THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 265, No. 10, Issue of April 5, pp. 5869-5874, 1990
Printed in U.S.A.

Isolation and Characterization of Calcium Binding Glycoproteins of Cardiac Sarcolemmal Vesicles*

(Received for publication, August 16, 1989)

Marek Michalak‡, Larry Fliegel§, and Ken Wlasichuk

From the Cardiovascular Disease Research Group and the Departments of Pediatrics and Biochemistry, University of Alberta, Edmonton, Alberta T6G 2S2, Canada

Two major Ca^{2+}-binding glycoproteins M_r 120,000 and 100,000 were isolated from 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid-solubilized bovine heart sarcolemma membrane. Peroxidase-conjugated concanavalin A and wheat germ agglutinin lectins bind strongly to the isolated 120- and 100-kDa glycoproteins. Treatment with endoglycosidase F resulted in conversion of the 120-kDa glycoprotein to a form migrating at about 97 kDa. Treatment of the 100-kDa band with endoglycosidase F produced form of about 80 kDa. Endoglycosidase H digestion removes only 5% of the mass of both glycoproteins. The carbohydrate structure of both glycoproteins, is therefore, predicted to be at least 75% complex structure and 25% high mannose or hybrid structure.

The 120- and 100-kDa glycoproteins are the major Ca^{2+}-binding proteins in the sarcolemma membranes. Intact and endoglycosidase-treated glycoproteins bind $^{45}Ca^{2+}$ as analyzed by a $^{45}Ca^{2+}$ overlay technique. Using polyclonal antibodies, the 120- and 100-kDa glycoproteins were identified in muscle plasma membranes (ventricles, atria, and uterus smooth muscle). They were, however, not present in non-muscle tissues such as pancreas, liver, and kidney.

The 120- and 100-kDa glycoproteins appear to be homologous molecules as judged by their similar V8 protease peptide maps, cross-reactivity with polyclonal antibody, and other physicochemical properties.

The sarcolemma (SL)[1] of the heart plays a key role in mediating the metabolic, electrophysiological, and contractile function of myocytes. A primary function of this membrane is the transport of ions into and out of the cell during the muscle excitation-contraction cycle (1). In addition, the SL membrane contains various hormone and drug receptors and thus provides signals for the modulation of heart metabolism and function (2–6).

Cardiac muscle is dependent on the presence of external Ca^{2+} for maintenance of contractile activity. Therefore, one of the most important functions of the SL is the control of Ca^{2+} movements. The cardiac cell is equipped with multiple mechanisms for trans-sarcolemmal Ca^{2+} transport (7). These include the electrogenic Na^+/Ca^{2+} exchange (8) and SL Ca^{2+}/calmodulin-dependent ATPase (9), a voltage-dependent Ca^{2+} channel (10) and Ca^{2+} binding activity (11). The relative contribution and importance of each of these mechanisms in maintaining cellular Ca^{2+} homeostasis in cardiac muscle have yet to be elucidated.

Langer's group (11, 12) has proposed, based on the physiological, biochemical, and cytochemical observations, that Ca^{2+} bound to SL membrane plays an important role in the control of the overall cellular Ca^{2+} homeostasis in the heart. Apart from its possible participation in one of the events of excitation-contraction coupling (11, 12), SL-bound Ca^{2+} might play an important role in the maintenance of homeostasis for Ca^{2+} and possibly other cations (13). The presence of different Ca^{2+}-binding sites in the heart SL have been reported (11, 12) and the association of Ca^{2+} with SL membrane confirmed by ultrastructural studies (14). It is not clear, however, how these Ca^{2+}-binding sites relate to specific molecules. The majority of Ca^{2+} (>70%) bound to SL membrane is associated with membrane acidic phospholipids (15). In earlier studies Limas (16) has indicated that proteins may also be involved in Ca^{2+} binding to SL vesicles but no specific Ca^{2+}-binding protein(s) was identified.

Recently, we have fractionated highly purified bovine cardiac SL vesicles by WGA agglutination method and studied the glycoprotein composition of this membrane (17). The majority of SL vesicles (75% of membrane preparation) were agglutinated in the presence of WGA, and they corresponded to the highly purified SL vesicles (17). The origin of the nonagglutinated vesicles is less clear but they may derive from a different part of SL membrane, possibly the transverse tubular system (17). During the course of this work, two major WGA-binding glycoproteins of M_r 120,000 and 100,000 were identified in the SL vesicles (17). In this report we describe the isolation and characterization of the 120- and 100-kDa glycoproteins of bovine cardiac SL membrane. The 120- and 100-kDa glycoproteins are the major Ca^{2+}-binding proteins in cardiac SL membrane. They appear to be homologous molecules as judged by their similar peptide maps, cross-reactivity with polyclonal antibodies, and similar mass of their carbohydrate. The 120- and 100-kDa glycoproteins are specific for muscle plasma membranes and are not detected with specific antibody in non-muscle tissues such as kidney, pancreas, and liver.

* This work was supported by the Medical Research Council of Canada, the Alberta Heart and Stroke Foundation, and the Alberta Heritage Foundation for Medical Research. 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.

‡ Scholar of the Medical Research Council of Canada and the Alberta Heritage Foundation for Medical Research. To whom correspondence should be addressed: Cardiovascular Disease Research Group, University of Alberta, 417 Heritage Medical Research Bldg., Edmonton, Alberta, Canada T6G 2S2.

§ Postdoctoral fellow of the Alberta Heritage Foundation for Medical Research.

[1] The abbreviations used are: SL, sarcolemma; WGA, wheat-germ agglutinin; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Endo F, endoglycosidase F; Endo H, endoglycosidase H; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

EXPERIMENTAL PROCEDURES

Materials—Hepes, histidine, Tris, lectins, peroxidase-conjugated lectins, Nonidet P-40, Triton X-100, Chaps, WGA-Sepharose and GlcNAc were purchased from Sigma. $^{45}CaCl_2$ was obtained from Du Pont-New England Nuclear. Endo F, Endo H, dimethyl sulfoxide, benzamidine, phenylmethylsulfonyl fluoride, *N*,[*N*-(L-3-trans-carboxyoxirane-2-carbonyl)-L-leucyl]-agmatine, aprotinin, leupeptin, pepstatin, L-1-chloro-3-(4-tosylamido)-7-amino-2-heptanone hydrochloride, L-1-chloro-3-(4-tosylamido)-4-phenyl-2-butanone, (4-amidinophenyl)-methansulfonyl fluoride, and *N*-(L-rhamnopyrnaosyl-oxyhydroxyphosphinyl)-L-leucyl-L-tryptophan were obtained from Boehringer-Mannheim. SDS-PAGE reagents and prestained molecular weight markers were purchased from Bio-Rad. *Staphylococcus aureus* protease V8 was from Miles Laboratories Ltd. Fresh bovine hearts, uterus, pancreas, kidney, and liver were obtained from a local slaughterhouse.

Preparation of Membrane Vesicles—SL membranes were isolated by sucrose flotation according to the method described by Jones (18). Initial homogenization of muscle tissue and subsequent membrane fractionation was performed in the presence of a mixture of the following protease inhibitors made in 2,000 times stock solution in 50% dimethyl sulfoxide, 0.5 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 μg of *N*-[*N*-(L-3-trans-carboxyoxirane-2-carbonyl)-L-leucyl]-agmatine/ml, 0.1 μg of aprotinin/ml, 0.5 μg of leupeptin/ml, 0.5 μg of pepstatin/ml, 50 μg of L-1-chloro-3-(4-tosylamido)-7-amino-2-heptanone hydrochloride and L-1-chloro-3-(4-tosylamido)-4-phenyl-2-butanone, 0.1 μg of (4-amidinophenyl)-methansulfonyl fluoride/ml, 50 μg of *N*-(L-rhamnopyranosyloxyhydroxyphosphinyl)-L-leucyl-L-tryptophan/ml. Vesicles were suspended at a concentration of 5 mg/ml in 250 mM sucrose and 20 mM Hepes, pH 7.4, frozen in liquid N_2 and stored at −80 °C. Atria SL vesicles were isolated by the procedure used for the purification of ventricle SL membrane (18). Pancreas, kidney, uterus, and liver microsomes were isolated by homogenizing the bovine tissues with a Polytron PT-20 in a buffer containing 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine, 120 mM NaCl, and 10 mM imidazole-HCl, pH 7.4. The homogenates were centrifuged at 1,600 × *g* for 10 min, and the supernatant was saved. The pellet was rehomogenized in the same buffer and centrifuged as above and both supernatants were combined. These were centrifuged at 10,000 × *g* for 15 min, and the pellets were discarded. The supernatants were pelleted at 100,000 × *g* for 60 min, and the microsomes were suspended in 250 mM sucrose, 10 mM Tris, pH 8.0, frozen in liquid N_2 and stored at −85 °C. Apical and basolateral plasma membrane vesicles were isolated from dog kidney (19, 20) and were a generous gift of Dr. R. A. F. Reithmeier, University of Toronto. Before use, samples of frozen membrane were thawed at 37 °C. Protein was determined by the method of Lowry *et al.* (21) or Bradford (22).

Membrane Solubilization—SL vesicles were solubilized with 8 mM Chaps (5 mg of Chaps/mg protein) in solubilization buffer containing 100 mM NaCl, 0.1 mM EGTA, and 20 mM Hepes, pH 7.4. After incubation at 4 °C for 15 min, nonsoluble material was separated by centrifugation at 150,000 × *g* for 45 min at 4 °C. Greater than 85% of membrane proteins were solubilized by this procedure.

WGA Affinity Chromatography—A WGA-Sepharose column with a bed volume of approximately 2 ml was regenerated before use with 50 ml of low salt buffer (100 mM NaCl, 0.1 mM EGTA, 2 mM $MgCl_2$, 0.05 mg of Chaps/ml, 20 mM Hepes, pH 7.4) containing 1% SDS followed by a wash with 100 ml of low salt buffer. SL membrane was solubilized as described above. $MgCl_2$ and NaCl were added to final concentrations of 2 and 500 mM, respectively. Two mg of SL protein were applied/2 ml of WGA-Sepharose. The column was then washed with (*a*, see Fig. 1) 40 ml of high salt buffer (500 mM NaCl, 0.1 mM EGTA, 2 mM $MgCl_2$, 0.05 mg of Chaps/ml, 20 mM Hepes, pH 7.4) followed by (*b*) 40 ml of low salt buffer, (*c*) 10 ml of low salt buffer containing 0.03% SDS (in some experiments SDS wash was omitted, see Fig. 3) and 10 ml of low salt buffer. The final 3 ml wash never contained greater than background absorbance at 280 nm. Specifically bound glycoproteins were eluted by (*d*) 10 ml wash of low salt buffer containing 200 mM GlcNAc and were concentrated to 500 μl using an Amicon Ultrafiltration Cell model 8200.

SDS-Polyacrylamide Gel Electrophoresis—SDS-PAGE was on 10% or 5–15% polyacrylamide gradient gels as described by Laemmli (23). After gel electrophoresis, gels were stained with either Coomassie Blue or silver and scanned using a DU-65 Beckman Spectrophotometer. Digestion of the isolated glycoproteins in SDS-PAGE was carried out with *S. aureus* protease V8 (Miles Laboratories Ltd.) according to Cleveland *et al.* (24). Molecular weight standards were myosin, 200,000; β-galactosidase, 116,300; phosphorylase *b*, 97,400; bovine serum albumin, 66,000; and ovalbumin, 42,000.

Analysis of Lectin and $^{45}Ca^{2+}$ Binding—For lectin and $^{45}Ca^{2+}$ binding analysis, proteins were transferred electrophoretically onto nitrocellulose membrane according to the method of Towbin *et al.* (25). For lectin binding, blots were blocked with 0.3% gelatin and subsequently incubated with appropriate peroxidase conjugated lectins (1 μg/ml) in a buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 0.05% Triton X-100, and 2 mM $MgCl_2$ (17). The peroxidase reaction was developed using 4-chloro-1-naphthol as the substrate. Identification of $^{45}Ca^{2+}$-binding proteins in SL membranes was carried out according to the procedures of Maruyama *et al.* (26).

Endoglycosidase Treatment—Glycoproteins purified by WGA chromatography were examined by SDS-PAGE on a 5–15% gradient gel as described above. Upon completion of the electrophoresis the sample lane was sliced, and slices containing the 120- and 100-kDa glycoproteins were transferred to the sample cup of an Isco model 1750 sample concentrator, overlaid with 10 mM NH_4HCO_3, pH 8.6, and 0.02% SDS, and electroeluted at 1 watt for 18 h at room temperature. The electroeluted material was dialyzed overnight against 100 mM sodium phosphate, pH 6.1, 50 mM EDTA, and 0.1% SDS at 4 °C and then concentrated by freeze-drying in a Speed-Vac concentrator. The isolated 120- and 100-kDa protein bands were divided into two equal aliquots, and the volume was adjusted to 90 μl with 100 mM sodium phosphate, pH 6.1, 50 mM EDTA, 1% β-mercaptoethanol, and 0.1% SDS and incubated at 37 °C for 30 min. The samples were then made to 1% with respect to Nonidet P-40, and 30 units/ml of Endo F, Endo H, or buffer alone was added. The samples were incubated at 37 °C for 16 h, after which they were examined by SDS-PAGE (23). In some experiments the purified glycoproteins were directly digested with Endo F and Endo H at 37 °C for 4 h in a buffer containing 100 mM sodium phosphate, pH 6.1, 50 mM EDTA, 1% β-mercaptoethanol, 0.1% SDS, and 30 units/ml of Endo F or H.

Preparation of Anti-120- and 100-kDa Glycoproteins Antibody—New Zealand rabbits were immunized intraperitoneally with 0.5 mg of bovine heart SL vesicles emulsified in Freund's complete adjuvent. After 2 and 4 weeks, the immunization was repeated but this time with protein bands excised from the SDS-PAGE gels of SL proteins and emulsified in Freund's incomplete adjuvant. Antibodies were screened by immunoblotting after electrophoretical transfer onto nitrocellulose membrane according to the method of Towbin *et al.* (25). For affinity purification of the antibody against either 120- and 100-kDa glycoprotein, the purified glycoproteins were separated by preparative SDS-PAGE and transferred to nitrocellulose (25). The nitrocellulose sheets were stained with Ponceau S, and the bands corresponding to either 120- or 100-kDa glycoprotein were cut out and incubated with a 1:25 dilution of the antiserum in phosphate-buffered saline containing 1% milk powder. Bound antibodies were eluted by incubation of the nitrocellulose strips in 200 mM glycine, pH 2.8. After 2 min of incubation, the pH of the antibody solution was adjusted to 8.0 by the addition of 1 M Tris-HCl, pH 8.8. In some experiments, the antibody solution was dialyzed overnight against phosphate-buffered saline and stabilized by the addition of bovine serum albumin to a final concentration of 1 mg/ml.

RESULTS

Lectin Chromatography—The chromatographic profile of solubilized SL proteins on WGA-Sepharose is shown in Fig. 1. More than 90% of applied proteins appeared in the column "run-through" or in the initial high salt, low salt, and SDS washes and, hence, failed to adsorb to the WGA-Sepharose. When this unadsorbed fraction was analyzed on SDS-PAGE almost all SL membrane proteins appeared in these fractions (Fig. 2*A*, *lanes 4–8*). About 0.6% of membrane protein adsorbed to the WGA-column and most (>60%) of the adsorbed protein was eluted with the specific sugar GlcNAc. SDS-PAGE of the GlcNAc fraction revealed that two glycoproteins, 120 and 100 kDa, were specifically eluted (Fig. 2, *A* and *B*). It was critical to wash a WGA column with a low concentration of SDS (0.03%) in order to achieve a good purification of both glycoproteins. Silver staining of SDS-PAGE of the GlcNAc fraction eluted from the WGA column not washed with SDS revealed the presence of additional protein bands

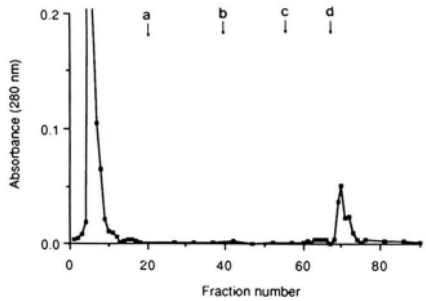

FIG. 1. **WGA-Sepharose chromatography of cardiac SL glycoproteins.** SL vesicles were solubilized with Chaps, centrifuged, and the Chaps-soluble fraction was separated on WGA-Sepharose according to the procedure described under "Experimental Procedures." WGA column was subsequently washed with different buffers as described under "Experimental Procedures"; *a*, high salt buffer; *b*, low salt buffer; *c*, low salt buffer containing 0.03% SDS followed by low salt buffer; and *d*, low salt buffer containing 200 mM GlcNAc. SDS-PAGE of the obtained fractions is presented in Fig. 2.

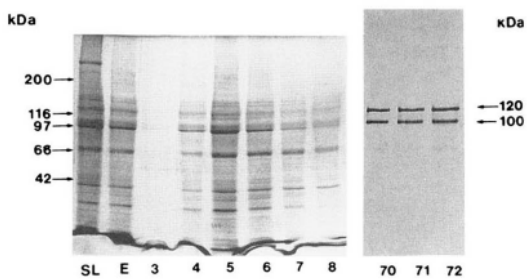

FIG. 2. **SDS-PAGE of cardiac SL proteins separated by lectin affinity chromatography.** WGA-Sepharose chromatography of cardiac glycoproteins was carried out as described under "Experimental Procedures" and the elution profile is presented in Fig. 1. Proteins (10 μg each, except for 2 μg in *lanes 70*, *71*, and *72* and 15 μg in *lane SL*) were subjected to 5–15% gradient SDS-PAGE and stained with silver. The *arrows* indicate the positions of Bio-Rad high molecular weight standard proteins. *120* and *100*, 120- and 100-kDa glycoproteins, respectively. The *lane numbers* correspond to the fraction numbers as depicted in Fig. 1. *SL*, SL vesicles; *E*, Chaps-solubilized SL applied on lectin column.

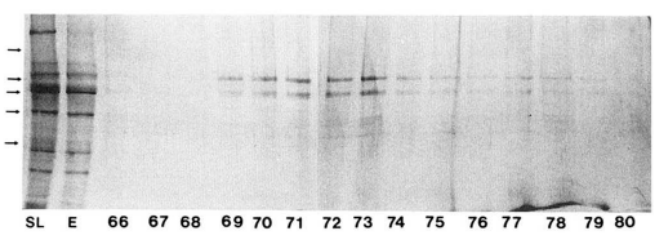

FIG. 3. **SDS-PAGE of cardiac SL proteins separated by lectin affinity chromatography not washed with SDS.** WGA-Sepharose chromatography of cardiac glycoproteins was carried out as described under "Experimental Procedures" except that wash with 0.03% SDS in low salt buffer (*c*, see Fig. 1) was omitted. Proteins were subjected to 5–15% gradient SDS-PAGE and stained with silver. Three-hundred-μl fractions were collected and a 25-μl sample of each fraction was separated on SDS-PAGE. The *arrows* indicate the positions of Bio-Rad high molecular weight standard proteins (myosin, 200,000; β-galactosidase, 116,300; phosphorylase *b*, 97,400; bovine serum albumin, 66,000; and ovalbumin, 42,000). The *lane numbers* correspond to the fraction numbers as depicted in Fig. 1. *SL*, SL vesicles; *E*, Chaps-solubilized SL applied on lectin column. Protein amounts were *lane SL*, 15 μg; *lane E*, 10 μg.

(Fig. 3). The major contaminant present was a protein band slightly smaller than 100-kDa glycoprotein (Fig. 3). Washing the WGA column with a low concentration of SDS prior to the GlcNAc elution removes these contaminants. In all subsequent experiments the 120- and 100-kDa glycoproteins were purified from the WGA column washed with 0.03% SDS as presented in Fig. 2. The yield of the 120- and 100-kDa glycoprotein complex was approximately 3–4 μg/mg SL vesicles. As judged from the yields of purified glycoproteins as well as from the SDS-PAGE of the proteins not bound to the WGA column (Fig. 2, compare *lanes 4–8* to *lanes 70–72*), we concluded that 120- and 100-kDa glycoproteins, which we purified, comprise only a small fraction of 120- and 100-kDa protein bands seen in native SL membrane. The ratio of SL membrane to WGA-Sepharose (2 mg of SL protein/2 ml of WGA Sepharose) was critical for the successful purification of the glycoproteins. Scaling up the procedure led to complete loss of column resolution as did the use of detergents other than Chaps (Triton X-100, octylglucosides, cholate, and deoxycholate were tested). When larger amounts of glycoproteins had to be purified a series of 2-ml WGA columns was used. Both glycoproteins eluted from WGA column simultaneously. This may be due to their similar physicochemical properties (see below) or to the formation of a protein complex under the conditions of membrane solubilization used in this study.

As a means of further characterizing the two glycoproteins, we digested purified proteins directly in SDS-PAGE. Fig. 4 shows the silver-stained, one-dimensional peptide map of the 120- and 100-kDa glycoproteins digested with V8 protease from *S. aureus*, it clearly showed that the 120- and 100-kDa glycoproteins are related or may even be identical. The 100-kDa glycoprotein may, therefore, be a degradation product of the 120-kDa glycoprotein or the 120- and 100-kDa glycoproteins may be highly related proteins present in the same membrane. In addition to the peptide map analysis, most of the physicochemical properties studied indicated that both the 120- and 100-kDa glycoproteins were very similar (see below).

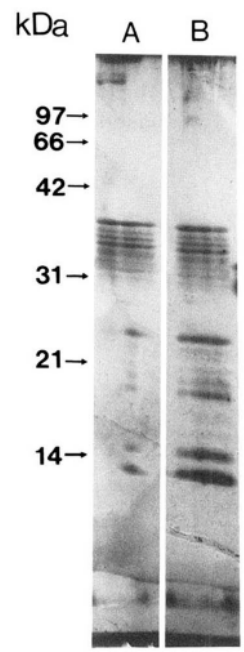

FIG. 4. **One-dimensional peptide maps of the isolated cardiac glycoproteins.** 120- (*A*) and 100-kDa (*B*) glycoproteins were separated on 10% SDS-PAGE, stained with Coomassie Brilliant Blue, eluted electrophoretically from the gel, digested with protease V8 from *S. aureus*, separated on 15% SDS-PAGE according to the method of Cleveland *et al.* (4) and stained with silver. The *arrows* indicate the positions of Bio-Rad low molecular weight standard proteins.

Analysis of Lectin Binding to SL Vesicles—Fig. 5 shows the binding of peroxidase labeled concanavalin A and WGA to SL proteins and to the isolated 120- and 100-kDa glycoproteins. The major concanavalin A-binding glycoproteins in SL vesicles were a doublet at 130 kDa, protein bands at 120, 100 kDa and diffused bands at 80, 60, 55, 50, and 40 kDa (Fig. 5 *ConA*). WGA binding was observed to 120-, 100-, 80-, 70-, 60-, and 55-kDa glycoproteins. Protein fractions isolated on the WGA column were also tested for their reactivity with peroxidase-conjugated WGA and concanavalin A (Fig. 5, *WGA*). The results obtained with lectin binding to SL showed a relatively complex glycoprotein composition of the membrane. However, under the conditions used in this study only two glycoproteins (120 and 100 kDa) were purified by WGA-Sepharose column chromatography. This may be due to the type of detergent as well as the conditions of WGA chromatography used in this study. Both lectins bind strongly to the isolated 120- and 100-kDa glycoproteins.

In order to confirm the presence of *N*-linked carbohydrate, as well as to provide more accurate information regarding the peptide mass of SL glycoproteins, the 120- and 100-kDa glycoproteins were individually treated with Endo F, as outlined under "Experimental Procedures." As shown in Fig. 6, treatment for 16 h with Endo F resulted in conversion of the 120-kDa glycoprotein to a form migrating at about 97 kDa. Treatment of the 100-kDa band with Endo F produced a form of about 80 kDa. Approximately 16–20% of the 120- and 100-kDa glycoproteins' mass consists of Endo F-sensitive carbohydrate.

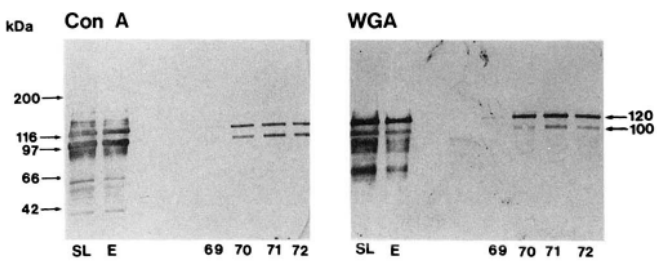

FIG. 5. **Lectin binding to SL proteins and purified glycoproteins.** SL membrane (*SL*), Chaps solubilized SL (*E*), and glycoprotein-containing fractions (*fractions number 69, 70, 71, 72*, see Fig. 1) eluted from lectin affinity column were separated on 10% SDS-PAGE, transferred electrophoretically to nitrocellulose membrane and reacted with peroxidase-labeled concanavalin A (*ConA*) and WGA (*WGA*) as described under "Experimental Procedures." The *arrows* indicate the positions of standard proteins. The positions of the 120- (*120*) and 100-kDa (*100*) glycoproteins are indicated.

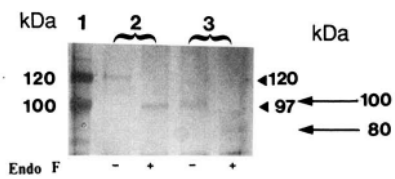

FIG. 6. **Endo F treatment of the purified cardiac SL glycoproteins.** 120- and 100-kDa glycoproteins were purified on a WGA affinity column, individual proteins were electroeluted out of the SDS-PAGE and treated with (+) and without (−) Endo F, separated on 10% SDS-PAGE and stained with silver as described under "Experimental Procedures." The *arrowheads* indicate the positions of 120-kDa glycoprotein (*120*) and the Endo F-digested form of 120-kDa glycoprotein, molecular weight of approximately 97,000 (*97*). The *long arrows* indicate the positions of 100-kDa glycoprotein (*100*) and the Endo F-digested form of 100-kDa glycoprotein, molecular weight of approximately 80,000 (*80*). The positions of the 120- (*120*) and 100-kDa (*100*) glycoproteins are indicated. For comparison the purified 120- and 100-kDa glycoproteins are shown in the first lane.

$^{45}Ca^{2+}$ Binding to SL Proteins—The major $^{45}Ca^{2+}$-binding proteins in the SL membrane, as analyzed by a $^{45}Ca^{2+}$ overlay technique (26), were 120 and 100 kDa (Fig. 7*B*, *lane 1*). Fig. 7*B*, *lane 2* shows that the purified 120- and 100-kDa glycoproteins bind $^{45}Ca^{2+}$. The results of $^{45}Ca^{2+}$-binding experiments indicated that the 120- and 100-kDa $^{45}Ca^{2+}$-binding proteins are the 120- and 100-kDa glycoproteins purified on WGA-Sepharose. In order to further analyze Ca^{2+} binding to the 120- and 100-kDa glycoproteins, as well as to establish if the Ca^{2+}-binding sites are in the polypeptide or carbohydrate parts of these molecules, isolated glycoproteins were treated with Endo F and H prior to SDS-PAGE, electrophoretical transfer, and $^{45}Ca^{2+}$ overlay technique. Fig. 8, *lanes 2* and *3*, show $^{45}Ca^{2+}$ binding to Endo F- and Endo H-digested glycoproteins. The binding was approximately equal for digested and undigested material. $^{45}Ca^{2+}$ bound to the purified Endo F- and Endo H-digested glycoproteins in amounts similar to those bound by the undigested proteins (Fig. 8). These results indicate that carbohydrates are not significantly involved in Ca^{2+} binding to the 120- and 100-kDa glycoproteins. These results also show that Endo H removes only 5% of the mass of both glycoproteins, which is equivalent to approximately ¼ of the carbohydrate that is sensitive to Endo F (compare *lanes 2* and *3* in Fig. 8, and *lanes 1* and *2* in Fig. 9*B*).

Identification of SL Glycoproteins Using Specific Antibodies—Antibodies raised against SDS-PAGE purified proteins reacted with both glycoproteins either from SL membrane or after purification by WGA-Sepharose column chromatography (Fig. 9*A*). Reactivity of this polyclonal antibody with both 120- and 100-kDa glycoproteins indicated that both proteins are closely related or may even be identical. This conclusion is further supported by the observations that either anti-120- or 100-kDa antibodies, affinity purified as outlined under "Experimental Procedures," cross-reacted with both glycoproteins as analyzed by immunoblotting after electrophoretical transfer of purified glycoproteins or SL proteins

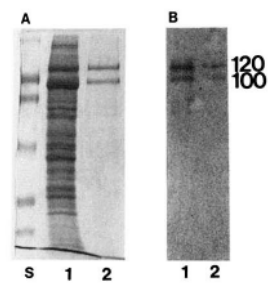

FIG. 7. **Identification of $^{45}Ca^{2+}$-binding proteins in SL membrane.** SL proteins and purified glycoproteins were separated by SDS-PAGE, transferred electrophoretically onto nitrocellulose membrane, and incubated with $^{45}Ca^{2+}$ as described under "Experimental Procedures." *A*, Coomassie Blue-stained SDS-PAGE of prestained molecular weight standards (*S*); 40 μg of SL membrane (*1*); 5 μg of purified glycoproteins. *B*, autoradiography of (*A*) after electrophoretically transferred to nitrocellulose membrane and incubation with $^{45}Ca^{2+}$. *1*, SL membrane; *2*, purified glycoproteins. The positions of the 120- (*120*) and 100-kDa (*100*) glycoproteins are indicated.

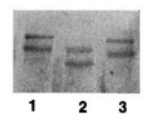

FIG. 8. **$^{45}Ca^{2+}$ binding to Endo F- and H-digested glycoproteins.** Purified 120- and 100-kDa glycoproteins were subjected to SDS-PAGE followed by electrophoretical transfer to nitrocellulose membrane and incubated with $^{45}Ca^{2+}$ as described under "Experimental Procedures." *1*, purified glycoproteins; *2*, purified glycoproteins treated with Endo F; *3*, purified glycoproteins treated with Endo H.

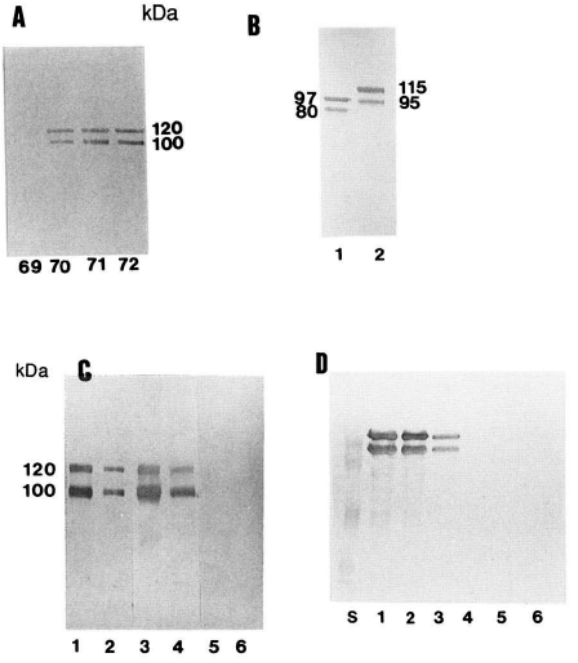

FIG. 9. **Immunoreactivity of antibody against cardiac SL glycoproteins.** Different plasma membrane preparations and purified cardiac SL 120- and 100-kDa glycoproteins were subjected to SDS-PAGE and transferred electrophoretically to nitrocellulose membrane. Immunoperoxidase staining of nitrocellulose blots with anti-120- and 100-kDa glycoprotein antibody was carried out as described under "Experimental Procedures." The positions of the 120- (*120*) and 100-kDa (*100*) glycoproteins are indicated and the relative molecular weight of immunoreactive protein bands is shown. *A*, GlcNAc-eluted fractions (*fractions 69*, *70*, *71*, *72*, see Fig. 1). *B*, purified 120- and 100-kDa glycoproteins digested with Endo F (*1*) or Endo H (*2*). *C*, immunoblot analysis of SL vesicles (ventricle) and plasma membranes from bovine uterus and atria. *1*, SL vesicles (ventricle); *2*, purified glycoproteins; *3*, SL vesicles (atria); *4*, plasma membrane vesicles (uterus); *5*, apical membrane vesicle (kidney); *6*, basolateral membrane vesicles (kidney). Protein amounts were *lane 1*, 20 μg; *lane 2*, 5 μg; *lane 3*, 20 μg; *lane 4*, 20 μg; *lanes 5* and *6*, 25 μg each. *D*, immunoblot analysis of microsomal fractions from bovine pancreas, liver, and kidney and plasma membrane from bovine uterus. *1*, SL vesicles (ventricle); *2*, SL vesicles (atria); *3*, plasma membrane vesicles (uterus); *4*, bovine pancreas microsomes; *5*, bovine liver microsomes; *6*, bovine kidney microsomes. Protein amounts were *lane 1*, 20 μg; *lane 2*, 20 μg; *lane 3*, 25 μg; *lanes 4*, *5*, and *6*, 20 μg each. *S*, prestained molecular weight standards.

onto nitrocellulose membrane (data not shown).

The polyclonal antibody used in this study reacted with proteins polypeptides which are devoid of the carbohydrate part of both glycoproteins (Fig. 9*B*), as shown by reactivity of the antibody with Endo F- (Fig. 9*B*, *lane 1*) and Endo H- (Fig. 9*B*, *lane 2*) digested glycoproteins.

The 120- and 100-kDa Glycoproteins Are Muscle Specific—Using polyclonal antibody reacting with 120- and 100-kDa glycoproteins, we studied the tissue distribution of both glycoproteins. Three different muscle tissues were tested. Fig. 9, *C* and *D*, shows that in addition to ventricle SL membrane, immunoreactive 120- and 100-kDa glycoproteins were also present in atria SL vesicles and uterus plasma membrane. When tested for cross-reactivity with non-muscle tissues anti-120- and 100-kDa did not react with bovine pancreas, liver, and kidney microsomes (Fig. 9*D*, *lanes 4* and *5*). It also did not react with either apical or basolateral membrane highly purified from kidney (Fig. 9, *C*, *lanes 5* and *6* and *D*, *lane 5*).

DISCUSSION

We have isolated and characterized two major glycoproteins of apparent M_r of 120,000 and 100,000 present in highly purified bovine heart SL membranes. The 120- and 100-kDa glycoproteins are the major Ca^{2+}-binding proteins in the membrane as shown by their $^{45}Ca^{2+}$ binding. Using polyclonal antibodies, we showed that both glycoproteins are specific for muscle plasma membranes. Immunoreactive glycoproteins are present in muscle tissue (smooth muscle, cardiac ventricles, and atria SL) but not in non-muscle tissues (pancreas, liver, and kidney).

It is surprising that after Chaps solubilization and a WGA column chromatography only two proteins can be identified by Silver-stained SDS-PAGE. There is no contamination in these protein bands since NH_2-terminal sequence analysis revealed only a single residue present at their respective NH_2-terminals (data not shown). There are a number of ion channels and receptor proteins present in the SL that are known to be glycosylated. However, they are not eluted under experimental conditions used in these studies. It is difficult to say if other SL glycoproteins are retained in the column or eluted during initial washes of the column. It has been noted in our laboratory that scaling up of the purification procedures led to absolute loss of the column resolution and a significant number of additional (glyco)proteins would elute from the column together with 120- and 100-kDa glycoproteins. This is also observed when detergents different than Chaps are used for solubilization of the SL vesicles. Most likely under the condition of solubilization and the condition of WGA column chromatography used in this study only the 120- and 100-kDa glycoproteins have their carbohydrate accessible for the interaction with lectins. This may be related to the type of micelles formed when SL vesicles are solubilized with Chaps. Another possibility is that the washing conditions used in this study led to the early elution of other glycoproteins present in this membrane. This subject will require a further study.

Both glycoproteins show similar sensitivity to endoglycosidase digestion. Sensitivities to Endo F and H, however, differ significantly from each other and allowed us to predict structures of the asparagine-linked oligosaccharide of the 120- and 100-kDa glycoproteins. Endo F cleaves only high mannose structures, biantennary structures, or biantennary complex structures of an oligosaccharide from a glycoprotein, leaving only single GlcNAc residues attached to the polypeptide (27, 28). Approximately 16–20% of the 120- and 100-kDa glycoproteins' mass consists of Endo F-sensitive carbohydrate, indicating that their oligosaccharides are of a high mannose and/or complex structure. Endo H, however, removes only 5% of the mass of both glycoproteins and only ¼ of the carbohydrate that is sensitive to Endo F. Endo H cleaves only high mannose or hybrid structure oligosaccharides but does not cleave sulfated high mannose or any complex structures (29, 30). These data taken together indicate that the carbohydrate structure of both glycoproteins must be at least 75% complex structures, with the remaining 25% high mannose or hybrid structures.

The 120- and 100-kDa glycoproteins appear to be homologous molecules as judged by their similar peptide maps, cross-reactivity with polyclonal antibody, and similar mass of their carbohydrate and physicochemical properties. The most logical explanation for this similarity is that the 100-kDa glycoprotein is a degradation product of the 120-kDa polypeptide. This is unlikely, since we have taken many precautions to avoid any protein degradation during membrane preparation. We have included in all the buffers a complex mixture of different protease inhibitors (see "Experimental Procedures"). Also, the ratio of the 120- and 100-kDa glycoproteins did not change whether SL vesicles were isolated in the

presence or absence of protease inhibitors, indicating that no significant degradation of SL proteins occurs during the membrane preparation procedure used in this study. Although unlikely, it is still possible that the 120-kDa glycoprotein was degraded to 100-kDa glycoprotein by an endogenous protease which is not inhibited by any of the protease inhibitors used in this study.

Another possibility is that the 120- and 100-kDa glycoproteins are closely related, highly homologous proteins present in the same membrane. This may be supported by completely different NH_2-terminal amino acid sequences of both proteins.The 15 NH_2-terminal amino acid residues of the 120- and 100-kDa glycoproteins are completely different (data not shown). Although it is still speculative, both glycoproteins may share some homology (or identity) at the COOH-terminal end but to a lesser extent at their NH_2-terminal end. This may be similar to the two related glycoproteins of the sarcoplasmic reticulum membrane, the 53- and 160-kDa glycoproteins (31–33). These two sarcoplasmic reticulum glycoproteins share, in part, the identical amino acid sequence, accounting for their immunological similarities and for some of their other properties (31–33). It is possible, therefore, that the 100-kDa glycoprotein is the same, or at least in part the same, as the 120 kDa except that the 100-kDa glycoprotein is missing the NH_2-terminal portion of the polypeptide. This is further supported by the fact that affinity purified antibodies against either 120- or 100-kDa glycoprotein cross-react with both glycoproteins by the Western blot analysis. We are currently isolating cDNA clones encoding these two polypeptides in order to directly address the question of their homology.

Both glycoproteins were identified as major Ca^{2+}-binding proteins in the SL membrane, suggesting they may be involved in Ca^{2+} binding and storage at the level of SL membrane. The majority of Ca^{2+} (>70%) bound to SL membrane has been shown to be associated with membrane acidic phospholipids (15). Limas (16), however, has indicated that proteins may also be involved in Ca^{2+} binding to SL vesicles. The 120- and 100-kDa glycoproteins may be the proteins involved in Ca^{2+} binding to the SL membrane. It is also possible that other Ca^{2+}-binding proteins are present in SL vesicles in addition to the 120- and 100-kDa glycoproteins but are not detected under the conditions used in this study. The SDS-PAGE and transfer to nitrocellulose membranes may have destroyed the Ca^{2+} binding domains of these proteins.

Acknowledgments—We would like to thank Dr. R. A. F. Reithmeier, University of Toronto, for providing samples of the apical and basolateral membrane of the kidney. We are grateful to Dr. J. M. H. Charuk, University of Toronto, for performing Western blot analysis of apical and basolateral membrane proteins. The technical help of E. Wandler and S. Baksh is greatly appreciated.

REFERENCES

1. Sulakhe, P. V., and St. Louis, P. J. (1980) *Prog. Biophys. Mol. Biol.* **35,** 135–195
2. Lopaschuk, G. D., Michalak, M., Wandler, E. L., Lerner, R. W., Piscione, T. D., Coceani, F., and Olley, P. M. (1989) *Circ. Res.* **65,** 538–545
3. Jones, L. R., Besch, H. R., Jr., Fleming, J. W., McConnaughey, M. M., and Watanabe, A. M. (1979) *J. Biol. Chem.* **254,** 530–539
4. Jones, L. R., Maddock, S. W., and Besch, H. R., Jr. (1980) *J. Biol. Chem.* **255,** 9971–9980
5. Manalan, A. S., and Jones, L. R. (1982) *J. Biol. Chem.* **257,** 10052–10062
6. Rogers, T. B. (1984) *J. Biol. Chem.* **259,** 8106–8114
7. Carafoli, E. (1987) *Annu. Rev. Biochem.* **56,** 395–433
8. Reeves, J. P., and Sutko, J. L. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 590–594
9. Caroni, P., and Carafoli, E. (1981) *Nature* **283,** 765–767
10. Reuter, H. (1983) *Nature* **301,** 569–574
11. Langer, G. A. (1986) *Can. J. Physiol. Pharmacol.* **65,** 627–631
12. Langer, G. A. (1984) *J. Mol. Cell. Cardiol.* **16,** 147–153
13. Langer, G. A., Frank, J. S., and Philipson, K. D. (1982) *Pharmacol. Ther.* **16,** 331–376
14. Borgers, M., Thone, F., Verheyen, A., and Ter Keurs, H. E. D. J. (1984) *Histochem. J.* **16,** 295–309
15. Philipson, K. D., Bers, D. M., Nishimoto, A. Y., and Langer, G. A. (1980) *Am. J. Physiol.* **238,** H373–H378
16. Limas, C. J. (1977) *Arch. Biochem. Biophys.* **179,** 302–309
17. Charuk, J. H. M., Hewlatt, S., and Michalak, M. (1989) *Biochem. J.* **264,** 885–892
18. Jones, L. R. (1988) *Methods Enzymol.* **157,** 85–91
19. Booth, A. G., and Kenny, A. J. (1974) *Biochem. J.* **142,** 575–581
20. Sacktor, B., Rosenbloom, I. L., Liang, C. T., and Cheng, L. (1981) *J. Membr. Biol.* **60,** 63–71
21. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
22. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
23. Laemmli, U. K. (1970) *Nature* **227,** 680–685
24. Cleveland, D. W., Fischer, S. G., Kirschner, M. W., and Laemmli, U. K. (1977) *J. Biol. Chem.* **252,** 1102–1106
25. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 4350–4354
26. Maruyama, K., Mikawa, T., and Ebashi, S. (1984) *J. Biochem. (Tokyo)* **95,** 511–519
27. Elder, J. H., and Alexander, S. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 4540–4544
28. Terentino, A. L., Gomez, C. M., and Plummer, T. H., Jr. (1985) *Biochemistry* **24,** 4665–4671
29. Freeze, H. H., and Wolgast, D. (1986) *J. Biol. Chem.* **261,** 127–134
30. Cladaras, M. H., and Kaplan, A. (1984) *J. Biol. Chem.* **259,** 14165–14169
31. Campbell, K. P., and MacLennan, D. H. (1981) *J. Biol. Chem.* **256,** 4626–4632
32. Leberer, E., Charuk, J. H. M., Clarke, D. M., Green, M. N., Zubrzycka-Gaarn, E., and MacLennan, D. H. (1989) *J. Biol. Chem.* **264,** 3484–3493
33. Leberer, E., Charuk, J. H. M., Green, M. N., and MacLennan, D. H. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 6047–6051

Isolation and characterization of calcium binding glycoproteins of cardiac sarcolemmal vesicles.

M Michalak, L Fliegel and K Wlasichuk

J. Biol. Chem. 1990, 265:5869-5874.
