Glycogen is the main storage form of glucose in mammals and plays an important role in whole body glucose metabolism. For example, a substantial proportion of ingested glucose is converted to muscle glycogen (1, 2). The rate of muscle glycogen synthesis is determined by its entry into muscle and the phosphorylation state of glycogen synthase, both of which processes are controlled by insulin (3, 4). The muscle isoform of glycogen synthase is phosphorylated at nine or more sites by multiple protein kinases (for reviews see Refs. 4–6). Phosphorylation leads to inactivation of glycogen synthase, but activity can be restored by the allosteric activator glucose 6-phosphate. In rabbit skeletal muscle glycogen synthase, the critical phosphorylation sites that control the enzyme activity are NH2-terminal residues Ser640 (site 2) and Ser644 (site 2a) and COOH-terminal residues Ser640 (site 3a) and Ser644 (site 3b) (7–10). Of these, site 3a is probably the most important. In vitro, glycogen synthase kinase-3 (GSK-3)1 phosphorylates sequentially sites 4, 3c, 3b, and 3a, where recognition of site 4 by GSK-3 requires that glycogen synthase has been previously phosphorylated at site 5 by casein kinase II (11–13). It was proposed that phosphate serves as part of the recognition determinant for GSK-3 in the sequence motif -S-X-X-S(P)− (14), a hypothesis that has been reinforced by the recent solution of the crystal structure of GSK-3 (15, 16). This mechanism of phosphorylation has been termed “hierarchical” (6). However, we also demonstrated that, for glycogen synthase expressed in COS cells and Rat1 fibroblasts, disruption of the recognition sequence for GSK-3 by Ser/Ala substitution at sites 3c, 4, and/or 5 did not preclude inactivation of the enzyme (9, 10, 17). Our work has shown also that sites 3a and 3b can be directly phosphorylated by as yet unidentified protein kinases (10, 18). In the present study, we purified a protein kinase that specifically phosphorylates site 3a in glycogen synthase. To confirm that DYRKs directly phosphorylate glycogen synthase, recombinant DYRK1A, DYRK2, and glycogen synthase were produced in bacterial cells. In the presence of Mg-ATP, both DYRKs inactivated glycogen synthase by more than 10-fold. The inactivation correlated with phosphorylation of site 3a in glycogen synthase. These results indicate that protein kinase(s) from the DYRK family may be involved in a new mechanism for the regulation of glycogen synthase.

Glycogen synthase, a key enzyme in the regulation of glycogen synthesis by insulin, is controlled by multisite phosphorylation. Glycogen synthase kinase-3 (GSK-3) phosphorolyses four serine residues in the COOH terminus of glycogen synthase. Phosphorylation of one of these residues, Ser640 (site 3a), causes strong inactivation of glycogen synthase. In previous work, we demonstrated in cell models that site 3a can be phosphorylated by an as yet unidentified protein kinase (3a-kinase) distinct from GSK-3. In the present study, we purified the 3a-kinase from rabbit skeletal muscle and identified one constituent polypeptide as HAN11, a WD40 domain protein with unknown function. Another polypeptide was identified as DYRK1A, a member of the dual-specificity tyrosine phosphorylated and regulated protein kinase (DYRK) family. Two isoforms of DYRK, DYRK1A and DYRK1B, co-immunoprecipitate with HAN11 when co-expressed in COS cells indicating that the proteins interact in mammalian cells. Co-expression of DYRK1A, DYRK1B, or DYRK2 with a series of glycogen synthase mutants with Ser/Ala substitutions at the phosphorylation sites in COS cells revealed that protein kinases cause phosphorylation of site 3a in glycogen synthase. To confirm that DYRKs directly phosphorylate glycogen synthase, recombinant DYRK1A, DYRK2, and glycogen synthase were produced in bacterial cells. In the presence of Mg-ATP, both DYRKs inactivated glycogen synthase by more than 10-fold. The inactivation correlated with phosphorylation of site 3a in glycogen synthase. These results indicate that protein kinase(s) from the DYRK family may be involved in a new mechanism for the regulation of glycogen synthesis.
prolin, the GAC codon for Asp$^\text{60}$ was replaced by the TGA stop codon in pCMV-GS vector as described previously. The cDNA for truncated glycogen synthase was digested with BamHI, and the 1.1-kb fragment was ligated into BamHI-cut pET-GbDum to produce pET-GSΔ. The pCMV-4-based constructs for expression of truncated glycogen synthase, which additionally contains phosphorylation site mutations, were described previously. The glycogen synthase mutants, designated pEB741 and pEB742, were eluted from the resin with 20 m M glutathione. The purified proteins were dialyzed against a buffer comprising 50 m M Tris-HCl (pH 7.5), 150 m M NaCl, 15 m M β-mercaptoethanol, and 30% glycerol to remove residual glutathione, and were stored at −80°C. The concentrations of protein kinases were determined by measuring optical density in the bands in Coomasie-stained gels using bovine serum albumin as a standard.

**Aspartate of Protein Kinase Activity**—Protein kinase activity was determined by measuring the incorporation of $\gamma^3$P phosphate into glycogen synthase mutants WT3; 2,AAΔ; 2,SA; or 2,AS (Fig. 1). Unless otherwise stated, assays contained, in a final volume of 20 m l, 0.2 μg of glycogen synthase, 10 μM ATP, 0.5–1 μCi of $\gamma^3$PATP, a portion of enzyme sample, and kinase assay buffer (10 m M Tris-HCl, pH 7.4, 10 m M MgCl$_2$, 1 m M dithiothreitol). After incubation at 37°C for 20 min, reactions were terminated by addition of SDS-PAGE sample buffer and boiling for 5 min. Polypeptides were separated by SDS-PAGE, gels were stained with Coomasie Blue and dried. The phosphoproteins were detected by autoradiography, then excised from gels and the radioactivity incorporated was quantitated by scintillation counting.

**Purification of 3α-Kinase from Rabbit Skeletal Muscle**—Approximately 250 g of rabbit skeletal muscle homogenate was homogenized in 20 m M Tris-HCl (pH 7.5), 0.7 M NaCl, 0.1 m M β-mercaptoethanol, and 0.1% Triton X-100 using a Polytron tissue homogenizer. The tissue homogenate was homogenized in 20 m M Tris-HCl (pH 7.5), 0.7 M NaCl, 0.1 m M β-mercaptoethanol, and 0.1% Triton X-100 using a Polytron tissue homogenizer. The tissue homogenate was homogenized in 20 m M Tris-HCl (pH 7.5), 0.7 M NaCl, 0.1 m M β-mercaptoethanol, and 0.1% Triton X-100 using a Polytron tissue homogenizer. The tissue homogenate was homogenized in 20 m M Tris-HCl (pH 7.5), 0.7 M NaCl, 0.1 m M β-mercaptoethanol, and 0.1% Triton X-100 using a Polytron tissue homogenizer. The tissue homogenate was homogenized in 20 m M Tris-HCl (pH 7.5), 0.7 M NaCl, 0.1 m M β-mercaptoethanol, and 0.1% Triton X-100 using a Polytron tissue homogenizer. The tissue homogenate was homogenized in 20 m M Tris-HCl (pH 7.5), 0.7 M NaCl, 0.1 m M β-mercaptoethanol, and 0.1% Triton X-100 using a Polytron tissue homogenizer. The tissue homogenate was homogenized in 20 m M Tris-HCl (pH 7.5), 0.7 M NaCl, 0.1 m M β-mercaptoethanol, and 0.1% Triton X-100 using a Polytron tissue homogenizer. The tissue homogenate was homogenized in 20 m M Tris-HCl (pH 7.5), 0.7 M NaCl, 0.1 m M β-mercaptoethanol, and 0.1% Triton X-100 using a Polytron tissue homogenizer. The tissue homogenate was homogenized in 20 m M Tris-HCl (pH 7.5), 0.7 M NaCl, 0.1 m M β-mercaptoethanol, and 0.1% Triton X-100 using a Polytron tissue homogenizer.
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Acid. Peptides detected by absorbance at 214 nm were collected and analyzed by Edman sequencing.

**MALDI-TOF Mass Spectrometry Analysis**—Protein bands were excised from gels, cut into small pieces, destained with 50% acetonitrile, 50 mM ammonium bichromate, reduced with 10 mM dithiothreitol (Sigma), and alkylated with 55 mM iodoacetamide (Sigma). After alkylation, the gel slices were digested with trypsin (Promega) in 50 mM ammonium bichromate (enzyme substrate ratio 1:50 to 1:100) overnight at 37 °C. The peptides were extracted from the gel with 0.1% trifluoroacetic acid in water and 0.1% trifluoroacetic acid in 60% acetonitrile for 30 min. One microliter of the extracted protein solution was spotted on a MALDI plate with 1 μl of α-cyano-4-hydroxycinnamic acid (Sigma) in a 50% acetonitrile, 50% methanol matrix solution. Mass spectra were recorded using the positive reflection mode of a MALDI-TOF mass spectrometer (Micromass, Manchester, UK). The time of flight was measured using the following parameters: 3400 V pulse voltage, 15000 V source voltage, 500 V reflector voltage, 1950 V MCP voltage, and low mass gate of 400 Da. For high accuracy mass measurement, the instrument was tuned to a resolution just over 15000 full width at peak half height. The MALDI-MS data obtained were used for data base searches.

**Antibodies**—HAN11 antibodies were generated against a peptide having sequence corresponding to residues 45–51 in HAN11. Rabbits were immunized with this peptide (NKVQLVGLDEESSEFIC) coupled to Injeqt® maleimide-activated maleic anhydride keyhole limpet hemocyanin (Pierce). After the third booster injection, serum was collected and incubated with an affinity resin prepared by coupling a peptide to Sulfo-Link beads (Pierce). After exhaustively washing the resin, the antigenic peptides of the antibody were eluted with 0.3 M glycine-HCl, pH 2.7, neutralized, and dialyzed against phosphate-buffered saline. Antibodies to M2 were from Sigma. Antibodies to Dyrk1A were from BD Biosciences. Antibodies p-GS/S641) (which specifically recognizes phosphorylated site 3α in glycogen synthase, were from Cell Signaling Technology.

**Northern Blot Analysis**—A human multiple tissue Northern blot (Clontech) was probed with a 32P-labeled 1.6-kb HAN11 fragment excised from EST clone AAA42821 by EcoRI and NotI digestion. The probe was prepared according to the manufacturer’s protocol.

**Electrophoresis and Immunoblotting**—To analyze protein expression, mouse tissues were homogenized in a buffer containing 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 100 mM NaF, 1 mM MgCl2, 1 mM dithiothreitol, and 0.01 mg/ml leupeptin. The homogenates were centrifuged for 20 min at 17,000 × g and the supernatants were retained for analysis. Approximately 50 μg of protein from each tissue was separated by SDS-PAGE followed by transfer to nitrocellulose, which was probed with affinity purified anti-HAN11 antibodies.

**Transient Transfection and Analysis of Expressed Proteins**—COS-M9 cells were transiently transfected by using LipofectAMINE® (Invitrogen). Briefly, 0.5–1.0 mg of plasmid DNA per 6 ml of LipofectAMINE® was used to transfect cells in a 35-mm dish. Cells were grown for 2 days and frozen in liquid nitrogen in 0.2 ml of buffer A, containing 50 mM Tris-HCl, pH 7.8, 10 mM EDTA, 2 mM EGTA, 100 mM NaF, 1 mM dithiothreitol, 1 mM FMSF, 0.1 mM TLCK, or in buffer B that contained 50 mM Tris-HCl, pH 7.8, 100 mM NaCl, 1 mM dithiothreitol, and protease inhibitors. Cells frozen in buffer A or buffer B were used for glycogen synthase assay or to analyze for interactions between expressed proteins, respectively. After thawing, cells were homogenized and centrifuged at 14,000 × g for 10 min. To analyze the interaction between S-tag HAN11 and FLAG-tagged DYRK isoforms, the supernatants were incubated with S-protein-agarose (Novagen) in the presence of 0.15 M NaCl. The precipitates were washed three times with buffer B containing 0.1% Triton X-100 and proteins were eluted with SDS-PAGE loading buffer. The supernatants, the pellet fractions, and the S-protein-agarose precipitates were separated by SDS-PAGE and analyzed by Western blot.

**Glycogen Synthase Assay**—Glycogen synthase activity was determined by the pellet fraction of COS cell homogenates by measuring the incorporation of [14C]glucose from UDP-[U-14C]glucose into glycogen (22) as described previously (9). The incorporation of glucose-6-P activity ratio was calculated after subtraction of endogenous glycogen synthase activity in COS cells (9).

**Activities of recombinant GST-DYRK1A and GST-DYRK2** were measured by the inactivation of added recombinant glycogen synthase protein as monitored by the decrease in the glucose-6-P activity ratio as a function of time. Reaction mixtures (total volume 0.1 ml) were composed of 20 mM Tris-HCl, pH 7.4, 10 mM Mgl2, 1 mM dithiothreitol, 0.1 mM ATP, and 3 μg of glycogen synthase. Reactions were started by adding 20 μl of GST-DYRK1A, GST-DYRK2, or protein kinase dilution buffer (20 mM Tris-HCl, pH 7.4, 0.1 mM NaCl, 1 mM dithiothreitol, 30% glycerol). After incubation at 30 °C for the indicated times the 10-μl aliquots were removed and mixed with 50 μl of glycogen synthase assay buffer containing 20 mM EDTA (9) to measure glycogen synthase activity.

**Analysis of Phosphorylation of Site 3α in Glycogen Synthase**—Glycogen synthase was incubated in the presence of GST-DYRK1A and GST-DYRK2 under the same conditions as described for time course inactivation of glycogen synthase. At the indicated times, 15-μl aliquots were removed, mixed with SDS-PAGE gel loading buffer, and analyzed by Western blot using phospho-specific antibodies that recognize phosphorylated site 3α (p-GS/S641) antibodies).

**Activity Assay of Recombinant DYRK1A and DYRK2**—Protein kinase activity was determined by measuring the incorporation of [32P]phosphate into protein substrates. The substrates, which included purified rabbit skeletal muscle glycogen synthase, glycogen phosphorylase b, phosphorylase kinase, protein phosphatase inhibitor-2, and recombinant targeting subunit (R) of the muscle-specific glycogen-associated type 1 protein phosphatase (exon 1), were kindly provided by Dr. Anna DePaoli-Roach (Indiana University School of Medicine, Indianapolis, IN). Assays contained, in a final volume of 20 μl: 0.8 μg of protein substrate, 10 μM ATP, 1 μCi of [γ-32P]ATP, 16 ng of GST-DYRK1A or 2 ng of GST-DYRK2, and kinase assay buffer (10 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 1 mM dithiothreitol). To estimate endogenous phosphorylation in protein substrates, GST-DYRK1A and GST-DYRK2 were excluded from the reaction mixture. After incubation at 37 °C for 10 min, reactions were terminated by addition of SDS-PAGE sample buffer and boiling for 5 min. Histones H1 and H2B were obtained from Roche. Individual caseins, α1-casein, β-casein, and κ-casein, obtained from Dr. P. J. Roach, Indiana University, were originally a gift from Dr. Elizabeth W. Bingham, USDA Eastern Regional Research Center, Philadelphia, PA. Polypeptides were separated by SDS-PAGE, gels were stained with Coomassie Blue and dried. The phosphorylated proteins were detected by autoradiography, then excised from gels and the radioactivity incorporated was quantitated by scintillation counting.

**Expression of HAN11 in Rat1 Fibroblasts**—Stable clones of Rat1 fibroblasts expressing StagHAN11 were generated by transfection of cells with pcDNA-S-HAN11 using LipofectAMINE and selection of the clones with 0.5 mg/ml Geneticin (Invitrogen).

**RESULTS**

**Design of Substrates for Purification of Glycogen Synthase Kinases**—To monitor 3α-kinase activity during purification, mutant forms of glycogen synthase were designed, expressed in E. coli, and purified. All the glycogen synthases are truncated at Lys652 to avoid phosphorylation at sites 1a and 1b and additionally contain Ser/Ala substitutions at sites 2, 3c, 4, and 5. Enzyme truncated at Lys652 but containing intact phosphorylation sites serves as “wild type” (Fig. 1). As shown previ-
uous, truncation did not have any effect on the activity, expression in COS cells, or the susceptibility of the enzyme to proteolysis indicating that the mutation does not significantly affect the overall conformation of glycogen synthase (10). The only difference between the mutants 2,AAΔ, 2,SAΔ, and 2,ASΔ is the availability of phosphorylation sites 3a and 3b (Fig. 1). The mutant 2,SAΔ served for detection of 3a-kinase and mutants 2,AAΔ and 2,ASΔ served as negative controls.

Purification and Characterization of 3a-Kinase from Rabbit Skeletal Muscle—Initial experiments were designed to identify protein kinase activity in skeletal muscle capable of phosphorylating the glycogen synthase mutant 2,SAΔ and not capable of phosphorylating the mutants 2,AAΔ and 2,ASΔ. This protein kinase was designated 3a-kinase. Although 3a-kinase activity cannot be assayed in skeletal muscle extracts, activity toward 2,SAΔ was detectable after initial ammonium sulfate precipitation and chromatography on phenyl-Sepharose. Proteins eluted from phenyl-Sepharose were chromatographed sequentially over multiple columns. Column fractions were collected and assayed for 3a-kinase activity, active fractions were pooled and prepared for loading in subsequent chromatographic steps. Proteins in pools and in column fractions were also analyzed by SDS-PAGE followed by silver staining. A typical purification is summarized in Table I. The 3a-kinase was purified about 3000-fold with a recovery of 1% from the phenyl-Sepharose eluate. Purified 3a-kinase is very active toward 2,SAΔ and wild type glycogen synthase but is incapable of phosphorylating 2,AAΔ (Fig. 2). A trace phosphorylation of 2,ASΔ was also observed. These data indicate a strong preference for the phosphorylation of site 3a. Incubation of the mutant 2,SAΔ with 3a-kinase in the presence of [γ-32P]ATP resulted in incorporation of −0.7 mol of [32P]phosphate/mol of glycogen synthase (not shown).

The final purified preparations contained two major species with apparent molecular weights of 39,000 and 68,000 (Fig. 3) on SDS-PAGE as well as several minor bands. Comparison of the protein composition with protein kinase activity in individual fractions of chromatography profiles suggested that the 54- and 39-kDa polypeptides best correlated with the 3a-kinase activity. The apparent Mr of 3a-kinase as determined by gel-filtration chromatography on Superose 12 was 138,000 (not shown). The distribution of 3a-kinase activity in the gel-filtration chromatography on Superose 12 was 138,000 (not shown).

![Fig. 2. Specificity of 3a-kinase.](image)

![Fig. 3. Purification of 3a-kinase.](image)

| Step                  | Protein Activity | Specific activity | Purification | Yield |
|-----------------------|------------------|-------------------|--------------|-------|
| 100,000 × g           | 114,534          |                   |              |       |
| Phenyl-Sepharose      | 8502             | 414               | 0.041        | 1     | 100  |
| SP-Sepharose          | 168              | 174               | 1.03         | 25    | 42   |
| Q-Sepharose           | 50               | 79                | 3.3          | 80    | 19   |
| Heparin-Sepharose     | 0.22             | 9.3               | 42.4         | 1034  | 2.3  |
| Hydroxyapatite        | 0.033            | 4.05              | 122.7        | 2993  | 1    |

**TABLE I**

**Purification of 3a-kinase from rabbit skeletal muscle**

One unit of 3a-kinase was the amount that catalyzed the incorporation of 1 nmol of phosphate into 200 ng of SA in 1 min.

| Step       | Protein Activity | Specific activity | Purification | Yield |
|------------|------------------|-------------------|--------------|-------|
| Phenyl-Sepharose | 8502 | 414 | 0.041 | 1 | 100 |
| SP-Sepharose | 168 | 174 | 1.03 | 25 | 42 |
| Q-Sepharose | 50 | 79 | 3.3 | 80 | 19 |
| Heparin-Sepharose | 0.22 | 9.3 | 42.4 | 1034 | 2.3 |
| Hydroxyapatite | 0.033 | 4.05 | 122.7 | 2993 | 1 |

**Novel Mechanism for the Control of Glycogen Synthesis**

One unit of 3a-kinase was the amount that catalyzed the incorporation of 1 nmol of phosphate into 200 ng of SA in 1 min.
with trypsin, and analyzed by MALDI-TOF mass spectrometry. The experimentally determined tryptic peptide masses were then subjected to analysis using ProFound, and the identity of protein band 9 (Fig. 4) was confirmed as DYRK1A (probability = 1.00 and Z score = 2.27). The protein of band 10 was identified as HAN11 (probability = 1.00 and Z score = 2.43) confirming the results of protein sequencing by automated Edman degradation. The protein of band 9 corresponds to a polypeptide with apparent molecular weight $M_r = 90,000$, whereas DYRK1A has $M_r = 90,000$. Therefore, the DYRK1A that was purified could be one of the alternatively spliced forms of DYRK1A that lacks COOH-terminal histidine repeats and a serine/threonine domain (24) or it might represent a proteolytic fragment of DYRK1A. Some other bands were identified as co-chaperone TPR2 (bands 6 and 7) and AMP-deaminase-1 (band 5).

Tissue Distribution of HAN11—The tissue distribution of HAN11 expression was analyzed by Northern blotting of poly(A)$^+$ RNA isolated from several different tissues. Two distinct transcripts for HAN11 (2 and 8.2 kb) were detected in all tissues (Fig. 5A). The 2-kb transcript was particularly abundant in heart, placenta, skeletal muscle, and pancreas. The significance of the difference in transcript size is not yet known. Analysis of mouse proteins revealed relatively high levels of HAN11 expression in brain, ovary, and testis (Fig. 5B). Lower levels were detected in placenta, liver, skeletal muscle, kidney, and pancreas.

Protein Kinases from DYRK Family Interact with HAN11—To determine whether HAN11 interacts with DYRK1A and other members of the DYRK family (25), we co-expressed NH$_2$-terminal FLAG-tagged mammalian DYRK isoforms, DYRK1A, DYRK1B, and DYRK2, with NH$_2$-terminal S-tagged HAN11 in COSM9 cells. DYRK1A, DYRK1B, and DYRK2 were detected as polypeptides with $M_r = 90,000$, 78,000, and 61,000, respectively, which is consistent with the predicted sizes of the protein kinases (Fig. 6A). After incubation of the lysates of COS cells with S protein-agarose, the precipitated proteins were detected with anti-HAN11 and anti-FLAG antibodies. As shown in Fig. 6B, S-tag HAN11 protein was detected in the S protein-agarose precipitates from cells transfected with S-tag HAN11 or cells co-transfected with both S-tag HAN11 and the different isoforms of DYRK. With anti-FLAG antibody, only DYRK1A and DYRK1B, but not DYRK2, were detected in precipitates from cells co-transfected with S-tag HAN11 and DYRK (Fig. 6C). DYRK2 did not co-precipitate with S-tag HAN11 even with a higher level of expression than shown in Fig. 6A (not shown). These data demonstrate that DYRK1A and DYRK1B interact with HAN11 and that the interaction can occur in mammalian cells. To identify proteins that interact with HAN11, stable clones of Rat1 fibroblasts expressing HAN11 and COOH-terminal histidine repeats and a serine/threonine domain (24) or it might represent a proteolytic fragment of DYRK1A. Some other bands were identified as co-chaperone TPR2 (bands 6 and 7) and AMP-deaminase-1 (band 5).

![Fig. 4. Analysis of proteins in purified 3a-kinase. Pooled fractions of partially purified 3a kinase from hydroxyapatite chromatography (Fig. 3) were concentrated and proteins were separated by SDS-PAGE. A Coomassie Blue-stained gel is shown. Molecular weight markers, lane 1; 3a-kinase fraction, lane 2. The numbers to the left indicate the molecular masses (kDa).](image1)

![Fig. 5. Analysis of HAN11 distribution. A, a human multiple tissue Northern blot (Clontech) was hybridized with the probe derived from cDNA for HAN11. The numbers to the left indicate the molecular weights (in kb) of standards. B, proteins from mouse tissues (50 μg/lane) were analyzed by Western blot using antibodies generated against HAN11.](image2)
indicating that both protein kinases phosphorylate site 3a but no other sites that affect glycogen synthase activity. However, DYRK2 further inactivated mutant 2,3a (Fig. 8C) presumably by phosphorylation of other COOH-terminal phosphorylation sites. To provide more evidence for phosphorylation of site 3a by DYRKs, two isoforms, DYRK1A and DYRK2, were expressed in E. coli as GST fusion proteins, purified, and incubated with the glycogen synthase mutant 2,SAΔ. Both protein kinases inactivated this form of glycogen synthase (Fig. 9A) but had no effect on the activation state of the glycogen synthase mutant 2,AAΔ (not shown). Inactivation of the mutant 2,SAΔ was accomplished by phosphorylation of site 3a as detected with phosphorylation site-specific antibodies (Fig. 9B). Phosphorylation of glycogen synthase by DYRK1A and DYRK2 was compared with phosphorylation of some other proteins, which are involved in regulation of glycogen synthesis, as well as some commonly used generic substrates of protein kinases. Relative to glycogen synthase, both protein kinases exhibited very low activity with glycogen phosphorylase b, phosphorylase kinase, targeting subunit (RGL/GM) of the muscle-specific glycogen-associated type 1 protein phosphatase (exon 1), protein phosphatase inhibitor-2, histones, and caseins (Table II). Based on these results, glycogen synthase would appear to be a relatively specific substrate for DYRKs.

**DISCUSSION**

The activity of glycogen synthase is under the control of hormones, such as insulin, catecholamines, and glucagon (4–6), and non-hormonal stimuli, such as the blood glucose level, amino acid availability, and exercise. The need to respond to multiple stimuli may necessitate the involvement of multiple signaling pathways to regulate enzyme activity and the rate of glycogen synthesis. Much of the control of glycogen synthase occurs via changes in the phosphorylation state of sites 2, 2a, 3a, and 3b (7–10), whereas phosphorylation of other sites has little or no effect on enzyme activity (9, 10). It was first demonstrated that sites 3a and 3b were phosphorylated by the protein kinase GSK-3 (11–13). Studies with cells and with animals have also shown that known inhibitors of GSK-3 can affect glycogen synthesis (26, 27). However, more recent studies indicated that alternative pathway(s) for the phosphorylation of these sites in glycogen synthase may operate (10, 18). Here we report that one alternative pathway for phosphorylation of site 3a may include one or more members of the DYRK family of protein kinases.

Mammalian DYRKs are a subfamily of mitogen-activated protein kinase-related protein kinases and were originally discovered on the basis of homology to the **Saccharomyces cerevisiae** Yak1 and **Drosophila** mini-brain kinases (25, 28). DYRKs possess Ser/Thr phosphorylation activity as well as autophosphorylation activity on Tyr residue(s). Autophosphorylation at
a conserved YXY motif located in the activation loop between consensus kinase subdomains VII and VIII is the mechanism for activation of DYRKs (29). In the DYRK family, DYRK1A has been the best characterized to date. The DYRK1A gene maps to the critical region of the Down’s syndrome locus (30) and causes learning and memory defects when overexpressed in transgenic mice (34), consistent with the gene dosage effect in Down’s syndrome. Targeted disruption of the DYRK1A gene in mice led to general growth delay and death during midgestation suggesting a non-redundant role of DYRK1A (32). Mice heterozygous for the mutation (DYRK1A+/−) have increased brain size in a region-specific manner (32). DYRK1A phosphorylates various substrates in vitro, including the signal transducer and activator of transcription 3 (STAT3) (33), the e subunit of eukaryotic initiation factor 2B, the microtubule-associated protein tau (34), the transcription factor of the forkhead family FKHR (35), and dynamin (36) indicating that DYRK1A might participate in several biochemical pathways.

**TABLE II**

| Substrate          | Relative kinase activity |
|--------------------|--------------------------|
|                    | DYRK1A | DYRK2 |
| Glycogen synthase  | 100    | 100  |
| Glycogen phosphorylase | 0.8    | 0.3  |
| Phosphorylase kinase | 1.5    | 0    |
| α-casein, exon 1   | 10.5   | 1.2  |
| Inhibitor-2        | 2.3    | 7.1  |
| Glycogen synthase  | 100    | 100  |
| Histone H1        | 0.6    | 2    |
| Histone H2B       | 2.6    | 3.3  |
| α-casein          | 0.7    | 0.8  |
| β-casein          | 10     | 20   |
| κ-casein          | 2.6    | 3.8  |

* The final concentration of the protein tested was 0.04 mg/ml.

* The final concentration of the protein was 0.6 μmol/liter.

* Phosphorylation of glycogen synthase and phosphorylase kinase by DYRKs was estimated after subtraction of endogenous phosphorylation.

**FIG. 9.** Phosphorylation and inactivation of glycogen synthase by DYRK. Glycogen synthase mutant 2,5SA (Fig. 1) was incubated with recombinant DYRK1A (2 μg/ml), DYRK2 (0.3 μg/ml), or without protein kinase (control) for the indicated times. Samples were analyzed for glycogen synthase activity ratio (A), for phosphorylation of site 3a (B), which was performed by immunoblot with phosphorylation site-specific antibodies, and for glycogen synthase protein (C), which was performed by immunoblot with antibodies raised against glycogen synthase.

**FIG. 8.** Inactivation of glycogen synthase by DYRK in COS cells. Glycogen synthase mutants 2,SA, 2,AA, and 2,3a (see Fig. 1) were expressed in COS cells in the presence (+) or absence (−) of DYRK1A (A), DYRK1B (B), or DYRK2 (C). The activities of glycogen synthase in COS cell lysates were measured in the presence and absence of glucose-6-P and the glycogen synthase activity ratio was determined. Data are mean ± S.E. of duplicate determinations from at least three experiments. *, p < 0.05 relative to values for glycogen synthase expressed in the absence of DYRK.
Although DYRK1A is formally a dual-specificity protein kinase because of its ability to autoprophosphorylate on tyrosine residue(s), it phosphorylates other known substrates only on serine/threonine residues, like GSK-3 and mitogen-activated protein kinase. Analysis of the phosphorylation of peptide substrates identified DYRK1A as a proline-directed kinase with a consensus recognition sequence Arg-Pro-Xxx-(Ser/Thr)-Pro, residue(s), it phosphorylates other known substrates only on nase because of its ability to autophosphorylate on tyrosine

ingly, Pro at than 3a, such as 3b, 3c, 4, and/or 5. Phosphorylation of site 3b of the glycogen synthase mutant 2,3a, but not 2,AA, in COS other sites in glycogen synthase. DYRK2-mediated inactivation

have not carefully analyzed whether DYRKs phosphorylate other sites in glycogen synthase. DYRK2-mediated inactivation of the glycogen synthase mutant 2,3a, but not 2,AA, in COS cells indicates that DYRK2 might phosphorylate site(s) other than 3a, such as 3b, 3c, 4, and/or 5. Phosphorylation of site 3b would directly inactivate glycogen synthase (9, 10). Interestingly, Pro at –2 in substrates is an important requirement for DYRK1A, but not for DYRK2 (41). Therefore, it is possible that DYRK2 might phosphorylate site 4, which is located in sequence Arg-His-Ser-Ser(site4)-Pro (Fig. 1). Phosphorylation of site 4 would “prime” glycogen synthase for GSK-3 action, which in turn would phosphorylate sites 3c and 3b. Alternatively, the phosphorylation of sites other than 3a following expression of DYRK in COS cells could be indirect and mediated by a kinase activated by DYRK.

The possibility of involvement of DYRKs in the regulation of glycogen synthesis in skeletal muscle is consistent with data about tissue distribution of these kinases. DYRK1A is ubiquitously expressed, with higher levels in brain, heart, placenta, and skeletal muscle (24, 39, 40). The closely related family member, DYRK1B, is expressed in skeletal muscle, testes (21, 41), and several types of cancer cells (41). Expression of DYRK2 was detected in many tissues, including skeletal muscle, with highest level in testes (42). DYRK1A and DYRK1B, but not DYRK2, contain a bipartite nuclear localization signal in the NH2-terminal domain. Most of a green fluorescent fusion protein of DYRK1A was found to accumulate in the nucleus of transfected COS-7 and HEK293 cells, whereas green fluorescent protein-DYRK2 was predominantly detected in the cytoplasm (21, 43). In contrast to green fluorescent protein-DYRK1A, green fluorescent protein-DYRK1B was found in both the nucleus and the cytoplasm of COS cells (21). Endogenous DYRK1B was found predominantly in the cytoplasm of colon carcinoma cells (41). Therefore, it is possible that the localization of DYRKs in cells is determined by cell type and/or the level of expressed protein. An important question raised by this study is of the relative importance of DYRK1A and DYRK2 as physiological glycogen synthase kinases. Cellular localization of course could be an important factor in dictating whether a protein kinase can act on its substrate and hence the cytosolic predominance of DYRK2 might favor this enzyme as a candidate. However, even nuclear localization does not preclude a physiological role as there is a report that glycogen synthase may have a nuclear localization until glucose is available (44). This issue will be an important focus of future work.

We have demonstrated that two isoforms of DYRK, DYRK1A and DYRK1B, interact with the protein HAN11. HAN11 is a human homolog of the plant protein AN11 that is involved in the regulation of transcription of anthocyanin biosynthetic genes in Petunia hybrida (23). Another homolog of AN11, TTG1, regulates several developmental and biochemical pathways in Arabidopsis, including the formation of hairs on leaves, stems, and roots, and the production of seed mucilage and anthocyanin pigment (45). AN11, TTG1, and HAN11 belong to the class of tryptophan and aspartic acid (WD) repeat proteins. This family of proteins has been shown to play a role in numerous cellular functions including signal transduction, mRNA processing, gene regulation, vesicular trafficking, and regulation of the cell cycle (46–48). The structure of the β-subunit of heterotrimeric G-proteins, one of the best characterized WD proteins, revealed that this class of proteins forms a β-γ-peller structure, which apparently creates a stable platform allowing simultaneous interaction with multiple proteins (49–52). If HAN11 is also able to interact with multiple proteins, it is possible that it targets the DYRK1 proteins to their substrates. In plant cells, AN11 is located in the cytosolic fraction (23). Therefore, HAN11 might target DYRKs to cytosolic locations for regulation of specific cellular functions. Interestingly, Yak1p, the DYRK homolog in S. cerevisiae, rapidly shuttles between the nucleus and the cytoplasm in response to glucose indicating that, in yeast, compartmentalization of Yak1p is regulated (53). It is possible that HAN11 provides targeting of DYRK1A and DYRK2 to cellular locations that are involved in glycogen synthesis and degradation. However, co-expression of HAN11 and glycogen synthase in COS cells did not significantly change the activation state of glycogen synthase (results not shown). These negative results could be explained if COS cells contain significant amounts of endogenous HAN11 (Fig. 6B), which already saturates endogenous DYRK. Alternatively, HAN11 might not be involved in DYRK-mediated phosphorylation of glycogen synthase and, although co-purified with DYRK1A, could be involved in other aspects of DYRK function. Further studies are under way to address this issue.

In summary, the phosphorylation of the functionally important site 3a in glycogen synthase by a novel category of protein kinase may represent a completely new pathway for the regulation of glycogen synthase activity and glycogen synthesis. Little is known of the control of DYRK and it will be of considerable interest to identify what physiological stimuli, hormonal, metabolic or other, lie upstream of DYRKs. Inhibitors of DYRK kinases, like inhibitors of GSK-3, would have the potential to promote glycogen synthesis and perhaps act as hypoglycemic agents.

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REFERENCES

1. Jue, T., Rothman, D. L., Tavitian, B. A., and Shulman, R. G. (1989) Proc. Natl. Acad. U. S. A. 86, 1439–1442
2. Shulman, G. I., Rothman, D. L., Jue, T., Stein, P., DeFronzo, R. A., and Shulman, B. G. (1990) N. Engl. J. Med. 322, 225–229
3. Lawrence, J. C., and Roach, P. J. (1997) Diabetes 46, 541–547
4. Skurat, A. V., and Roach, P. J. (2000) in DePaoli-Roach, A. A., Ahmad, Z., Camici, M., Lawrence, J. C., Jr., and Roach, P. J. (1987) J. Biol. Chem. 262, Lippincott-Raven Publishers, Philadelphia, PA
5. Cohen, P. (1986) in The Enzymes (Boyer, P. D., and Krebs, E. G., eds) Vol. 17, 264, Academic Press, Inc., Orlando, FL
6. Roach, P. J. (1990) FASEB J. 4, 2961–2968
7. Plotow, H., and Roach, P. J. (1989) J. Biol. Chem. 264, 9126–9128
8. Nakielny, S., Campbell, D. G., and Cohen, P. (1991) Eur. J. Biochem. 199, 713–722
9. Skurat, A. V., Wang, Y., and Roach, P. J. (1994) J. Biol. Chem. 269, 25534–25541
10. Skurat, A. V., and Roach, P. J. (1995) J. Biol. Chem. 270, 12491–12497
11. Picton, C., Aitken, A., Bilham, T., and Cohen, P. (1992) Eur. J. Biochem. 124, 37–45
12. DePaoli-Roach, A. A., Ahmad, Z., Camici, M., Lawrence, J. C., Jr., and Roach, P. J. (1983) J. Biol. Chem. 258, 10702–10709
13. Zhang, W., DePaoli-Roach, A. A., and Roach, P. J. (1993) Arch. Biochem. Biophys. 304, 219–225
14. Pidul, C. J., Mahrenholz, A. M., Wang, Y., Roeseke, R. W., and Roach, P. J. (1987) J. Biol. Chem. 262, 14042–14048
15. Dajani, R., Fraser, E., Roe, S. M., Young, N., Good, V., Dale, T. C., and Pearl,
