Identification of glycogen synthase as a new substrate for stress-activated protein kinase 2b/p38β

Yvonne KUMA, David G. CAMPBELL and Ana CUENDA[1]
MRC Protein Phosphorylation Unit, School of Life Sciences, University of Dundee, Dundee DD1 5EH, U.K.

The endogenous glycogen synthase in extracts from mouse skeletal muscle, liver and brain bound specifically to SAPK2b (stress-activated protein kinase 2b)/p38β, but not to other members of the group of SAPK/p38 kinases. Glycogen synthase was phosphorylated *in vitro* more efficiently by SAPK2b/p38β than by SAPK2a/p38α, SAPK3/p38γ or SAPK4/p38δ. SAPK2b/p38β phosphorylated glycogen synthase *in vitro* at residues Ser^{644}, Ser^{652}, Thr^{718} and Ser^{724}, two of which (Ser^{644} and Ser^{652}) are also phosphorylated by glycogen synthase kinase 3. Thr^{718} and Ser^{724} are novel sites not known to be phosphorylated by other protein kinases. Glycogen synthase becomes phosphorylated at Ser^{644} in response to osmotic shock; this phosphorylation is prevented by pretreatment of the cells with SB 203580, which inhibits SAPK2a/p38α and SAPK2b/p38β activity. *In vitro*, phosphorylation of glycogen synthase by SAPK2b/p38β alone had no significant effect on its activity, indicating that phosphorylation at residue Ser^{644} itself is insufficient to decrease glycogen synthase activity. However, after phosphorylation by SAPK2b/p38β, subsequent phosphorylation at Ser^{640} by glycogen synthase kinase 3 decreased the activity of glycogen synthase. This decrease was not observed when SAPK2b/p38β activity was blocked with SB 203580. These results suggest that SAPK2b/p38β may be a priming kinase that allows glycogen synthase kinase 3 to phosphorylate Ser^{640} and thereby inhibit glycogen synthase activity.

Key words: glycogen, glycogen synthase, phosphorylation, stress, stress-activated protein kinase 2b/p38β (SAPK2b/p38β).

INTRODUCTION

SAPKs (stress-activated protein kinases) are MAPK (mitogen-activated protein kinase) family members that are activated by cellular stresses, bacterial lipopolysaccharide and cytokines [1]. A major challenge in this field is to identify the physiological substrates and functions of each SAPK. The group of SAPK/p38 kinases comprises SAPK2a/p38α, SAPK2b/p38β, SAPK3/p38γ [also known as ERK6 (extracellular-signal-regulated kinase 6)] and SAPK4/p38δ. Identification of physiological substrates for SAPK2a/p38α and SAPK2b/p38β has been greatly facilitated by the availability of specific inhibitors of these enzymes, such as the cell-permeant pyridyl imidazole SB 203580 and related compounds [2,3]. Substrates for SAPK2a/p38α and SAPK2b/p38β include other protein kinases, as well as several transcription factors [4]. SAPK3/p38γ and SAPK4/p38δ are not inhibited by SB 203580 [5,6], and consequently only a little is known about their substrates. Stathmin and the elongation factor 2 kinase have been proposed as physiological substrates for SAPK4/p38δ [7–9], whereas the transcription factor ATF2 (activating transcription factor 2), microtubule-associated protein tau and α1-syntrophin are *in vitro* substrates of SAPK3/p38γ [10–13].

Each member of the SAPK/p38 group may have different biological functions and different physiological substrates, but all SAPK/p38s can phosphorylate substrates containing the minimal consensus sequence Ser/Thr-Pro. In recent years, it has been shown that, to achieve substrate specificity, kinases rely on direct docking interactions with their substrate, using sites distinct from the phospho-acceptor sequence, and that these interactions also contribute to the specificity and regulation of protein kinase activity [12,14]. Recently, we have shown that the phosphorylation of SAP90/PSD95 and α1-syntrophin by SAPK3/p38γ is dependent on the interaction of the C-terminal sequence -Glu-Thr-Xaa-Leu of the kinase with the PDZ domains of these proteins. PDZ domains are modular protein–protein interaction domains that serve to localize proteins to specific subcellular sites. The finding that SAPK3/p38γ binds through its C-terminal sequence to the PDZ domain of α1-syntrophin, and that the phosphorylation of these proteins by SAPK3/p38γ depends on this interaction, identified a novel mechanism for targeting a protein kinase to its substrate [12]. In our search for the physiological roles of SAPK/p38s, we decided to look for binding proteins that could be substrates or substrate-binding proteins for these kinases. In the present paper we show that GS (glycogen synthase) from skeletal muscle, brain and liver binds specifically to GST (glutathione S-transferase)–SAPK2b/p38β, but not to fusion proteins containing the other SAPK isoforms. We also show that GS is phosphorylated efficiently by SAPK2b/p38β *in vitro* and that this phosphorylation allows GS kinase 3 (GSK3) to phosphorylate other residues, which then cause partial inactivation of GS activity.

MATERIALS AND METHODS

Materials

Precast polyacrylamide gels, running buffer and transfer buffer were from Invitrogen (Paisley, U.K.). SB 203580 was obtained from Calbiochem (Nottingham, U.K.) and MBP (myelin basic protein) from GIBCO-BRL (Paisley, U.K.). Complete proteinase inhibitor cocktail tablets were from Roche. Other chemicals were of the highest purity available and were purchased from Merck or Sigma-Aldrich.

Abbreviations used: ERK, extracellular-signal-regulated kinase; GS, glycogen synthase; GSK3, glycogen synthase kinase 3; GST, glutathione S-transferase; HA, haemagglutinin; HEK, human embryonic kidney; MALDI-TOF, matrix-assisted laser-desorption ionization–time-of-flight; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MKK, MAPK/ERK kinase; SAPK, stress-activated protein kinase.

[1] To whom correspondence should be addressed (e-mail a.i.cuenda@dundee.ac.uk).

© 2004 Biochemical Society

Antibodies

Antibodies against SAPK2b/p38β, SAPK2a/p38α, HA (haemagglutinin) tag and GS were obtained from the Division of Signal Tranduction Therapy (Dundee, U.K.). These antibodies were affinity purified as described in [10], and used for immunoprecipitation and immunoblotting. A mouse anti-SAPK2b/p38β antibody used for immunoblotting was purchased from Zymed (San Francisco, CA, U.S.A.).

Antibodies that recognize SAPK2a/p38α phosphorylated at Thr^{180} and Tyr^{182} (these antibodies also recognize phosphorylated SAPK2b/p38β) were obtained from New England Biolabs (Hitchin, U.K.). Antibodies that recognize GS phosphorylated at Ser^{640} and Ser^{644} were raised in sheep against the phosphopeptides RYPRPVpSVPPSPSLSR and RYPRPVSVPPpSPSLSR respectively, corresponding to residues 634–649 from human GS. The peptides were coupled to BSA and keyhole limpet haemocyanin and injected into sheep at Diagnostics Scotland (Pennicuik, U.K.). The antisera were affinity purified on a phosphopeptide antigen–Sepharose column, then passed through another column to which the unphosphorylated form of the peptide was bound. The flow-though fractions were collected and used at 1 μg/ml for immunoblotting. All secondary antibodies used in the present study were obtained from Perbio Science UK (Tattenhall, Cheshire, U.K.).

DNA constructs

For overexpression, GST-tagged GS was in the vector pEBG-2T, whereas HA–SAPK2a/p38α and HA–SAPK2b/p38β were in pcMV5.

Protein expression, purification and activity

All GST–SAPK/p38s were prepared by the protein production team of the Division of Signal Transduction Therapy (Dundee, U.K.) [2,15].

GS was purified from rabbit skeletal muscle as described [16]. GS preparations are contaminated by traces of another protein kinase capable of phosphorylating GS [18,19], identified recently as AMP-dependent kinase [20]. To partially resolve GS from the co-purifying protein kinase activity, the preparation was chromatographed on a Mono-Q column pre-equilibrated in 50 mM Tris/HCl, pH 7.5, 1 mM EDTA, 0.1 % (v/v) 2-mercaptoethanol and 10 % (v/v) glycerol. The column was developed with a linear gradient of 0–2 M NaCl. GS eluted at 0.4 M NaCl. The leading half of the peak of GS activity was pooled and used in further experiments.

SAPK/p38 family members were assayed routinely using MBP as substrate [10]. To study the relative rates of phosphorylation of GS or α1-syntrophin by active GST–SAPK/p38s, the kinases were matched for activity against MBP. Each GST–SAPK/p38 (1 unit/ml) was incubated for 15 min at 30 °C with Mg[γ-^{32}P]ATP plus 1 μM GS from rabbit skeletal muscle or 1 μM recombinant GST–α1-syntrophin. The samples were denatured in SDS, electrophoresed and autoradiographed. The phosphorylated GS or α1-syntrophin was excised from the gel and the incorporated ^{32}P was counted. GS activity was determined by measuring incorporation of [^{14}C]glucose from UDP-[^{14}C]glucose into glycogen [16,17].

Cell culture, transfection and lysis

HEK293 (human embryonic kidney 293) cells were cultured at 37 °C (in an atmosphere of 5 % CO_2) in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) fetal calf serum and 2 mM glutamine (Biowhittaker). Transfection of HEK293 cells was carried out using the calcium phosphate method [10]. After 16–24 h, cells were stimulated with agonists as indicated below.

Mouse C2C12 myoblasts were cultured at 37 °C (in an atmosphere of 5 % CO_2) in Dulbecco's modified Eagle's medium supplemented with 20 % (v/v) fetal calf serum, 0.5 % (v/v) chick embryo extract and antibiotics. C2C12 myoblasts were induced to differentiate to myotubes as described in [21].

Cells were exposed to 0.5 M sorbitol as indicated in the Figure legends, then lysed in buffer A [50 mM Tris/HCl, pH 7.5, 1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM NaF, 50 mM sodium β-glycerophosphate, 5 mM pyrophosphate, 0.27 M sucrose, 0.1 mM PMSF, 1 % (v/v) Triton X-100] plus 0.1 % (v/v) 2-mercaptoethanol and Complete Proteinase Inhibitor Cocktail. Lysates were centrifuged at 13 000 ***g*** for 5 min at 4 °C, then the supernatants were removed, quick frozen in liquid nitrogen and stored at − 80 °C until used. When required, cells were preincubated for 1 h with 10 μM SB 203580, prior to stimulation with sorbitol.

Immunoprecipitation

Extracts from HEK293 cells or C2C12 myotubes (0.5 mg of protein) were incubated with 5 μg of anti-GS or sheep anti-SAPK2b/p38β coupled to Protein G–Sepharose. After incubation for 2 h at 4 °C, the captured proteins were centrifuged at 13 000 ***g***; the supernatant was discarded and the beads were washed in buffer A containing 0.5 M NaCl, then twice in buffer A alone. Samples were denatured, electrophoresed and then immunoblotted with the appropriate antibodies.

Preparation of mouse tissue extract and GST–SAPK/p38 pull-down

The skeletal muscle, liver and brain were extracted rapidly from adult mice, freeze-clamped in liquid nitrogen, stored at − 80 °C and homogenized to powder in liquid nitrogen. A 3-fold mass excess of ice-cold lysis buffer (buffer A) was added to the powdered tissue, which was briefly vortexed and then centrifuged at 4 °C for 10 min at 13 000 ***g*** to remove insoluble material. The supernatant was snap-frozen in aliquots in liquid nitrogen and stored at − 80 °C. Tissue extracts (10 mg) were incubated twice for 60 min with GSH–Sepharose beads on a shaking platform. After centrifugation, the final supernatants were incubated for 90 min with 20 μg of either GST alone or GST–SAPK/p38, and then for another 90 min with GSH–Sepharose on a shaking platform to couple the GST fusion proteins to the beads. All procedures were performed at 4 °C. After centrifugation at 13 000 ***g*** for 1 min, the pellets were washed twice with buffer A, denaturated in SDS and electrophoresed. SAPK/p38-interacting proteins were visualized by colloidal Coomassie Blue staining.

Proteins from skeletal muscle that were specifically pulled down by one SAPK/p38, but not the others, were excised from the gel for identification by tryptic mass fingerprinting. Tryptic peptides were analysed on a Perseptive Biosystems (Framingham, MA, U.S.A.) Elite STR MALDI-TOF (matrix-assisted laser-desorption ionization–time-of-flight) mass spectrometer with saturated α-cyanocinnamic acid as the matrix. The mass spectrum was acquired in the reflector mode and was mass-calibrated internally. The tryptic peptide ions obtained were scanned against the Swiss-Prot and NCBI databases using the MS-FIT program of Protein Prospector.

Identification of phosphorylation sites in GS

Rabbit skeletal muscle GS (∼ 1 μM) was incubated for 30 min at 30 °C with activated GST–SAPK2b/p38β (2.5 units/ml), 10 mM magnesium acetate and 100 μM [γ-^{32}P]ATP in 50 mM Tris/HCl,

© 2004 Biochemical Society

pH 7.5, 0.1 mM EGTA, 0.1 mM sodium orthovanadate and 0.1 % (v/v) 2-mercaptoethanol, to obtain complete phosphorylation. The procedure for mapping the phosphorylation sites is detailed elsewhere [22]. The ^{32}P-labelled protein was incubated with 0.5 % (v/v) 4-vinylpyridine to alkylate cysteine residues and then subjected to SDS/PAGE. The band corresponding to ^{32}P-labelled GS was excised, digested with trypsin and chromatographed on a Vydac C_{18} column equilibrated in 0.1 (v/v) trifluoroacetic acid. The column was developed with gradients of acetonitrile in 0.1 (v/v) trifluoroacetic acid: 0–30 % acetonitrile (0–90 min), 30–50 % acetonitrile (90–110 min) and 50–100 % acetonitrile (110–120 min). The flow rate was 0.8 ml/min. Fractions of 0.4 ml were collected and the ^{32}P radioactivity was determined by Čerenkov counting. Briefly, sites of phosphorylation within the peptides were determined by solid-phase Edman sequencing of peptides coupled to a Sequelon-arylamine membrane. The ^{32}P radioactivity released after each cycle of Edman degradation was counted.

RESULTS

SAPK2b/p38β interacts with GS

In order to identify new proteins that interact with SAPK/p38, recombinant GST-fused forms of SAPK2a/p38α, SAPK2b/p38β, SAPK3/p38γ and SAPK4/p38δ were incubated with mouse skeletal muscle extracts. The extracts first had been preincubated with GSH–Sepharose, as described in the Materials and methods section. GST–SAPK/p38s were pulled down with GSH–Sepharose and the pellets were subjected to electrophoresis on a polyacrylamide gel, which was stained with colloidal Coomassie Blue (Figure 1A). GST–SAPK/p38 isoforms not incubated with lysate were also electrophoresed as controls (Figure 1A). Several bands were observed which were present in both GST and GST–SAPK/p38 pellets; however, only one band, of ~85 kDa, was present in the GST–SAPK2b/p38β pellets but absent from the rest of the samples (Figure 1A). The identity of this band was established by tryptic peptide mass-spectral fingerprinting procedures (20/30 peptide matches; sequence coverage 44 %) as the protein GS (NCBI accession number P54859). The specific association of muscle GS with GST–SAPK2b/p38β was also confirmed by immunoblotting with anti-GS antibody (Figure 1B). GS was also present in GST–SAPK2b/p38β precipitates when similar experiments were carried out with brain and liver extracts (Figure 1B).

SAPK2b/p38β phosphorylates GS *in vitro*

We then examined whether GS is an *in vitro* substrate for SAPK2b/p38β. As shown in Table 1, studies of initial rates of phosphorylation showed that GS was a good substrate for SAPK2b/p38β. SAPK2a/p38α also phosphorylated GS, although at a lower rate, whereas SAPK3/p38γ and SAPK4/p38δ phosphorylated GS less well (Table 1).

At higher concentrations (2.5 units/ml; see the Materials and methods section), SAPK2b/p38β phosphorylated GS purified from rabbit skeletal muscle with a stoichiometry of more than 2 mol of phosphate/mol of protein (results not shown). The GS phosphorylated by SAPK2b/p38β was then digested with trypsin and the resulting peptides were chromatographed on a Vydac C_{18} column. Five major peaks of ^{32}P radioactivity, termed P1–P5, were observed (Figure 2A). A sixth peak of radioactivity was also observed eluting at a high concentration of acetonitrile (Figure 2A; indicated by *). The radioactivity of this peak was due to phosphorylation on residue Ser^{7} (results not shown) by a contaminant kinase in the GS purification, since it was also present when samples of GS were incubated with [γ-^{32}P]ATP in the absence of SAPK2b/p38β (upper panel in Figure 2A). The minor peak P1 contained a peptide corresponding to residues 650–663 of GS phosphorylated at Ser^{652} (Figure 2B). Peaks P2 (residues 638–649) and

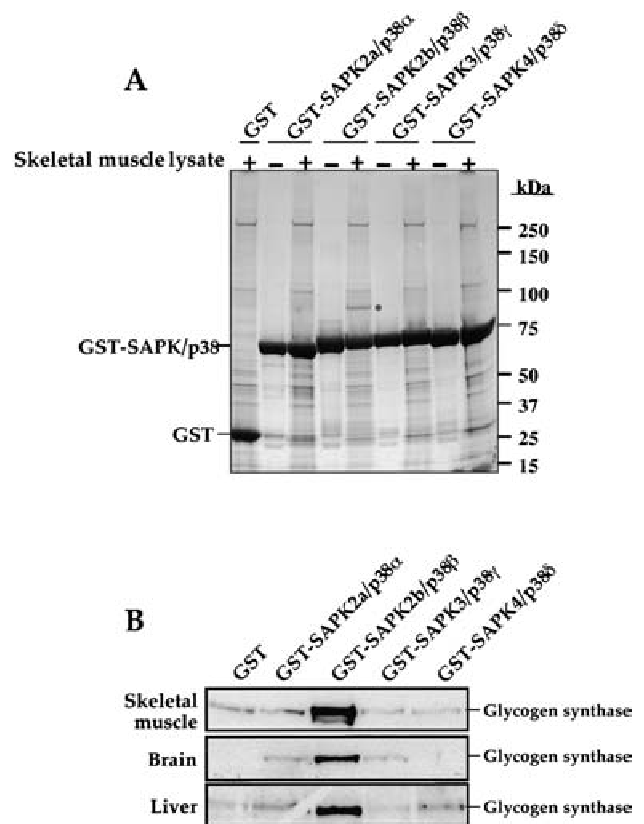

Figure 1 Association of SAPK2b/p38β with GS

(**A**) Lysates from mouse skeletal muscle were incubated first with GSH–Sepharose to remove all proteins that bind to the beads, as described in the Materials and methods section. After centrifugation, the resulting supernatants (skeletal muscle lysates) were incubated with the different GST–SAPK/p38s or GST alone. GST–proteins were pulled down with the GSH–Sepharose beads, and the pellets were washed twice with buffer A and then electrophoresed on a polyacrylamide gel. The protein bands were visualized following colloidal Coomassie Blue staining. As a control, recombinant proteins alone were also electrophoresed. A protein band unique for SAPK2b/p38β is indicated (*). The protein band was excised from the gel and digested in-gel with trypsin, and its identity was determined by tryptic peptide mass-spectral fingerprinting. (**B**) SAPK2b/p38β interacts with GS from different tissues. Lysates prepared as in (**A**) from skeletal muscle, brain or liver were incubated with GST alone or GST–SAPK/p38s, the recombinant proteins were pulled down with GSH–Sepharose beads and the pellets were immunoblotted with an anti-GS antibody.

Table 1 Comparison of substrate specificities of different SAPK/p38 family members

Each enzyme was present at 1 unit/ml, and the substrate concentration was 1 μM. Assays were carried out under initial-rate conditions, as described in the Materials and methods section. Phosphorylation is relative to that of MBP (100 %).

	Phosphorylation relative to MBP (%)	
Kinase	GS	α1-Syntrophin
SAPK2b/p38β	100 ± 2	12 ± 4
SAPK2a/p38α	52 ± 2	10 ± 2
SAPK3/p38γ	9 ± 4	100 ± 8
SAPK4/p38δ	24 ± 3	40 ± 5

© 2004 Biochemical Society

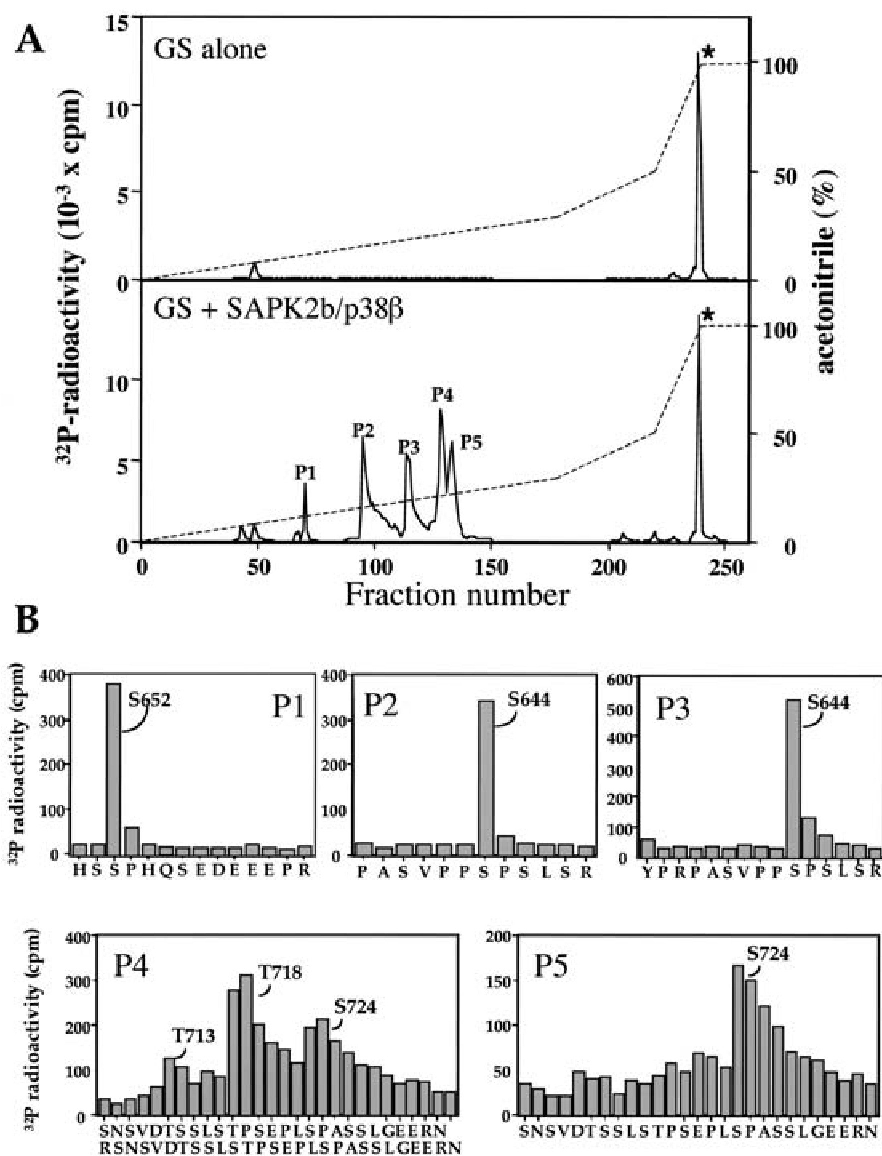

Figure 2 Identification of residues in GS phosphorylated by SAPK2b/p38β

(**A**) GS from rabbit skeletal muscle was incubated for 30 min at 30 °C with Mg[γ-^{32}P]ATP in the absence (upper panel) or in the presence (lower panel) of 2.5 units/ml SAPK2b/p38β and then subjected to SDS/PAGE. The phosphorylated GS was excised from the gel, digested with trypsin and the peptides separated by chromatography on a Vydac C_{18} column. The column was developed with an acetonitrile gradient (broken line), and ^{32}P radioactivity is shown (solid line). Phosphopeptides P1 to P5 and (*) are indicated (see the text). (**B**) The major peaks (P1–P5) of ^{32}P radioactivity were analysed by MALDI-TOF and Edman degradation as described in the Materials and methods section.

P3 (residues 634–649) both contained peptides mono-phosphorylated at residue Ser^{644}. Peak P4 was a mixture of three phosphopeptides, corresponding to residues 708–733, 708–734 and 707–734. MS (mass spectrometry) confirmed the presence of diphosphopeptides. The phosphorylated amino acids were Thr^{718} and Ser^{724}; a trace of phosphorylation on Thr^{713} was also observed. Peak P5 contained a peptide mono-phosphorylated at residue Ser^{724} (Figure 2B).

Phosphorylation of GS by SAPK2a/p38α, SAPK3/p38γ or SAPK4/p38δ was also analysed by chromatography on a Vydac C_{18} column after tryptic digestion. The same five peptides phosphorylated by SAPK2b/p38β were seen, although the proportions varied. The pattern with SAPK2a/p38α was similar to that with SAPK2b/p38β, except that there was only a trace of phosphorylation of P1. SAPK3/p38γ and SAPK4/p38δ phosphorylated P5 to a greater extent (Figure 3).

Phosphorylation of GS by SAPK/p38s in cells

To examine whether GS is phosphorylated *in vivo* under conditions where SAPK/p38s are activated, we decided to study the phosphorylation at Ser^{644}, since this residue was phosphorylated to the highest levels by SAPK2b/p38β *in vitro*, and it has been shown, together with residue Ser^{640}, to be the most important for regulation of GS activity [25]. HEK293 cells were transfected with GST–GS and HA–SAPK2a/p38α or HA–SAPK2b/p38β constructs, exposed to osmotic stress (0.5 M sorbitol) to trigger the activation of both SAPK/p38s, and the phosphorylation of GS was analysed using the phospho-Ser^{644} antibody. The SAPK2/p38s were both activated after sorbitol treatment, and GS became phosphorylated at Ser^{644} (Figure 4A). This phosphorylation on Ser^{644} was prevented by incubating the cells with the compound SB 203580 (Figure 4A), indicating that

© 2004 Biochemical Society

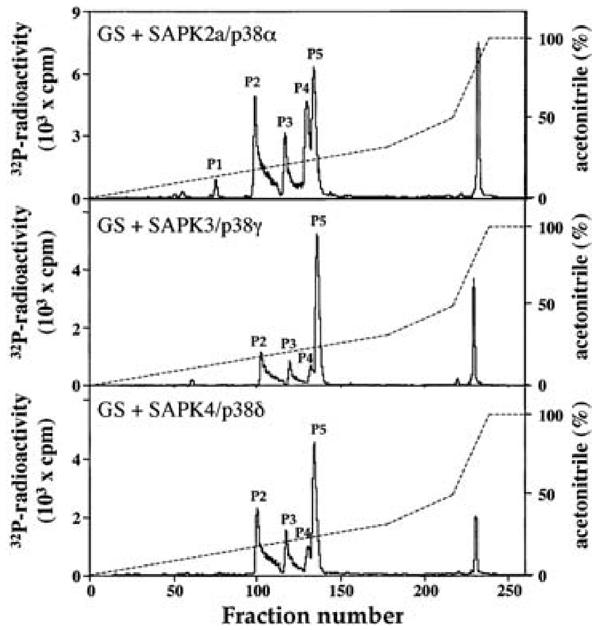

Figure 3 Phosphorylation of GS by different SAPK/p38 isoforms

GS from rabbit skeletal muscle was incubated for 30 min at 30 °C with Mg[γ-^{32}P]ATP in the presence of 2.5 units/ml SAPK2a/p38α, SAPK3/p38γ or SAPK4/p38δ, and subjected to SDS/PAGE. The phosphorylated GS was excised from the gel, digested with trypsin and the peptides separated by chromatography on a Vydac C_{18} column. The column was developed with an acetonitrile gradient (broken line), and ^{32}P radioactivity is shown (solid line). Phosphopeptides P1–P5 are indicated.

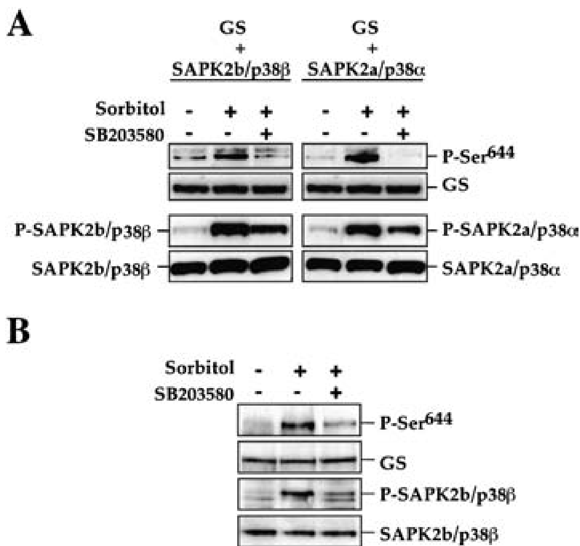

Figure 4 Phosphorylation of GS by SAPK/p38 in HEK293 cells and C2C12 myotubes

(**A**) After transfection of HEK293 cells with plasmids encoding GST–GS and HA–SAPK2a/p38α or HA–SAPK2b/p38β, the cells were incubated for 1 h with or without 10 μM SB 203580 and then exposed for 15 min to 0.5 M sorbitol. GS was immunoprecipitated from 0.5 mg of cell lysate with anti-GS antibody, denatured, electrophoresed and immunoblotted using an antibody that recognizes GS phosphorylated at Ser^{644} (P-Ser^{644}) and an antibody that recognizes unphosphorylated and phosphorylated GS equally. To examine SAPK/p38 activation, 50 μg of cell lysate was used in the immunoblot. The SAPK2a/p38α phospho-specific antibody also recognizes phosphorylated SAPK2b/p38β. (**B**) C2C12 myotubes were incubated for 1 h with or without 10 μM SB 203580 and then exposed for 15 min to 0.5 M sorbitol. GS and SAPK2b/p38β were immunoprecipitated from 0.5 mg of cell lysate as in (**A**) (see the Materials and methods section). Total SAPK2a/p38α and SAPK2b/p38β were identified using an anti-HA antibody in (**A**), and anti-SAPK2a/p38α or anti-SAPK2b/p38β antibodies in (**B**).

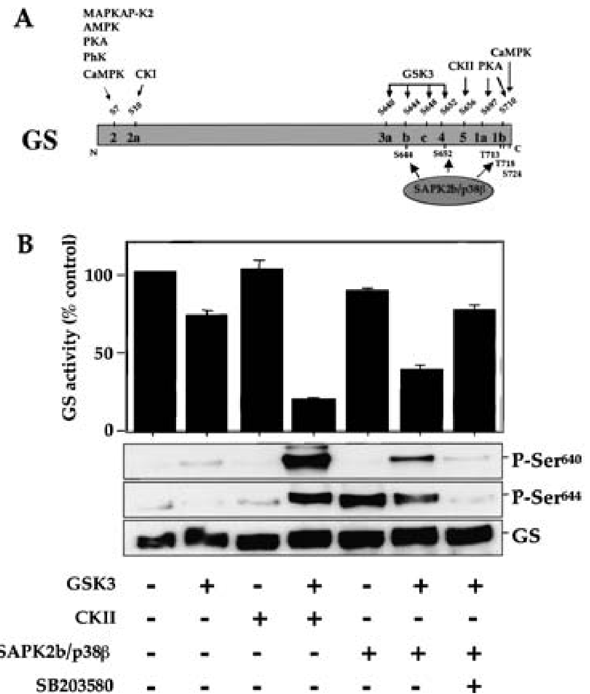

Figure 5 Inhibition of GS activity by phosphorylation

(**A**) Phosphorylation sites on rabbit skeletal muscle GS and the protein kinases that phosphorylate them *in vitro*. MAPKAP-K2, MAPK-activated protein kinase 2; AMPK, AMP-dependent kinase; PKA, protein kinase A; PhK, phosphorylase kinase; CaMPK, Ca^{2+}/calmodulin-dependent protein kinase. (**B**) GS from rabbit skeletal muscle or GST–GS was incubated for 30 min with 150 m-units/ml of the protein phosphatase PP1γ, prior to incubation for another 30 min with 0.4 unit/ml CK2 (CKII) or 1 unit/ml SAPK2b/p38β in the absence or presence of 10 μM SB 203580, and then for 30 min with 1 unit/ml GSK3, as indicated. GS activity was assayed as described in the Materials and methods section or immunoblotting was performed using antibodies that recognize GS phosphorylated at Ser^{640} or at Ser^{644}, or an antibody that recognizes both phosphorylated and unphosphorylated GS. Results in (**B**) are means $\pm$ S.E.M. for at least triplicate activity determinations from three experiments.

SAPK2a/p38α and/or SAPK2b/p38β phosphorylate GS under these conditions.

We also examined the phosphorylation of endogenous GS at Ser^{644} in the skeletal muscle cell line C2C12. Cells were differentiated to myotubes as described in the Materials and methods section [21], and exposed to osmotic shock as before. The phosphorylation of endogenous immunoprecipitated GS was analysed using the phospho-Ser^{644} antibody. GS was phosphorylated at Ser^{644} (Figure 4B), and this phosphorylation was prevented by incubating the cells with SB 203580 (Figure 4B), indicating that SAPK2a/p38α and/or SAPK2b/p38β phosphorylate endogenous GS under these conditions in myotubes.

Inactivation of GS by phosphorylation

The activity of GS is controlled by multisite phosphorylation and the binding of allosteric ligands [23,24]. Phosphorylation leads to inactivation of GS [17]. In mammalian skeletal muscle GS, phosphorylation sites are located at both the N- and C-termini of the molecule (Figure 5A). Of the seven C-terminal phosphorylation sites, Ser^{640} (site 3a) and Ser^{644} (site 3b) are thought to be the most important for regulation of GS [25]. At the N-terminus, both Ser^{7} (site 2) and Ser^{10} (site 2a) influence GS activity [17,26]. *In vitro*, GSK3 phosphorylates, sequentially, sites 4 (Ser^{652}), 3c (Ser^{648}), 3b (Ser^{644}) and 3a (Ser^{640}) in GS, but recognition of site 4 by GSK3 requires that GS has first been phosphorylated at site 5 (Ser^{656}) by the protein kinase CK2 [27].

© 2004 Biochemical Society

Two of the sites phosphorylated by SAPK2b/p38β, Ser[644] and Ser[652], are also phosphorylated by GSK3 (Figure 5A). In order to study the effect of phosphorylation by SAPK2b/p38β on GS activity, the GS preparation was chromatographed on Mono-Q as described in the Materials and methods section, which resolved GS from the co-purifying protein kinase that phosphorylates site 2 (Figure 2A), thought to be AMP-dependent kinase [20]. Fractions containing GS, and not the contaminating kinase, were then treated with the protein phosphatase PP1γ to completely dephosphorylate GS. The dephosphorylated GS was then phosphorylated with different kinases (Figure 5B). Under the conditions examined, GSK3 alone inhibited GS activity by approx. 30 %, whereas CK2 or SAPK2b/p38β alone had no effect or a very small effect respectively on GS activity (Figure 5B). However, when GS was first phosphorylated by CK2 or SAPK2b/p38β, subsequent incubation with GSK3 decreased GS activity by 80 % and 60 % respectively (Figure 5B). The decrease in GS activity caused upon phosphorylation by SAPK2b/p38β followed by GSK3 was abolished when the SAPK2b/p38β inhibitor SB 203580 was present.

We examined the phosphorylation state of residues Ser[640] and Ser[644] using phospho-specific antibodies. The specificity of these antibodies was established by the finding that they only recognized GS after it had been phosphorylated *in vitro* by SAPK2b/p38β and GSK3, and did not recognize the non-phosphorylated form of GS (Figures 4 and 5B). Furthermore, the recognition of phosphorylated GS was abolished when the antibody was incubated with the phosphopeptide used to raise it, but not the non-phosphorylated form of this peptide or the phosphopeptide corresponding to the other phosphorylation site (results not shown). We found that SAPK2b/p38β phosphorylated Ser[644] only, and that phosphorylation of Ser[640] occurred following incubation with GSK3 when GS had been phosphorylated first at Ser[644] by CK2 or SAPK2b/p38β (Figure 5B). However, when SAPK2b/p38β was preincubated with the compound SB 203580, which specifically inhibits its activity [3], phosphorylation at both residues was blocked (Figure 5B). GS was not significantly phosphorylated by CK2 or GSK3 alone (Figure 5B).

DISCUSSION

In the present study, we show that endogenous GS from mouse skeletal muscle, liver and brain interacted specifically with GST–SAPK2b/p38β, but not with other members of the group of SAPK/p38s. We also show that GS was phosphorylated *in vitro* by all SAPK/p38s, although SAPK2b/p38β and SAPK2a/p38α were more efficient than SAPK3/p38γ and SAPK4/p38δ. SAPK2b/p38β phosphorylated GS in the C-terminal part of the molecule, at residues Ser[644], Ser[652], Thr[713], Thr[718] and Ser[724]. Two of these sites, Ser[644] and Ser[652], are also phosphorylated by GSK3, whereas Thr[713], Thr[718] and Ser[724] are sites that were not known previously to be phosphorylated. Interestingly, residue Thr[713] on GS that is phosphorylated by SAPK2b/p38β is not followed by a proline residue, although nearly all MAPK family members are highly specific for serine and threonine residues that precede a proline residue. This is not the first time that this has been observed, since SAPK2a/p38α and ERK5, another member of the MAPK family, have also been shown to phosphorylate serine and threonine residues that are not followed by a proline [28,29]. Phosphorylation at Thr[713] was very weak compared with phosphorylation at residues Thr[718] and Ser[724] in the same phosphopeptide (Figure 2B). This phosphorylation of GS was not due to MKK6 (MAPK/ERK kinase 6), the enzyme used to activate GST–SAPK/p38s, since no phosphopeptides were found in control samples where GS was incubated with active MKK6 under phosphorylating conditions and then chromatographed on a Vydac C_{18} column (results not shown).

Using phospho-specific antibodies that recognize phosphorylated Ser[644], we showed that endogenous GS from the skeletal muscle cell line C2C12 became phosphorylated at that residue in response to osmotic shock. This phosphorylation was prevented by pretreatment of the cells with SB 203580, indicating that SAPK2a/p38α and/or SAPK2b/p38β are responsible for this phosphorylation *in vivo*. The generation and exploitation of new phospho-specific antibodies that recognize the other sites phosphorylated specifically by SAPK/p38s will be required in order to investigate whether these phosphorylations occur *in vivo*.

GS can be inactivated by sequential phosphorylation at the C-terminal residues site 4 (Ser[652]), site 3c (Ser[648]), site 3b (Ser[644]) and site 3a (Ser[640]) catalysed by GSK3. Effective recognition of GS by GSK3 occurs only after the phosphorylation of Ser[656] (site 5) by CK2 [30–32]. In mammalian skeletal muscle GS, phosphorylation sites are located at both the N- and C-termini of the molecule. At the N-terminus, phosphorylation of both Ser[7] (site 2) and Ser[10] (site 2a) decrease GS activity [17,26]. Of the seven C-terminal phosphorylation sites, sites 3a and 3b are thought to be the most important for regulation of GS [25]. It has been shown that mutation of the phosphorylation sites of GS to alanine residues, in particular site 2 combined with either site 3a or 3b, prevents the inactivation of transfected GS in transfected COS cells. In contrast, single mutations at each of the known sites or a multiple mutation of sites 3a, 3b and 3c has only a partial effect [24,25].

On the other hand, it has been inferred that sites 3a and 3b of GS must also be phosphorylated directly by as yet unidentified protein kinases [24,32], because mutation of site 5, or the combined mutation of sites 3c, 4 and 5, barely affected the ability of sites 3a and 3b to be phosphorylated in COS cells transfected with GS [25,26]. Therefore it was proposed that, in COS cells and some other cultured cells, another priming kinase must act at site 3b to enable site 3a to be phosphorylated by GSK3. The identity of the putative site 3b priming kinase is unknown, although a ‘proline-directed’ protein kinase(s) must be involved, since mutation of Pro[645] to alanine eliminates phosphorylation at site 3b [33]. We show here that one of the five residues of GS phosphorylated by SAPK2b/p38β was site 3b, and that this residue was phosphorylated more efficiently than any of the other four (Figure 3A). Phosphorylation of GS by SAPK2b/p38β alone had no effect on its activity, demonstrating for the first time that phosphorylation of site 3b does not inactivate GS directly. However, when it was phosphorylated first by SAPK2b/p38β and then by GSK3, the activity of GS decreased to a much greater extent. This decrease was not observed when SAPK2b/p38β activity was blocked by its inhibitor, SB 203580. These results suggest that phosphorylation of GS by SAPK2b/p38β provides the priming phosphate(s) that allows GSK3 to phosphorylate other residues, which causes partial inactivation of GS activity. They also suggest that SAPK/p38 could be the unknown kinase that phosphorylates site 3b *in vivo* and perhaps site 4 under certain conditions.

A substantial number of studies have investigated the mechanism by which GS activity is regulated by hormones [24]. In contrast, very little is known about the cellular events leading to changes in GS activity following exercise or skeletal muscle contraction. GS is regulated by both allosteric factors (primarily glucose 6-phosphate) and reversible phosphorylation. Exercise activates both stimulatory and inhibitory regulators of GS, and it is thought that the resultant activity of GS during exercise depends on the relative strength of opposing signals [34]. It is now well established that exercise or muscle contractile activity can activate

© 2004 Biochemical Society

three of the MAPK signalling pathways, including ERK1/2, JNK (c-Jun N-terminal kinase) and SAPK/p38 [35,36].

In summary, our results show that GS interacts specifically with SAPK2b/p38β. To our knowledge, this is the first time it has been shown that GS is phosphorylated by this kinase *in vitro* and in cells, and that this promotes subsequent inactivation of GS by GSK3. Further work is needed to address the question of whether phosphorylation of GS by SAPKs could play a role in the regulation of glycogen synthesis during exercise and other stressful conditions in skeletal muscle.

We thank Philip Cohen and Tricia Cohen for helpful advice, Moustapha Aoubala [Division of Signal Tranduction Therapy (DSTT), University of Dundee] for preparation of antibodies, Mark Peggie and Rachel Toth for the GST–GS DNA, James Hastie for help with the purification of GS from rabbit skeletal muscle, and Leanne Brown for help in culturing the cells. All SAPK/p38s, GSK3 and CK2 were purified by the Protein Production Team (DSTT), co-ordinated by Hilary McLauchlan and James Hastie. The work was supported by the U.K. Medical Research Council (A. C. and Y. K.).

REFERENCES

1 Cohen, P. (1997) The search for physiological substrate of MAP and SAP kinases in mammalian cells. Trends Cell Biol. **7**, 353–361
2 Cuenda, A., Rouse, J., Doza, Y. N., Meier, R., Cohen, P., Gallagher, T. F., Young, P. R. and Lee, J. C. (1995) SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. FEBS Lett. **364**, 229–233
3 Davies, S. P., Reddy, H., Caivano, M. and Cohen, P. (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem. J. **351**, 95–105
4 Kyriakis, J. M. and Avruch, J. (2001) Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol. Rev. **81**, 807–869
5 Goedert, M., Cuenda, A., Craxton, M., Jakes, R. and Cohen, P. (1997) Activation of the novel stress-activated protein kinase SAPK4 by cytokines and cellular stresses is mediated by SKK3 (MKK6); comparison of its substrate specificity with that of other SAP kinases. EMBO J. **16**, 3563–3571
6 Eyers, P. A., Craxton, M., Morrice, N., Cohen, P. and Goedert, M. (1998) Conversion of SB 203580-insensitive MAP kinase family members to drug-sensitive forms by a single amino-acid substitution. Chem. Biol. **5**, 321–328
7 Parker, C. G., Hunt, J., Diener, K., McGinley, M., Soriano, B., Keesler, G. A., Bray, J., Yao, Z., Wang, X. S., Kohno, T. and Lichenstein, H. S. (1998) Identification of stathmin as a novel substrate for p38 delta. Biochem. Biophys. Res. Commun. **249**, 791–796
8 Knebel, A., Morrice, N. and Cohen, P. (2001) A novel method to identify protein kinase substrates: eEF2 kinase is phosphorylated and inhibited by SAPK4/p38delta. EMBO J. **20**, 4360–4369
9 Knebel, A., Haydon, C. E., Morrice, N. and Cohen, P. (2002) Stress-induced regulation of eukaryotic elongation factor 2 kinase by SB 203580-sensitive and -insensitive pathways. Biochem. J. **367**, 525–532
10 Cuenda, A., Cohen, P., Buee-Scherrer, V. and Goedert, M. (1997) Activation of stress-activated protein kinase-3 (SAPK3) by cytokines and cellular stresses is mediated via SAPKK3 (MKK6); comparison of the specificities of SAPK3 and SAPK2 (RK/p38). EMBO J. **16**, 295–305
11 Goedert, M., Hasegawa, M., Jakes, R., Lawler, S., Cuenda, A. and Cohen, P. (1997) Phosphorylation of microtubule-associated protein tau by stress-activated protein kinases. FEBS Lett. **409**, 57–62
12 Hasegawa, M., Cuenda, A., Spillantini, M. G., Thomas, G. M., Buee-Scherrer, V., Cohen, P. and Goedert, M. (1999) Stress-activated protein kinase-3 interacts with the PDZ domain of alpha1-syntrophin. A mechanism for specific substrate recognition. J. Biol. Chem. **274**, 12626–12631
13 Buée-Scherrer, V. and Goedert, M. (2002) Phosphorylation of microtubule-associated protein tau by stress-activated protein kinases in intact cells. FEBS Lett. **515**, 151–154
14 Biondi, R. M. and Nebreda, A. R. (2003) Signalling specificity of Ser/Thr protein kinases through docking-site-mediated interactions. Biochem. J. **372**, 1–13
15 Bain, J., McLauchlan, H., Elliott, M. and Cohen, P. (2003) The specificities of protein kinase inhibitors: an update. Biochem. J. **371**, 199–204
16 Nimmo, H. G., Proud, C. G. and Cohen, P. (1976) The purification and properties of rabbit skeletal muscle glycogen synthase. Eur. J. Biochem. **68**, 21–30
17 Nakielny, S., Campbell, D. G. and Cohen, P. (1991) The molecular mechanism by which adrenalin inhibits glycogen synthesis. Eur. J. Biochem. **199**, 713–722
18 Nimmo, H. G. and Cohen, P. (1974) Glycogen synthetase kinase 2 (GSK 2); the identification of a new protein kinase in skeletal muscle. FEBS Lett. **47**, 162–166
19 Cohen, P., Yellowlees, D., Aitken, A., Donella-Deana, A., Hemmings, B. A. and Parker, P. J. (1982) Separation and characterisation of glycogen synthase kinase 3, glycogen synthase kinase 4 and glycogen synthase kinase 5 from rabbit skeletal muscle. Eur. J. Biochem. **124**, 21–35
20 Hojlund, K., Staehr, P., Hansen, B. F., Green, K. A., Hardie, D. G., Richter, E. A., Beck-Nielsen, H. and Wojtaszewski, J. F. (2003) Increased phosphorylation of skeletal muscle glycogen synthase at NH2-terminal sites during physiological hyperinsulinemia in type 2 diabetes. Diabetes **52**, 1393–1402
21 Cuenda, A. and Cohen, P. (1999) Stress-activated protein kinase-2/p38 and a rapamycin-sensitive pathway are required for C2C12 myogenesis. J. Biol. Chem. **274**, 4341–4346
22 Campbell, D. G. and Morrice, N. A. (2002) Identification of protein phosphorylation sites by a combination of mass spectrometry and solid phase Edman sequencing. J. Biomol. Tech. **13**, 119–130
23 Cohen, P. (1986) Muscle glycogen synthase. Enzymes 3rd edn. **17A**, 461–497
24 Lawrence, J. C. and Roach, P. J. (1997) New insights into the role and mechanism of glycogen synthase activation by insulin. Diabetes **46**, 541–547
25 Skurat, A. V. and Roach, P. J. (1995) Phosphorylation of sites 3a and 3b (Ser640 and Ser644) in the control of rabbit muscle glycogen synthase. J. Biol. Chem. **270**, 12491–12497
26 Skurat, A. V., Wang, Y. and Roach, P. J. (1994) Rabbit skeletal muscle glycogen synthase expressed in COS cells. Identification of regulatory phosphorylation sites. J. Biol. Chem. **269**, 25534–25542
27 Cohen, P. and Frame, S. (2001) The renaissance of GSK3. Nat. Rev. Mol. Cell. Biol. **2**, 769–776
28 Cheung, P. C., Campbell, D. G., Nebreda, A. R. and Cohen, P. (2003) Feedback control of the protein kinase TAK1 by SAPK2a/p38alpha. EMBO J. **22**, 5793–5805
29 Mody, N., Campbell, D. G., Morrice, N., Peggie, M. and Cohen, P. (2003) An analysis of the phosphorylation and activation of extracellular-signal-regulated protein kinase 5 (ERK5) by mitogen-activated protein kinase kinase 5 (MKK5) *in vitro*. Biochem. J. **372**, 567–575
30 Picton, C., Aitken, A., Bilham, T. and Cohen, P. (1982) Multisite phosphorylation of glycogen synthase from rabbit skeletal muscle. Organisation of the seven sites in the polypeptide chain. Eur. J. Biochem. **124**, 37–45
31 DePaoli-Roach, A. A., Ahmad, Z., Camici, M., Lawrence, Jr, J. C. and Roach, P. J. (1983) Multiple phosphorylation of rabbit skeletal muscle glycogen synthase. Evidence for interactions among phosphorylation sites and the resolution of electrophoretically distinct forms of the subunit. J. Biol. Chem. **258**, 10702–10709
32 Zhang, W., DePaoli-Roach, A. A. and Roach, P. J. (1993) Mechanisms of multisite phosphorylation and inactivation of rabbit muscle glycogen synthase. Arch. Biochem. Biophys. **304**, 219–225
33 Skurat, A. V. and Roach, P. J. (1996) Multiple mechanisms for the phosphorylation of C-terminal regulatory sites in rabbit muscle glycogen synthase expressed in COS cells. Biochem. J. **313**, 45–50
34 Nielsen, J. N. and Richter, E. A. (2003) Regulation of glycogen synthase in skeletal muscle during exercise. Acta Physiol. Scand. **178**, 309–319
35 Haydon, C. E., Watt, P. W., Morrice, N., Knebel, A., Gaestel, M. and Cohen, P. (2002) Identification of a phosphorylation site on skeletal muscle myosin light chain kinase that becomes phosphorylated during muscle contraction. Arch. Biochem. Biophys. **397**, 224–231
36 Sakamoto, K. and Goodyear, L. J. (2002) Intracellular signaling in contracting skeletal muscle. J. Appl. Physiol. **93**, 369–383

Received 10 October 2003/10 December 2003; accepted 17 December 2003
Published as BJ Immediate Publication 17 December 2003, DOI 10.1042/BJ20031559

© 2004 Biochemical Society