Multiple mechanisms for the phosphorylation of C-terminal regulatory sites in rabbit muscle glycogen synthase expressed in COS cells

Alexander V. SKURAT* and Peter J. ROACH

Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, IN 46202-5122, U.S.A.

Glycogen synthase can be inactivated by sequential phosphorylation at the C-terminal residues Ser^{652} (site 4), Ser^{648} (site 3c), Ser^{644} (site 3b) and Ser^{640} (site 3a) catalysed by glycogen synthase kinase-3. *In vitro*, glycogen synthase kinase-3 action requires that glycogen synthase has first been phosphorylated at Ser^{656} (site 5) by casein kinase II. Recently we demonstrated that inactivation is linked only to phosphorylation at site 3a and site 3b, and that, in COS cells, modification of these sites can occur by alternative mechanisms independent of any C-terminal phosphorylations [Skurat and Roach (1995) J. Biol. Chem. **270**, 12491–12497]. To address these mechanisms multiple Ser → Ala mutations were introduced in glycogen synthase such that only site 3a or site 3b remained intact. Additional mutation of Arg^{637} → Gln eliminated phosphorylation of site 3a, indicating that Arg^{637} may be important for recognition of site 3a by its corresponding protein kinase(s). Similarly, additional mutation of Pro^{645} → Ala eliminated phosphorylation of site 3b, indicating a possible involvement of 'proline-directed' protein kinase(s). Mutation of Arg^{637} alone did not activate glycogen synthase as expected from the loss of phosphorylation at site 3a. Rather, mutation of both Arg^{637} and the Ser → Ala substitution at site 3b was required for substantial activation. The results suggest that sites 3a and 3b can be phosphorylated independently of one another by distinct protein kinases. However, phosphorylation of site 3b can potentiate phosphorylation of site 3a, by an enzyme such as glycogen synthase kinase-3.

INTRODUCTION

Rabbit skeletal muscle glycogen synthase is phosphorylated at nine or more sites by multiple protein kinases [1–3] (see Figure 1). The critical phosphorylation sites that control the enzyme activity are N-terminal sites 2 (Ser^{7}) and 2a (Ser^{10}) [4–7] and C-terminal sites 3a (Ser^{640}) and 3b (Ser^{644}) [6–8]. Phosphorylation of either sites 2 and 2a or sites 3a and 3b leads to inactivation of glycogen synthase. After inactivation, full activity of glycogen synthase can be restored in the presence of the allosteric activator glucose 6-phosphate. Therefore, the $-/+$ glucose 6-phosphate activity ratio is a parameter that reflects the extent of glycogen synthase phosphorylation [9]. *In vitro*, phosphorylation of sites 3a, 3b, 3c (Ser^{648}) and 4 (Ser^{652}) is catalysed by glycogen synthase kinase-3 [10,11]. These sites are phosphorylated sequentially in the order 4, 3c, 3b and 3a, but recognition of site 4 by glycogen synthase kinase-3 requires that glycogen synthase has first been phosphorylated at site 5 (Ser^{656}) [12–14]. The molecular basis appears to be that glycogen synthase kinase-3 modifies residues in the sequence motif -S-X-X-X-S(P)- [11].

Recent studies, utilizing rabbit muscle glycogen synthase expressed in COS cells, have demonstrated the operation of an alternative mechanism for the phosphorylation of sites 3a and 3b [6,7]. Despite the extremely low activity ratio of the expressed glycogen synthase, disruption of the recognition sequence for glycogen synthase kinase-3 by Ser → Ala substitutions at sites 3c, 4 or 5 did not increase the activity of glycogen synthase [6]. Phosphorylation of site 3a and site 3b was shown to occur even if all other adjacent sites were mutated [7]. In the present study, we show that the amino acid residues Arg^{637} and Pro^{645} are important for the direct phosphorylation of sites 3a and 3b respectively. The results also suggest that phosphorylation of site 3b can potentiate phosphorylation of site 3a, thus providing more effective inactivation of glycogen synthase.

EXPERIMENTAL

Vectors and site-directed mutagenesis

Vectors for expression of glycogenin (pCMV-GN) and glycogen synthase (pCMV-GS) have been described previously [15]. Mutations were introduced into rabbit muscle glycogen synthase using the PCR strategy described by Higuchi [16]. A 32-base DNA oligonucleotide identical to the glycogen synthase cDNA sequence at bases 1930–1961, which contains an *Afl*III restriction site (arbitrarily designated AflIII), and a 25-base oligonucleotide complementary to the cDNA sequence at bases 2317–2334, which contains additional sequence to generate a *Xba*I site immediately after the stop codon (designated XbaIa), were designed as outside primers for mutagenesis of Arg^{637}. Two 29-base primers were designed to be identical or complementary to the glycogen synthase sequence at bases 2014–2042 (designated R637 or R637a respectively) except that the codon for Arg^{637} (CGC) was replaced by a codon for glutamine (CAG). By using AflIII with R637a and R637 with XbaIa in separate PCR reactions with pCMV-GS as a template, we amplified 113- and 328-bp fragments respectively. The purified fragments were then mixed together and PCR was performed using primers AflIII and XbaIa. The final product was digested with *Afl*III and *Xba*I and purified. The pCMV-GS vector was digested with *Afl*III and *Xba*I and then the fragments *Afl*III–*Afl*III and *Xba*I–*Afl*III were isolated. These two fragments and the PCR-generated fragment *Afl*III–*Xba*I were ligated together to produce a vector expressing the glycogen synthase mutant R637Q. To introduce both the Arg → Gln substitution at position 637 and the Ser → Ala substitution at position 644 (site 3b), we followed the same strategy but now using glycogen synthase cDNA mutated at site 3b [6] as the starting template. To mutagenize simultaneously both Ser^{7} (site 2) and Arg^{637}, *Afl*III fragments encoding an Arg^{637} mutant of glycogen synthase were religated into a pCMV-GS vector con-

* To whom correspondence should be addressed.

taining the $Ser^{7} \rightarrow Ala$ mutation from which the same *Afl*III fragment had been removed.

To make an R637Q mutant, which is additionally truncated at Lys^{682}, we used a 42-base primer complementary to the glycogen synthase cDNA from bases 2146–2172 and containing additional sequence that encodes the TGA stop codon to replace the GAC codon for Asp^{683} and an *Xba*I site located 3′ to the stop codon [7]. This primer was used in PCR instead of the primer XbaIa. As a template we used cDNA encoding mutant SAAAAΔ682 [7] which is a truncated glycogen synthase containing $Ser \rightarrow Ala$ substitutions at sites 3b, 3c, 4 and 5. The construct obtained after PCR and ligation was designated R637Q, SAAAAΔ682.

To introduce the mutation $Pro^{645} \rightarrow Ala$, we used a 41-base mutagenic primer which is identical to the glycogen synthase sequence at bases 2026–2066 except that the codon for Pro^{645} (CCC) was changed to a codon for Ala (GCC). This primer was used in PCR in conjunction with the primer XbaIa. The PCR product was digested with *Sac*II and *Xba*I and ligated into the pCMV-GS vector containing a $Ser^{7} \rightarrow Ala$ mutation from which the same *Sac*II–*Xba*I fragment had been removed. To construct a truncated mutant which contains $Ser \rightarrow Ala$ substitutions at sites 3a, 3c, 4 and 5 and a $Pro \rightarrow Ala$ substitution at codon 645, we used an identical 41-base mutagenic primer which additionally changes the codon TCT for Ser^{640} to a codon (GCT) for Ala. As a template, we used cDNA encoding the mutant ASAAAΔ682 [7]. The PCR product was ligated into pCMV-GS after digestion with *Sac*II and *Xba*I and the mutant was designated as P645A, ASAAAΔ682. The DNA sequence corresponding to the *Afl*III–*Xba*I and *Sac*II–*Xba*I regions in each mutant was confirmed by sequencing.

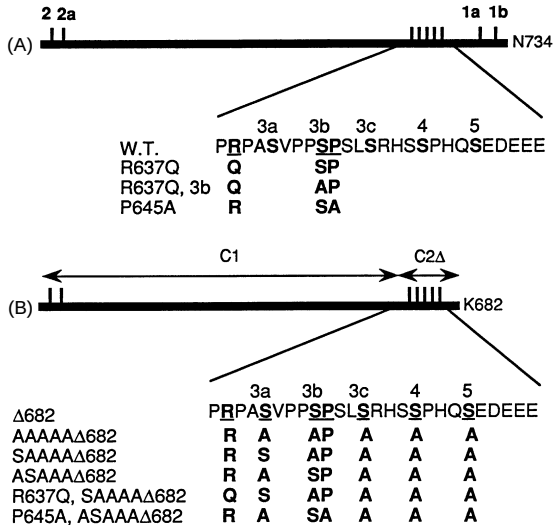

Figure 1 Mutations introduced in rabbit muscle glycogen synthase

The lines represent full-length (A) or truncated (B) glycogen synthase with phosphorylation sites indicated by vertical tick markers. The amino acid sequence of the region containing phosphorylation sites from 3a to 5 is given. The residues subjected to mutagenesis are underlined and the replacement amino acids are indicated underneath. The designations of each mutant are indicated to the left of the sequence. The two fragments generated by chymotrypsin treatment of truncated glycogen synthase are designated C1 and C2Δ. In some cases, site 2 was also mutated to Ala, as in mutants 2, R637Q, 2, R637Q, 3e and 2, P645A.

Expression and analysis of glycogen synthase mutants

Recombinant plasmids were purified twice by CsCl/ethidium bromide centrifugation [17]. COS M9 cells were grown in 35-mm-diam. plates and transfected with 0.5 μg of pCMV vector encoding wild-type or mutant glycogen synthase in the presence of 0.5 μg of pCMV-GN using LipofectACE (Life Technologies, Inc.) as described previously [6]. Cells were lysed, homogenized and centrifuged at 16000 ***g*** for 15 min as described previously [15]. The supernatants and resuspended pellets so obtained are designated as the 'soluble' and 'pellet' fractions respectively. Glycogen synthase activity was measured by incorporation of [^{14}C]glucose from UDP-[U-^{14}C]glucose into glycogen with or without 7.2 mM glucose 6-phosphate using the filter paper assay of Thomas et al. [18]. The activity of the pellet fraction was expressed in terms of the protein in the corresponding soluble fraction; the $-/+$ glucose 6-phosphate activity ratio was calculated after subtraction of the endogenous glycogen synthase activity of COS cells [6].

Western blot analyses were performed using anti-(glycogen synthase) antibodies kindly provided by Dr. John C. Lawrence, Jr. (Washington University, St. Louis, MO, U.S.A.). After separation on SDS/7.5 %-PAGE, immunoblotting was performed as described previously [15].

Analysis of phosphorylation of glycogen synthase

Incubation of COS cells with [^{32}P]phosphate and immunoprecipitation of truncated mutants of glycogen synthase were performed as described previously [6,7]. Immunoprecipitates were treated with 0.1 μg/ml chymotrypsin and the released small C-terminal fragments (C2Δ-fragments) were separated from Protein A–agarose beads (Pierce) by centrifugation at 12000 ***g*** for 5 min. The beads, either without cleavage or after treatment with chymotrypsin leaving the large N-terminal C1 fragment attached (Figure 1), were boiled in SDS-sample buffer. Then ^{32}P-labelled polypeptides were separated by SDS/7.5 %-PAGE according to Laemmli [19] and subjected to autoradiography. The C2Δ-fragments were analysed by gel electrophoresis in the Tricine-based system of Schagger and von Jagow [20] using a 10 % spacer gel and 16.5 % polyacrylamide gel with 6 M urea [7]. After electrophoresis, gels were dried and exposed to X-ray films.

Miscellaneous methods

Protein was quantified by the method of Bradford [21] using bovine γ-globulin as standard.

RESULTS

Analysis of phosphorylation of glycogen synthase mutated at protein kinase recognition determinants

In previous work, we had constructed a series of glycogen synthase mutants truncated at Lys^{682} [7]. Truncated glycogen synthase was expressed in COS cells followed by labelling of the cells with [^{32}P]phosphate and immunoprecipitation to isolate ^{32}P-labelled glycogen synthase. When analysed by SDS/PAGE, all the expressed truncated proteins were clearly separated from the co-immunoprecipitating full-length endogenous glycogen synthase which is present in control COS cells and which has a lower electrophoretic mobility (Figure 2A). Treatment of the immunoprecipitates with chymotrypsin at low concentrations cleaves the Tyr^{633}-Arg^{634} bond and releases a C-terminal fragment (C2Δ, see Figure 2C) from the immune complex [7]. This fragment contains phosphorylation sites 3a, 3b, 3c, 4 and 5 but not the other C-terminal sites, 1a and 1b, which have been eliminated by the truncation (Figure 1B). All samples from control and transfected COS cells contained the same amounts of polypeptide with molecular mass of $\approx$22.4 kDa which represents the C-

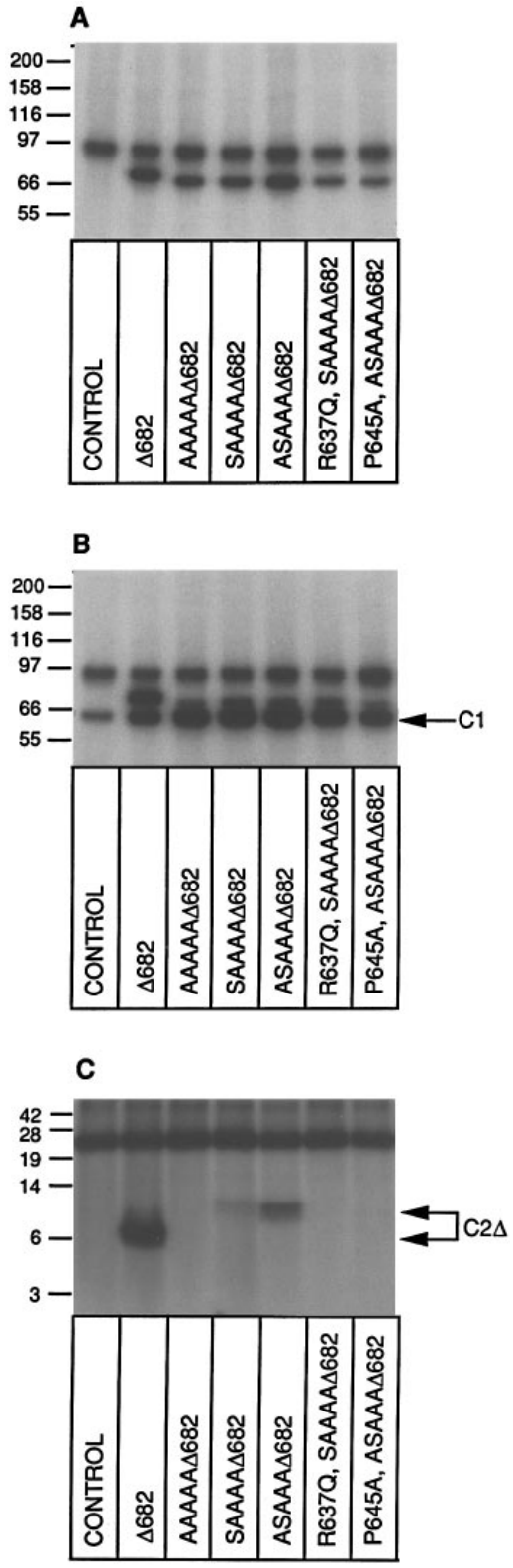

Figure 2 Electrophoretic analysis of expressed and ^{32}P-labelled truncated glycogen synthase mutants and their proteolytic fragments

Control cells or COS cells expressing truncated glycogen synthase mutants were incubated with [^{32}P]phosphate as described in the Experimental section. Glycogen synthase was purified by immunoprecipitation and aliquots removed. The rest of the immunoprecipitate was digested with 0.1 μg/ml chymotrypsin for 16 h at 30 °C. The C2Δ fragments were released from the immune complex and separated from C1 fragment by centrifugation. The uncleaved glycogen synthases (**A**) and C1 fragments (**B**) were analysed by electrophoresis according to Laemmli [19] in a 7.5% gel. The C2Δ fragments (**C**) were analysed by electrophoresis according to Schagger and von Jagow [20] in 16.5% gels in the presence of urea. Gels were dried and exposed to X-ray film.

terminal fragment of the endogenous COS cell glycogen synthase (see [7] and Figure 2C). The large fragment (C1), containing phosphorylation sites 2 and 2a, remains bound to the anti-(glycogen synthase) antibodies after chymotrypsin treatment and is therefore associated with immunoprecipitate (Figure 2B). The uncleaved endogenous and expressed glycogen synthases could be distinguished from the C1 fragments which have higher electrophoretic mobilities (Figure 2B). Introduction of Ser → Ala substitutions at all five remaining C-terminal phosphorylation sites (mutant AAAAAΔ682) leads to loss of the phosphorylation of the C2Δ fragment (Figure 2C). Re-instating a serine residue at either site 3a (SAAAAΔ682) or site 3b (ASAAAΔ682) restores labelling of the fragment, indicating the re-establishment of phosphorylation at these sites in COS cells (see [7] and Figure 2C).

To investigate further the molecular basis for the phosphorylation of the critical sites 3a and 3b of glycogen synthase, we mutated amino acids potentially relevant to the recognition of sites 3a or 3b by site 3a- or site 3b-kinases. Thus, an Arg → Gln substitution at position 637 and a Pro → Ala substitution at position 645, respectively, were introduced into SAAAAΔ682 and ASAAAΔ682. These new mutants R637Q, SAAAAΔ682 and P645A, ASAAAΔ682 (see Figure 1B) were expressed in COS cells, labelled with [^{32}P]phosphate, isolated by immunoprecipitation and subjected to limited proteolysis. It is important to note that replacement of Arg^{637} by Gln did not affect the cleavage of the Tyr^{633}-Arg^{634} bond by chymotrypsin (Figure 2B). In the mutants R637Q, SAAAAΔ682 and P645A, ASAAAΔ682 phosphorylation of site 3a and site 3b was lost, indicating the importance of these residues for phosphorylation of the respective sites (Figure 2C).

Expression of full-length mutants in COS cells

In order to analyse the role of site 3a- and site 3b-kinases in the regulation of the enzyme activity by phosphorylation, the substitutions Arg^{637} → Gln and Pro^{645} → Ala were introduced into full-length glycogen synthase protein. The expressed wild-type and mutant glycogen synthase was associated with both the soluble and pellet fractions of COS cells (Figure 3). As was previously shown, Ser → Ala substitutions at sites 3a, 3b, 3c, 4 or 5 increase the electrophoretic mobility of the expressed protein, whereas mutations of N-terminal phosphorylation sites has no such effect [6]. Mutation of Arg^{637} → Gln, either in wild-type glycogen synthase or in enzyme additionally mutated at site 2, did not change the electrophoretic mobility of the protein as judged by Western blot analysis (Figure 3). Therefore, the mobility shift observed for the mutants R637Q, 3b and 2, R637Q, 3b (see Figure 1) is a result of the Ser → Ala substitution at site 3b. Interestingly, a similar increase in mobility was caused by the Pro^{645} → Ala substitution even though the expressed protein does not contain mutations at the phosphorylation sites (Figure 3).

Effect of Arg^{637} → Gln and Pro^{645} → Ala mutations on glycogen synthase activity

Based on Western blot analysis (Figures 3A and 3B), as well as our earlier observations [6,7], the activity of glycogen synthase expressed in COS cells was distributed between the soluble and

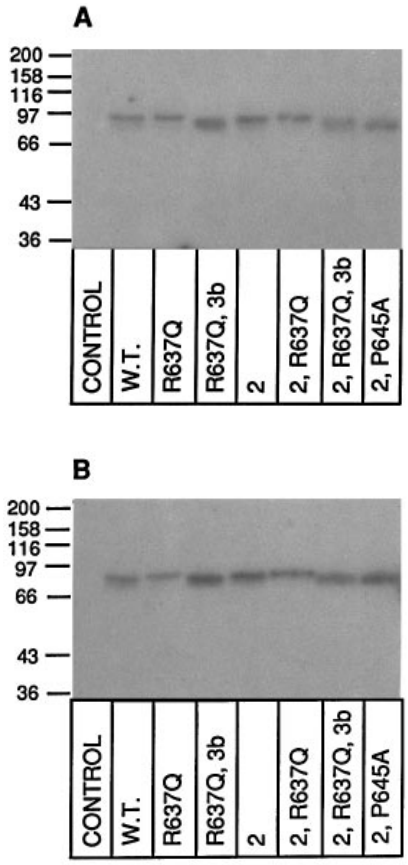

Figure 3 Expression of full-length glycogen synthase in COS M9 cells

Cells transfected with wild-type or mutated glycogen synthase were lysed and centrifuged to generate soluble (**A**) and pellet (**B**) fractions. Proteins were separated by SDS/PAGE according to Laemmli [19] followed by transfer to nitrocellulose, which was probed with anti-(glycogen synthase) antibodies and ^{125}I-labelled Protein A. The migration of molecular mass markers (in kDa) is indicated. Abbreviation: W.T., wild type.

pellet fractions (Table 1). As expected, the mutation $Arg^{637} \rightarrow Gln$ did not increase the activity ratio of expressed glycogen synthase because of the residual inactivating effect of phosphorylation at sites 2 and 2a. However, if the mutation $Arg^{637} \rightarrow Gln$ was introduced into enzyme which already carried a $Ser \rightarrow Ala$ substitution at site 2 (mutant 2, R637Q), no activation of glycogen synthase was observed. This can be explained by phosphorylation of site 3a by an alternative protein kinase which does not use Arg^{637} for recognition of this site. The obvious candidate for this alternative site 3a-kinase is glycogen synthase kinase-3. Indeed, glycogen synthase kinase-3 does phosphorylate site 3a *in vitro*, but with an obligatory requirement for prior phosphorylation of site 3b [11]. Therefore, we eliminated any phosphorylation at site 3b by mutating it to Ala. The activity ratio of this mutant enzyme (2, R637Q, 3b; Table 1) was significantly greater than the activity ratio of glycogen synthase mutated only at sites 2 and 3b and was equal to the ratio of the enzyme mutated at sites 2 and 3a, suggesting the lack of phosphorylation at site 3a. The low activity ratio of the mutant R637Q, 3b, which has no additional mutation at site 2 (Table 1), confirms the occurrence of a strong inactivation of glycogen synthase due to N-terminal phosphorylation [6,7]. The substitution $Pro^{645} \rightarrow Ala$ in conjunction with a mutation at site 2 (Table 1) caused the same activating effect on the glycogen synthase activity ratio as elimination of the adjacent phosphorylation site 3b. The mutation itself could affect the activity ratio of the enzyme, although we have no precedent for such an occurrence in previous mutational analyses. More likely, the mutation has suppressed phosphorylation at site 3b, whether by glycogen synthase kinase-3 or by a separate site 3b-kinase.

DISCUSSION

Phosphorylation of the C-terminal serine residues of mammalian glycogen synthase is a key regulatory mechanism for controlling the enzyme activity [1–3]. The spatial location of phosphorylation sites 3a, 3b, 3c, 4 and 5 precisely matches a recognition motif for glycogen synthase kinase-3, namely -S-X-X-X-S(P)- [3,11]. It was thus proposed that phosphorylation of site 5 by casein kinase II triggers a sequential phosphorylation of sites 4, 3c, 3b and 3a by glycogen synthase kinase-3 [11,14]. During the past 2 years, it has been found that phosphorylation at only two of the sites, site 3a and site 3b, causes significant inactivation of glycogen synthase [6–8]. Moreover, it was shown that site 3a and site 3b can be phosphorylated directly in rabbit muscle glycogen synthase expressed in COS cells via a mechanism that is independent of prior phosphorylation at sites 3c, 4 and 5 [7]. This observation

Table 1 Effect of mutation R637Q and P645A on the activity of glycogen synthase expressed in COS cells

Full-length glycogen synthase mutated at C-terminal amino acid residues alone or in conjunction with Ser → Ala substitution at the N-terminal phosphorylation site 2 were expressed in COS cells. Activities in the presence and in the absence of glucose 6-phosphate (G-6-P) were analysed in soluble and pellet fractions of cell homogenate as described in the Experimental section. Results are means ± S.E.M. for the number of experiments indicated. The data for control, wild type (W.T.) and mutants 2, 2, 3b and 2, 3a (asterisks) were obtained previously [6].

	Glycogen synthase activity (−G-6-P)		Glycogen synthase activity (+G-6-P)		−/+G-6-P activity ratio	
Mutation	Soluble	Pellet	Soluble	Pellet	Soluble	Pellet
Control*	0.10 ± 0.06	0.07 ± 0.03	4.7 ± 0.8	1.7 ± 0.6	0.022 ± 0.010	0.034 ± 0.014
W.T.*	0.24 ± 0.15	0.41 ± 0.33	30 ± 14	25 ± 12	0.009 ± 0.004	0.013 ± 0.006
R637Q (4)	0.08 ± 0.03	0.10 ± 0.03	23.0 ± 6.3	22 ± 5	0.004 ± 0.002	0.004 ± 0.001
R637Q, 3b (3)	0.67 ± 0.09	0.42 ± 0.15	36.0 ± 8.6	31 ± 15	0.019 ± 0.006	0.015 ± 0.002
2*	1.30 ± 0.71	1.60 ± 1.37	46 ± 25	46 ± 31	0.033 ± 0.013	0.029 ± 0.011
2, R637Q (4)	0.50 ± 0.16	0.50 ± 0.23	23 ± 13	28 ± 15	0.025 ± 0.008	0.019 ± 0.004
2, R637Q, 3b (4)	4.1 ± 1.0	10.20 ± 3.36	7 ± 3	27 ± 13	0.615 ± 0.125	0.405 ± 0.115
2, P645A (3)	2.40 ± 0.43	5.40 ± 0.65	11 ± 2	30 ± 8	0.210 ± 0.010	0.190 ± 0.053
2, 3b*	5.5 ± 1.3	19.5 ± 2.3	23 ± 7	94 ± 11	0.245 ± 0.030	0.208 ± 0.020
2, 3a*	7.6 ± 2.5	26.9 ± 8.8	13 ± 4	72 ± 17	0.590 ± 0.045	0.366 ± 0.060

suggests the involvement of protein kinase(s) other than glycogen synthase kinase-3 in the phosphorylation of these sites. Substrate recognition by many protein kinases is determined by specific sequences surrounding the phosphorylation site [22,23]. In the present study, we modified amino acid residues in glycogen synthase potentially important for recognition by putative site 3a- and site 3b-kinase(s).

The inability of site 3a-kinase to phosphorylate mutant R637Q, SAAAAΔ682 (Figure 2C) indicates the importance of the Arg at position 637 for direct phosphorylation of site 3a. Such a requirement would make several known protein kinases possible candidates to act as a site 3a-kinase. For example, a basic amino acid residue at position −3 relative to the phosphorylated amino acid residue (position 0) is a substrate specificity determinant for cyclic AMP-dependent protein kinase, cyclic GMP-dependent protein kinase, protein kinase C [22,23], and calmodulin-dependent protein kinases II and IV [22–24]. In addition, the amino acid sequence around site 3a resembles sites recognized by phosphorylase kinase, namely K/R-X-X-S-V/I [23]. However, of the protein kinases listed, only cyclic AMP-dependent protein kinase has been reported to modify site 3a *in vitro* [25]. This occurred only at high levels of total phosphorylation of glycogen synthase, indicating that site 3a is poorly favoured as a site for cyclic AMP-dependent protein kinase *in vitro* [25].

The lack of phosphorylation of the C2Δ fragment in the mutant P645A, ASAAAΔ682 (Figure 2C) suggests that the protein kinase directly phosphorylating site 3b may be generally classified as 'proline-directed'. The involvement of glycogen synthase-3 in direct phosphorylation of site 3b or site 3a is unlikely because neither the α- nor β-isoform of glycogen synthase kinase-3 was able to phosphorylate recombinant mutants SAAAAΔ682 and ASAAAΔ682 which were expressed in *Escherichia coli* and purified (A. V. Skurat, Q. Wang and P. J. Roach, unpublished work). Another family of protein kinases which recognize the sequence S/T-P [26] and which, therefore, could phosphorylate site 3b *in vitro*, is that of the mitogen-activated protein kinases. Activation of these protein kinases by insulin and growth factors is accompanied by activation of glycogen synthase [27,28], an outcome that is in the opposite sense to what should result from increased phosphorylation at site 3b. A third group of proline-directed protein kinases that could phosphorylate site 3b is that of the cyclin-dependent protein kinases. These protein kinases are active in many cell lines and their amount is increased severalfold in cells expressing simian virus 40 (SV-40) large T antigen [29]. A proline-directed protein kinase, which phosphorylates site 3b in synthetic peptide *in vitro*, has been partially purified from PC12 rat pheochromocytoma [30] and later identified as a complex between $p34^{cdc2}$ and cyclin A [31]. Thus, despite the fact that several known protein kinases can potentially recognize the sequence around sites 3a and 3b in glycogen synthase, their physiological relevance is uncertain and, possibly, novel protein kinases are implicated in the control of the enzyme.

A major outcome of this work is to emphasize the possibility that multiple mechanisms for the regulation of cellular proteins can co-exist in the same cell. The $Arg^{637} \rightarrow Gln$ substitution, which abolishes direct phosphorylation of site 3a, does not activate glycogen synthase. Additional Ser → Ala mutation at site 3b is required for significant activation (Table 1). This observation suggests that site 3a is still phosphorylated in the R637Q mutant but via a mechanism consistent with the hierarchal phosphorylation proposed for glycogen synthase kinase-3 action [3]. According to this mechanism, the $Arg^{637} \rightarrow Gln$ substitution would not prevent phosphorylation of site 3a by glycogen synthase kinase-3 which recognizes the sequence -S-X-X-X-S(P)-

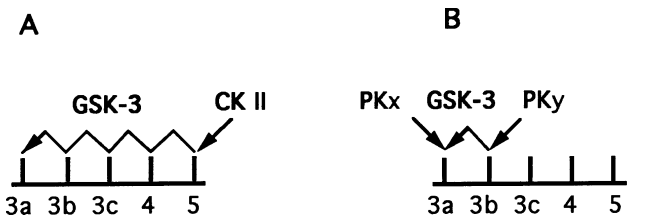

Figure 4 Mechanisms of phosphorylation of sites 3a and 3b in muscle glycogen synthase

The sequential mechanism for the phosphorylation of glycogen synthase by casein kinase II (CK II) and glycogen synthase 3 (GSK 3) (**A**) and alternative mechanisms of phosphorylation observed in the enzyme expressed in COS cells (**B**) are discussed in the text. PKx and PKy represent protein kinases that would independently phosphorylate site 3a or site 3b, respectively.

(Figure 4; [11]). Only elimination of phosphoserine as a recognition determinant at site 3b would prevent phosphorylation of site 3a by glycogen synthase kinase-3. In this model, phosphorylation of site 3a would be mediated by at least two different protein kinases (Figure 4). A site 3a-kinase (PKx) would recognize Arg^{637} and phosphorylate site 3a directly, whereas phosphorylation by glycogen synthase kinase-3 would require a preliminary phosphorylation at site 3b by a site 3b-kinase (PKy).

Previous results [6,7] and the data presented in this paper indicate that glycogen synthase expressed in COS cells is extensively phosphorylated at all key regulatory phosphorylation sites. At least two different protein kinases must be involved in phosphorylating sites 2 and 2a to provide substantial inactivation of glycogen synthase [6,7]. Now we demonstrate that at least three distinct protein kinases combine to inactivate glycogen synthase in COS cells by phosphorylation of sites 3a and 3b. The reason for the existence of redundant mechanisms of inactivation of glycogen synthase is unknown. However, the main consequence of such control would be the ability to integrate incoming stimuli from several signal transduction pathways. We should emphasize that our studies have addressed phosphorylation of muscle enzyme in COS cells and we cannot be certain that identical controls exist in skeletal muscle. However, this idea that different cells might vary as to the regulatory pathways operating is itself of interest and potential importance. Thus, tissue-specific distribution and regulation of the relevant signal transduction pathways could specify both the extent of glycogen accumulation as well as the mechanisms regulating its metabolism.

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REFERENCES

1 Cohen, P. (1982) Nature (London) **296**, 613–620
2 Cohen, P. (1986) in The Enzymes (Boyer, P. D. and Krebs, E. G., eds.), vol. 17, pp. 461–497, Academic Press, Orlando, FL
3 Roach, P. J. (1990) FASEB J. **4**, 2961–2968
4 Flotow, H. and Roach, P. J. (1989) J. Biol. Chem. **264**, 9126–9128
5 Nakielny, S., Campbell, D. G. and Cohen, P. (1991) Eur. J. Biochem. **199**, 713–722
6 Skurat, A. V., Wang, Y. and Roach, P. J. (1994) J. Biol. Chem. **269**, 25534–25542
7 Skurat, A. V. and Roach, P. J. (1995) J. Biol. Chem. **270**, 12491–12497
8 Wang, Y. and Roach, P. J. (1993) J. Biol. Chem. **268**, 23876–23880
9 Roach, P. J., Takeda, Y. and Larner, J. (1976) J. Biol. Chem. **251**, 1913–1919
10 Picton, C., Aitken, A., Bilham, T. and Cohen, P. (1982) Eur. J. Biochem. **124**, 37–45
11 Fiol, C. J., Mahrenholz, A. M., Wang, Y., Roeske, R. W. and Roach, P. J. (1987) J. Biol. Chem. **262**, 14042–14048

12 Picton, C., Woodgett, J., Hemmings, B. and Cohen, P. (1982) FEBS Lett. **150**, 191–196
13 DePaoli-Roach, A. A., Ahmad, Z., Camici, M., Lawrence, J. C., Jr. and Roach, P. J. (1983) J. Biol. Chem. **258**, 10702–10709
14 Zhang, W., DePaoli-Roach, A. A. and Roach, P. J. (1993) Arch. Biochem. Biophys. **304**, 219–225
15 Skurat, A. V., Cao, Y. and Roach, P. J. (1993) J. Biol. Chem. **268**, 14701–14707
16 Higuchi, R. (1989) in PCR Technology: Principles and Applications for DNA Amplification (Erdich, H. A., ed.), pp. 61–70, Stockton Press, New York
17 Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, pp. 1.42–1.43, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
18 Thomas, J. A., Schlender, K. K. and Larner, J. (1968) Anal. Biochem. **25**, 486–499
19 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
20 Schagger, H. and von Jagow, G. (1987) Anal. Biochem. **166**, 368–379
21 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
22 Kennelly, P. J. and Krebs, E. G. (1991) J. Biol. Chem. **266**, 15555–15558
23 Pearson, R. B. and Kemp, B. E. (1991) Methods Enzymol. **200**, 63–81
24 Nairn, A. C. and Picciotto, M. R. (1994) Semin. Cancer Biol. **5**, 295–303
25 Sheorain, V. S., Juhl, H., Bass, M. and Soderling, T. R. (1984) J. Biol. Chem. **259**, 7024–7030
26 Davis, R. J. (1993) J. Biol. Chem. **268**, 14553–14556
27 Robinson, L. J., Razzack, Z. F., Lawrence, J. C., Jr. and James, D. E. (1993) J. Biol. Chem. **268**, 26422–26427
28 Lin, T.-A. and Lawrence, J. C., Jr. (1994) J. Biol. Chem. **269**, 21255–21261
29 Oshima, J., Steinmann, K. E., Campisi, J. and Schlegel, R. (1993) Oncogene **8**, 2987–2993
30 Vulliet, P. R., Hall, F. L., Mitchell, J. P. and Hardie, D. G. (1989) J. Biol. Chem. **264**, 16292–16298
31 Hall, F. L., Braun, R. K., Mihara, K., Fung, Y.-K. T., Derndt, N., Carbonaro-Hall, D. A. and Vulliet, P. R. (1991) J. Biol. Chem. **266**, 17430–17440

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