Purification, Characterization, and Molecular Cloning of a 60-kDa Phosphoprotein in Rabbit Skeletal Sarcoplasmic Reticulum Which Is an Isoform of Phosphoglucomutase*

(Received for publication, May 26, 1992)

Young Sup Lee‡§, Andrew R. Marks¶¶, Nerija Gureckas*, Ronald LaCroix†, Bernardo Nadal-Ginardi‡, and Do Han Kim‡¶**

From the ‡Cardiology Division, Department of Medicine, University of Connecticut Health Center, Farmington, Connecticut 06030-1305, and the ¶¶Department of Cardiology, the Children's Hospital, and the Howard Hughes Medical Institute, Harvard Medical School, Boston, Massachusetts 02115

A 60-kDa substrate of calmodulin-dependent protein kinase in rabbit "heavy" skeletal sarcoplasmic reticulum (SR) was characterized by purification and cDNA cloning. Purification was achieved by column chromatography using DEAE-Sephadex, heparin-agarose, and hydroxylapatite at 0.5% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid (CHAPS). Analyses of amino acid sequence and composition indicated that the CHAPS-soluble 60-kDa protein is an isoform of phosphoglucomutase (PGM). cDNAs encoding two isoforms of PGM were isolated from rabbit skeletal muscle. The translated amino acid sequences show that the isoforms, PGM1 and PGM2, differ in the N-terminal 77 amino acids and that PGM2 is identical to the 60-kDa protein in the SR. Northern blot analysis showed that the size of the mRNA encoding PGM2 is 2.4 kilobases. The PGM enzyme activity was markedly inhibited in SR membranes, while perturbation of the membranes with CHAPS or guanidine-HCl recovered the enzyme activity. KCl (0.15–1 M) led to a partial recovery of the enzyme activity suggesting that the charge interaction is not the primary force for PGM-SR interaction. PGM is localized in the heavy fraction of SR, where calsequestrin and Ca²⁺ release channel are enriched. Our results demonstrate that an isoform of PGM localized in junctional skeletal SR is the 60-kDa substrate of calmodulin-dependent protein kinase.

The presence of a Ca²⁺/CaM²⁺-dependent protein kinase in isolated skeletal muscle SR vesicles was first reported by Campbell and MacLennan (1982) who found that three proteins with a molecular mass of 85, 60, and 20 kDa were phosphorylated in the presence of Ca²⁺ and CaM. Chiesi and Carafoli (1982) reported Ca²⁺/CaM-dependent phosphorylation of three proteins having a molecular mass of 57, 35, and 20 kDa. Chu et al. (1990) recently reported that multiple junctional protein components could be phosphorylated by the membrane-bound CaM-dependent kinase. Although the profiles of the phosphoproteins varied depending on experimental conditions (e.g. concentrations of ATP, Ca²⁺, and CaM) and types of SR preparations, it is generally accepted that a ~60-kDa protein in junctional SR is the most rapidly and extensively phosphorylated protein in the Ca²⁺/CaM-dependent phosphoprotein group (Campbell and MacLennan, 1982; Chiesi and Carafoli, 1982; Seiler et al., 1984; Kim and Ikemoto, 1986; Chu et al., 1990; Witcher et al., 1991). Phosphorylation of the 60-kDa protein in junctional SR has been correlated with Ca²⁺ release function by comparing extents of the phosphorylation and Ca²⁺ fluxes in the SR (MacLennan et al., 1984; Kim and Ikemoto, 1986).

None of the protein kinases involved in the phosphorylation of the junctional SR proteins and their substrates has been purified. In the present study, we have purified and cloned the 60-kDa phosphoprotein and presented evidence demonstrating that the CHAPS-soluble 60-kDa phosphoprotein is an isoform of phosphoglucomutase (PGM), which is preferentially localized to junctional SR. The hydrophobic nature of this enzyme (Ray et al., 1983) and its highly specific interaction with and localization in junctional SR suggest that this enzyme is an "amphitropic" protein, as suggested by Burn (1988). As several groups have shown, various glycogenic and glycolytic enzymes are associated with both cardiac and skeletal SR membranes. The identification and characterization of PGM and its interaction with junctional SR should be significant in terms of understanding how such enzymes interact with muscle membrane system and how it affects the membrane functions (Meyer et al., 1970; Entman et al., 1976, 1980; Horl et al., 1978; Pierce and Philipson, 1985; Caswell et al., 1989).

* This work was supported by National Institutes of Health Grant HL 35026, a grant-in-aid from the American Heart Association Connecticut Affiliate, and a research grant from the University of Connecticut Research Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) M97963 and M97964.

§ Current address: Dept. of Biochemistry, College of Natural Sciences, Kyungbuk National University, Taegu, Korea.

¶¶ A Systen Scholar. Current address: Mount Sinai School of Medicine, Brookside Center for Molecular Biology, One Gustave Place, New York, NY 10029.

** Established Investigator of the American Heart Association. To whom correspondence and reprint requests should be addressed: Cardiology Division, Dept. of Medicine, University of Connecticut Health Center, 263 Farmington Ave., Farmington, CT 06030-1305.

The abbreviations used are: CaM, calmodulin; SR, sarcoplasmic reticulum; PGM, phosphoglucomutase; SR-PGM, sarcoplasmic reticulum associated phosphoglucomutase; MOPS, 3-[N-morpholino]propanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate acid; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; kb, kilobase; bp, base pair; aa, amino acid.
EXPERIMENTAL PROCEDURES

Materials— [γ-32P]ATP and “CaCl₂ were obtained from Du Pont-New England Nuclear. DEAE-Sephacel, heparin-agarose, CHAPS, and molecular weight standards were purchased from Sigma. Hydroxylapatite (Bio-Gel HTP) was obtained from Bio-Rad and Sephacryl S-300 from Pharmacia LKB Biotechnology Inc. Oligonucleotides were synthesized by a Cyclone oligonucleotide synthesizer (Biosearch, Inc.). Taq-polymerase was obtained from Perkin-Elmer Cetus and reverse transcriptase from Pharmacia. The oligos for rapid amplification of cDNA ends (RACE) experiments (Frohman et al., 1983) were a generous gift from Dr. Hyun-Duck Nah (University of Connecticut Health Center, Farmington, CT) and all other reagents used were of analytical grade.

SR Preparation—Fast-twitch muscles from hind limb and back of rabbit were extracted in 0.3 M sucrose for preparation of “heavy” SR by differential centrifugation as described previously (Kim et al., 1983), and SR vesicles in the final pellets were washed twice with excess suspension buffer containing the protease inhibitors (Kim and Ikemoto, 1986) to eliminate cytotoxic contamination. To obtain “light” SR, the supernatant after pelleting heavy SR (Kim et al., 1983) was centrifuged at 12,500 rpm in a Sorvall GSA rotor for 30 min and the recovered supernatant was recentrifuged at 30,000 rpm in Beckman 50 Ti for 30 min. The pellets were suspended in the SR suspension buffer (Kim and Ikemoto, 1986). Purification of the CHAPS-soluble 60-kDa Phosphoprotein—5 ml of CHAPS extract were phosphorylated in 0.5 ml of mM [γ-32P]ATP (300 cpm/pmol), 0.5 mM MgCl₂, 20 mM MOPS (pH 6.8), 5 mM CaCl₂, 1 mM CaM, and 0.1 mM PMSF and then diluted with 5 ml of solution containing 20 mM MOPS (pH 6.8) and 1% CHAPS at 20 °C. The suspension was centrifuged at 35,000 rpm for 30 min in a Beckman 50 Ti rotor. Approximately 1.8 ml of the CHAPS-soluble 60-kDa extract were loaded onto a DEAE-Sephacel column (1 × 2 cm) equilibrated with a solution containing 20 mM MOPS (pH 6.8) and 0.5% CHAPS (Buffer I). The flow-through was loaded on a heparin-agarose column (0.7 × 1 cm) equilibrated with Buffer I. The flow-through from the heparin-agarose column was then loaded onto hydroxylapatite column equilibrated with Buffer I. The column was washed with Buffer I until no protein was detected in the eluate. The bound proteins to the column were eluted in 40 ml of a solution containing 20 mM MOPS (pH 6.8) and a linear gradient of KPO₄ (0–100 mM). The 60-kDa phosphoprotein appeared in 45–65 mM KPO₄.

Identification of Phosphoproteins—Phosphorylation of the SR proteins was quenched by a 10-fold dilution with a solution containing 2% SDS, 62.7 mM Tris-HCl (pH 8.8), 5% β-mercaptoethanol, 10% glycerol, and 3.3 μg/ml bromophenol blue. The proteins in quenched solution were separated by SDS-polyacrylamide gel electrophoresis according to Laemmli (1970), and gels were stained by Coomassie Blue, silver staining, or both. After destaining, the gels were dried with cellophane paper backing. The dried gels were autoradiographed using Kodak X-Omat film. The amounts of phosphorylated incorporated into the proteins of the dried gels were determined by scintillation counting. For scintillation counting, the gels were sliced, and the gel slices were incubated in 0.2 ml of 7.5% H₂O₂ solution overnight at 50 °C. Determination of Native Molecular Weight—The isolated 60-kDa phosphoprotein was loaded on a Sephacryl S-300 column (1.0 × 50 cm) equilibrated with 0.5% CHAPS, 20 mM MOPS (pH 6.8), and 0.1 mM PMSF and standardized with molecular weight markers (e.g., bovine serum albumin). The eluate was fractionated by a fraction collector to estimate the native molecular weight.

Amino Acid Analysis and Peptide Sequencing—The amino acid composition of the 60-kDa protein was determined on acid hydrolysates of the samples using an updated single column Beckman model 7300 automatic amino acid analyzer. Proteins were hydrolyzed in 6 N HCl at 105 °C in evacuated tubes for 18 h. No corrections were made for the destruction of seryl and threonyl residues. For the determination of cysteine content, the protein was performic acid oxidized in the presence of 88% formic acid and then hydrolyzed with 6 N HCl at 110 °C for 20 h (Korza and Ozols, 1988). Sequence analysis of the proteins was performed on a Beckman 890C Sequencer using the 0.1 M quadrol program 030176. Microsequence analysis of peptides was performed on a Model 470A, gas-phase sequencer, equipped with model 120A PTH analyzer according to the manufacturer’s instructions.

Polyacrylamide Gel Electrophoresis—Gel electrophoresis was performed in the presence of SDS using a conventional gel electrophoresis system on a Pharmacia Phast System with the buffer system of Laemmli (1970). Molecular weight standards included carbonic anhydrase (M, 29,000), egg albumin (M, 45,000), bovine albumin (M, 66,000), phosphorylase b (M, 97,400), β-galactosidase (M, 116,000), and myosin (M, 205,000).

Measurements of PGM Enzyme Activity in SR—The reaction was started by addition of SR (233 μg/ml) to the reaction mixture containing 84 mM triethanolamine (pH 7.6), 3.5 mM glucose 1-phosphate (potassium salt), 0.02 mM glucose 1,6-diphosphate, 0.9 mM EGTA, 17 mM MgCl₂, 0.19 mM NADP (sodium salt), and 0.6 mM glucose-6-phosphate dehydrogenase, and the slope of the optical change at 340 nm was monitored by a spectrophotometer (Lowry and Passonneau, 1959). In order to examine the effect of salt, detergent, or EDTA on the activity of PGM in SR, the SR was incubated with each of them for 2 min before the measurements of the enzyme activity. Control experiments were conducted with the purified PGM in the absence of SR. The results showed the addition of each of the compounds alone did not significantly affect the enzyme activity.

Library Construction and Screening of the cDNAs—Construction of the rabbit fast twitch skeletal muscle cDNA library was carried out as described elsewhere (Marks et al., 1989). To screen the cDNA library for the 60-kDa protein, a portion of the identified amino acid sequence of rabbit skeletal PGM (Raw et al., 1985) (468–477) was synthesized on siliconized oligonucleotides (TCTAGTGACTTCTCGAAGTGCTTCGCTTT).

Rapid Amplification of 5’ cDNA End—Total RNA from rabbit skeletal muscle was extracted by the guanidium extraction method and mRNA by oligo-dT celluose chromatography (Sambrook et al., 1989). The 5’ end of SR-PGM (PGM type 2b) clone was cloned utilizing anchored polymerase chain reaction (PCR) following the protocol of Frohman et al. (1988). Poly(A)+ RNA from rabbit fast skeletal muscle was hybridized to dATP (22.4 μl μl PGM cDNA) for 30 min and reverse transcribed by adding 4 μl of First Strand Buffer (U. S. Biochemical Corp.) 1.5 µl of dX-dT to 22.4 μl of PGM cDNA. 50 pmol of PGM specific primer, and 40 units of Moloney murine leukemia virus reverse transcriptase (U. S. Biochemical Corp.). The reaction mixture was incubated at 122 °C for 5 min and reverse transcribed primer was removed by centrifugation of the reverse transcription product in Amicon SS-34 at 2,800 rpm for 20 min and by centrifugation in Speed-Vac. ATP tailing was carried out by adding 1 μl of X-tailing buffer (1 X-tailing buffer contained 0.1 μM potassium cacodylate, pH 7.2, 2 mM CaCl₂, and 0.2 mM dithiothreitol) and 0.6 μl of 10 mM dATP to 22.4 μl DNA for 2 min at 37 °C. The reaction mixture was incubated at 85 °C for 4 min. The reaction mixture was cooled to 4 °C, and diluted with TE buffer (pH 8.0) to 1 ml. Excess 5’ reverse transcription primer was removed by centrifugation of the reverse transcription product in Amicon Centricor 100 in a Sorval SS-34 at 2,800 rpm for 20 min and by centrifugation in Speed-Vac. The reaction mixture was incubated with the purified PGM specific primer (10 pmol, 5’-GCTCTTCATGTAGAACCTG-3’), 1.5 μl of adaptor-dT primer (80 pmol/ml, 5’-GACTCGAGTCGACTGAACT-3’), and 20 μl of dATP-tailed DNA. The PCR mixture was denatured at 95 °C for 5 min, cooled to 72 °C for 2 min, and combined with 2.5 units of Taq polymerase (Perkin-Elmer Cetus). Sixty μl of mineral oil overlay the PCR mixture. The PCR mixture was annealed at 55 °C for 5 min and extended at 72 °C for 4 min. The reaction underwent 40 PCR cycles at 96 °C for 1 min, 50 °C for 2 min, and 72 °C for 2 min. Positive PCR product was verified by Southern analysis using an internal PGM probe (5’-GCGGAGAACCTTCAGCA-3’).

Analysis of the cDNA Sequence—Potential transmembrane segments were identified by the methods of Kyte and Doolittle (1982) as implemented in the University of Wisconsin Genetics Computer Group program package for DNA and protein sequence analysis. The use of 9 kDa of a 15-kDa protein was used to synthesize substrates for the type II CaM kinase (Bennett and Kennedy, 1987).

Other Methods—The free Ca²⁺ concentrations were calculated with the use of a computer program and constants described previously (Ikemoto, 1974). Protein concentrations were determined by the Lowry method using bovine serum albumin as a standard (Lowry et al., 1951).
RESULTS

Identification and Purification of a 60-kDa Phosphoprotein—Three phosphoprotein bands (108, 82, and 60 kDa) were identified after phosphorylation of heavy SR proteins in the presence of 5 mM CaCl₂, 1 μM CaM, 0.5 mM MgCl₂, and 0.5 mM [γ-32P]ATP (500 cpm/mmol) (Fig. 1). The extent of the phosphorylation was highest in the 60-kDa protein band, as reported previously (Kim and Ikemoto, 1986). In an attempt to isolate proteins in the 60-kDa region, the solubility of the 60-kDa protein in various concentrations of CHAPS was examined by autoradiography of 32P incorporated into the soluble and insoluble proteins. Fig. 1 depicts the protein profiles (lanes 2-5) and 32P-autoradiographs (lanes 6-9) of the soluble (lanes 4 and 8) and insoluble fractions (lanes 5 and 9) at 1:1 CHAPS:SR ratio, where approximately 35% of the total SR proteins were solubilized. The apparent molecular mass of the CHAPS-soluble 60-kDa phosphoprotein was increased to 73 kDa (lanes 4 and 8). At 1:1 CHAPS:SR ratio, the extent of phosphorylation of the 60-kDa protein in the CHAPS-soluble fraction was approximately 40% of that in the CHAPS-insoluble pellets. The time between addition of [γ-32P]ATP and stopping the reaction by sample buffer was fixed at 30 min for all samples shown in Fig. 1 to avoid time-dependent appearance or disappearance of new bands.

The relative amount of the newly appearing 73-kDa protein band in Fig. 1 (lane 4), estimated by densitometry scanning was 3% in the CHAPS-soluble fraction. It appears that the mobility change of the CHAPS-soluble 60-kDa protein was due to the large quantity of casein and an electrophoretic mobility change of the CHAPS-soluble 60-kDa protein was estimated by densitometry scanning. The soluble fraction was approximately 40% of that in the total SR proteins were solubilized. The apparent molecular mass of the CHAPS-soluble 60-kDa phosphoprotein was increased to 73 kDa (lanes 4 and 8). At 1:1 CHAPS:SR ratio, the extent of phosphorylation of the 60-kDa protein in the CHAPS-soluble fraction was approximately 40% of that in the CHAPS-insoluble pellets. The time between addition of [γ-32P]ATP and stopping the reaction by sample buffer was fixed at 30 min for all samples shown in Fig. 1 to avoid time-dependent appearance or disappearance of new bands.

The protein profiles (lanes 2-5) and 32P-autoradiographs (lanes 6-9) of the soluble (lanes 4 and 8) and insoluble fractions (lanes 5 and 9) at 1:1 CHAPS:SR ratio, where approximately 35% of the total SR proteins were solubilized. The apparent molecular mass of the CHAPS-soluble 60-kDa phosphoprotein was increased to 73 kDa (lanes 4 and 8). At 1:1 CHAPS:SR ratio, the extent of phosphorylation of the 60-kDa protein in the CHAPS-soluble fraction was approximately 40% of that in the CHAPS-insoluble pellets. The time between addition of [γ-32P]ATP and stopping the reaction by sample buffer was fixed at 30 min for all samples shown in Fig. 1 to avoid time-dependent appearance or disappearance of new bands.

Characterization of the 60-kDa Protein—The native molecular mass of the 60-kDa protein in the presence of 0.5% CHAPS was determined using molecular sieve chromatography (Sephacryl S-300). Intact SR vesicles were phosphoryl-

![Fig. 1. Protein profiles (lanes 2–5) and autoradiographs (lanes 6–9) of CHAPS-solubilized heavy SR vesicles phosphorylated by Ca²⁺/CaM-dependent protein kinase. To phosphorylate the SR components, SR vesicles (10 mg/ml) were incubated in a solution containing 20 mM MOPS (pH 6.8), 5 mM CaCl₂, 0.5 mM [γ-32P]ATP, 0.5 mM MgCl₂, and 1 μM CaM. After 2 min of incubation, an aliquot of the reaction solution was mixed with an equal volume of a solution containing 1% CHAPS and 20 mM MOPS (pH 6.8). The suspension was centrifuged at 35,000 rpm for 15 min in a Beckman 50 Ti rotor and the supernatant separated from the pellets. At 30 min after the initial phosphorylation reaction started, proteins in each of the fractions were mixed with Laemmli sample buffer and separated in 5–15% gradient SDS-polyacrylamide gel according to Laemmli (1970), the gel stained with Coomassie Blue, and the dried gel autoradiographed as described under "Experimental Procedures." Lane 1, 40 μg of molecular weight standard proteins stained with Coomassie Blue (β-galactosidase, 116,000; phosphorylase b, 97,400; bovine plasma albumin, 66,000; egg albumin, 45,000); lane 2, phosphorylated heavy SR (300 μg); lane 3, phosphorylated heavy SR in 0.5% CHAPS (300 μg); lane 4, the supernatant in 0.5% CHAPS (90 μg); lane 5, the pellets in 0.5% CHAPS (210 μg). The autoradiographs shown in lanes 6–9 were obtained from the gel shown in lanes 2–5. The upper and lower arrows represent the molecular masses of 73 and 60 kDa. Note the appearance of a new 73-kDa protein band in lane 4 and a matched 32P-band in lane 8.

![Fig. 2. Protein profiles and autoradiographs of the flow-throughs of DEAE-Sephacel and successive heparin-agarose columns. Phosphorylation and solubilization of heavy SR were conducted as described in the legend to Fig. 1. The soluble fraction was loaded on DEAE-Sephacel column equilibrated with 0.5% CHAPS and 20 mM MOPS (pH 6.8). The flow-through from the DEAE column was loaded on heparin-agarose column equilibrated with 0.5% CHAPS, and the bound proteins were eluted with 0–100 mM phosphate gradient (Fig. 3). The 60-kDa protein appeared in 45–65 mM KPO₄, fractions. Occasionally, a minor band appeared underneath the 60-kDa protein.

The native molecular mass of the 60-kDa protein in the presence of 0.5% CHAPS was determined using molecular sieve chromatography (Sephacryl S-300). Intact SR vesicles were phosphoryl-

1 2 3 4 5 6 7 8 9

1 2 3 4 5 6 7 8 9

1 2 3 4 5 6 7 8 9

1 2 3 4 5 6 7 8 9
**TABLE I**

Summary of the characteristics of SR-PGM

<table>
<thead>
<tr>
<th>Molecular mass (kDa)</th>
<th>SDS-polyacrylamide gel electrophoresis</th>
<th>73</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoelectric point</td>
<td>S-DNA</td>
<td>62</td>
</tr>
<tr>
<td>Two-dimensional gel</td>
<td>6.5-7.0</td>
<td></td>
</tr>
<tr>
<td>cDNA</td>
<td>7.04</td>
<td></td>
</tr>
</tbody>
</table>

**TABLE II**

Effects of various compounds on PGM enzyme activity in heavy skeletal muscle SR

<table>
<thead>
<tr>
<th>Addition</th>
<th>No. of observations</th>
<th>Enzyme activity (nmol/min/mg)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4</td>
<td>1.5 ± 0.9</td>
<td>3.1 ± 1.9</td>
</tr>
<tr>
<td>0.15 mM KCl</td>
<td>4</td>
<td>10.5 ± 4.3</td>
<td>21.1 ± 8.7</td>
</tr>
<tr>
<td>1 mM KCl</td>
<td>3</td>
<td>7.3 ± 1.7</td>
<td>15.4 ± 5.7</td>
</tr>
<tr>
<td>CHAPS (1:1 SR, w/w)</td>
<td>5</td>
<td>27.1 ± 3.3</td>
<td>55.3 ± 6.7</td>
</tr>
<tr>
<td>CHAPS (0.2:1 SR, w/w)</td>
<td>3</td>
<td>9.9 ± 1.1</td>
<td>20.3 ± 2.3</td>
</tr>
<tr>
<td>0.15 mM KCl + CHAPS (1:1 SR, w/w)</td>
<td>6</td>
<td>49.0 ± 6.8</td>
<td>100.0 ± 15.9</td>
</tr>
<tr>
<td>1 mM KCl + CHAPS (1:1 SR, w/w)</td>
<td>4</td>
<td>43.3 ± 5.3</td>
<td>88.5 ± 10.9</td>
</tr>
<tr>
<td>5 mM EDTA</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1 mM Guanidine HCl</td>
<td>3</td>
<td>48.4 ± 2.5</td>
<td>98.8 ± 5.2</td>
</tr>
</tbody>
</table>

FIG. 4. Partial restriction endonuclease maps and sequencing strategies for the rabbit skeletal phosphoglucomutase cDNA clones. A linear composite map of the cDNAs is shown, with the coding region indicated by the darloured box and the positions of several diagnostic restriction endonuclease sites marked. Each of the separate clones analysed is shown by a solid bar below the map. The clones PGM1 and PGM2a were obtained from a Xgt11 cDNA library and PGM2b using the RACE method, as described under "Experimental Procedures." The arrow indicates direction and extent of sequencing. The wavy line (AAAA..) indicates the identical region between PGM1 and PGM2.

**FIG. 3.** Further purification of the 60-kDa protein using hydroxylapatite. The flow-through from the heparin-agarose column (see the legend to Fig. 2) was loaded on hydroxylapatite column equilibrated with the washing buffer containing 0.5% CHAPS and 20 mM MOPS (pH 6.8). The unbound proteins remaining in the column were washed with Buffer 1 and the bound proteins were eluted with 40 ml of KPO4 linear gradient (0-100 mM). The unbound proteins remaining in the column by 7% in lanes 7-11 were obtained from the gel shown in lanes 2-6.
A

-342

-342

-252

-72

21084

60-kDa Phosphoprotein in Skeletal Sarcoplasmic Reticulum

-73

-1

FIG. 5. The nucleotide and deduced amino acid sequences of the rabbit skeletal phosphoglucomutase. The nucleotides are numbered negatively in the 5' noncoding region with amino acid residue 1 corresponding to the first residue of the translated proteins. The different nucleotide and amino acid sequences between PGM1 and PGM2 are underlined. The identified amino acids in PGM2 which differ from the published sequence are shown by an asterisk. The predicted Ca2+/CaM-dependent phosphorylation sites are double underlined.
60-kDa Phosphoprotein in Skeletal Sarcoplasmic Reticulum

![Image of the document content](image-url)

Fig. 5—continued

twice in an excess amount of the final suspension buffer containing 150 mM KCl (see “SR Preparation”) and once with KCl-free final suspension buffer. Table II shows that the untreated heavy SR membranes had substantially lower PGM enzyme activity than the SR treated with 150 mM KCl and CHAPS (1.5 versus 49 nmol/mg/min). Addition of 0.15-1 M KCl to the SR partially restored the enzyme activity up to about 20%. Addition of 1 M guanidine HCl led to nearly maximal activation of the enzyme activity (99%). CHAPS (1:1) alone restored 55% of the enzyme activity, whereas lower amount of CHAPS (0.2:1) showed partial activation of the enzyme activity. Addition of 5 mM EDTA inhibited the PGM enzyme activity (Lowry and Passonneau, 1969).

The relative protein amounts of PGM in light and heavy fractions of SR were deduced by measurement of PGM enzyme activity in the presence of 150 mM KCl and 1:1 CHAPS:SR which led to the maximal recovery of the enzyme activity (Table III). The relative protein amounts of the major SR proteins (Ca²⁺ release channel, Ca²⁺ pump, and calsequestrin) in the two SR fractions were also calculated by densitometry scanning of SDS gels and compared with the estimated protein contents of PGM. The heavy SR fraction had 4 times higher PGM content than the light SR. The ratio (4) for PGM is similar to that for Ca²⁺ release channel (3.3) or calsequestrin (2.7), but not to that for Ca²⁺ ATPase (0.7).

cDNA Isolation—In order to isolate cDNAs encoding PGM, oligonucleotides (5' TCATGGTTACCTCCTCTCCTTCG-CCTTCAGACGTGAGTTCAGGAAAGCTCACCC...3') complementary to amino acid sequence numbers 468-477 of PGM (Ray et al., 1983) were synthesized, end-labeled, and used to screen a Xgtll cDNA library of rabbit fast skeletal muscle. Positively identified clones after tertiary screening were amplified by PCR using two universal primers and subjected to Southern analysis using the end-labeled oligonucleotide probes described above. The four largest clones (2.0-2.9 kb) were selected and subcloned into pBluescript or PUC19. The nucleotide sequence of the largest clone (2.9 kb) designated as type 1 was different from that of the other three clones (designated as type 2). Type 1 PGM cDNA was cut with HindIII and PstI and subcloned into pBluescript or PUC19, and the nucleotide sequence was determined, as...
shown in Fig. 4. The rest of the sequence of type 1 PGM was determined on both strands using synthetic oligonucleotide primers as shown in Fig. 4. The N-terminal portion of type 2 PGM was amplified using the RACE method (Frohman et al., 1988) as described under "Experimental Procedures." DNA sequence of the amplified RACE fragment subcloned in PUC9 was determined using universal primer, the adapter primer and a PGM type 2 specific oligonucleotide used for the RACE method.

cDNA Sequence—In Fig. 5, we present the nucleotide and deduced amino acid sequences. The initiator methionine was found 1698 and 1686 bp upstream of the the termination code for type 1 and type 2 PGM, respectively. The initiation methionine codon in type 2 PGM present in the longer sequence, CCACCATGG was identical to the consensus initiation sequence (Kozak, 1984), whereas the consensus initiation sequence was not found in type 1 PGM. The 176 bp upstream of the initiator methionine of type 2 PGM clone contained approximately 75% G + C residues, characteristic of 5'-untranslated sequences in other SR protein cDNAs (MacLennan et al., 1985; Takeshima et al., 1990; Zorzato et al., 1990; Nakai et al., 1990), whereas type 1 PGM clone contained about 50% G + C residues.

Amino Acid Sequence Analysis—Type 2 PGM cDNA sequence encoded a protein of 562 amino acids. The amino acid sequence deduced from the cDNA sequence in this study differs from the reported amino acid sequence (Ray et al., 1983) in that the initiation methionine is added to the N-terminal end and the amino acid at position 392 was altered from Glu to Asp. The deduced isoelectric point is 7.04. On the other hand, type 1 PGM clone has 566 amino acids. The 77 amino acids in the N-terminal region of type 2 PGM were substituted with 81 amino acids found in type 1. The deduced pI is 6.17. There are no amino acid sequence differences beyond the altered region of N-terminal end, although we found that the base pair at position 474 was altered from G to A in PGM type 2 clone.

Five potential sites of Ca\(^{2+}\)/CaM-dependent phosphorylation (indicated by double underline) (Fig. 5B) were predicted on the basis of the consensus sequence, Arg/Lys-Xaa-Xaa-Ser/Thr characteristic of phosphorylation sites on several substrate of the type II CaM kinase (Bennett and Kennedy, 1987). The known active site of PGM is aa 116 serine which is located in the long stretch of hydrophobic region (aa 65–120) (Fig. 7).

Northern Blot Analysis—Fast twitch skeletal muscle-specific expression of PGM was studied by Northern blot analyses of RNA isolated from rabbit fast twitch skeletal muscle (Fig. 6). P3'-Labeled PGM2a clone was used to identify the size of the PGM gene. The size of mRNA hybridizing to PGM type 2 isoform was estimated to be 2.4 kb.

**DISCUSSION**

The goal of this study was to characterize a 60-kDa phosphoprotein which is known to be a major CaM-dependent phosphoprotein in junctional skeletal SR vesicles (Campbell and MacLennan, 1982; Chiesi and Carafoli, 1982; Seiler et al., 1984; Kim and Ikemoto, 1986; Chu et al., 1990; Witcher et al., 1991). Our results show that an isoform of PGM, an enzyme known to be involved in the conversion of glucose 1-phosphate to glucose 6-phosphate (Ray, 1983) is a 60-kDa substrate of CaM-dependent protein kinase in the junctional SR. The specific nature of the association of PGM with SR is evidenced by the finding that the PGM activity in SR, which was not removed by extensive washing, substantially decreased upon membrane association. In contrast, the PGM-SR upon solubilizing in the presence of detergent regained considerable enzyme activity.

**Purification and Characterization of a 60-kDa Phosphoprotein**—Fig. 1 shows a partition of the 60-kDa phosphoprotein band into CHAPS-soluble supernatant and CHAPS-insoluble pellets. The unique mobility change of the CHAPS-soluble phosphoprotein from 60 to 73 kDa (Fig. 1, lanes 4 and 8) probably reflects the large amount of calsequestrin in the heavy SR fraction. The CHAPS-soluble 60-kDa protein was homogeneous, as identified by the analyses of protein composition and N-terminal amino acid sequence. A part of the CHAPS-insoluble 60-kDa protein band could contain also the subunit of Ca\(^{2+}\)/CaM-dependent protein kinase reported by Tuana and MacLennan (1988) and Vale (1988), who reported that the 60-kDa phosphoprotein is the major CaM-binding protein in the SR, as would be predicted if a 60-kDa protein was a part of the Ca\(^{2+}\)/CaM-dependent protein kinase system. The finding that the 60-kDa protein consists of a single polypeptide chain with sequence homology to PGM is signif-

---

**Fig. 6. Northern blot analysis of total mRNA from rabbit skeletal muscle.** Total rabbit skeletal muscle RNA (30 μg) was fractionated on formaldehyde gels as described under "Experimental Procedures." After transfer to nitrocellulose paper, the samples were hybridized to 32P-labeled PGM2a clone and washed as described under "Experimental Procedures." Positions of the 28S and 18S ribosomal subunits are indicated. The size of mRNA hybridizing to the PGM type 2 isoform was estimated to be 2.4 kb.

**Fig. 7. Hydropathy analysis of PGM2.** The hydropathy characteristics of PGM2 was evaluated using the procedures of Kyte and Doolittle (1982) with a window of 20 amino acids.
ciant in light of evidence that various enzymes involved in glycolytic passageways are specifically associated with SR membranes (Meyer et al., 1970; Entman et al., 1976, 1980; Horl et al., 1978; Caswell et al., 1989). Binding of PGM to the SR membrane caused the protein to lose substantial portion of its enzyme activity (Table I). The loss or inhibition of glycolytic enzyme activity upon membrane binding has been reported by other groups (Pierce and Philipson, 1985; Brandt et al., 1990; Rossi et al., 1990; Waddell and Burchell, 1991). The loss of the enzyme activity could be caused by substantial conformational changes upon protein-membrane interactions. If binding of PGM to the SR membrane were due to a simple charge interaction, as suggested for glyceraldehyde phosphate dehydrogenase and phosphoglycerate kinase (Pierce and Philipson, 1985), PGM should be released from the SR membranes upon treatment with high concentration of salt. However, we found that PGM was associated with the SR even after extensive washing with 150 mM KCl buffer during the isolation step (see "Experimental Procedures"). Treatment of the SR membranes with 1 M guanidine HCl suggesting that PGM is associated with phospholipids and/or protein(s) located in the junctional SR, as predicted by hydrophobic aspect of PGM molecules (Ray et al., 1983). In this context, it is interesting to note that the active site of PGM is aa 116 serine located in the N-terminal hydrophobic region (aa 65-120) (Fig. 7). Therefore, it is tempting to speculate that the hydrophobic stretch of PGM may interact with SR membranes, and thereby the resulting conformational change could lead to loss of the enzyme activity.

Molecular Cloning of PGM Isoforms—We have identified two types of PGM cDNAs having 85% sequence homology. PGM type 2 clone represents the 62-kDa PGM, previously identified by Ray et al. (1983), and this clone also appears to encode the SR-PGM. We have found 1 amino acid difference between the 62-kDa PGM (Ray et al., 1983) and the translated PGM clone 2 (at aa 392, Glu→Asp). The initial Met may not have been detected in sequencing reactions. Most of the substitutions in PGM1 clone were found in the N-terminal coding region and noncoding region, while 1-bp alteration was found at cDNA postion 474 (type 2). The functional role of PGM type 1 enzyme remains to be clarified. The consensus G-C content (75%) in the N-terminal noncoding region for SR proteins was found in PGM type 2 clone, but not in PGM type 1 clone (50%) further suggesting that PGM type 2 is SR-PGM and related to SR functions. The result of the Northern analysis (Fig. 6) showed that the size of the mRNA encoding PGM type 2 is approximately 2.4 kDa which agrees with the identified cDNA sequence.

There are five putative phosphorylation sites for Ca2+/CaM-dependent protein kinase, but there is no predicted CaM-binding site, further suggesting that this protein is a substrate of, but not the Ca2+/CaM-dependent protein kinase.

Role of Glycolytic Enzymes in SR Function—The specific association of glycolytic and glycogenolytic enzymes with SR membranes and the evidence that the enzymes may participate in striated muscle excitation-contraction coupling have been shown by various groups (Meyer et al., 1970; Entman et al., 1976, 1980; Horl et al., 1978; Pierce and Philipson, 1985; Caswell et al., 1989). Ca2+-dependent phosphorylase b kinase and its substrate phosphorylase in SR membranes have been suggested to be involved in the regulation of Ca2+-pump in SR (Hori et al., 1978). Association of phosphoglycerate kinase, glycogen synthase, glycogen debranching enzyme, and glycose 6-phosphatase with SR or endoplasmic reticulum have been also reported (Pierce and Philipson, 1985; Waddell and Burchell, 1991). It appears that the functions of these enzymes in SR are related to production of glycolytic ATP and thereby supplying energy to Ca2+ ATPase (Entman et al., 1976, 1980). However, it has been suggested that aldolase and glyceraldehyde phosphate dehydrogenase directly participate in the association between transverse tubule and junctional SR (Brandt et al., 1990). The specific binding of inositol trisphosphate to aldolase which is located in junctional SR suggests that phosphatidylinositol metabolism is regulated by glycolytic enzyme(s) in SR (Thieleczek et al., 1989; Heimleyer et al., 1990).

Although the enrichment of protein kinase systems in junctional SR has been found, the functional consequence of the protein phosphorylation has not been well resolved (Campbell and MacLennan, 1982; Chiesi and Carafoli, 1982; Seiler et al., 1984; Kim and Ikemoto, 1986; Chu et al., 1990; Witcher et al., 1991). A hypothesis that a 60-kDa phosphoprotein is involved in the regulation of Ca2+ release from the SR was proposed on the basis of the findings that the extent of phosphorylation of the 60-kDa protein paralleled the inhibition of Ca2+ release from the SR (Campbell and MacLennan, 1982; Kim and Ikemoto, 1986) and that the 60-kDa protein is the main phosphoprotein in SR under the variety of experimental conditions (Campbell and MacLennan, 1982; Chiesi and Carafoli, 1982; Seiler et al., 1984; and Chu et al., 1990).

Our study does not establish a role for SR-PGM in the regulation of Ca2+ release from the skeletal SR. Elucidation of such a relationship must await more direct functional studies including expression of the cloned cDNAs in a system where it is possible to examine the functions of each of the proteins.

Acknowledgments—We thank Drs. Arnold M. Katz, Achilles Papano, Noriaki Ikemoto, and John Gergely for valuable suggestions and encouragement. We express our special thanks to Dr. Hyoung-Soon Duck Nah for generously providing protocols and reagents to carry out the RACE method. We thank Enjoo Lee, Paul Stabach, Drs. Steve Padula, Nikolaus Spoerel, and Paul Primakoff for advice on various experimental protocols and Dr. Edwin G. Krebs for providing purified myosin light chain kinase. The Northern blot data shown in Fig. 6 were carried out by Dr. William Barry. Determinations of 5′ and 3′ termini of the amino acid composition and N-terminal amino acid sequence of the studied proteins were carried out by George Korza at the Amino Acid Facility (Director: Dr. J. Ozols).

REFERENCES

60-kDa Phosphoprotein in Skeletal Sarcoplasmic Reticulum


Witte, D. R., Kovacs, R. J., Schulman, H., Cefali, D. C., and Jones, L. R. (1991) J. Biol. Chem. 266, 11144-11152