A novel protein was cloned from a rat liver cDNA library by interaction with the liver glucokinase. This protein contained 339 residues and possessed a canonical consensus sequence for a dual specificity phosphatase. The recombinant protein was able to dephosphorylate phosphotyrosyl and phosphoseryl/threonyl substrates. We called this protein the glucokinase-associated phosphatase (GKAP). The GKAP partially dephosphorylated the recombinant glucokinase previously phosphorylated, in vitro, by protein kinase A. The GKAP fused with green fluorescent protein was located in the cytosol, where glucokinase phosphorylates glucose, and not in the nucleus where the glucokinase is retained inactive by the glucokinase regulatory protein. More importantly, the GKAP accelerated the glucokinase activity in a dose-dependent manner and with a stoichiometry close to the nucleus of hepatocytes from starved rats, and after 1 and 2 h of refeeding, glucokinase is translocated into the cytosol (8). The role of the glucokinase regulatory protein is thus to sequester the enzyme in a compartment where it might be inactive (5, 9). Nevertheless, at the present time, it is not known if a protein is able to retain glucokinase in the cytoplasm either at the plasma membrane or at the mitochondria, close to the glucose or ATP sources. Moreover, post-translational protein modifications might also alter the activity of glucokinase.

The aim of this study was to identify proteins expressed in the liver implicated in the regulation of glucokinase activity after interaction with glucokinase. We performed a two-hybrid screen using rat liver glucokinase as a bait, and showed that glucokinase was interacting not only with the glucokinase regulatory protein, but also with a newly identified dual specificity phosphatase called GKAP.1 The function of this interaction was analyzed.

MATERIALS AND METHODS

Plasmid Constructions—All manipulations were carried out by standard techniques and plasmid structures verified by DNA sequencing. The entire coding sequence of rat hepatic glucokinase was recovered (BamHI/AuII fragment) from a plasmid (10) and inserted in frame at the BamHI site of the yeast expression vector pLex9 (pLex-GK) and of the bacterial expression vector pQE-32 (pQE-32-GK). pBSK-GKRP contains the cDNA of glucokinase regulatory protein (GKRP) (a gift from E. Van Schaftingen). Then, GKRP was subcloned in frame into pLex9. Hexokinase II was similarly constructed in pLex9, in fusion with the yeast LexA DNA binding domain (pLex-HKII). The cDNA encoding GK was excised (EcoRI/Xho1 fragment) from the pGAD plasmid of the two-hybrid screen and subcloned into pBSK to produce pBSK-GKAP. An EcoRI/Not1 fragment containing the GKAP insert was then isolated from pBSK-GKAP and inserted into pGEX-4T2 vector (Amersham Pharmacia Biotech), in frame with GST coding sequence (pGEX–4T2-GKAP) and into an pEGFPc vector (CLONTECH), in frame with green fluorescent protein (pEGFP-GKAP).

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1 The abbreviations used are: GKAP, glucokinase-associated phosphatase; PTP, protein-tyrosine phosphatase; dsPTP, dual specificity protein-tyrosine phosphatase; pNPP, p-nitrophenyl phosphate; GK, glucokinase; bp, base pair(s); PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; GFP, green fluorescent protein; PBS, phosphate-buffered saline; BSA, bovine serum albumin; RT, reverse transcriptase; PCR, polymerase chain reaction; PKA, cAMP-dependent protein kinase; GKRP, glucokinase regulatory protein.
cDNA Library Screening—A yeast two-hybrid screen was performed as described previously (11), using the entire coding sequence of glucokinas e as the bait. The rat liver cDNA library was constructed in pGADT72X plasmid. The plasmids were tested for specificity using pLex-HKII. Then, their cDNA sequences were sequenced using an Applied Biosystems model 373A sequencer. Specific for the sequence of cDNA the BLAST program was used to search for sequence homology in the GenBank data base.

Production and Purification of Proteins Expressed in Bacteria—Recombinant GKAP was expressed as a glutathione S-transferase (GST) fusion protein, using the plasmid pGEX-4T2-GKAP to transform Escherichia coli BL21. These cells were grown in Luria-Bertani (LB) medium supplemented with penicillin, streptomycin, 0.001 M insulin, 1 ppm dexamethasone, 1 ppm triiodothyronine, and 5% fetal calf serum. GKAP3T3F heptoma cell line was derived from transgenic mice synthesizing the SV40 large T and small antigens under the control of antithrombin III promoter (14). Cells were grown on glass-four-chamber slides (Falcon, CultureSlides) in Dulbeccos modified Eagles medium/Ham`s F12, Glutamax (Life Technologies), 5% FCS, 50 units/ml penicillin, and 50 units/ml streptomycin. The inducer isopropyl-1-thio-D-\(\beta\)-galactopyranoside was utilized for His-GK. The radiolabeled peptides were separated from the fully dehydrogenase-coupled assay (described below). Protein concentrations were determined by the method of Bradford using BSA as a standard and the integrity of the fusion proteins were verified by SDS-PAGE.

Phosphorylation of Glucokinase—Bacterially expressed His-tagged GK (2 mg) was incubated with 10 units of the catalytic subunit of protein kinase A (Sigma) for 1 or 2 h at 30 °C in a 25-mL reaction mixture containing 20 mM HEPES, pH 7.4, 10 mM MgCl\(_2\), 8 mM \(\beta\)-mercaptoethanol, 50 \(\mu\)M ATP, and 20 \(\mu\)Ci of \(\gamma\)-\(32\)P-ATP (5000 Ci/mmol, Amer sham Pharmacia Biotech). The reaction was stopped by the addition of 25 \(\mu\)l of 10 mM sodium phosphate, 10 mM sodium pyrophosphate, pH 8.0, and the phosphorylated glucokinase was affinity-purified on nickel-nitrilotriacetic acid-agarose beads (Qiagen). Bound protein was eluted with 30 \(\mu\)l of 1 M NaOH and the absorbance at 405 nm was measured. For peptide assays, radiolabeled peptides were separated from free \(\gamma\)-\(32\)P-ATP using 1 × 1-cm P-81 sheets of phosphocellulose paper essentially as described in Ref. 12. Dephosphorylation with GST-GKAP was carried out for the indicated time at 30 °C in a 200-\(\mu\)l reaction mixture containing 50 mM imidazole, pH 7.0, 0.1% \(\beta\)-mercaptoethanol, 50 \(\mu\)M 

Phosphatase Assays—Hydrolysis of pNPP by GKAP was carried out in a reaction volume of 800 \(\mu\)l containing 50 mM imidazole, pH 7.0, 0.1%\(\beta\)-mercaptoethanol, and 20 \(\mu\)M pNPP at 37 °C for 2 h. The reaction was stopped by addition of 200 \(\mu\)l of 1 M NaOH and the absorbance at 405 nm was measured. For peptide assays, radiolabeled peptides were separated from free \(\gamma\)-\(32\)P-ATP using 1 × 1-cm P-81 sheets of phosphocellulose paper essentially as described in Ref. 12. Dephosphorylation with GST-GKAP was carried out for the indicated time at 30 °C in a 200-\(\mu\)l reaction mixture containing 50 mM imidazole, pH 7.0, 0.1% \(\beta\)-mercaptoethanol, 50 \(\mu\)M ATP, and 20 \(\mu\)l of cell lysate. The reaction volume was 800 \(\mu\)l containing 10 mM sodium phosphate, 10 mM sodium pyrophosphate, pH 8.0, and the phosphorylated glucokinase was affinity-purified on nickel-nitrilotriacetic acid-agarose beads (Qiagen). Bound protein was eluted with 30 \(\mu\)l of 1 M NaOH and the absorbance at 405 nm was measured. For peptide assays, radiolabeled peptides were separated from free \(\gamma\)-\(32\)P-ATP using 1 × 1-cm P-81 sheets of phosphocellulose paper essentially as described in Ref. 12. Dephosphorylation with GST-GKAP was carried out for the indicated time at 30 °C in a 200-\(\mu\)l reaction mixture containing 50 mM imidazole, pH 7.0, 0.1% \(\beta\)-mercaptoethanol, 50 \(\mu\)M 

RESULTS

Identification of Proteins Interacting with Hepatic Glucokinase—To identify novel proteins that interact with hepatic glucokinase, we have used the two-hybrid system (16). A fusion between the LexA DNA-binding domain and the full-length glucokinase was used as a bait to screen a library of rat liver cDNA fused with the Gal4 activation domain (Promega). 10 \(\mu\)l of the phosphorylation mix was then used for the phosphatase assay.

Tissue-specific Expression of GKAP—Total RNA was purified from rat tissues using the method of Chomczynski and Sacchi (13). Northern blot analysis was performed as described previously (11) using as probe the full-length GKAP cDNA. The presence of GKAP in \(\beta\) versus other pancreas tissue was assessed by RT-PCR analysis. The rat pancreatic islets were isolated after collagenase digestion of the pancreas, and resuspended in PBS-EGTA supplemented with 0.1 mg/ml trypsin. The \(\beta\) cells were sorted by the autofluorescence for FAD and the light scatter parameter (cell size) using a FACStar plus (Becton Dickinson) with an argon laser (163, Spectra Physics) at 488 nm. The quality of the sorting was verified by subsequent FACS analysis of samples of the purified cells. RNA from \(\beta\) cells were about 40–50-fold enriched in specific mRNA when compared with RNA from total pancreas (data not shown).

Reverse transcription was performed using 5 \(\mu\)g of RNA from the sorted \(\beta\) cells (and white adipose tissue) using a random primer, and then PCR was achieved using two oligonucleotides: cegtrtacagg and gttgtg. The amplifications were done following the accumulation of NADH at 340 nm using a spectrophotometer.

The glucokinase phosphorylates glucose into glucose 6-phosphate (G-6-P), this substrate is transformed into 6-phosphogluconate and NADH by the G-6-P dehydrogenase (from Leuconostoc mesenteroides). This last reaction depends directly on the glucokinase activity. The glucokinase activity was measured in the presence of 0.1 \(\mu\)g glucose, 50 mM HEPES, pH 7.4, 0.1 \(\mu\)g KCl, 75 mM dithiothreitol, 0.5 mM NAD, 2 units of G-6-P dehydrogenase, and 1 or 2 \(\mu\)g of recombinant GK, during 15 min at 30 °C. The base line was recorded for 2 min, and then the reaction was initialized by the addition of ATP-Mg\(^2+\) (5 mM). The glucose phosphorylating activity was also tested without recombinant glucokinase, without glucose, without ATP, or with 1 \(\mu\)g glucose or with 0.1 unit glucose 6-phosphatase. In these conditions, no activity was detected. 10 \(\mu\)l of recombinant glucokinase was phosphorylated directly after elution from nickel beads by incubation during 30 min at 30 °C in 90 \(\mu\)l of medium containing protein kinase A buffer (1 × Bioslab), 8 mM \(\beta\)-mercaptoethanol, 200 \(\mu\)M ATP-Mg\(^2+\), 50 mM NaCl, 5% glycerol, and in absence or presence of 25 units of the protein kinase A catalytic subunit (Bioslab). We verified by autoradiography that the recombinant glucokinase was phosphorylated by protein kinase A in presence of 10 \(\mu\)Ci of \(\gamma\)-\(32\)P-ATP. Then, the glucokinase activity was measured on 100 \(\mu\)l of the phosphorylation reaction. The GKAP effect on glucokinase activity was recorded after the addition of various amounts of the fusion protein 10 min after the beginning of the reaction. The results were expressed as enzymatic units per milligram of protein.

RESULTS

Identification of Proteins Interacting with Hepatic Glucokinase—To identify novel proteins that interact with hepatic glucokinase, we have used the two-hybrid system (16). A fusion between the LexA DNA-binding domain and the full-length glucokinase was used as a bait to screen a library of rat liver cDNA fused with the Gal4 activation domain. Approximately 5 × 10\(^6\) yeast transformants were tested, and 70 clones were classified as positive, since they interacted strongly with glucokinase and not with an unrelated protein such as lamin.

Restriction mapping and sequence analysis revealed five different groups of positive clones. One of these groups corresponded to the glucokinase regulatory protein, whose specific interaction with glucokinase is known. Another group contained the cDNA of an unknown protein, which was studied here.

The deduced amino acid sequence of the new protein possesses the calcium module (CXXGXXYX/5'T), a catalytic, 0.1 \(\mu\)M protein-tyrosine phosphatases (PTPs) (see below), suggesting that it may be a novel protein phosphatase. We called it GKAP (glucokinase-associated phosphatase). GKAP cDNA extends 1314 bp with a possible translation start site (17) at bp 19, followed by a 1017-bp coding region (Fig. 1). Searches of missing sequence upstream of this initiating methionine, using the
GKAP was present in pancreatic cells, confirming earlier studies showing that GKAP is associated with glucokinase in this tissue (23). Among pancreatic cells, we verified by RT-PCR analysis that GKAP mRNA was also detected in kidney and white adipose tissue. Lower levels of GKAP expression were seen in lung, brain, and pancreas, whereas moderate levels were found in heart, liver, intestine, skeletal muscle, and brown adipose tissue. Lower levels of GKAP mRNA were also detected in kidney and white adipose tissue. Lower levels of GKAP mRNA were also detected in kidney and white adipose tissue. Lower levels of GKAP mRNA were also detected in kidney and white adipose tissue. Lower levels of GKAP mRNA were also detected in kidney and white adipose tissue. Lower levels of GKAP mRNA were also detected in kidney and white adipose tissue. Lower levels of GKAP mRNA were also detected in kidney and white adipose tissue. 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fied glucokinase expressed in E. coli as a His-tagged fusion protein (Fig. 6B). Then, labeled glucokinase was incubated with GST-GKAP. Recombinant GST-GKAP (Fig. 6D) dephosphorylated glucokinase in a concentration-dependent manner, as shown in Fig. 6C (lanes 1–4). The dephosphorylation of labeled glucokinase by GST-GKAP was not complete even when the GST-GKAP treatment was greater than 2 h (data not shown). Moreover, neither E. coli lysate (data not shown) nor GST alone purified in the same conditions as GST-GKAP (Fig. 6C, lane 5) were able to dephosphorylate glucokinase. Thus, proteins copurified with GST-GKAP are unlikely to be responsible for the glucokinase dephosphorylation observed. To further assess the specificity of the reaction, the GKAP was produced by using an in vitro transcription/translation system (Fig. 6F). This GKAP generated an identical dephosphorylation of glucokinase (Fig. 6E, lane 2). The glucokinase regulatory protein, produced by reticulocyte lysate (Fig. 6F, lane 3), was unable to dephosphorylate glucokinase (Fig. 6E, lane 4). This eliminated the possible dephosphorylating activity of proteins present in the reticulocyte lysate. Taken together these experiments suggest that glucokinase dephosphorylation was due to
**A Phosphatase Accelerates Glucokinase Activity**

GKAP. We also showed that the dephosphorylating activity was sensitive to inhibition by a phosphatase inhibitor, sodium orthovanadate (2 mM) (Fig. 6E, lane 1).

**Effect of GKAP on GK Activity**—We further investigated the role of GKAP by studying its effect on the glucokinase activity. The basal recombinant glucokinase activity was about 1–4 units/mg of protein, in our experimental conditions, as described under “Materials and Methods.” No hexokinase or glucokinase activity could be detected in GST-GKAP or in GST preparations (data not shown). Adding GST-GKAP (6 μg) to the active glucokinase led to a 2-fold augmented production of NADH quantified by recording the optical density at 340 nm (Fig. 7A). This was not observed when GST alone was added. A second addition of 6 μg of GST-GKAP similarly induced an increase in GK activity (Fig. 7A). The 2.5 stimulatory effect of GKAP on GK activity was dependent on the quantity of GKAP added and it was saturated over 20 μg of added GKAP (Fig. 7B). The glucokinase activity was significantly decreased by 31 ± 9% (n = 5) when glucokinase was previously phosphorylated by protein kinase A (Fig. 7C). The addition of 3 μg of GKAP on phosphorylated GK induced a 73 ± 26% (n = 8) stimulation of its activity, whereas a similar amount of GKAP (3 μg) increased only by 50 ± 19% the unphosphorylated glucokinase activity.

**DISCUSSION**

The association between glucokinase and a new protein (GKAP) was observed by using the two-hybrid system. GKAP mRNA was detected in all the rat tissues tested. Although these results do not correlate with the limited distribution pattern of glucokinase, GKAP mRNA appears in all tissues expressing glucokinase, suggesting that GKAP may have a specific role in these tissues. Furthermore, GKAP specifically interacts with GK and not with the closely related protein HKII. Because a dual specificity phosphatase consensus sequence was revealed after computer analysis of the cloned...

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**Fig. 5. Phosphatase activity of purified GKAP.** A, the ability of GST-GKAP to hydrolyze pNPP, either in the absence (closed circles) or presence (open circles) of 2 mM sodium vanadate, and of GST control (closed squares) was assayed at the indicated concentrations and expressed as increased absorbance at 405 nm. B, time course of labeled raytide dephosphorylation by GKAP (10 μg), in the absence (closed circles) or presence (open circles) of 2 mM sodium vanadate. The control assays were performed with 10 μg of GST (closed squares). Data are the mean S.E. of two or three experiments performed in duplicate.

**Fig. 6. Phosphorylation and dephosphorylation of glucokinase.** A, phosphorylation of His-glucokinase by protein kinase A is shown on a representative autoradiograph of SDS-polyacrylamide gel. The controls were performed in the absence of catalytic subunit of protein kinase A or glucokinase, shown in lanes 1 and 2, respectively. The length of the reaction was 60 min (lane 3) or 120 min (lane 4). B, production in bacteria, purification, and quantification of His-glucokinase (BSA, lanes 1 and 2, respectively; 2 and 4 μg; His-glucokinase, lanes 3 and 4, 1 and 3 μg). C, a representative autoradiograph of dephosphorylation of glucokinase by recombinant GKAP. Increasing amounts of purified recombinant GST-GKAP (0, 1, 5, and 10 μg) were assayed for their ability to dephosphorylate radiolabeled His-glucokinase (lanes 1, 2, 3, and 4). The control is performed with 10 μg of GST (lane 5) or E. coli lysate (data not shown). D, production in bacteria, purification, and quantification of GST-GKAP. BSA, lanes 1 and 2, respectively; 2 and 6 μg; GST-GKAP, lanes 3 and 4, respectively, 5 and 10 μg. E, the dephosphorylation of labeled glucokinase by GKAP produced by a transcription/translation system (TNT). The dephosphorylation of glucokinase by GKAP (10 μl of TNT mix) was performed in the presence (lane 1) or absence (lane 2) of 2 mM sodium vanadate, or in the presence of unlabeled glucokinase (lane 3). The GKR (10 μl of TNT mix) was unable to dephosphorylate glucokinase (lane 4). F, production in vitro by a transcription/translation system (TNT) in the presence of [35S]methionine of GKAP (lane 1) or GKR (lane 3). [32P]His-glucokinase was also loaded (lane 2). The experiments were reproduced three to four times independently. The produced proteins were at the expected size.
sequence, the function of this new protein was studied.

It has been shown that purified glucokinase from rat liver is phosphorylated by PKA on serine residue(s) and that the phosphorylation reduced (50%) the enzyme activity (18). In our hands, recombinant glucokinase activity was also diminished by PKA phosphorylation. The yeast hexokinase HK2, the isoform homologous to glucokinase, has two phosphorylation sites. The serine 15, belonging to a protein kinase A consensus phosphorylation sequence, is phosphorylated after a shift to a medium with low glucose concentration (19). This phosphorylation would affect the oligomeric state of HK2 (20) and is essential for glucose signaling (21). On serine 158, a transitory phosphorylation might occur during the transfer of γ-phosphate from ATP to glucose (22). In human β cell glucokinase, serine 151 corresponding to serine 158 of yeast hexokinase 2 is also phosphorylated, as a product of ATP hydrolysis (23). Thus, a phosphorylated-dephosphorylated state of glucokinase is observed. Nevertheless, the identity of the amino acid residues bearing the phosphate, transitorily or not, is still an open question (23–25).

GKAP is related to a subclass of PTPs commonly referred to as dePTP (reviewed in Ref. 26), hydrolyzing phosphate from Ser/Thr and Tyr residues. Importantly, all of the amino acids previously shown to participate in the catalytic mechanism underlying dual specificity phosphatase activity are conserved in GKAP, including Cys-114, the catalytic cysteine that functions as the active site nucleophile, and Asp-83 and Ser-121, both involved in hydrolysis of the thiol phosphate intermediate (27, 28). This protein has a long C-terminal amino acid domain of unknown function. By comparison, the recently cloned MKP-5 dual specificity phosphatase for p38 possesses a long N-terminal domain of unknown function (29), suggesting that the position of the catalytic domain is not the signature for this family of proteins. We confirmed experimentally that the recombinant protein is indeed a phosphatase because it dephosphorylated a model substrate PNP and the phosphotyrosine raytide and its activity is blocked by phosphatase inhibitor, vanadate. Since glucokinase is interacting with GKAP and, generally a phosphatase is associated to its own substrate, glucokinase might be the phosphosubstrate for this phosphatase. Indeed, the recombinant glucokinase, phosphorylated by PKA (on serine residues, according to Ref. 18), was dephosphorylated by GKAP produced by two techniques. However, the dephosphorylation was not complete presumably because the in vitro phosphorylation of glucokinase by protein kinase A occurred also on residues outside the specific motif recognized by GKAP. Similarly, it was reported that MKP dephosphorylates only partially the activated MAPK (30, 31). Our results supported the notion that GKAP is a dual (phosphoserine/threonine and phosphotyrosine) phosphatase and may be a glucokinase-specific phosphatase.

GKAP is located in the cytoplasm and not in the nucleus. This is the cell compartment where the glucokinase is catalytically active and where glycolysis occurs. Thus, it can be hypothesized that the interaction between glucokinase and GKAP regulates glucokinase activity. We showed that GKAP stimulated glucokinase activity in a dose-dependent manner. This effect occurred at a stoichiometry of GK/GKAP of 1/3 to 1/5, compatible with a physiological phenomenon. The glucokinase activity, diminished by a previous phosphorylation of the recombinant protein by PKA, was augmented by the presence of the phosphatase GKAP to a higher extent than that obtained with unphosphorylated glucokinase. The mechanism by which glucokinase activity is stimulated by GKAP remains unknown. The dephosphorylation of the glucokinase might help the phosphotransfer, as suggested in the yeast hexokinase where a transitory phosphorylated state exists as the result of the phosphotransference activity of the enzyme (21). The dephosphorylation of glucokinase by GKAP could be implicated in the proteolysis of glucokinase. Indeed, it has recently been reported that mice bearing an inactivated glucokinase regulatory protein gene exhibit decreased levels of liver glucokinase. Since similar amounts of glucokinase are produced by control and knockout mice, the retention of glucokinase in the nucleus by its interaction with GKR might protect it from degradation in the cytosol (9).

In conclusion, by interaction with glucokinase, we cloned a novel dual specificity phosphatase (GKAP), which is located into the cytoplasm. We described a regulatory mechanism that
accelerates the glucose phosphorylation. This new step might control the glycolysis rate, and as a consequence, the glucose uptake by the liver and the insulin secretion by pancreatic β cell.

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