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Glycogen- and PP1c-targeting Subunit G_M Is Phosphorylated at Ser^{48} by Sarcoplasmic Reticulum-bound Ca^{2+}-Calmodulin Protein Kinase in Rabbit Fast Twitch Skeletal Muscle*

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Roberta Sacchetto‡§¶, Elisa Bovo‡, Arianna Donella-Deana‖, and Ernesto Damiani‡**

From the Departments of ‡Experimental Biomedical Sciences and ‖Biochemistry, University of Padova, viale Giuseppe Colombo 3, 35121 Padova, Italy and the §Department of Experimental and Diagnostic Medicine, University of Ferrara, via Luigi Borsari, 46, 44100 Ferrara, Italy

Multifunctional Ca^{2+}-calmodulin-dependent protein kinase (CaMKII) is a Ser/Thr protein kinase uniformly distributed within the sarcoplasmic reticulum (SR) of skeletal muscle. In fast twitch muscle, no specific substrates of CaMKII have yet been identified in nonjunctional SR. Previous electron microscopy data showed that glycogen particles containing glycogen synthase (GS) associate with SR at the I band level. Furthermore, recent evidence implicates CaMKII in regulation of glucose and glycogen metabolism. Here, we demonstrate that the glycogen- and protein phosphatase 1-targeting subunit, also known as G_M, selectively localizes to the SR membranes of rabbit skeletal muscle and that G_M and GS co-localize at the level of the I band. We further show that G_M, GS, and PP1c assemble in a structural complex that selectively localizes to nonjunctional SR and that G_M is phosphorylated by SR-bound CaMKII and dephosphorylated by PP1c. On the other hand, no evidence for a structural interaction between G_M and CaMKII was obtained. Using His-tagged G_M recombinant fragments and site-directed mutagenesis, we demonstrate that the target of CaMKII is Ser^{48}. Taken together, these data suggest that SR-bound CaMKII participates in the regulation of GS activity through changes in the phosphorylation state of G_M. Based on these findings, we propose that SR-bound CaMKII participates in the regulation of glycogen metabolism, under physiological conditions involving repetitive raises elevations of $[Ca^{2+}]_i$.

In skeletal muscle, a regulatory subunit called G_M (also known as G, R_{Gl}, R3, and PP1R3A) (1, 2) is responsible for the targeting of glycogen and PP1c (protein phosphatase 1 catalytic subunit) to the sarcoplasmic reticulum (SR)[1] (3–6). G_M is a striated muscle-specific protein expressed at higher levels in skeletal than in cardiac muscle (7, 8). G_M (glycogen- and PP1c-targeting subunit) belongs to a family of mammalian glycogen- and PP1c-binding proteins (PPP1R6, PTG, and GL) (9). G_M rabbit skeletal muscle cDNA sequence (7) predicts a protein of 1109 amino acids with a calculated M_r of 124,257. However, on SDS-PAGE, G_M displays an apparent M_r of ~160,000 (7, 10), likely because of its acidic pI. Binding sites for PP1c (aa 64–69; see Ref. 11) and glycogen (aa 150–159 in the case of rabbit G_M; see Ref. 12) have been identified in the NH_2-terminal domain of G_M. On the other hand, G_M is characterized by the unique presence, at the COOH terminus, of a stretch of hydrophobic residues (aa 1063–1097; see Ref. 7). It has been proposed that this amino acid sequence is indicative of a transmembrane helix that mediates its binding to SR membranes (7, 8). Interestingly, this transmembrane region appears to be involved in the interaction of G_M with the SERCA2-regulatory protein phospholamban (13).

A key role for G_M phosphorylation in regulation of glycogen synthase (GS) is suggested by a number of observations: (i) cAMP-dependent protein kinase (PKA) phosphorylates G_M on Ser^{48} and Ser^{67} both *in vitro* (3–5) and *in vivo* in response to adrenaline catecholamines (10, 14). Notably, both phosphorylation sites are conserved between rabbit and human G_M (7–8). Ser^{67} lies within the PP1c-binding motif of G_M (11), and PKA-dependent phosphorylation triggers dissociation of PP1c from G_M, thereby inactivating its phosphatase activity (4, 5). (ii) G_M is also phosphorylated by GSK3 at Ser^{40} and Ser^{44} (15). (iii) G_M is possibly phosphorylated by insulin-dependent protein kinase at Ser^{48} (14, 16).

Experiments using G_M null mice (17, 18) further support the possibility that G_M may be involved in regulation of GS activity. Recently, using COS7 cells and C_2C_{12} myotubes transfected with G_M, Liu and Brautigan (19) found that transfected G_M co-immunoprecipitated with endogenous GS. The interaction between G_M and GS occurs at specific sites of G_M, localized between aa 77–118 and 219–240 (19). However, thus far, there is no experimental evidence supporting a direct, structural association between G_M and GS in native SR membranes.

Here, we provide clear evidence that G_M associates with nonjunctional SR membranes, where it forms a complex with GS and PP1c. In addition, we demonstrate that G_M is phosphodephosphorylated by SR-bound CaMKII and PP1c. Using His-tagged G_M recombinant protein corresponding to the aa 40–338 sequence, we also demonstrate that endogenous, as well as exogenous CaMKII are able to phosphorylate serine residue(s) in the NH_2-terminal region of G_M. The finding that CaMKII is unable to phosphorylate both a truncated G_M (aa 69–338) lacking Ser^{48} and Ser^{67} and a point-mutated form in which Ser^{48} is replaced by Ala identifies this residue as a

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** To whom correspondence should be addressed. Tel.: 390-498276038; Fax: 390-498276040; E-mail: damiani@civ.bio.unipd.it.

[1] The abbreviations used are: SR, sarcoplasmic reticulum; CaMKII, Ca^{2+}-calmodulin protein kinase; GS, glycogen synthase; JFM, junctional face membrane of sarcoplasmic reticulum; PKA, cAMP-dependent protein kinase; SERCA, sarcoendoplasmic reticulum Ca^{2+}-ATPase; aa, amino acid(s); Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; TRITC, tetramethylrhodamine isothiocyanate.

CaMKII target in G_M. We thus suggest that Ca^{2+}-calmodulin activation of SR-bound CaMKII might play a pivotal role in the regulation of glycogen metabolism during physical exercise through phosphorylation of G_M.

EXPERIMENTAL PROCEDURES

Materials—Molecular mass standards were purchased from BDH Laboratories (Poole, UK; molecular weight range 200,000–43,000 or 77,000–12,000). [γ-^{33}P]ATP (3000–6000 Ci/mmol) was purchased from PerkinElmer Life Sciences. Exogenous Ca^{2+}-calmodulin-dependent protein kinase (500,000 units/ml) was purchased from New England Biolabs. Uridine-diphospho-D-[6-^{3}H]glucose, NH_4 salt (14.1 Ci/mmol) was purchased from Amersham Biosciences. Hog brain calmodulin was purchased from Roche Applied Science. CaMKII inhibitor KN-93 was purchased from Calbiochem (San Diego, CA). Protein kinase A from bovine heart (1–2 units/μg of protein kinase), DL-propranolol, protein A-Sepharose, and glucose assay kit were purchased from Sigma-Aldrich. Okadaic acid was purchased from Calbiochem-Novabiochem (Bad Soden, Germany). All other chemicals were analytical grade and were purchased
from Sigma-Aldrich.

Animals and Muscles—New Zealand male adult rabbits were lawfully acquired and properly housed, fed, and taken care of in the Animal Colony of the Department of Experimental Biomedical Sciences of the University of Padova in compliance with Italian Law (Decreto Legge, September 27, 1992, no. 116). The adductor magnus was used as a representative fast twitch muscle (20).

Generation and Purification of His_6-G_M Fusion Proteins—Total cellular RNA extraction from rabbit adductor and cDNA synthesis were performed as described (21). G_M cDNA was amplified to generate two forms of G_M: G_M-(40–338) and G_M-(69–338). We used an identical cloning strategy with the same reverse primer and a different forward primer. The primers were designed based on the rabbit G_M sequence (7) (GenBank™ accession number M65109) and are: 40 Forward (5′-CTC TTC ATA TGG CAC ATC ACC ATC ACC ATC ACA TTG ATG ACG ACG ACA AGT CCC CTC AAC CGA GAC GAG-3′, containing the NdeI restriction enzyme and a His_6 encoding sequences); 96 Forward (5′-CGA CAT ATG CAC CAC CAC CAC CAC CAC GTC GAC AAC TTT GGA TTC AAT-3′, containing the NdeI restriction enzyme and a His_6 encoding sequences); and 338 Reverse (5′-CTT CCT GAA TTC CTA TTC AGA AGC AGC ACA TCT AG-3′, containing the EcoRI restriction enzyme sequence). The first set of primers amplifies the region that encodes for a 299-aa fragment protein (NH_2-terminal residues from aa 40 to 338). The second, amplifies the region from aa 69 to 338. PCR conditions were as follow: 94 °C for 3 min, 94 °C for 1 min, 65 °C for 1 min, and 72 °C for 2.5 min for 5 cycles followed by 30 cycles of 94 °C for 1 min, 57 °C for 1 min, and 72 °C for 2.5 min. The extension step was performed using the *Pfu* DNA polymerase by Promega. The PCR products, purified using a Qiagen kit, were digested with NdeI and EcoRI enzymes and cloned into the pET 21a+ vector (Novagen). The amplified mutated vector was used for heat-pulsing transformation of Novablue cells. Vector extraction was performed using a QIAprep Miniprep kit (Qiagen) After confirmation of the sequences, the plasmid was heat pulse transferred into the *Escherichia coli* strain BL21 (DE). Cultures were grown at 37 °C in a medium containing ampicillin. When cultures reached an A_{550} of 0.5 units, the His-tagged fusion protein fragments were induced with 0.5 mM isopropyl β-D-thiogalactopyranoside for 3 h at 37 °C. His-tagged protein fragments were purified from bacterial lysate by nickel-nitrilotriacetic acid affinity chromatography (Qiagen) according to the manufacturer's instructions. Purified protein was quantified by densitometry of Coomassie Blue-stained gels, with reference to a calibration curve of bovine serum albumin (0.5–10 μg).

Site-directed Mutagenesis—Alanine substitution in G_M-(40–338) was generated by introducing a mutation with the use of the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's specifications. The mutagenic primers were designed complementary to opposite strands of the vector and containing two bases mismatched (bold letters) in correspondence of serine 48 to obtain alanine. They were as follow: sense primer 5′-CCG AGC AGA CGA GGT **GC**G GAA TCT TCT GAA GAG GTC-3′; and antisense primer 5′-GAC CTC TTC AGA AGA TTC **CG**C ACC TCG TCT GCT CGG-3′. PCR conditions were as follows: 1 cycle of 95 °C for 30 s and 16 cycles of 95 °C for 30 s, 65 °C for 1 min, and 72 °C for 7 min. The amplified mutated vector was used for heat-pulsing transformation of Novablue cells. Vector extraction was performed using a QIAprep Miniprep kit (Qiagen). Mutation was confirmed by DNA sequencing of the region containing the mutation. The cloning strategy of the mutated G_M-(40–338)-S48A was identical of that described under "Purification of His-G_M Fusion Proteins. Mutated His tag G_M-(40–338)-S48A was purified from bacterial lysate by nickel-nitrilotriacetic acid affinity chromatography (Qiagen) according to the manufacturer's instructions.

Preparative Procedures—To avoid β-adrenergic stimulation and to maximize association of glycogen to SR, rabbits were anesthetized with Zoletil 100 (10 mg/kg per vena) and injected intravenously with propranolol (3 mg/kg), as described by Walker *et al.* (14). After 10 min, the animals were killed by intravenous injection of 1.5 ml of Tanax (Intervet International B.V., Boxmee, The Netherlands) in accordance with procedure approved by the Animal Committee of the University of Padova. Bilateral adductor muscles (a representative fast twitch muscle) were homogenized in 0.3 M sucrose (20% weight/volume), 5 mM imidazole, pH 7.0, 1 mM EGTA, 1 μg/ml leupeptin, and 100 μM phenylmethylsulfonyl fluoride. Muscle microsomes enriched in content of nonjunctional SR were isolated by the method of Chu *et al.* (22) by centrifuging muscles homogenates at 7700 × *g* for 10 min. The post-myofibrillar supernatant was recentrifuged for 20 min at 15,000 × *g*. The final post-mitochondrial supernatant was centrifuged at 150,000 × *g* for 90 min to obtain soluble cytoplasm and a microsomal fraction. SR membranes were further purified by isopycnic sucrose density centrifugation (23) to yield four distinct fractions, labeled F1–F4 from top to bottom of gradients. The Ca^{2+} pump membrane and the junctional face membrane (JFM) were dissociated by incubating F4 membranes with 0.25% detergent Chaps at low Ca^{2+} and centrifuging, as described (24). Ca^{2+} pump membrane corresponded to the solubilized material. Crude JFM was further purified by the method of Sacchetto *et al.* (25). The membrane fractions were resuspended in 0.3 M sucrose, 5 mM imidazole, pH 7.4, 100 μM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, divided in aliquots, and stored at −80 °C, until used. Protein concentration was determined by the method of Lowry *et al.* (26), using bovine serum albumin as a standard.

Measurement of Glycogen—Glycogen content of membrane fractions was measured under the general conditions described by Gomez-Lechon *et al.* (27) and Lees *et al.* (28). Membranes resuspended (0.5–1 mg/ml) in 0.2 M sodium-acetate buffer, pH 4.8, were incubated at 40 °C with 1750 milliunits/100 μl of amyloglucosidase. At the end of 2 h of incubation, distilled water was added to the assay mixture up to final volume of 1 ml and neutralized with 5.5 μl of 1 N NaOH. Glucose liberated after enzymatic hydrolysis of glycogen was determined by a colorimetric method, using a glucose assay kit (Sigma). A blank of the reaction was performed by incubating SR membranes without amyloglucosidase. A calibration curve was constructed with known amounts of glycogen.

Assay of Glycogen Synthase Activity—GS activity was assayed at 37 °C, by the method of Thomas *et al.* (29), measuring the incorporation of [^{3}H]glucose from UDP[^{3}H]glucose into glycogen. Membranes (final protein concentration, 0.4 mg/ml) were incubated in duplicate in a standard assay mixture (final volume, 50 μl), containing 50 mM Tris-HCl, pH 7.8, 10 mM EDTA, 50 mM NaF, 15 mg/ml glycogen, 2 μM okadaic acid, 10 mM UDP[^{3}H]glucose (specific activity, 0.15 μCi/μmol), and 10 mM glucose 6-phosphate. At the end of 2 min of incubation, the assay mixture was spotted on Whatman C glass filters. The filters were washed twice for 30 min with ice-cold 70% ethanol (10 ml/filter) and dried. [^{3}H]Glucose incorporated into glycogen was measured by liquid scintillation. Background radioactivity was measured in the absence of the enzyme. Zero time controls were prepared by mixing membranes and test mixture on ice and immediately spotting the assay mixture on filters.

Phosphorylation Assays—CaMKII phosphorylation assay: Phosphorylation of SR proteins and of G_M-(40–338) by endogenous CaMKII was carried out, as described previously (21), using a standard medium containing 100 μM free Ca^{2+} and 1 μM CaM, unless otherwise stated. Incubation was at 30 °C for 30 min. Phosphorylation by exogenous CaMKII of His-tagged G_M recombinant fragments was carried out using a commercially available CaMKII (New England Biolabs). Prior to substrate phosphorylation, CaMKII was activated by incubation with ATP/Mg^{2+} in the presence of $CaCl_2$ and calmodulin as indicated by the manufacturer. Activated CaMKII enzyme was then added to the assay mixture (250 units/50 μl) containing substrate proteins. Composition of phosphorylation assay medium was identical to that used for endogenous phosphorylation. Incubation was at 30 °C for 30 min.

PKA Phosphorylation Assay—Phosphorylation by exogenous PKA of SR membranes and of His-tagged G_M fusion proteins was carried out at 30 °C as described by Damiani *et al.* (30), except that the incubation time was 30 min. Incubation medium contained 5 μM cAMP (final concentration) and 2.6 μM PKA (5–10 units/50 μl). All of the phosphorylation reactions were started by the addition of 50 μM [γ-^{33}P]ATP (specific radioactivity, 0.10 Ci/mmol) and were terminated by adding

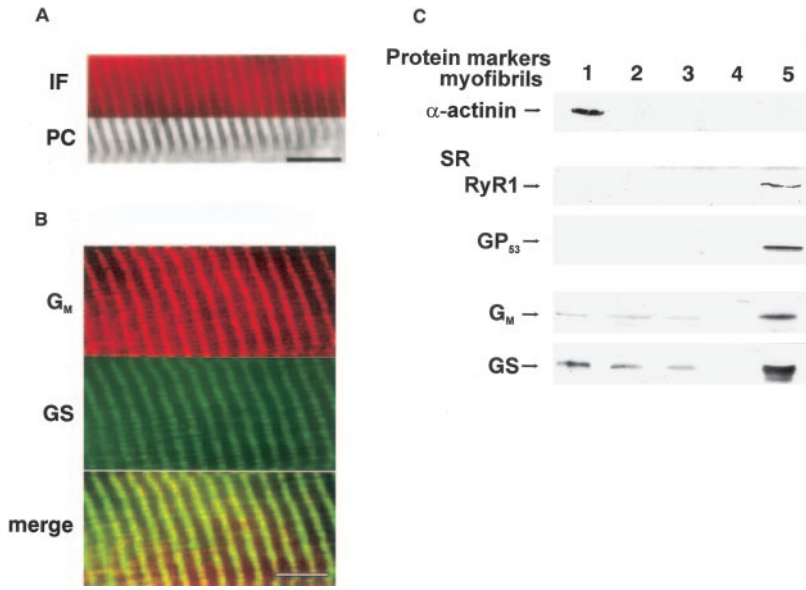

FIG. 1. **Localization of G_M to I band in rabbit skeletal muscle and co-enrichment of G_M and GS in SR membranes.** *A*, comparison between immunofluorescence (*IF*) and phase contrast (*PC*) images of rabbit adductor fibers labeled with guinea pig anti-G_M antibody. *Bar*, 10 μm. *B*, comparison of immunofluorescence localization of G_M and GS. Longitudinal cryosections were double-labeled with antibodies to G_M (*red fluorescence*) and to GS (*green fluorescence*), as indicated. Simultaneous visualization by image analysis (see "Experimental Procedures") of the two fluorochromes (merge) shows an extensive overlapping (*yellow signal*). *Bar*, 10 μm. *C*, muscle homogenates were subfractionated by differential centrifugation, as described under "Experimental Procedures," and aliquots of the different fractions subjected to 5–10% SDS-PAGE, followed by Western blot analysis with the organelle-specific antibody RyR1 and GP_{53}. Protein loading was 50 μg/lane. *Lane 1*, total homogenate; *lane 2*, post-myofibrillar supernatant obtained at 6.600*g*; *lane 3*, post-mitochondrial supernatant obtained at 15,000 $\times$ *g*; *lane 4*, post-microsomal supernatant obtained at 150,000 $\times$ *g*; *lane 5*, microsomes.

SDS-solubilizing buffer to samples. ^{33}P-Labeled protein were detected by autoradiography (Hyperfilm Amersham) or by a Bio-Rad model GS-250 Molecular Imager. Bound radioactivity was quantified by using a β-particle sensitive screen and a model GS-250 Molecular Imager (Bio-Rad). One unit of exogenous CaMKII and PKA is defined as the amount of enzyme that will transfer 1 pmol of phosphate to specific substrate in 1 min at 30 °C.

Protein Dephosphorylation—Isolated SR membranes were incubated at 30 °C with 50 μM [γ-^{33}P]ATP in assay medium for phosphorylation by endogenous CaMKII. After 30 min of incubation, CaMKII was inhibited by 2 μM staurosporin. As reported in detail in the legends to Fig. 5, protein dephosphorylation by endogenous PP1c was carried out in the same medium for 30 min at 30 °C.

Phosphoamino acid Analysis—G_M, ^{33}P-phosphorylated by exogenous CaMKII as described above, was blotted to nitrocellulose membrane, localized by autoradiography, and digested by trypsin. The tryptic peptides were subjected to acidic hydrolysis (6 M HCl for 4 h at 110 °C), and the radiolabeled phosphoamino acids were separated by high voltage paper electrophoresis at pH 1.9, as described by Perich *et al.* (31).

Preparation of Antisera against G_M Fusion Proteins—Chicken polyclonal antibodies against the purified His-tagged G_M-(40–338) fusion protein were raised, as previously reported by Damiani *et al.* (32). Guinea pig polyclonal antibodies were raised against a 11.2-kDa peptide corresponding to aa 754–852 of rabbit G_M (7). The immunogen was expressed and purified by on-column purification through an IMAC column and was then used to raise the antiserum (Harlan Sera-Lab Ltd., Bicester Oxon, UK).

Immunoprecipitation—Immunoprecipitation experiments were carried out as described (21). 250 μg of F4 protein, after solubilization, was incubated for 2 h in cold room with the guinea pig anti-G_M antibody to (1:1000 dilution). Protein A-Sepharose (40 μl) was added to the mixture, incubated for 1 h, and sedimented using an Eppendorf centrifuge. After washing, the pellets were solubilized with SDS solubilization buffer.

Gel Electrophoresis and Immunoblotting—SDS-PAGE (33) and immunoblotting were carried out, as described (21). Slab gels were stained with Coomassie Blue and then with Stains All. Apparent M_r values were calculated from a graph of relative mobilities *versus* log M_r of standard proteins. The blots were probed with: (i) mouse monoclonal antibodies to α-actinin (Sigma), RyR1 (sarcoplasmic reticulum Ca^{2+} release channel, skeletal isoform; BioMol, Plymouth Meeting, PA), GP_{53} (a SR glycoprotein of 53 kDa) (ABR, Golden, CO), GS (Chemicon International, Temecula, CA), and PP1c (Santa Cruz Biotechnology, CA); (ii) guinea pig polyclonal antibodies to G_M; or (iii) chicken polyclonal antibodies to G_M. Antibody binding was detected by immunoenzymic staining (21). Densitometry of blotted proteins, after immunostaining, was carried out using a Bio-Rad model GS-670 imaging densitometer. GS content was determined by densitometry of proteins immunostained with mouse monoclonal antibody GS, with reference to a calibration curve of purified GS. G_M content was determined by densitometry of blots immunostained with chicken polyclonal antibodies to G_M, with reference to a calibration curve obtained with purified G_M-(40–338).

Immunofluorescence Microscopy—Muscle cryosections were fixed with paraformaldehyde and immunostained by incubating with primary antibodies to G_M and GS (dilution 1:100), followed by incubation with the appropriate secondary antibody conjugated with TRITC or fluorescein isothiocyanate (Dako), as described by Sacchetto *et al.* (34). The images were captured by a B/W chilled CCD camera (Hamamatsu, Japan), transmitted to an interactive image analysis system equipped with image memory (High Fish Beta, version 2.0) and image processing software (Image Processing, version 3.4, Casti Imaging, Venice, Italy). The images were printed on a CP-D1E printer (Mitsubishi, Japan).

RESULTS

Localization of G_M to SR—When longitudinal cryosections from rabbit adductor muscle were probed with a guinea pig anti-G_M antibody, a cross-striated pattern was observed (Fig. 1*A*). By comparison with phase contrast microscopy, the fluorescent striations were found to correspond to the I band of the sarcomere. Fig. 1*B* shows that G_M co-localized with GS, which also is resident in the I band (35). The overlapping between the two proteins was virtually complete. It was verified that nonspecific antibody or secondary alone did not give this fluorescence pattern (not shown).

The subcellular localization of G_M was further investigated by differential centrifugation of muscle homogenates. The immunoblot analysis shown in Fig. 1*C* clearly shows that most G_M sedimented mostly in the microsomal fraction, which was devoid of contamination by myofibrillar proteins and highly enriched in SR membranes, as demonstrated by the localization of the SR-specific protein markers, RyR1 and GP_{53}. It is noteworthy that G_M and GS co-fractionated throughout the entire preparation.

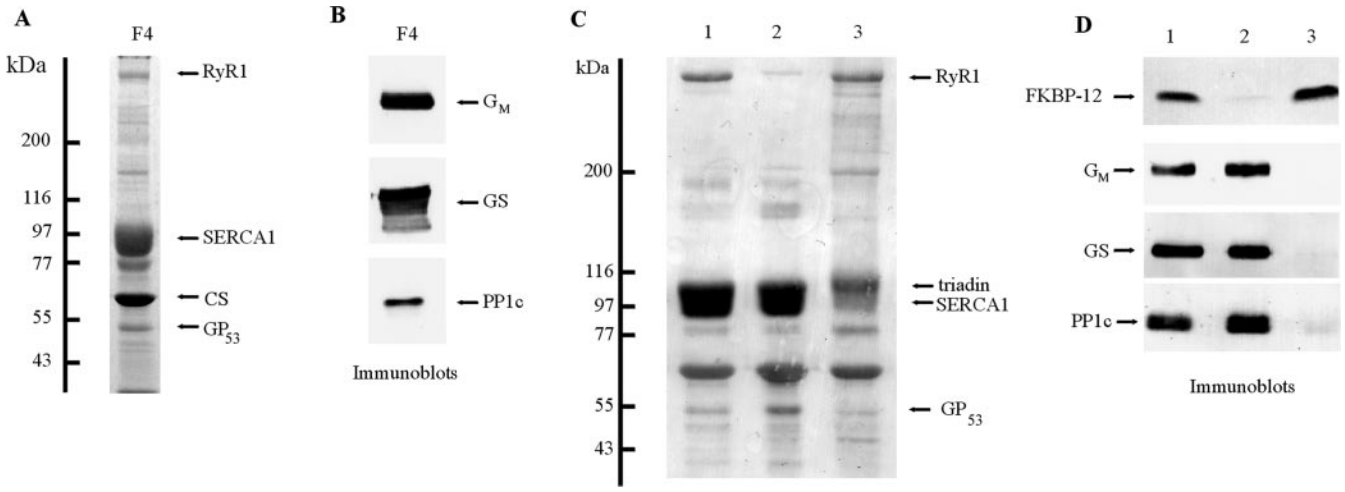

FIG. 2. **G_M associates to the Ca^{2+} pump membrane of nonjunctional SR.** *A*, 5–10% SDS-PAGE analysis, followed by Stains All staining, of nonjunctional SR fraction purified from total microsomes by isopycnic sucrose density centrifugation (see "Experimental Procedures"). Protein loading was 50 μg/lane. The positions of specific proteins are indicated on the *right*. The positions of molecular mass markers are indicated on the *left*. *B*, immunoblot analysis of F4 membranes for content of G_M, GS, and PP1c. The blots were incubated with the indicated specific antibody. Protein loading was 30 μg/lane. *C* and *D*, F4 membranes were treated with 0.25% detergent Chaps and centrifuged to separate Ca^{2+} pump membrane from JFM vesicles. The samples were analyzed by 5–10% SDS-PAGE. The proteins were either stained with Coomassie Blue (*C*) or blotted onto nitrocellulose (*D*). The blots were incubated with the indicated antibodies. Protein loading was 30 μg/lane. *Lanes 1*, F4, starting material; *lanes 2*, Ca^{2+} pump membrane; *lanes 3*, JFM.

A fraction enriched in nonjunctional SR membranes was obtained from muscle microsomes by isopycnic-sucrose density centrifugation (22). This procedure, at variance with that of Saito *et al.* (23), which yields a much higher proportion of membranes deriving from the terminal cisternae of junctional SR, yields mainly vesicles derived from nonjunctional SR (22). The nonjunctional SR origin of this fraction was confirmed by [^{3}H]ryanodine binding measurements, carried out at optimal conditions of free Ca^{2+} (100 μM) and ionic strength (1 M KCl). The specific SR illustrated in Fig. 2*A* gave a B_{max} value of 1.1 pmol/mg protein, which is almost 20-fold lower than that previously reported for purified terminal cisternae (19.0 pmol/mg protein) (24). The same fraction was consistently found to be highly enriched in glycogen (927 ± 207 μg/mg protein, average ± S.E. of three determinations), as well as in GS activity (0.82 ± 0.04) and in GS protein (179 ± 20 μg/mg protein). Of interest, fraction F4 was also characterized by a high density of G_M and PP1c (Fig. 2*B*). Based on this evidence, henceforth all experiments were carried out using the F4 fraction.

Differential Distribution of G_M between Ca^{2+} Pump Membrane and JFM—The treatment of SR membranes with low concentrations of the detergent Chaps can be used (24) to separate the Ca^{2+} pump membrane, *i.e.* the highly specialized membrane l of nonjunctional SR consisting of SERCA1 protein (22), from the membrane domains of the SR that are enriched in Ca^{2+} release channels, RyR1. The RyR1-enriched SR region is named JFM (24), because of its structural association with transverse tubules. We took advantage of this preparative procedure to investigate the partition of G_M associated to F4 membranes between Ca^{2+} pump membrane and the JFM. In agreement with the prevalent nonjunctional SR origin of membranes forming the F4 fraction, more than 80% of total SR protein was solubilized. Fig. 2 shows that well defined junctional SR markers, such as RyR1 (Fig. 2*C*) and FKBP-12 (Fig. 2*D*), were present only in the JFM (Fig. 2, *C* and *D*, *lanes 3*) (24). On the other hand, the Ca^{2+} pump membrane was completely devoid of these proteins, while being enriched in SERCA1 and GP_{53} content (Fig. 2, *C* and *D*, *lanes 2*). Western blot analysis with antibodies to G_M, GS, and PP1c showed that all of these proteins localized exclusively to the Ca^{2+} pump membrane (Fig. 2*D*).

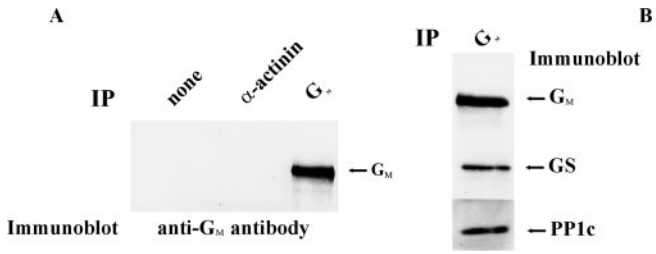

FIG. 3. **G_M co-immunoprecipitates with GS and PP1c from SR membranes.** *A*, F4 membranes were solubilized and immunoprecipitated (*IP*) with guinea pig anti-G_M antibody, as described under "Experimental Procedures." The immunocomplexes were probed with antibodies to G_M. Negative controls were incubated without antibody or with monoclonal antibody to α-actinin as indicated. Only the molecular weight region between 200,000 and 116,000 is shown. *B*, solubilized F4 membranes were immunoprecipitated with guinea pig polyclonal anti-G_M antibody, as in *A*. Blots of immunoprecipitates were probed with the indicated specific antibodies.

Co-immunoprecipitation of G_M, GS, and PP1c—F4 membranes were solubilized and immunoprecipitated with a guinea pig polyclonal antibody to G_M protein. Fig. 3*A* shows that a prominent G_M band was specifically immunoprecipitated by this antibody. G_M protein neither was bound to protein A in the absence of antibody nor was immunoprecipitated by a nonrelated anti-α-actinin antibody (Fig. 3*A*). Fig. 3*B* shows that G_M co-immunoprecipitated not only with PP1c, as expected (36), but also with GS. The specificity of this result was validated as in Fig. 3*A* (data not shown).

Phosphorylation-Dephosphorylation of G_M by SR-bound CaMKII and PP1c—G_M is phosphorylated by PKA at Ser^{48} and Ser^{67} (Refs. 4 and 5; see also Ref. 7). Based on the consensus sequence (37), another potential PKA target site of G_M is Thr^{215}. The analysis of these potential phosphorylation sites reveals that the amino acid sequence (R*XX*(S/T)) also corresponds to a consensus sequence for CaMKII (37, 38). Because rabbit SR membranes contain CaMKII (see Ref. 40 for a review), we tested whether that G_M is phosphorylated by SR-bound CaMKII.

To address this point, F4 membranes were incubated with [γ-^{33}P]ATP in the presence of Ca^{2+}-calmodulin in the assay

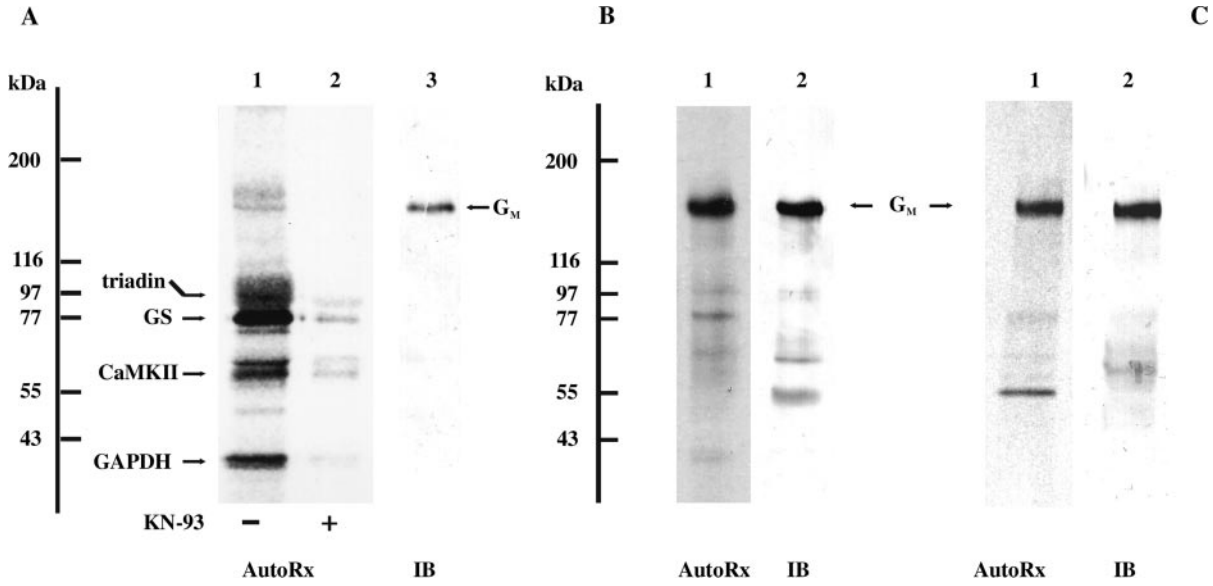

FIG. 4. **G_M is phosphorylated by endogenous CaMKII.** *A*, F4 vesicles were phosphorylated for 30 min at 30 °C with 50 μM [γ-^{33}P]ATP in the presence of 100 μM Ca^{2+} and 0.2 μM calmodulin without or with 100 μM KN-93. ^{33}P-Labeled proteins were resolved by 5–12.5% SDS-PAGE and either identified by autoradiography of dried gels (*lanes 1* and *2*), or blotted. The blots were then immunostained with antibodies to G_M (*lane 3*). Protein loading was 10 μg/lane. The expected mobilities of triadin, 60-kDa CaMKII, and glyceraldheyde-3-phosphate dehydrogenase are indicated. An *arrow* indicates the 84-kDa phosphoprotein identified as GS. *B*, F4 proteins phosphorylated 30 min at 30 °C with 50 μM [γ-^{33}P]ATP in the presence of 100 μM Ca^{2+} and 0.2 μM calmodulin were solubilized and immunoprecipitated with the guinea pig anti-G_M antibody. The immunocomplexes were resolved by 5–12.5% SDS-PAGE and blotted onto nitrocellulose. The blots, after autoradiography (*AutoRx*) (*lane 1*), were immunostained with antibody to G_M (*lane 2*). *C*, F4 vesicles were phosphorylated for 30 min at 30 °C with 50 μM [γ-^{33}P]ATP by exogenous PKA and processed as described for *B*. *Lane 1*, autoradiography (*AutoRx*); *lane 2*, immunoblot (*IB*) after autoradiography with anti-G_M antibody. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

medium. As we have already reported (39, 24, 21), the incorporation of radiolabeled phosphate into SR proteins is poor, if it occurs at all, in the absence of exogenously added Ca^{2+}-calmodulin. On the contrary, in the presence of these effectors several proteins were phosphorylated, including a 60-kDa protein corresponding to autophosphorylated CaMKII (21, 40). Additional SR proteins phosphorylated by CaMKII were 95-kDa triadin (39) and 36-kDa glyceraldheyde-3-phosphate dehydrogenase (41) (Fig. 4*A*, *lane 1*). As already reported (39), a prominent ^{33}P-labeled protein was also detected at about 85 kDa. This phosphoprotein proved to be GS, based on Western blot analysis with anti-GS antibody.[2] The addition of 100 μM KN-93, a specific inhibitor of CaMKII that competes for calmodulin binding to the enzyme (42), almost completely abolished the phosphorylation of SR proteins (Fig. 4*A*, *lane 2*) (see also Ref. 21). Taken together, these results strongly support the notion that the Ca^{2+}-calmodulin-dependent phosphorylation of SR proteins is mediated by endogenous CaMKII.

Fig. 4*A* shows that ^{33}P-labeled protein bands were also detectable in the M_r range of 160,000–170,000, including a protein that displayed the same mobility of G_M as judged by Western blot analysis (Fig. 4*A*, *lane 3*). The low extent of the protein phosphorylation is consistent with the low content of G_M in F4 membranes (about 0.5 μg/mg of SR protein; see "Experimental Procedures"). Because another SR protein, called HRC, with the apparent M_r of 170,000 has been described to be phosphorylated by endogenous CaMKII (39, 40) to confirm the phosphorylation of G_M, ^{33}P-labeled SR proteins were solubilized and immunoprecipitated with the guinea pig anti-G_M antibody. Fig. 4*B* shows that the ^{33}P-labeled protein of about 160 kDa was indeed G_M. As a positive control, we performed parallel experiments in which F4 membranes were phosphorylated with exogenous PKA. Also in this case, the anti-G_M antibody immunoprecipitated a 160-kDa ^{33}P-labeled protein that was immunostained with anti-G_M antibody (Fig. 4*C*).

To investigate whether G_M could be dephosphorylated by endogenous PP1c, F4 proteins were first ^{33}P-phosphorylated in the presence of Ca^{2+} and calmodulin (Fig. 5, *lane 1*). After 30 min of incubation, endogenous CaMKII was inhibited by adding 2 μM staurosporin to the assay medium. In this particular case, we ought to use staurosporin instead of KN-93, because, once autophosphorylated, CaMKII remains active even in the absence of Ca^{2+}-calmodulin (autonomous form). The membranes were then solubilized and immunoprecipitated with anti-G_M antibody. Fig. 5 clearly demonstrates that radiolabeled G_M was completely dephosphorylated by endogenous PP1c (*lanes 1* and *2*). Indeed, the level of G_M phosphorylation remained constant when the PP1c inhibitor okadaic acid was also present in the incubation medium (Fig. 5, *lane 3*).

Identification of CaMKII Phosphorylation Sites of G_M—All of the phosphorylation sites thus far identified in G_M are clustered within 28 residues (Ser^{40} to Ser^{67}) located at the NH_2 terminus of the molecule. Therefore, we investigated the possibility that CaMKII might phosphorylate G_M within this phosphoregulatory region. A His-tagged recombinant protein corresponding to aa 40–338 of G_M (G_M-(40–338)) was expressed in *E. coli* and purified according to the manufacturer's instructions. Fig. 6*A* shows that, when added to SR fraction, G_M-(40–338) was phosphorylated by endogenous CaMKII (*lane 5*) and that this phosphorylation was almost completely abolished by KN-93 (*lane 6*). Fig. 6*B* shows that the G_M-(40–338) fusion protein was also phosphorylated by exogenous, commercially available CaMKII. No phosphorylation of G_M-(40–338) fusion protein occurred in the absence of activated CaMKII (not shown).

Based on the known consensus sequence for CaMKII (37, 38), there are six potential Ser or Thr phosphoacceptor sites in G_M-(40–338) fusion protein: Ser^{48}, Ser^{67}, Ser^{205}, Thr^{215}, Ser^{263}, and Ser^{267}. The analysis of the phosphorylated residues obtained from G_M-(40–338) ^{33}P-phosphorylated by the commer-

[2] R. Sacchetto, L. Salviati, E. Damiani, and A. Margreth, unpublished data.

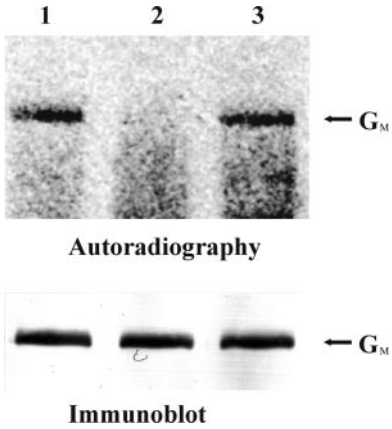

FIG. 5. **SR-bound PP1c dephosphorylates G_M.** F4 proteins were phosphorylated for 30 min at 30 °C with [γ-^{33}P]ATP by endogenous CaMKII, as described in the legend to Fig. 4*A*. CaMKII activity was then inhibited by adding 2 μM staurosporin to the medium (*lane 1*), or otherwise the incubation was prolonged for 30 min at 30 °C in the absence (*lane 2*) or presence (*lane 3*) of 2 μM okadaic acid. The membranes were then solubilized and immunoprecipitated with the guinea pig anti-G_M antibody. Immunoprecipitated G_M was detected by autoradiography of blots. The blots were then immunostained with the guinea pig anti-G_M antibody. Only the molecular weight region between 200,000 and 116,000 is shown.

cial exogenous CaMKII showed that only serine residue(s) were affected by the kinase (results not shown).

Further experiments were carried out to identify the target residues of CaMKII. To this purpose, an additional His-tagged G_M fusion protein lacking aa 40–68 (G_M-(69–338)) was generated. Fig. 7*A* shows that the phosphorylability of G_M-(69–338) by both CaMKII and PKA was completely lost upon deletion of the sequence containing Ser^{48} and Ser^{67} (*lanes 6*, 8). These results suggest that the serine residue(s) phosphorylated by CaMKII are located in the 40–68-amino acid sequence. It is noteworthy that PKA phosphorylation of G_M-(40–338), which reached a stoichiometry of 1 mol/mol protein, induced a substantial shift of the Coomassie Blue band toward higher molecular mass (Fig. 7*A*, *lane 1*), whereas CaMKII phosphorylation did not affect the protein mobility (lane 3).

To discriminate between the two potential CaMKII phosphorylation sites, a recombinant G_M-(40–338) protein was generated in which Ser^{48} was mutated to alanine (G_M-(40–338)$^{S48\text{-}A}$). As expected, when G_M-(40–338)$^{S48\text{-}A}$ was incubated with PKA, a substantial decrease of ^{33}P incorporation into the protein was observed, as compared with wild-type G_M-(40–338) (Fig. 7*B*, *lanes 3* and *4*). The inability of CaMKII to phosphorylate the point-mutated G_M-(40–338)^{S48A} (Fig. 7*B*, *lanes 5* and *6*) demonstrated that the only target residue affected by CaMKII is Ser^{48}.

Phosphorylation of Ser^{48} by PKA does not dissociate PP1c from G_M. Therefore, we compared the effect of CaMKII and PKA phosphorylation on the interaction of PP1c with SR membranes. To maximize the phosphorylation by CaMKII, SR membranes were incubated in a medium containing not only Ca^{2+} and calmodulin but also exogenous CaMKII. Under these experimental conditions and as determined by densitometry of radiolabeled proteins, we observed that the extent of phosphorylation by CaMKII of G_M protein was comparable with that obtained after PKA phosphorylation of SR proteins (Fig. 8*A*). As a further indication of the increased efficiency of CaMKII phosphorylation under these experimental conditions, we found that the amount of radioactivity incorporated into GS was more than 2-fold higher (22.050 arbitrary counts) than that observed for PKA-dependent phosphorylation (9859 arbitrary counts). When the association of PP1c to G_M was monitored by Western blot analysis, a substantial amount of PP1c was released from SR membranes into the supernatant, following phosphorylation by PKA (Fig. 8, *lanes 3* and *4*). On the contrary, CaMKII-mediated phosphorylation of G_M only negligibly affected the association of PP1c to SR (*lanes 5* and *6*). It was verified by Western blot analysis that association of GS to G_M was unaffected under either condition (not shown).

DISCUSSION

In skeletal muscle CaMKII is targeted to SR by a specific anchoring protein, αCaMKII association protein (43). Even though both the αCaMKII association protein and CaMKII are uniformly distributed within junctional and nonjunctional SR (40), the function of nonjunctional SR CaMKII is still unknown in fast twitch muscle.

Previous electron microscopy studies in resting skeletal muscle (44) showed that glycogen particles associate to SR at the level of the I band, where the highest density of nonjunctional SR tubules is observed. This association is preserved under conditions of sucrose density centrifugation (45), likely because of the presence of the glycogen- and PP1c-targeting subunit G_M. It is also well established that glycogen granules contain enzymes involved in their metabolism, such as GS and glycogen phosphorylase (46). Recent studies implicated CaMKII in regulation of glucose metabolism in skeletal muscle (41, 47). This involvement seems reasonable, because during physical exercise cytosolic [Ca^{2+}] increases, thereby activating Ca^{2+}-calmodulin-dependent enzymes. As a matter of fact, activity of CaMKII is increased by exercise in human skeletal muscle (48).

Based on immunofluorescence and subfractionation studies, here we demonstrated that G_M, one of the key regulatory components of glycogen metabolism, selectively localizes to SR. Furthermore we show that G_M is a component of the highly specialized Ca^{2+} pump membrane forming nonjunctional tubules of SR.

Our subcellular localization studies do not support the previous report by Walker *et al.* (14) suggesting the presence of two pools of G_M in skeletal muscle, one cytosolic and one bound to the SR. Our experiments demonstrate unequivocally that G_M previously detected in the low speed, post-mitochondrial supernatant was due to the contamination by unsedimented SR vesicles. Based on this evidence, we conclude that in skeletal muscle only one form of G_M is present that localizes to the nonjunctional SR.

Our work also shows that a structural complex containing G_M, GS, and PP1c exists in native SR membranes. Using COS7 cells or C_2C_{12} myotubes transiently expressing G_M, Liu and Brautigan (19) reported that transfected G_M immunoprecipitated with endogenous GS. However, our data provide the first direct experimental evidence for the presence of this complex in native nonjunctional SR membranes isolated from fast twitch skeletal muscle.

The main goal of the present study was to investigate whether nonjunctional SR-bound CaMKII might phosphorylate key proteins of glycogen metabolism. We have shown that endogenous CaMKII phosphorylates G_M and that phospho-G_M is dephosphorylated by endogenous PP1c. By using G_M recombinant fragments and site-directed mutagenesis, we demonstrate that CaMKII phosphorylated Ser^{48} in the NH_2 terminus of G_M. This finding is consistent with previous observations (10, 15) reporting high levels of Ser^{48} phosphorylation *in vivo*, as a consequence of phosphorylation by muscle contraction-activated protein kinase(s) (14). This serine residue is phosphorylated by PKA *in vivo* (14) and *in vitro* (4), as well as, possibly, by an insulin-dependent protein kinase (14, 19, 49). When G_M is phosphorylated at Ser^{48}, PP1c activity toward GS and phosphorylase kinase is much higher than that of unphosphoryl-

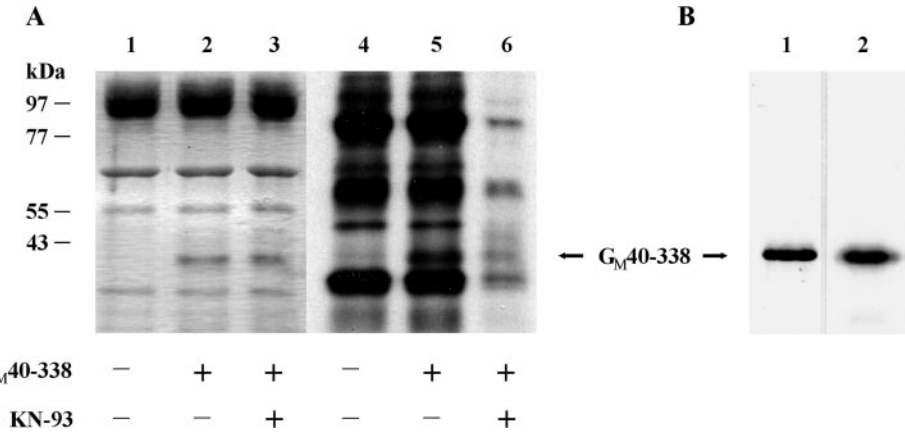

FIG. 6. **G_M-(40–338) fusion protein is phosphorylated by endogenous and exogenous CaMKII.** *A*, F4 vesicles (50 μg) were incubated for 30 min at 30 °C with 50 μM [γ-^{33}P]ATP in the presence of 100 μM Ca^{2+} and 0.2 μM calmodulin without (*lane 1* and *4*) or with (*lanes 2*, *3*, *5*, and *6*) 2 μg of His-tagged G_M-(40–338) fusion protein. Where indicated, 100 μM KN-93 was added to the incubation medium. After 5–10%,SDS-PAGE, ^{33}P-labeled proteins were detected by autoradiography of dried gels. Only the molecular weight region between 97,000 and 30,000 is shown. The position of G_M-(40–338) is indicated. *Lanes 1–3*, Coomassie Blue staining; *lanes 4–6*, autoradiography. *B*, G_M-(40–338) fusion protein (4 μg) was incubated for 30 min at 30 °C with 50 μM [γ-^{33}P]ATP in assay medium containing activated CaMKII (see "Experimental Procedures"). Phosphorylated G_M-(40–338) fusion protein was detected by autoradiography. *Lane 1*, Coomassie Blue; *lane 2*, autoradiography.

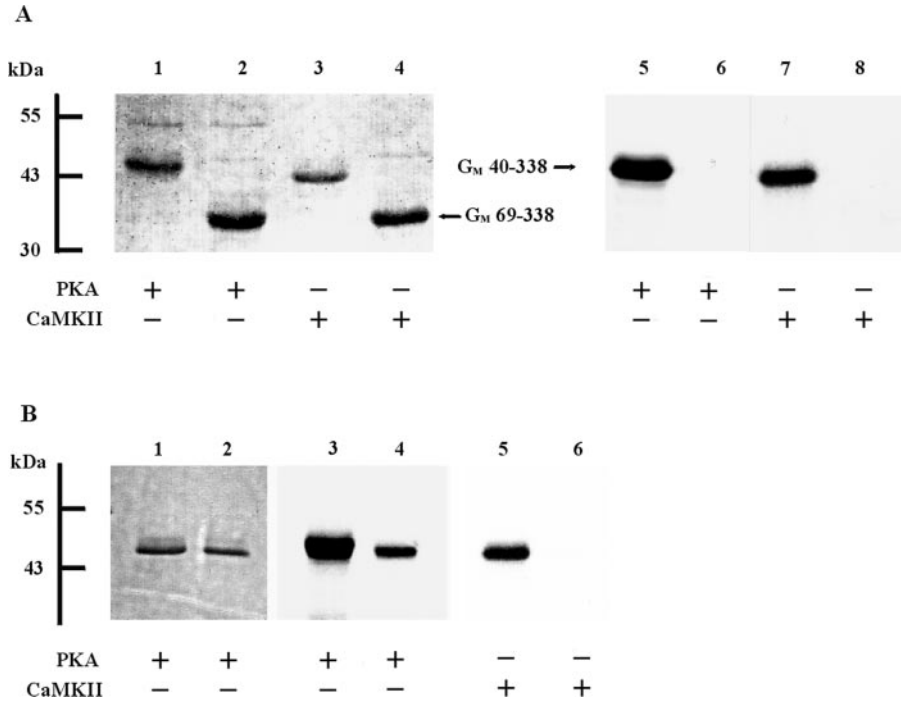

FIG. 7. **Identification of phosphorylation site of G_M phosphorylated by CaMKII.** *A*, G_M-(40–338) (*lanes 1*, *3*, *5*, and *7*) and G_M-(69–338) (*lanes 2*, *4*, *6*, and *8*) fusion proteins were incubated for 30 min at 30 °C with 50 μM [γ-^{33}P]ATP in assay medium containing either PKA (*lanes 1*, *2*, *5*, and *6*) or activated CaMKII (*lanes 3*, *4*, *7*, and *8*) (as detailed under "Experimental Procedures"). After 5–10% SDS-PAGE, ^{33}P-labeled proteins were detected by autoradiography of dried gels. The position of G_M-(40–338) is indicated by *two arrows*, because phosphorylation by PKA and CaMKII differentially affects the protein mobility (see "Results"). The position of G_M-(69–338) is also indicated. Only the molecular weight region between 55,000 and 30,000 is shown. *Lanes 1–4*, Coomassie Blue staining; *lanes 5–8*, autoradiography. *B*, wild-type G_M-(40–338) (*lanes 1*, *3*, and *5*) and point-mutated G_M-(40–338)^{S48A} (*lanes 2*, *4*, and *6*) were phosphorylated by PKA (*lanes 3* and *4*) or activated CaMKII (*lanes 5* and *6*), as described for *A*. *Lanes 1* and *2*, Coomassie Blue staining; *lanes 3–6*, autoradiography.

ated G_M (19, 49). This, in turn, should lead to reactivation of GS and hence to the resynthesis of glycogen (14).

Our results support the concept that G_M, like GS, is a multisite phosphorylated protein and that the domain of the protein critical for regulation by reversible phosphorylation is the region near the amino terminus, termed the phosphoregulatory domain (49). In this study, we considered the possibility that G_M might directly interact with CaMKII, via the transmembrane domain of G_M, on the analogy of what was observed for the slow twitch muscle integral SR membrane protein, phospholamban (13). However, co-immunoprecipitation experiments failed to provide evidence supporting a direct protein-protein interaction between CaMKII and the heteromeric G_M, GS, and PP1c protein complex (not shown).

The physiological significance of the CaMKII-mediated phosphorylation of G_M Ser^{48} is complex. Because CaMKII is activated during exercise, a role for CaMKII in inhibition of GS should be conceivable. However, it is also possible that phosphorylation of Ser^{48} of G_M by CaMKII plays a role during the recovery period. On this respect, it is interesting to remember the unique regulatory features of CaMKII, which, once autophosphorylated, becomes independent on regulation by Ca^{2+} and calmodulin. Therefore the signal conveyed by this autonomous form of CaMKII might well outlast that of adrenergic

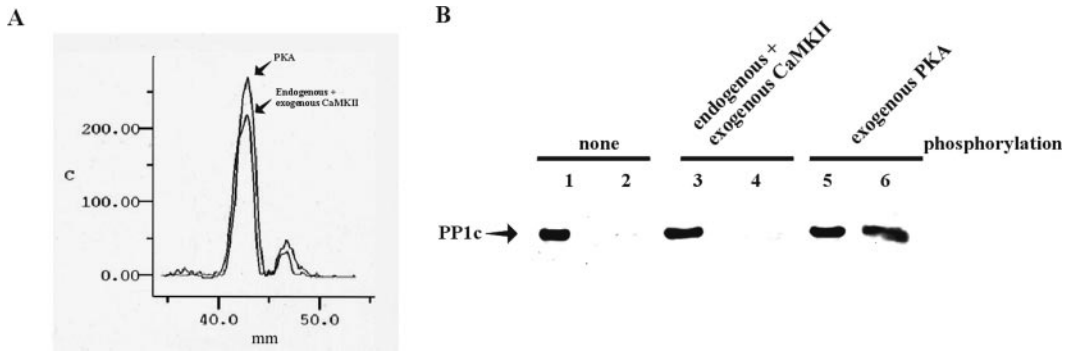

FIG. 8. **Phosphorylation of G_M by CaMKII does not dissociate PP1c from SR membranes.** *A*, F4 membranes were incubated for 30 min at 30 °C with 50 μM [γ-^{33}P]ATP either with exogenous PKA or in assay medium containing 100 μM Ca^{2+}, 1.0 μM calmodulin, and activated exogenous CaMKII. After 5–10% SDS-PAGE, ^{33}P-labeled proteins were detected by autoradiography of dried gels, and the radioactive areas corresponding to radiolabeled G_M were determined by densitometry (see "Experimental Procedures"). *B*, F4 membranes were incubated for 30 min at 30 °C with 50 μM unlabeled ATP as in *A*, in assay medium without addition (*lanes 1* and *2*), or containing 100 μM Ca^{2+}, 1.0 μM calmodulin and activated exogenous CaMKII (*lanes 3* and *4*) or PKA (*lanes 5* and *6*). At the end of the incubation, the membrane suspensions were centrifuged with an air-driven ultracentrifuge at 22 p.s.i. for 30 min to separate pellets and supernatants. The proteins were analyzed by 5–10% SDS-PAGE, and the blots were probed with the anti-PP1c antibody. *Lanes 1*, *3*, and *5*, pellets; *lanes 2*, *4*, and *6*, supernatants.

stimulation. CaMKII-dependent phosphorylation of Ser^{48} might provide a potential mechanism for reactivating glycogen resynthesis, by increasing the rate of dephosphorylation of GS during the recovery period after exercise, when adrenergic stimulus is terminated.

Ser^{48} phosphorylation might also provide a mechanism for regulation of autophosphorylated Ca^{2+}-calmodulin-independent CaMKII. In fact, we observed that, in addition to G_M and GS, PP1c also dephosphorylates CaMKII (not shown). As a matter of fact, this finding identifies PP1c as the major CaMKII phosphatase thus far identified in skeletal muscle SR. This observation is even more striking, because PP1c activity seems to be specific for glycogen-associated substrates (49), such as GS, glycogen phosphorylase, and G_M, as also shown here. Therefore, activation of PP1c secondary to CaMKII-dependent phosphorylation of G_M Ser^{48} might result in dephosphorylation of autonomous CaMKII and hence termination of CaMKII signal.

It is well known that direct phosphorylation of GS by several protein kinases, such as PKA, CK1, or CK2, poorly inhibits GS activity (50, 51). On this respect, the most relevant kinase is GSK3 (50, 51). Like PKA, CK1, and CK2, SR-bound CaMKII also phosphorylates GS.[3] In preliminary experiments we found that the inhibition of GS activity by this phosphorylation was minimal (not shown). It is also known that GS activity can be regulated by the synergic action of two protein kinases. This is the case of phosphorylase kinase and CK1 (52), PKA and CK1 (53), and CK2 and GSK3 (50, 51). Therefore, the possibility exists that CaMKII and PKA may act synergistically in the regulation of GS activity in a complex fashion also depending on the intensity and duration of exercise (54). It is well known that levels of circulating catecholamines are directly related to the intensity of the exercise.

Finally, the finding that CaMKII phosphorylates G_M at site 1 suggests a synergy with GSK3 because it is well known that GSK3 can phosphorylate G_M at Ser^{40} and Ser^{44} only following the primary phosphorylation of Ser^{48}. Thus far, this was demonstrated only for PKA (15). Our results provide that CaMKII might substitute for PKA in this priming role for GSK3. It should be remembered that the functional role of GSK3 phosphorylation of G_M is still undefined.

In summary, our data indicate the existence of a functional relationship between SR-bound CaMKII and the heteromeric protein complex formed by G_M, GS, and PP1c. This interaction takes place in the Ca^{2+} pump membrane of nonjunctional SR. The finding that Ser^{48} of G_M is phosphorylated by CaMKII supports the hypothesis that SR-bound CaMKII is involved in the modulation of glycogen metabolism in skeletal muscle during exercise.

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[3] R. Sacchetto, E. Bovo, A. Donella-Deana, A. Margreth, and E. Damiani, present results and unpublished data

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