, weak, nonspecific bands were detected, with an apparent molecular weight corresponding to the light chains of mouse immunoglobulins used in the immunoprecipitation. C: solubilized extracts immunoprecipitated with mouse anti-HA immunoglobulins were blotted with rabbit anti-Sgk1 antiserum. Sgk was detected only in lane 4, just above nonspecific bands also detected using the rabbit preimmune serum (D).

**Fig. 4. Colocalization of Sgk1 and PMM2.** Monolayers of COS-7 cells grown on glass coverslips at ~50% confluence were transfected with expression vectors encoding the full-length Myc-tagged Sgk1 (pCINeo-Myc Sgk) and the full-length HA-tagged PMM2 (pCELF-HA PMM2). Serum-starved cells were fixed as indicated in MATERIALS AND METHODS and stained with rabbit anti-Myc immunoglobulins diluted 1:200 in PBS to visualize Sgk1 (A) and mouse anti-HA immunoglobulins diluted 1:200 in PBS to visualize PMM2 (B). The staining for Sgk1 was detected with Alexa Fluor 488 goat anti-rabbit IgG. The staining for PMM2 was detected with Alexa Fluor 568 donkey anti-mouse IgG (Molecular Probes). Image shown in C is an overlay of A and B.
Because neither insulin nor Sgk1 affected the quantity of HA-PMM2 contained in the immunoprecipitates (Fig. 5B, lanes 3, 4, 7, and 8), we conclude that insulin increased the enzymatic activity of PMM and that Sgk1 decreased the enzymatic activity of PMM in the basal state and upon insulin stimulation in intact COS-7 cells.

**DISCUSSION**

Previous data in A6 cells, a well-characterized model of the principal cells of the distal nephron, suggested that PDK1/2-dependent phosphorylation of Sgk1 is essential for the activation of the enzyme and that it is indeed the active enzyme responsible for insulin-, vasopressin-, and aldosterone-dependent sodium transport through the amiloride-sensitive ENaC (2). We then decided to perform yeast two-hybrid screening to identify molecular partners involved in the transduction of signals through Sgk1. We used a kidney library as a source of prey cDNA because the hormonal regulation of sodium transport takes place in the principal cells of the distal nephron.

### In Vitro Interaction between Sgk1 and PMM2

**Two-hybrid screening.** Using two-hybrid screening, we identified several independent clones coding for putative Sgk1 interacting proteins. One of them, transportin–H9251, is related to importin–α, previously shown by others (8) to interact with the nuclear localization sequence of Sgk1 and to cause phosphatidylinositol 3-kinase-dependent nuclear import of Sgk1. Three more clones are under evaluation.

Four of the clones coding for putative Sgk1 interacting proteins contained cDNA inserts of similar size, with the full sequence of PMM2, an enzyme that catalyzes the interconversion between mannose-6-phosphate and mannose-1-phosphate (20).

**PMM2 phosphorylation.** PMM2 sequence contains several serine and threonine residues close enough to arginine residues to identify potential targets of phosphorylation by different serine/threonine kinases, such as protein kinase A (PKA residues 115–118), casein kinase II (residues 47–50, 118–121, 137–140, and 232–235), and protein kinase C (PKC 237–239) (ExPASy; Swiss protein no. 015305). Residues 134–137 are also part of a consensus sequence for Sgk and PKB (3). We were able to show that PMM2 can be an in vitro substrate for Sgk1 kinase activity, although with low efficiency. This phosphorylation experiment adds more evidence to the hypothesis that Sgk1 and PMM2 may indeed have molecular interaction, although it is not intended to prove that PMM2 activity is regulated by phosphorylation.

**Interaction between Sgk1 and PMM2 in intact cells.** The interaction between Sgk1 and PMM2 occurs also in eukaryotic cells. In fact, we were able to detect Sgk1 in HA immunoprecipitates from Sgk1 and PMM2 transfected COS-7 cells. PMM2 activity was measured in HA immunoprecipitates from cells transfected with empty vectors (pCELF-HA and pcDNA4TO), Myc-tagged Sgk1 (pcDNA4TO-Myc Sgk1), empty vector pCeLF-HA (columns 1 and 2), HA-tagged PMM2 (pCELF-HA PMM2), empty vector pcDNA4TO (columns 3 and 4), Myc-tagged Sgk1 (pcDNA4TO-Myc Sgk1), and HA-tagged PMM2 (pCELF-HA PMM2) (columns 5 and 6). Serum-starved cells were stimulated with insulin as indicated. PMM2 activity was calculated by subtracting the background immunoprecipitated from cells transfected with empty vectors and was expressed as a fraction of the mean activity immunoprecipitated from insulin-stimulated cells (A). The protein G pellets used in the enzymatic assay for PMM2 activity were boiled in Laemmli sample buffer. The proteins were separated by performing SDS-PAGE, followed by immunoblotting with rabbit anti-HA immunoglobulins (B).

### Table 1. Optical density of transiently transfected COS-7 cells

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<tr>
<th>Cell Group</th>
<th>Without Insulin</th>
<th>With Insulin</th>
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<tr>
<td>Untransfected cells</td>
<td>42 ± 1.8</td>
<td>60 ± 2.35</td>
</tr>
<tr>
<td>Sgk-transfected cells</td>
<td>50 ± 1.8</td>
<td>64.5 ± 2.35</td>
</tr>
<tr>
<td>PMM2-transfected cells</td>
<td>72.5 ± 0.7</td>
<td>136.5 ± 14.83</td>
</tr>
<tr>
<td>PMM2/Sgk-transfected cells</td>
<td>46 ± 2.8</td>
<td>63 ± 3.7</td>
</tr>
</tbody>
</table>

Data are means ± SE of optical density (×1,000) at 340 nm.
Cystic Fibrosis

Sodium transport through the ENaC is related to chloride transport through the cystic fibrosis transmembrane regulator. In particular, the activation of chloride transport has been described as inhibiting sodium transport in epithelial cells (19). The lack of inhibition in sodium absorption and the consequent hyperviscosity of secretions are thought to have a role in the pathogenesis of cystic fibrosis (9). Increased expression of Sgk1 has been described in the bronchial epithelial cells of patients with cystic fibrosis (21), and this increased expression may also have a pathogenetic role, although the relationship between cystic fibrosis transmembrane regulator and Sgk1 has only recently become partially understood (5, 17). Interestingly, decreased glycosylation has been described in the bronchial epithelia of patients with cystic fibrosis, and this has been associated with increased susceptibility to bacterial infection (15). It is possible that the increased expression of Sgk1 is indeed the mechanism by which decreased glycosylation occurs through inhibition of PMM activity.

Finally, the regulation of PMM2 activity and protein glycosylation by a kinase involved in transducing serum- and steroid-dependent survival signals may be relevant for cell adhesion and interaction as well as for the development of renal complications of diabetes and hypertension.

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REFERENCES


