Regulation of Glutamine:Fructose-6-Phosphate Amidotransferase by cAMP-Dependent Protein Kinase

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Glutamine:fructose-6-phosphate amidotransferase (GFA) is the rate-limiting enzyme in hexosamine biosynthesis, an important pathway for cellular glucose sensing. Human GFA has two potential sites for phosphorylation by cAMP-dependent protein kinase A (PKA). To test whether GFA activity is regulated by cAMP-dependent phosphorylation, rat aortic smooth muscle cells were treated in vivo with cAMP-elevating agents, 10 µmol/l forskolin, 1 mmol/l 8-Br-cAMP, or 3-isobutyl-1-methylxanthine. All treatments resulted in rapid and significant increases (2- to 2.4-fold) in GFA activity assayed in cytosolic extracts. Maximal effects of forskolin were observed at 10 µmol/l and 60 min. Preincubation of cells with cycloheximide did not abolish the effect of forskolin. Incubation of cytosolic extracts at 37°C for 10 min in a buffer without phosphatase inhibitors led to a 79% decrease of GFA activity. This loss of activity was inhibited by the addition of phosphatase inhibitors (5 mmol/l sodium orthovanadate, 50 mmol/l sodium fluoride, or 5 mmol/l EDTA, but not 100 nmol/l okadaic acid), suggesting that GFA undergoes rapid dephosphorylation by endogenous phosphatases. Purified GFA is phosphorylated in vitro by purified PKA, resulting in a 1.7-fold increase in GFA activity. Treatment of GFA with purified protein kinase C had no effect. We conclude that GFA activity may be modulated by cAMP-dependent phosphorylation. *Diabetes* 47:1836–1840, 1998

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Received for publication 3 April 1998 and accepted in revised form 13 August 1998.

Q.K.H and E.A.G. hold stock in Monsanto.

DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; FCS, fetal calf serum; F-6-P, fructose-6-phosphate; GlcN-6-P, glucosamine-6-phosphate; GFA, glutamine:fructose-6-phosphate amidotransferase; IBMX, isobutylmethylxanthine; PKA, protein kinase A; PMSF, phenylmethylsulfonyl fluoride; PP, protein phosphatase; RASM, rat aortic smooth muscle.

Glucose at high concentrations has multiple deleterious effects on cell metabolism and growth. For instance, hyperglycemia worsens and, in fact, may cause insulin resistance in type 1 and 2 diabetes by leading to decreased glucose uptake and glucose utilization (1,2). Hyperglycemia is also the primary factor in the pathogenesis of diabetic microvascular complications (3). The mechanism for this so-called glucose toxicity is unknown, but recent studies have shown that the hexosamine biosynthesis pathway apparently mediates some of these deleterious effects of high glucose (4–8).

The first and rate-limiting step for hexose entry into the hexosamine pathway is catalyzed by glutamine:fructose-6-phosphate amidotransferase (GFA, EC 2.6.1.16). GFA catalyzes the transfer of the amido group from glutamine to fructose-6-phosphate (F-6-P) to produce glucosamine-6-phosphate (GlcN-6-P). We have previously shown that the overexpression of GFA results in a decrease in the insulin sensitivity of glycogen synthase in transgenic rat-1 fibroblasts (9,10) and desensitization of insulin-stimulated glucose transport in skeletal muscle of transgenic animals (11). These data suggest that GFA plays an important role in the regulation of glucose homeostasis and that the hexosamine pathway functions as a "glucose sensor" for aspects of glucose-dependent metabolic regulation (12).

Cloning of bacterial (13), yeast (14), and human (15,16) GFA has shown it to be highly conserved through evolution. In the fungus *Blastocladiella emersonii*, GFA enzyme activity is developmentally regulated (17); GFA remains dephosphorylated and constitutively active during vegetative growth, but during sporulation GFA is phosphorylated and feedback-inhibited by the end product of the pathway, UDP-*N*-acetyl-glucosamine. Furthermore, an in vitro phosphorylation assay suggested that partially purified fungal GFA could be desensitized to feedback inhibition by cAMP-dependent protein kinase A (PKA).

Amino-acid sequence analysis reveals that human GFA has two potential PKA-phosphorylation sites, $_{202}$RRGS$_{205}$ and $_{232}$KKGS$_{235}$; one of these consensus sites is conserved in yeast GFA, whereas bacterial GFA has neither. In the present work, we have studied the effects of cAMP-elevating agents, phosphatase inhibitors, and PKA itself on GFA activity to characterize regulation of GFA activity in mammals.

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RESEARCH DESIGN AND METHODS

Materials and cell culture. Forskolin, 8-bromo-cAMP, isobutylmethylxanthine (IBMX), sodium fluoride, and sodium orthovanadate were obtained from Sigma (St. Louis, MO). Purified PKA catalytic subunit was obtained from Promega (Madison, WI), protein kinase C (PKC) catalytic fragment from rat brain and okadaic acid were from Calbiochem (La Jolla, CA), and microcystin-LR was from Life Technologies (Gaithersburg, MD).

Rat aortic smooth muscle (RASM) cells were prepared as described previously (18). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) and routinely passed every 4 days at confluence. Cells were equilibrated in DMEM–0.1% FCS for 1 h before treatment in vivo.

Preparation of cytosolic extracts and assay of GFA activity. GFA activity was assayed using the method published previously (10). Briefly, at the end of treatment, cells were rinsed twice with ice-cold Krebs-Ringer phosphate buffer–10 mmol/l HEPES, pH 7.6, and harvested with a rubber policeman. Cells were pelleted and sonicated in extraction buffer containing 100 mmol/l KCl, 1 mmol/l EDTA, and 50 mmol/l sodium phosphate, pH 7.5. Cytosolic extracts were obtained by centrifugation at 16,000*g* for 10 min at 4°C. Extracts, 25–30 µg of protein in 50 µl volume, were then incubated with 50 µl substrate mix (final concentrations: 12 mmol/l F-6-P, 12 mmol/l glutamine, 40 mmol/l sodium phosphate, pH 7.4, 1 mmol/l EDTA, and 1 mmol/l dithiothreitol [DTT]) at 37°C for 45 min. GFA enzyme activity was measured by the generation of GlcN-6-P and quantified by fluorescence after derivatization by *o*-phthaldialdehyde and fractionation by reverse-phase high-performance liquid chromatography as described (19). GFA activity was expressed as units per milligram of protein where 1 unit represents the generation of 1 picomole of GlcN-6-P per minute.

In vitro dephosphorylation of GFA. Cells were cultured and harvested as described above, except cytosolic extracts were prepared by sonicating cells in 25 mmol/l Tris (pH 7.4), 1 mmol/l EDTA, 0.5 mmol/l EGTA, 200 µmol/l phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml leupeptin, and 20 µg/ml aprotinin. Cytosolic extracts (25–30 µg protein) were preincubated at 37°C for 10 min in the presence or absence of phosphatase inhibitor, cooled on ice for 5 min, and then assayed for GFA activity by adding substrate mix.

Treatment of purified GFA with PKA and PKC. GFA was purified from rat liver, with all purification procedures being carried out at 4°C. Rat liver (20 g) was minced and suspended in 100 ml of 25 mmol/l HEPES buffer (pH 7.5) containing 5 mmol/l EDTA, 100 mmol/l KCl, 1 mmol/l DTT, 5 mmol/l glucose-6-phosphate, and protease inhibitors (Complete Inhibitor Cocktail; Boehringer Mannheim, Indianapolis, IN). The suspension was homogenized using a Tekmar homogenizer and centrifuged for 45 min at 20,000*g*. The supernatant solution was collected and further centrifuged at 105,000*g* for 1 h. The supernatant solution containing GFA activity was then subjected to ammonium sulfate fractionation. The fraction isolated from 40 to 55% ammonium sulfate was collected and stored at –80°C. This fraction was later thawed and dissolved in 9 ml of 50 mmol/l potassium phosphate buffer, pH 7.5, containing 50 mmol/l KCl, 1 mmol/l DTT, 2 mmol/l F-6-P, and protease inhibitors and applied to PD-10 gel filtration columns (Pharmacia, Piscataway, NJ). The active high-molecular-weight fraction was collected. Glucose-6-phosphate was added to a final concentration of 5 mmol/l, and the solution was applied to a column of DEAE-sepharose (20 ml) that was previously equilibrated with 50 mmol/l potassium phosphate buffer. The column was washed with 80 ml of the same buffer and then with 40 ml of 150 mmol/l KCl and 30 ml of 250 mmol/l KCl, each in the 50 mmol/l potassium phosphate buffer. The enzyme activity was eluted with 250 mmol/l KCl buffer (20). The eluate was then applied to a column of hydroxyapatite (10 ml) previously equilibrated with the 50 mmol/l potassium phosphate buffer (21). The column was washed with 10 ml of the same buffer, 20 ml of 150 mmol/l potassium phosphate buffer, and 10 ml of 350 mmol/l potassium phosphate buffer, each pH 7.5 and containing 50 mmol/l KCl, 2 mmol/l F-6-P, 1 mmol/l DTT, and protease inhibitors. The enzyme activity was found in the last wash. The enzyme solution was concentrated to 5 ml and stored at –80°C. The purification resulted in a greater than 500-fold increase in specific activity, and the final product contained a single protein band of the appropriate molecular weight visualized by Coomassie blue staining after SDS-PAGE.

To demonstrate phosphorylation of GFA by PKA, 0.36 µg GFA was incubated in 40 mmol/l Tris (pH 7.4), 20 mmol/l $MgCl_2$, 2 mmol/l PMSF, 2 mmol/l DTT, 50 µmol/l ATP, 10 µCi γ-[^{32}P]ATP, and 62 U PKA catalytic subunit for 30 min at 37°C. Phosphoproteins were fractionated by SDS-PAGE and visualized by autoradiography. To analyze the effect of PKA treatment on GFA activity, GFA was treated in the same fashion except that the labeled ATP was omitted and the concentration of ATP was increased to 200 µmol/l. Control samples were incubated in parallel without PKA. GFA activity was subsequently assayed as described above. PKC treatment proceeded in a similar fashion using the catalytic fragment of PKC from rat brain. Treatment with PKC was for 30 min at 30°C in 10 µl of a buffer containing 50 mmol/l morpholinoethane sulfonic acid, pH 6.1, 6 mmol/l EGTA, 60 mmol/l $MgCl_2$, and 20 ng PKC.

RESULTS

GFA activity is increased by treatment of cells with cAMP-elevating agents. To investigate whether cAMP-dependent pathways play a role in the regulation of mammalian GFA enzyme activity, RASM cells were treated for 30 min with 10 µmol/l forskolin, an activator of adenylate cyclase. Cytosolic extracts were then prepared and assayed for GFA activity. As shown in Fig. 1, GFA activity from forskolin-treated cells was increased 2.2-fold compared with activity from control cells (903 vs. 410 U/mg, $P < 0.001$). Preincubation of cells with 2 µg/ml cycloheximide for 2 h did not abolish the effect of forskolin. Other intracellular cAMP-elevating agents such as 8-Br-cAMP (1 mmol/l) and IBMX (1 mmol/l), an inhibitor of phosphodiesterase, also led to 2-fold (828 U) and 2.4-fold (1,010 U) increases of GFA activity, respectively, at the end of the 30-min incubation period ($P < 0.001$).

The effect of cAMP-elevating agents on GFA activity was time and dose dependent. As soon as 15 min after treating cells with 10 µmol/l forskolin, a twofold increase in GFA activity was seen, with maximal stimulation observed at 60 min (2.3-fold) (Fig. 2*A*). GFA activity was maximally stimulated at 10 µmol/l forskolin (Fig. 2*B*).

Although human GFA also contains several potential sites for phosphorylation by protein kinase C, we failed to observe any change of GFA activity in RASM cells treated with 100 nmol/l to 10 µmol/l phorbol-12-myristate,13-acetate for 15–60 min or for 24 h in vivo (data not shown).

Effects of phosphatase inhibitors on GFA activity in vitro. Further evidence for the importance of phosphorylation in the regulation of GFA was obtained by incubating crude cellular extracts in the presence or absence of phosphatase inhibitors. Incubation of RASM cytosolic extracts at 37°C for 10 min in a Tris buffer that contained EGTA, EDTA, and protease inhibitors but no phosphatase inhibitors before the assay of GFA activity resulted in a 79% decrease of GFA activity (Fig. 3*A*). The presence of the substrate F-6-

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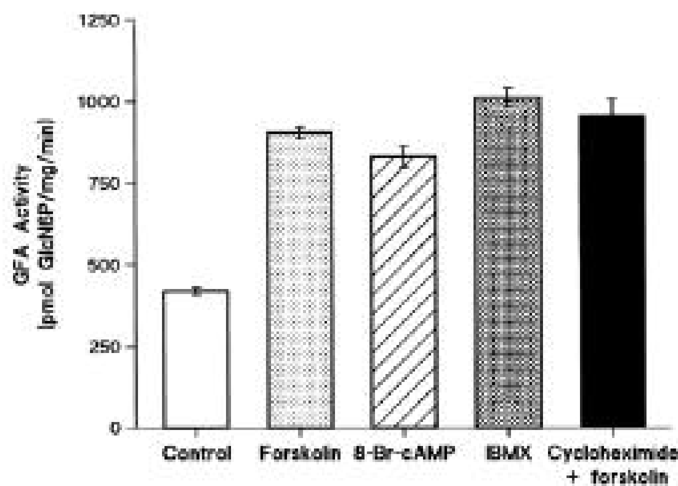

FIG. 1. Effect of forskolin, 8-Br-cAMP, and IBMX on GFA enzyme activity. RASM cells were grown in DMEM containing 10% FCS for 3 days. Cells were then equilibrated in DMEM–0.1% FCS for 1 h and treated with 10 µm forskolin, 1 mmol/l 8-Br-cAMP, or 1 mmol/l IBMX for 30 min. GFA activities were assayed as described in METHODS and calculated as production of picomoles GlcN-6-P per milligram protein per minute of reaction. All treatment groups differ from controls by Student's *t* test ($P < 0.001$).

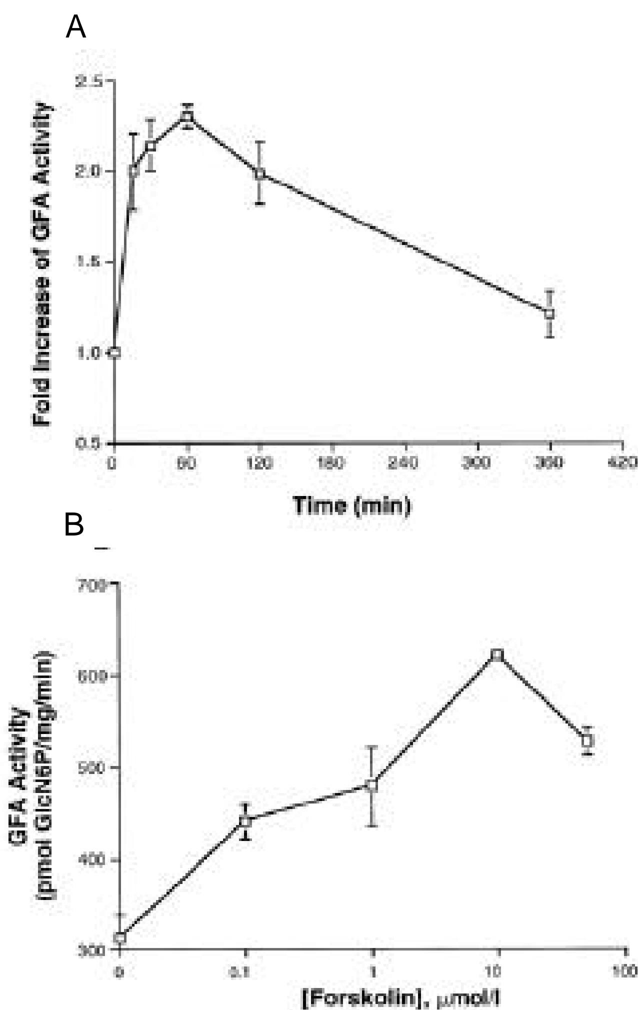

FIG. 2. Time- and dose-dependent effect of forskolin on GFA enzyme activity. *A*: RASM cells were treated with 10 μmol/l forskolin for 0, 15, 30, 60, 120, or 360 min. *B*: Cells were incubated for 30 min with 0, 0.1, 1, 10, or 50 μmol/l forskolin before harvest. Each point represents the average of three (*A*) or two (*B*) independent experiments.

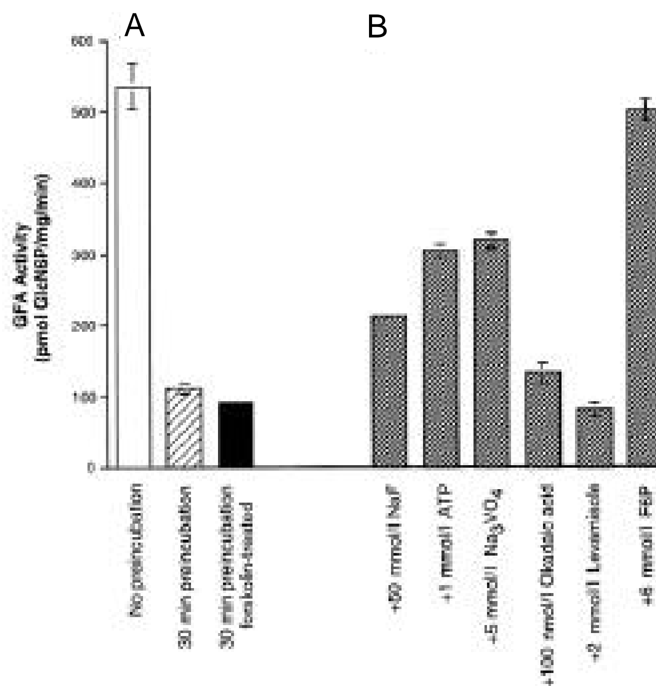

FIG. 3. Effects of endogenous phosphatases and phosphatase inhibitors on GFA activity. RASM cytosolic extracts were prepared in extraction buffer containing 25 mmol/l Tris (pH 7.4), 1 mmol/l EDTA, 0.5 mmol/l EGTA, 200 mmol/l PMSF, 10 μg/ml leupeptin, and 20 μg/ml aprotinin. *A*: GFA activities were assayed with or without preincubation at 37°C for 10 min. *B*: Phosphatase inhibitors or F-6-P were added before preincubation. All the data were obtained from cells without forskolin treatment unless specifically noted.

P (but not glutamine) prevented this loss of activity during incubation (Fig. 3*B*), consistent with earlier reports (20) and with the fact that the loss of activity is not seen during the routine assay of GFA when F-6-P is present.

The twofold higher GFA activity in cells treated first with forskolin was reduced by this preincubation to equal that of non–forskolin-treated cells, suggesting that the loss of activity was not simply nonspecific but might be due to dephosphorylation during the incubation. In support of that suggestion, the loss of activity could be inhibited 23, 47, and 49% by treatment with the phosphatase inhibitors NaF (50 mmol/l), ATP (1 mmol/l), and sodium orthovanadate (5 mmol/l), respectively (Fig. 3*B*). The latter agent is known to inhibit serine/threonine phosphatases and, more potently (at micromolar concentrations), tyrosine phosphatases (22,23). Involvement of serine/threonine phosphatases is suggested by two observations: vanadate's effects were seen only at millimolar concentrations, and an anti-phosphotyrosine antibody failed to immunoprecipitate any GFA activity. The addition of up to 100 nmol/l okadaic acid (Fig. 3*B*) had very little effect on the decrease of GFA activity, indicating that the okadaic acid–sensitive serine/threonine phosphatases, protein phosphatase (PP)1 and PP2A (24), are not involved in this regulatory process. Consistent with this conclusion, microcystin-LR, a cyclic heptapeptide inhibitor of PP1 and PP2A but not of PP2C (25), did not protect GFA from deactivation during the 37°C incubation (data not shown). Levamisole (2 mmol/l), a specific inhibitor of alkaline phosphatase (26), had no effect on the loss of GFA activity. Presence of the protease inhibitors in the extraction buffer had no significant effect on the loss of GFA activity during the preincubation.

Effects of PKA treatment on purified GFA. To demonstrate direct effects of cAMP-dependent phosphorylation on GFA, purified GFA was treated in vitro with the catalytic subunit of PKA. As shown in Fig. 4, this treatment led to phosphorylation of GFA (Fig. 4*A*) as well as to a 1.7-fold increase in GFA activity (Fig. 4*B*, $n = 3$, $P < 0.005$). The change in GFA activity was manifest mainly as a change in the V_{max} of the enzyme, and the K_m values for GFA's substrates increased slightly with PKA treatment. The K_m for F-6-P increased from 0.15 to 0.19 mmol/l with PKA treatment, and the K_m for glutamine increased from 0.43 to 0.61 mmol/l. The K_i for the feedback inhibitor of GFA, UDP-*N*-acetyl-glucosamine (0.64 mmol/l), was not altered by PKA treatment. Purified GFA underwent a 30% decrease in activity when incubated at 37°C (212 U preincubation vs. 149 U after 45 min at 37°C) under conditions identical to those under which crude GFA lost 79% of its activity.

Treatment of the purified GFA with PKC was without significant effect (125 ± 6 U untreated, 117 ± 17 U treated with PKC).

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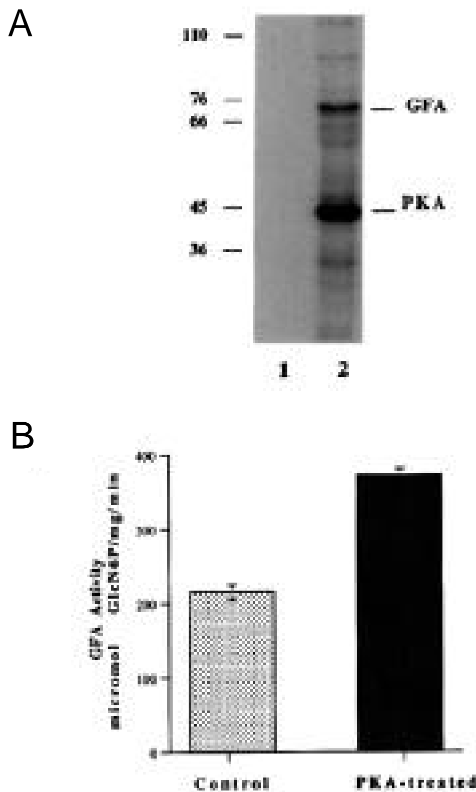

FIG. 4. Effect on GFA of phosphorylation by PKA. Purified GFA was incubated for 30 min at 37°C with the purified catalytic subunit of PKA as described. *A*: Demonstration of PKA-dependent incorporation of ^{32}P from ATP into GFA (*lane 1*, control GFA without PKA; *lane 2*, sample with PKA). *B*: Effect of PKA on GFA activity.

DISCUSSION

Glutamine:fructose-6-phosphate amidotransferase is a protein conserved through evolution; human GFA has 55% amino acid identity with yeast GFA and 35% with *Escherichia coli* GFA. Previous studies on *E. coli* GFA and other glutamine-dependent amidotransferases have shown that the glutamine binding domain is at the NH_2-terminus, while F-6-P binding domain is at the COOH-terminus (27–29). Amino acid sequence analysis reveals a much less conserved "hinge" region between these two well-conserved substrate binding domains. Interestingly, we also found that all the consensus sites (R-R/K-X-S/T) for PKA phosphorylation are located in this hinge region: there are two in human GFA ($_{202}$RRGS$_{205}$ and $_{232}$KKGS$_{235}$) and one in yeast GFA ($_{196}$RKGS$_{199}$); *E. coli* GFA has none (Fig. 5).

We therefore hypothesized that this poorly conserved hinge region may serve as a regulatory element for GFA activity through a cAMP-dependent pathway. In the present work, we have shown that in RASM cells the cAMP-elevating agents forskolin, 8-Br-cAMP, and IBMX all stimulated GFA activity 2- to 2.4-fold. Similar results, but with a smaller increase, are seen in COS-7 cells (data not shown). Direct treatment of purified GFA with the catalytic subunit of PKA led to equivalent changes in GFA activity.

The cAMP-dependent effect on GFA activity is probably posttranslational. First, the increase of GFA activity induced by forskolin was very rapid, being near maximal 15 min after in vivo treatment (Fig. 2). Second, pretreatment by cycloheximide did not affect the forskolin-induced increase of GFA activity. Finally, GFA activity from control extracts was rapidly reduced by simply incubating cytosolic extracts at 37°C for 10 min, and the increment of GFA activity seen in forskolin-treated cells was abolished by the same incubation of the cell extract at 37°C. This decrease in GFA activity could be inhibited by the addition of phosphatase inhibitor. Most importantly, the effect could be reproduced by in vitro phosphorylation of GFA by PKA.

Dramatic decreases in GFA activity such as were seen in the preincubations at 37°C are not seen during routine GFA assays, as evidenced by the linearity of GFA activity measured at 37°C for up to 1 hour (19). The reasons for this discrepancy are not wholly clear. The loss of activity is not affected by the presence of protease inhibitors, nor is it seen to the same degree with purified GFA, suggesting that the decrease is not completely due to inherent enzyme instability or degradation. The presence of the substrate F-6-P is known to stabilize the enzyme (Fig. 3*B*) (20,30), accounting for the lack of appreciable loss of activity in the course of its normal assay, but the mechanism for this stabilization is not known.

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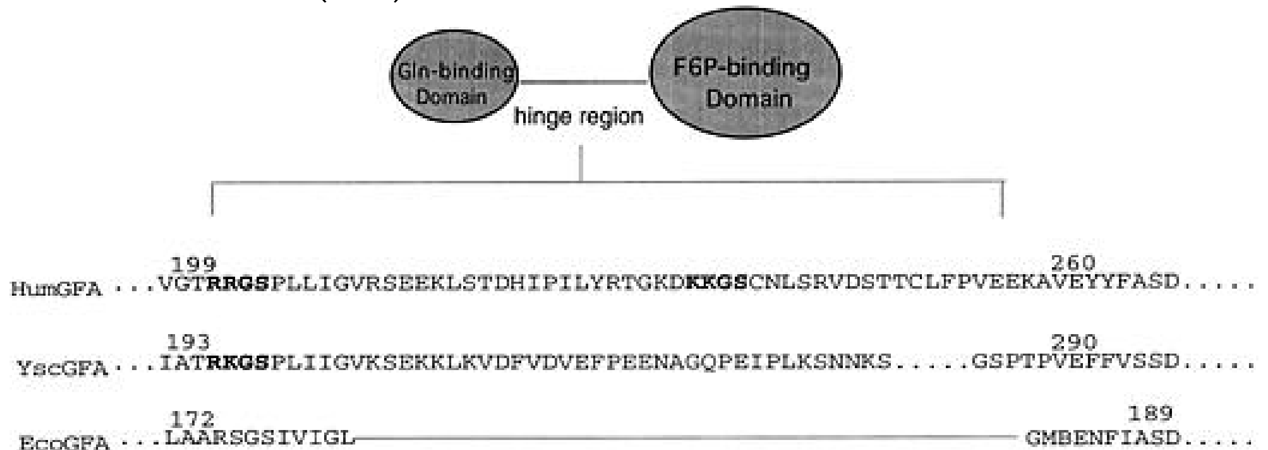

FIG. 5. Comparison of GFA hinge regions containing the consensus sequences for cAMP-dependent protein kinase phosphorylation. In *E. coli* (EcoGFA), *Saccharomyces cerevisiae* (YscGFA), and human (HumGFA), GFA proteins consist of two conserved substrate-binding domains and a poorly conserved hinge region, in which the consensus sites (R-R/K-X-S/T) for cAMP-dependent PKA are located (bold type).

The endogenous protein phosphatase (or phosphatases) responsible for downregulation of GFA activity remains unidentified. Our data (Fig. 3) suggest that GFA is dephosphorylated by serine/threonine phosphatases, consistent with upregulation of GFA activity by cAMP-dependent phosphorylation. The activity of endogenous phosphatases in RASM cytosolic extracts was not inhibited by okadaic acid, consistent with the suggestion that in the fungus *B. emersonii* GFA is developmentally regulated primarily by PP2C (31). In the case of mammalian GFA phosphorylation, however, PKA-induced phosphorylation did not alter the sensitivity of GFA to feedback inhibition by UDP-*N*-acetyl-glucosamine as was the case in *B. emersonii* (31).

Regulation of GFA activity is only beginning to be examined but may be complex. For example, GFA activity increases significantly with high glucose or insulin treatment of cultured human skeletal muscle cells (19,32) but is reduced by glucose and glucosamine in adipocytes (33). In hyperglycemic and hypoinsulinemic streptozotocin-treated rats, muscle GFA activity was decreased (34). In the present work, we have shown that GFA activity is stimulated by a cAMP-dependent pathway in RASM cells. Thus, GFA is regulated by both insulin- and glucose-dependent, as well as by counter-insulin (cAMP), pathways. These data are consistent with the hypothesis that GFA is an actively regulated molecule with an important role in mediating glucose uptake and cellular utilization of glucose (4,11,12,19).

ACKNOWLEDGMENTS

This work was supported by the American Diabetes Association, the National Institutes of Health (NIH) (DK-43526), the Veterans Administration, and G.D. Searle and Company.

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