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Evidence for the lumenal location of the 53 kDa glycoprotein of sarcoplasmic reticulum

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The 53 kDa glycoprotein from sarcoplasmic reticulum was shown to be protected from proteolysis by trypsin, V8 proteinase and proteinase K in intact vesicles yet readily digested in the presence of the non-denaturing detergent $C_{12}E_8$. Competitive ELISAs with a library of seven monoclonal antibodies raised against the 53 kDa glycoprotein showed that the epitopes for these antibodies were only accessible in $C_{12}E_8$ solubilised and not intact sarcoplasmic reticulum. When the monoclonal antibodies against the 53 kDa glycoprotein were assessed for their effect on the uptake of Ca^{2+} by sarcoplasmic reticulum no effect was detected; neither were these antibodies able to augment the inhibitory influences of anti-(Ca^{2+}-Mg^{2+})-ATPase monoclonal antibodies on Ca^{2+} uptake. These data indicate that the 53 kDa glycoprotein is located in the lumen of the sarcoplasmic reticulum.

Introduction

Sarcoplasmic reticulum (SR) of fast twitch muscle contains three major proteins, (Ca^{2+}-Mg^{2+})-ATPase, calsequestrin and a 53 kDa glycoprotein. The ATPase constitutes 60–70% of SR protein and calsequestrin and the 53 kDa glycoprotein make up most of the remaining 20–30% [1]. Although the roles of the ATPase and calsequestrin are well established [2–4] the function of the 53 kDa glycoprotein is still controversial. Based on the finding that SR depleted of 53 kDa glycoprotein following treatment with detergent had a reduced rate of calcium accumulation it was suggested that the 53 kDa glycoprotein played a role in modulating ATPase activity [5]. However it can be shown that the conditions required for the removal of the 53 kDa glycoprotein from SR vesicles also result in a randomising of the orientation of the ATPase in SR vesicles [6] which would account for the reduced calcium accumulation. In addition cross-linking experiments failed to show any association between the ATPase and the 53 kDa glycoprotein [7] and the dependence of ATPase activity on the concentrations of Ca^{2+} and ATP were identical for purified ATPase and A23187 uncoupled SR [7] indicating that there were no modulatory influences on the ATPase in SR. Martin [8] was also unable to find any difference between the efficiency of Ca^{2+} transport in SR and in reconstituted ATPase, and the coexpression of the 53 kDa glycoprotein and (Ca^{2+}-Mg^{2+})-ATPase in COS-1 cells also failed to show any modulatory influence of the 53 kDa glycoprotein [15].

A number of experiments using antibodies raised against the 53 kDa glycoprotein have been presented which appear to show a possible role for the 53 kDa glycoprotein in modulating the (Ca^{2+}-Mg^{2+})-ATPase in SR. Thus, binding of antibodies directed against the 53 kDa glycoprotein to SR vesicles has been reported to reduce Ca^{2+} accumulation by SR vesicles [9]. Mahaney et al. [10] reported that antibodies directed against the 53 kDa glycoprotein affected the rotational dynamics of both the 53 kDa glycoprotein and the ATPase as measured by saturation transfer electron paramagnetic resonance and resulted in a change in ATPase activity [11]. Only if the 53 kDa glycoprotein has epitopes exposed on the cytoplasmic surface of the SR vesicles can addition of antibodies against the 53 kDa glycoprotein directly affect the properties of the vesicles, but the precise location of the 53 kDa glycoprotein, whether transmembranous or entirely intralu-

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Abbreviations: ELISA, enzyme linked immunosorbant assay; KLH, keyhole limpet haemocyanin; mAbs, monoclonal antibodies; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulphate; SR, sarcoplasmic reticulum; MBS, *m*-maleimidobenzoyl-*N*-hydroxy succinimide.

menal is unclear [12,13,15]. If the 53 kDa turns out to be an intralumenal protein the evidence for any interaction between the 53 kDa glycoprotein and the (Ca^{2+}-Mg^{2+})-ATPase will remain unproven.

In this study we have raised monoclonal and antipeptide antibodies directed against the 53 kDa glycoprotein and determined the transmembranous location of the epitopes by competitive ELISA. Also we have used proteolytic enzymes and Western blotting to determine the location of the 53 kDa glycoprotein. These studies indicate that the 53 kDa glycoprotein is located in the SR lumen and it is therefore inaccessible to antibodies in intact SR.

Materials and Methods

Preparation of sarcoplasmic reticulum and (Ca^{2+}-Mg^{2+})-ATPase

Sarcoplasmic reticulum and purified (Ca^{2+}-Mg^{2+})-ATPase were prepared from female rabbit (New Zealand White) hind-leg and back muscle as described previously [16].

Preparation of monoclonal antibodies

Monoclonal antibodies against the 53 kDa glycoprotein, calsequestrin and the (Ca^{2+}-Mg^{2+})-ATPase were prepared as outlined previously [7]. MAbs against calsequestrin and the (Ca^{2+}-Mg^{2+})-ATPase were prepared by immunisation of BALB/C mice with the pure protein. MAbs against the 53 kDa glycoprotein were raised by initially injecting purified ATPase followed by an immunisation schedule with sarcoplasmic reticulum 3 weeks later. This protocol favoured the production of mAbs against components of the sarcoplasmic reticulum other than the (Ca^{2+}-Mg^{2+})-ATPase [7]. Antipeptide antibodies against the (Ca^{2+}-Mg^{2+})-ATPase were made as described elsewhere [17,18]

Peptide synthesis

Peptides are named according to the residues to which they correspond in the sequence of the 53 kDa glycoprotein [15]. Peptides 1–11 (EETEDVSEEVPC) and 442–452 (CGETPKNRYKK) correspond to the N- and C-terminals of the 53 kDa glycoprotein, respectively. Peptides were synthesized by the method of Merrifield [19] and checked for purity by HPLC on a reversed phase C8 column. Peptide 1–11 had an additional Cys residue at its C-terminus to allow coupling to the carrier protein keyhole limpet haemocyanin (KLH) with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide (MBS) as cross-linker according to the method of Green et al. [20]. KLH (8 mg) in 10 mM sodium phosphate buffer (pH 7.8; 0.25 ml) was reacted with MBS (1.4 mg in 20 μl dimethylformamide) for 30 min at room temperature. The KLH-MBS (5 mg), separated by gel exclusion chromatography on a 10 ml Sephadex G-10 column which had been equilibrated with buffer (10 mM sodium phosphate, pH 6.5), was then mixed with peptide (5 mg) dissolved in buffer (0.5 ml; 10 mM sodium phosphate, pH 6.5) and the coupling reaction was allowed to proceed overnight. The product was assayed for free -SH groups by the Ellman reaction [21] to check for complete coupling.

Immunisation

Primary immunisations were carried out with approx. 0.5 mg peptide-KLH in Freund's complete adjuvant injected by the intramuscular route into New Zealand white rabbits on day 1. A booster injection of 0.3 mg peptide-KLH was given in Freund's incomplete adjuvant by the same route after 28 days. Several booster injections were given every two weeks using 0.3 mg of peptide-KLH or peptide in Freund's incomplete adjuvant and blood taken for the production of antisera 5–10 days later. Antisera were stored at −70°C. In some cases antibodies were purified from antisera on a protein A-Sepharose column followed by dialysis against 1 litre of phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2).

Enzyme linked immunosorbant assays (ELISAs)

ELISAs were performed using the method outlined in Hudson and Hay [22]. Polystyrene ELISA plates (Dynatech, Immulon 1) were coated overnight with peptides or SDS denatured SR (80°C for 10 min in 0.5% SDS before dilution) at 1 μg per well in 50 mM sodium carbonate/bicarbonate buffer (pH 9.0), at 4°C. Incubations with antisera or purified antibody were carried out in 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, and 0.05% Tween (pH 7.2) (PBS-Tween) for 1 h at 37°C and following extensive washing with PBS-Tween the plate was incubated with sheep anti-rabbit or rabbit anti-mouse IgG,A,M (as appropriate) conjugated with horseradish peroxidase (Sera-Tec) diluted 1 in 5000 with PBS-Tween (200 μl/well). After further washing the plate was incubated for 30 min at room temperature with 0.34 mg/ml *o*-phenylenediamine and 0.003% H_2O_2 in 0.15 M citrate-phosphate buffer (pH 5.0) (200 μl/well). The reaction was stopped by adding 50 μl 12.5% H_2SO_4 to each well. Competitive assays were carried out by incubating the conditioned culture media (diluted 1:350–500) for 2 h at room temperature in 200 μl 50 mM sodium phosphate, 0.1 M KCl, 0.25 M sucrose (pH 7.4), containing 0–30 μg native SR or SR initially solubilised in 0.5% $C_{12}E_8$ and diluted to 0.005% $C_{12}E_8$. The concentration of detergent used was shown to be without effect on the ELISA. The unbound antibody was then detected by transferring the incubates to the SDS-denatured ATPase coated ELISA plates and carrying out the ELISA as described above.

Immunoblotting

Proteins (300 μg per gel) were separated on 10–14% polyacrylamide gels [23] and transferred to nitrocellulose paper (0.45 μm, Schleicher and Schuell) electrophoretically as described by Towbin et al. [24]. The nitrocellulose was cut into strips and exposed sequentially in PBS-Tween to antipeptide antibodies (diluted 1:50 with PBS-Tween, 1 h) or monoclonal antibodies in conditioned media (diluted 1:5 with PBS-Tween), horseradish peroxidase conjugated to sheep anti-rabbit or rabbit anti-mouse IgG, A, M (diluted 1:3000) and substrate (Bio-Rad, 4-chloro-1-naphthol 0.5 mg/ml plus 0.15% H_2O_2 in PBS) washing extensively between each step with PBS-Tween.

Preparation and analysis of proteolytic fragments of the 53 kDa glycoprotein and 160 kDa glycoprotein

Digestion of SR was performed as described in Colyer et al. [25]. SR (150–300 μg) was digested with 10 μg of proteinase K or V8 proteinase or trypsin (Sigma) for 30 min in 50–80 μl of either hypotonic buffer, 10 mM Tris-HCl buffer (pH 7.4), or isotonic buffer, 50 mM sodium phosphate, 0.1 M KCl, 0.2 M sucrose (pH 7.4), at room temperature. The reactions were terminated by adding phenylmethylsulphonyl fluoride (PMSF) to give a final concentration of 2 mM. Samples were solubilised in Laemmli sample buffer containing 2% SDS and 2% β-mercaptoethanol and processed by SDS polyacrylamide gel electrophoresis [23].

Measurement of Ca^{2+} accumulation by SR vesicles

Uptake and release of Ca^{2+} was monitored by the change in the absorbance of murexide (0.45 mM) with a wavelength pair of 507–542 nm in an SLM-Aminco DW2000 dual-wavelength spectrophotometer, as described previously [26]. SR (0.3 mg) was incubated in 1 ml 40 mM Hepes/KOH, 100 mM KCl, 5 mM $MgSO_4$, 50 μM $CaCl_2$, 5 mM K_2HPO_4, pH 6.3. Uptake was initiated by adding 0.5 mM ATP and after calcium accumulation was essentially complete 10 μg of A23187 was added to show release of the Ca^{2+} from the vesicles. The effects of the monoclonal antibodies were investigated by preincubating SR (0.3 mg) for 30 min on ice with the appropriate amount of antibody; 1.5 mg of ammonium sulphate purified antibody or 0.9 mg of protein A purified antibody. When 2 antibodies were used in succession the second antibody was added 30 min after the first and the SR incubated for a further 30 min in the presence of both antibodies.

Results

Seven monoclonal antibodies (designated L/1B6, L/1E12, L/1G3, L/1G7, L/2E5, L/5F8 and M/6A11) were raised against the 53 kDa glycoprotein of SR as outlined previously [7]. That they were against the 53 kDa glycoprotein and not another protein with a similar M_r was confirmed by the finding that they all recognised the immuno-related 160 kDa glycoprotein (not shown, though see L/5F8 in Fig. 3A) [15] and by the observation that the SR component recognised by the antibodies was also recognised by concanavalin A which is known to bind to the 53 kDa glycoprotein [13] (data not shown). The antibodies also recognised fragments generated by Endo H treatment of the 53 kDa glycoprotein in Western blots (data not shown).

Epitopes for the antibodies on the 53 kDa glycoprotein were located by limited proteolysis. Fig. 1A shows the effect of limited proteolysis of the 53 kDa glycoprotein by proteinase K. The Western blot was probed with mAbs, Con A-HRP and an antipeptide antibody directed against the C-terminus; an antipeptide antibody raised against the N-terminus failed to recognise the 53 kDa glycoprotein in Western blots. Proteinase K treatment produced a series of fragments identified by the mAbs. Three profiles were discernible. MAbs L/5F8, L/1B6, M/6A11, L/2E5, and L/1G7 bound to the intact glycoprotein and to a fragment with apparent molecular weight of 40 kDa. These bands on the gel were also recognised by the anti-C-terminus antibodies. The mAb L/1E12 recognised the intact glycoprotein and the 40 kDa fragment and also a fragment of around 28 kDa, which was not recognised by the other mAbs, Con A or the anti-C-terminus antibodies. The remaining mAb, L/1G3, only recognised undigested 53 kDa glycoprotein. Since the 40 kDa fragment contains the C-terminus and is not recognised by L/1G3, this mAb must have an epitope located within 13 kDa of the N-terminus. The epitope for L/1E12 must be located on the N-terminal side of residue N261 (one of the two glycosylation sites) because the 28 kDa fragment to which it binds is not recognised by Con A, or the anti-C-terminus antibody and therefore this fragment cannot have been generated from the C-terminal part of the 53 kDa glycoprotein (see Fig. 2). Calculated M_r for fragments from residues 1 to 260 and from residues 261 to 452 (the C-terminus) are 30 kDa and 26.2 kDa respectively, suggesting that the C-terminus of the 28 kDa fragment must be close to N261. Since the other mAbs (L/5F8, L/1B6, M/6A11, L/2E5 and L/1G7) bind at a site separate from that for L/1E12; i.e., they do not bind to the 28 kDa fragment, these antibodies must be located within at least 25 kDa of the C-terminus.

Binding to fragments generated by V8 proteinase is shown in Fig. 1B. A group of three fragments at 20/22 kDa were detected by all mAbs (except L/1G3) and by the anti-C-terminal antibodies and Con A indicating that, with the exception of L/1G3, the epitopes for the mAbs lie within 22 kDa of the C-terminus. Therefore, combining the information for the two digests it can be

deduced that the epitope for L/1E12 must be very close to the glycosylation site at N261. A further band at 18/19 kDa is detected by Con A. Since this fragment does not contain the epitope for L/1E12, L/5F8, L/1B6, M/6A11, L/2E5 and L/1G7 it must be a truncated form of the 22 kDa fragment. This means that the epitope(s) for L/5F8, L/1B6, M/6A11, L/2E5 and L/1G7 must lie within 3 kDa of the C-terminus. A more precise location for L/1G3 can be obtained using the very short trypsin digestion (Fig. 1C). All mAbs except L/1G3 detect 2 proteolytic fragments at 51 and 49 kDa, indicating that the epitope for this antibody

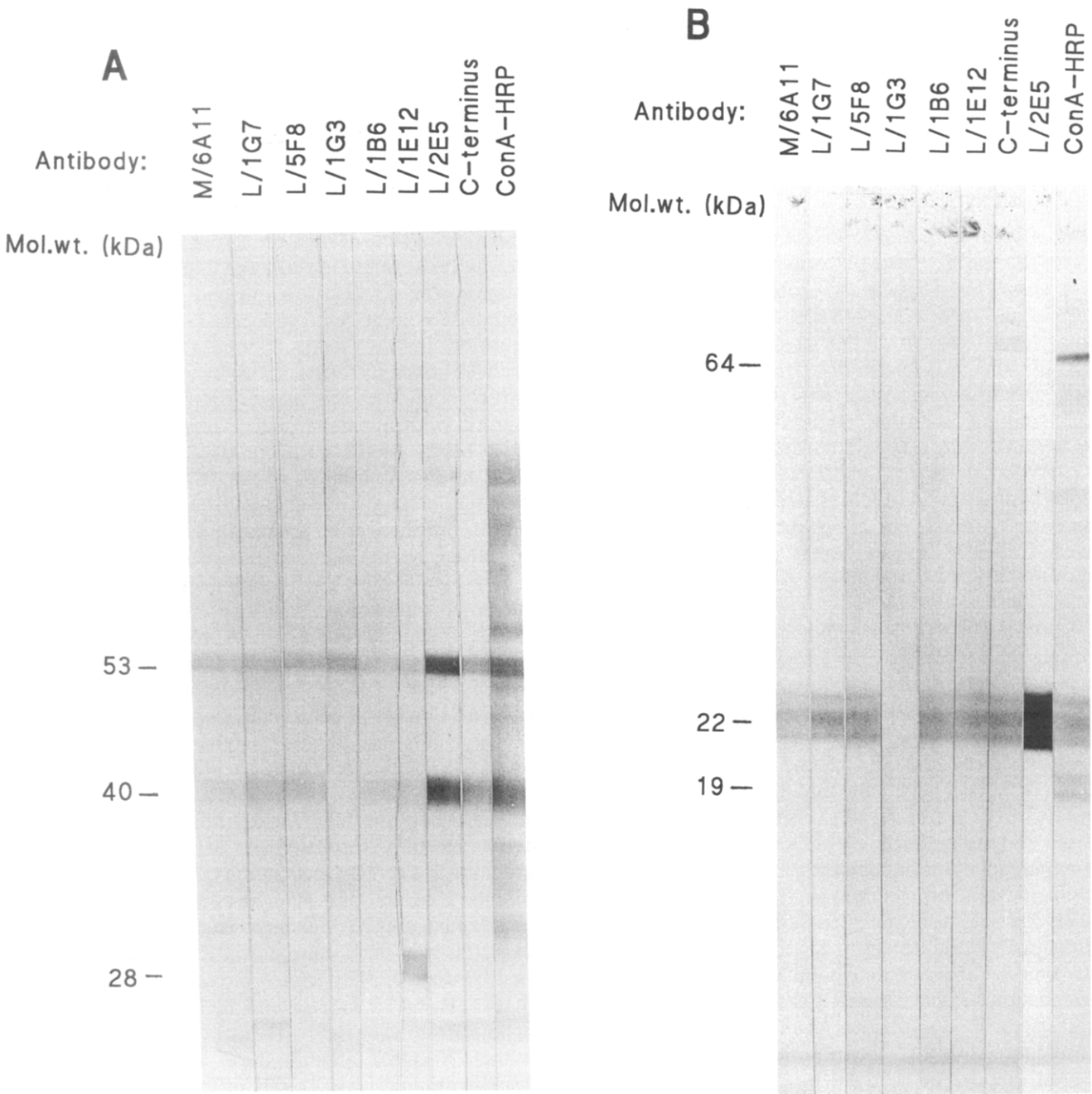

Fig. 1. Epitope mapping of antibodies to the 53 kDa glycoprotein by limited proteolysis. SR (300 μg) in 80 μl 10 mM Tris-HCl (pH 7.4) was incubated with (A) proteinase K (10 μg), (B) V8 proteinase (10 μg) for 30 min at 25°C or (C) trypsin (5 μg) for 5 min at 25°C. The reaction was stopped by adding PMSF to a final concentration of 2 mM. The digests were separated by SDS polyacrylamide gel (15%) electrophoresis and transferred electrophoretically onto nitrocellulose. Strips of nitrocellulose were incubated with the monoclonal antibodies L/1B6, L/1E12, L/1G3, L/1G7, L/2E5, L/5F8 and M/6A11 (conditioned media diluted 1:5) or antipeptide antibodies raised in rabbits against the C-terminus of the 53 kDa glycoprotein, designated 53C-term (serum diluted 1:50) or Con A-HRP (Sigma, 5 μg/ml). After extensive washing the strips were incubated with rabbit anti-mouse IgG, A, M horseradish peroxidase conjugate or sheep anti-rabbit IgG, A, M horseradish peroxidase conjugate (diluted 1:1000), followed by incubation with H_2O_2 and 4-chloro-1-naphthol.

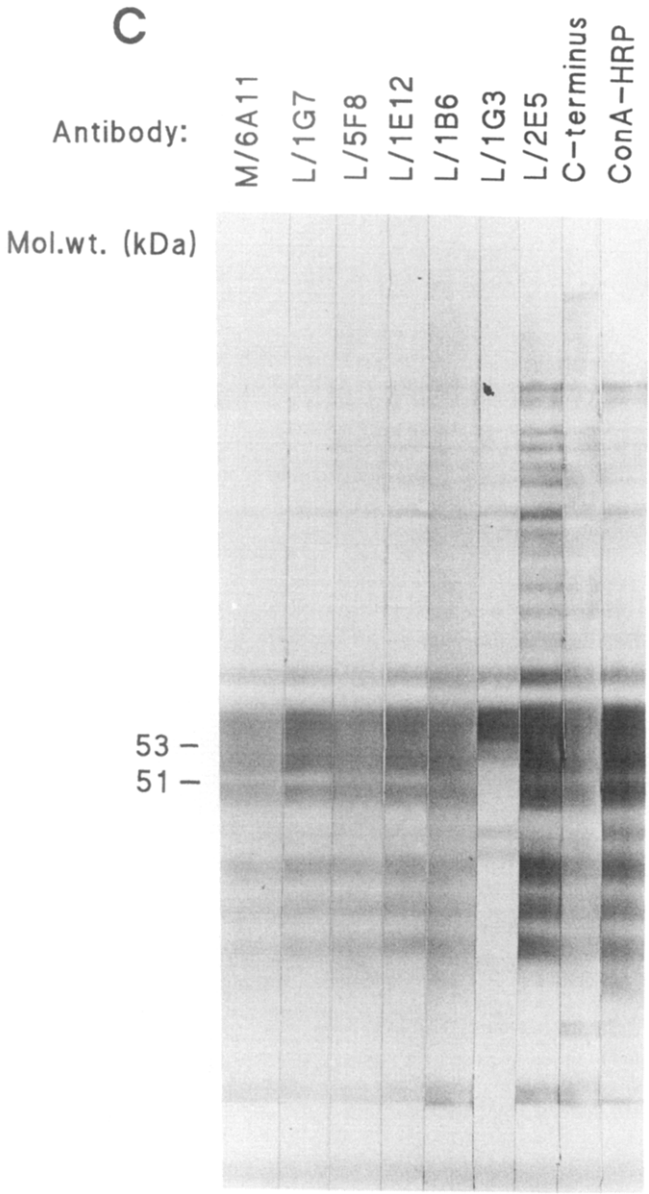

Fig. 1 (continued).

must be within 2 kDa of the N-terminus (since it is already established that the L/1G3 epitope is not located at the C-terminus). These data are summarised in Fig. 2.

If the 53 kDa glycoprotein is exposed on the outer surface of the SR membrane then it would be expected to be susceptible to proteolysis in intact SR vesicles, whereas if it is located in the lumen of the vesicles it will be protected. Fig. 3 shows the time course of digestion of SR vesicles by proteinase K, probed by Western blotting using an antipeptide antibody against residues 877–888 of the ATPase [17] and the monoclonal antibody L/5F8 directed against the 53 kDa glycoprotein. It can be seen that the ATPase has been completely digested within 10 min (Fig. 3B) but significant amounts of the 53 kDa glycoprotein remain intact even after 2 h. After digestion of intact SR with proteinase K for 10 min a breakdown product of the 53 kDa glycoprotein of 40 kDa became apparent. A similar profile is seen for the breakdown of calsequestrin, i.e., no breakdown of the calsequestrin for around 10 min followed by a slow breakdown, but with significant amounts of calsequestrin remaining after 2 h (data not shown). If 0.1% SDS is used to solubilize the vesicles, the 53 kDa glycoprotein is broken down within 2 min by proteinase K (data not shown).

Fig. 4 shows the results of an experiment in which intact vesicles, vesicles lysed with hypotonic buffer (10 mM Tris pH 7.2), or vesicles solubilised with the detergent $C_{12}E_8$ are treated with trypsin or proteinase K. As shown, proteinase K has no effect on the 53 kDa glycoprotein in intact vesicles, but causes extensive proteolysis in vesicles made leaky by addition of $C_{12}E_8$ or by lysis with hypotonic buffer. For trypsin treated vesicles, the 53 kDa glycoprotein appears to run anomalously in SDS gels, presumably as a result of the extensive breakdown of the $(Ca^{2+}$-$Mg^{2+})$-ATPase, which produces large amounts of two fragments with molecular weights around 50–55 kDa [17,18]. There are small amounts of some proteolysis products of the 53 kDa glycoprotein seen with the intact vesicles in Fig. 4 but since there is always a small percentage of disrupted vesicles in these SR preparations [14] this is to be expected. However, the clear difference between the results for intact and leaky vesicles again suggests a lumenal location for the 53 kDa glycoprotein.

The competitive ELISAs (Fig. 5), where the ability of intact and solubilised SR to bind monoclonal antibodies was assessed, shows that monoclonal antibodies

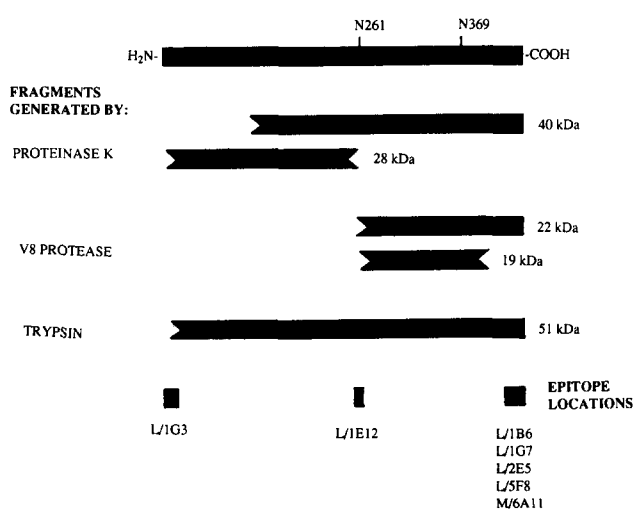

Fig. 2. Summary of epitope mapping. The probable location of fragments generated by treatment with proteinase K, V8 proteinase and trypsin is displayed below the line representing the primary structure of the 53 kDa glycoprotein. Also shown are the potential glycosylation sites and the deduced locations of the epitopes for the mAbs.

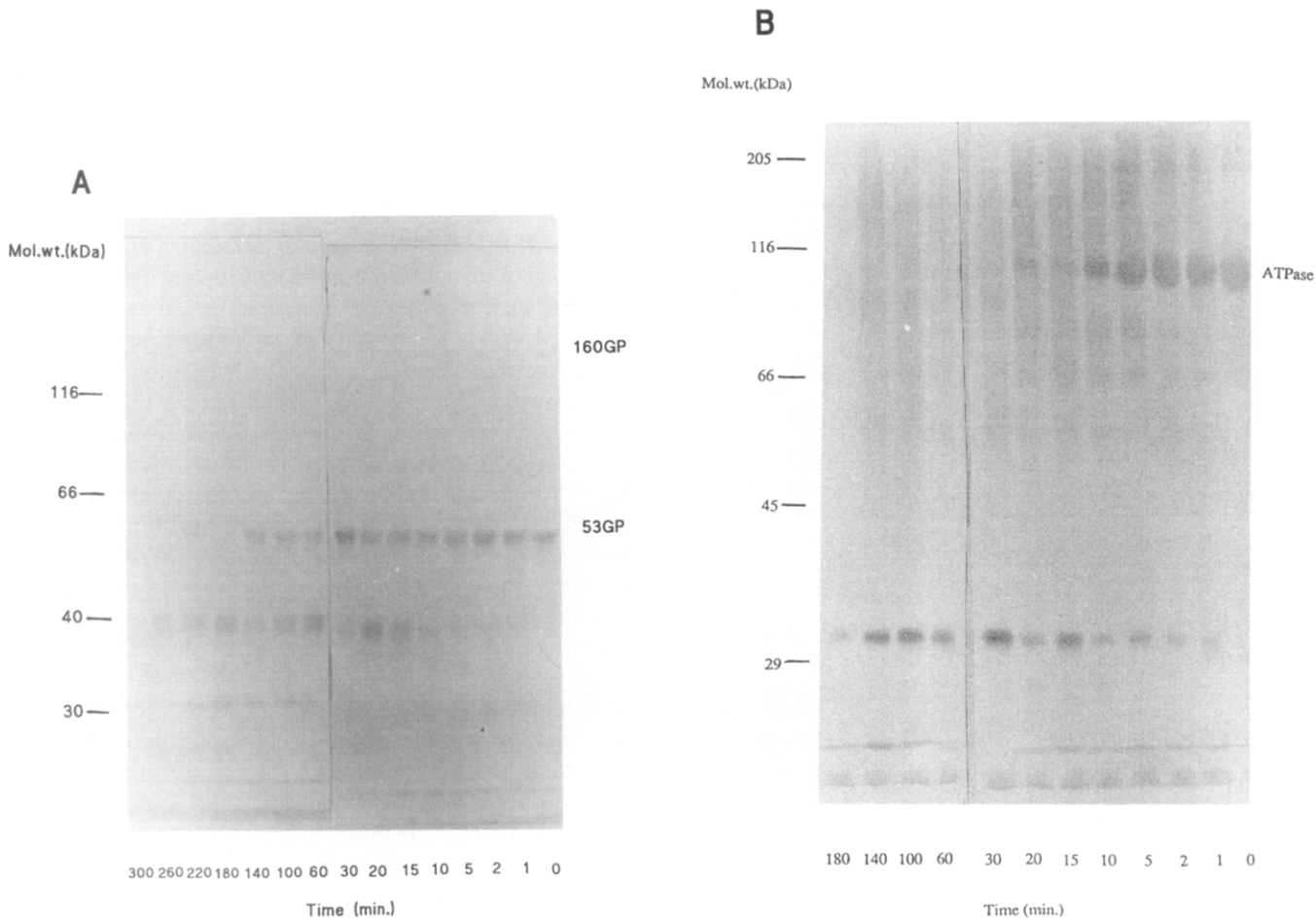

Fig. 3. Time course of SR digestion by proteinase K. SR vesicles (600 μg) were incubated in 300 μl 50 mM sodium phosphate, 0.1 M KCl, 0.25 M sucrose (pH 7.4) for up to 300 min with proteinase K (20 μg). The reaction was stopped by adding 20 μl aliquots to 20 μl of 4 mM PMSF. The digests were separated by SDS polyacrylamide gel (15%) electrophoresis and transferred electrophoretically onto nitrocellulose. Strips of nitrocellulose were incubated with either, (A) L/5F8 or, (B) an antipeptide antibody raised in rabbit against residues 877–888 of the (Ca^{2+}-Mg^{2+})-ATPase. After extensive washing the strips were incubated with rabbit anti-mouse IgG,A,M horseradish peroxidase conjugate or sheep anti-rabbit IgG,A,M horseradish peroxidase conjugate (diluted 1:1000), followed by incubation with H_2O_2 and 4-chloro-1-naphthol.

L/1G3, L/1E12 and L/5F8 do not bind to intact SR. Only when the vesicles are solubilised is the medium depleted of these monoclonal antibodies as seen by the reduction in absorbance at elevated SR concentrations (i.e., around 1–20 μg/well). These results also argue for a lumenal location for the 53 kDa glycoprotein.

The effects of monoclonal antibodies against the (Ca^{2+}-Mg^{2+})-ATPase and the 53 kDa glycoprotein on uptake of Ca^{2+} by SR vesicles is shown in Fig. 6. In the absence of antibodies, Ca^{2+} is rapidly accumulated by SR over a period of 3 min; the accumulated Ca^{2+} can be released by addition of the Ca^{2+} ionophore A23187 (Fig. 6). Antibodies L/2E5, L/1E12 and L/1G3 against the 53 kDa glycoprotein are without effect on the uptake of Ca^{2+} by SR vesicles. Two antibodies against the (Ca^{2+}-Mg^{2+})-ATPase which affect the steady state hydrolysis of ATP by the purified ATPase, Y/2E9 and Y/1F4, reduced the rate and extent of Ca^{2+} accumulation but antibody 1/2H7 which is without effect on ATPase activity is also without effect on Ca^{2+} accumulation (Fig. 6). When antibodies against the glycoprotein and against the ATPase were used in combination, i.e, every combination of either L/2E5, L/1E12 or L/1G3 with either Y/2E9, Y/1F4 or 1/2H7, effects seen were identical to those seen with anti-ATPase antibodies alone. Thus, there was no evidence of any interaction between the antibodies in terms of their effect on Ca^{2+} uptake.

Discussion

The three major proteins of the SR are the (Ca^{2+}-Mg^{2+})-ATPase, calsequestrin and the 53 kDa glycoprotein [1]. The function of the glycoprotein is unknown, but in a number of papers it has been suggested that it could interact with the (Ca^{2+}-Mg^{2+})-ATPase, serving to modulate the activity of the ATPase, and to couple ATP hydrolysis to transport of

Ca^{2+} [5,9–11]. Such an interaction, if it occurred, would obviously be an important factor in understanding the function of the ATPase. Evidence in favour of an interaction between the 53 kDa glycoprotein and the ATPase first came from experiments in which SR

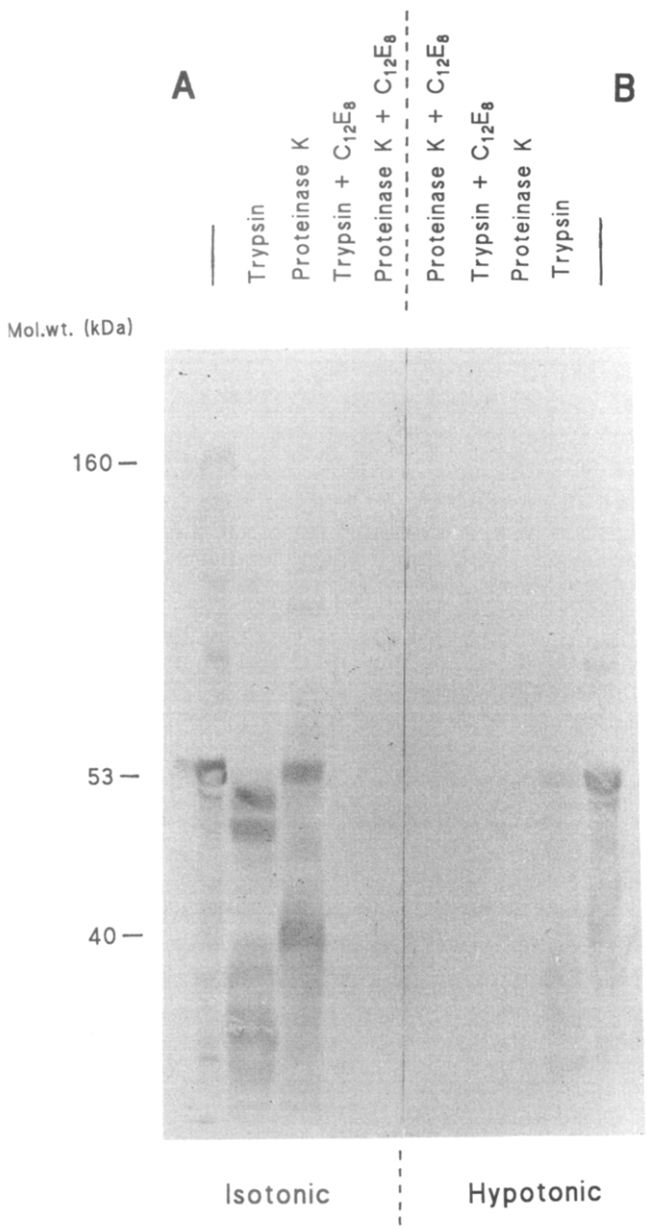

Fig. 4. Proteolysis of the 53 kDa glycoprotein by trypsin and proteinase K in intact, osmotically shocked and $C_{12}E_8$ solubilised SR. SR (150 μg) in either (A) 50 μl isotonic buffer (50 mM sodium phosphate, 0.1 M KCl, 0.2 M sucrose, pH 7.4) or (B) hypotonic buffer (10 mM Tris-HCl, pH 7.4) was treated with either trypsin (5 μg) or proteinase K (5 μg) for 30 min at 25°C in the presence or absence of 0.5% $C_{12}E_8$. The reactions were stopped with 2 mM PMSF and the digests separated by SDS polyacrylamide gel (10%) electrophoresis and transferred to nitrocellulose. The nitrocellulose was incubated with L/1E12 (conditioned culture medium diluted 1:5). After extensive washing the strips were incubated with rabbit anti-mouse IgG,A,M horseradish peroxidase conjugate followed by incubation with H_2O_2 and 4-chloro-1-naphthol.

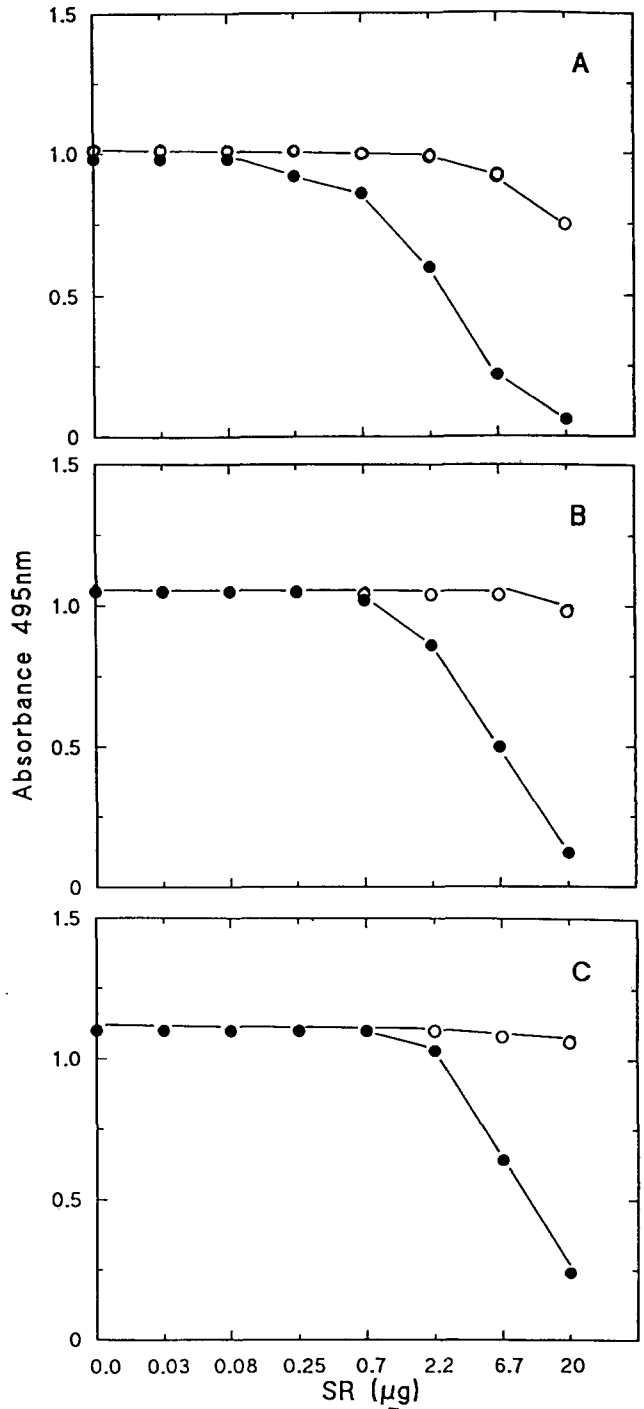

Fig. 5. Determination of the transmembranous location of monoclonal antibody epitopes by competitive enzyme linked immunosorbant assay. MAbs L/5F8, diluted 1:400 (A), L/1E12 diluted 1:500 (B) and L/1G3 diluted 1:350 (C) were pre-incubated for 2 h at 25°C in 50 mM sodium phosphate, 0.1 M KCl, 0.25 M sucrose (pH 7.4), with native SR (○) or SR solubilised in $C_{12}E_8$ (●). The incubate was then transferred to an ELISA plate coated with denatured SR in order to estimate the amount of unbound antibody, which was detected by sequential incubations with horseradish peroxidase conjugated second antibody followed by H_2O_2 and *o*-phenylenediamine. The absorbance was measured at 490 nm.

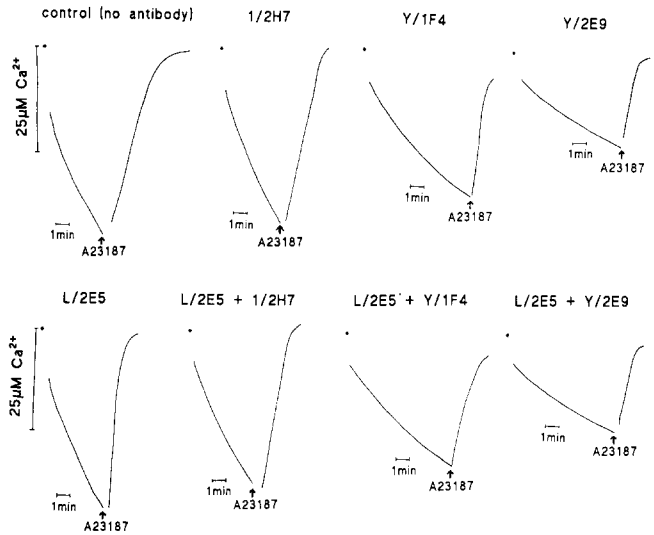

Fig. 6. The effect of monoclonal antibodies against the 53 kDa glycoprotein and the (Ca^{2+}-Mg^{2+})-ATPase on calcium transport by SR. Calcium uptake was monitored by the change in the absorbance of murexide (0.45 mM) using dual beam spectrophotometry (wavelength pair 507 and 542 nm). SR (0.3 mg) was incubated in 1 ml 40 mM Hepes/KOH, 100 mM KCl, 5 mM $MgSO_4$, 50 μM $CaCl_2$, 5 mM K_2HPO_4 (pH 6.3). Following the addition of Ca^{2+} (50 μM final concentration to calibrate the absorbance signal uptake was initiated by adding 0.5 mM ATP indicated by (*). There is a small addition artefact as the sample is stirred. After calcium accumulation was essentially complete 10 μg of A23187 was added to show release of the Ca^{2+} from the vesicles. The effects of the monoclonal antibodies were investigated by preincubating SR (0.3 mg) for 30 min on ice with incubation buffer above as a control or 1.5 mg of ammonium sulphate purified anti-ATPase mAbs (1/2H7, B/3D6, Y/1F4 or Y/2E9) and or 0.9 mg of protein A purified L/2E5.

vesicles were reconstituted following treatment with cholate at low and high concentrations of K^+ [5]. We have shown that these experiments can also be interpreted in terms of differing degrees of solubilisation of the SR vesicles at the different concentrations of K^+, with no need to invoke interactions involving the glycoprotein [6]. Cross-linking experiments also failed to find evidence for any interaction between the 53 kDa glycoprotein and the ATPase [7] and experiments in which either the ATPase alone or both the glycoprotein and the ATPase were expressed in COS cells failed to find any difference in function attributable to the presence of the glycoprotein [15].

Other evidence in favour of an interaction between the 53 kDa glycoprotein and the ATPase came from experiments in which SR vesicles were treated with antibodies against the glycoprotein, resulting in changes in the function of the ATPase or in rotational motion of the ATPase [9,10,11]. For an antibody against the glycoprotein to affect SR vesicles, it is necessary for the glycoprotein to be at least partly exposed on the outer surface of the vesicles, but the sequence of the glycoprotein shows no clear hydrophobic trans-membranous stretches [15]. We have therefore investigated the effects of a number of mAbs raised against the glycoprotein on the function of SR.

Epitopes for the mAbs were defined by studying binding to proteolytic fragments of the 53 kDa glycoprotein separated in SDS gels. As shown in Fig. 2, we have mAbs to both ends of the molecule and to a centrally located epitope. We also produced an anti-peptide antibody to the C-terminus of the glycoprotein. Binding of the mAbs to the glycoprotein in intact SR vesicles was investigated by competitive ELISA. As shown in Fig. 5, mAb L/1G3, which has an epitope close to the N-terminus of the 53 kDa glycoprotein (Fig. 2), binds to SR vesicles only when the vesicles have been solubilised with $C_{12}E_8$ indicating that it is unlikely that the N-terminus is extralumenal. MAbs L/1E12 and L/5F8, which bind in the region of the C-terminus of the glycoprotein, also only bind to SR in the presence of solubilising concentrations of detergent, suggesting a lumenal location for the C-terminus. Unfortunately, although the antibody raised to peptide 442–452 (the anti-C-terminus antibody) recognised the 53 kDa glycoprotein in Western blots it did not bind sufficiently well to be of use in competitive ELISA. Monoclonal antibody L/1E12, binding to an epitope towards the middle of the glycoprotein molecule also bound to SR vesicles only after solubilisation with $C_{12}E_8$ (Fig. 5). We and others have shown that the concanavalin A binding sites, which are probably 2 high mannose sugar chains attached to the 53 kDa glycoprotein at residues N261 and N369 [15] are also intralumenal [13,14].

The mAb experiments, demonstrating lumenal locations for the C- and the N-termini and the middle of the 53 kDa glycoprotein molecule, strongly argue against a single trans-membranous spanning segment which would leave a significant amount of the N- or the C-terminus exposed on the cytoplasmic surface of the SR. However as outlined by Leberer et al. [15] there are two weakly hydrophobic stretches (see Fig. 7)

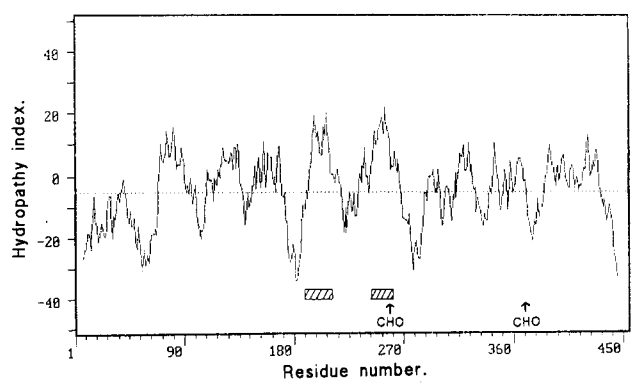

Fig. 7. Hydropathy analysis of the 53 kDa glycoprotein sequence [15], based on the method of Kyte and Doolittle [29], using a 15 amino acid residue window. Hatched bars represent the slightly hydrophobic regions and the arrows the glycosylation sites.

which could give rise to a hairpin like configuration for the 53 kDa glycoprotein. Since neither the C- or the N-termini appear to be exposed on the cytoplasmic surface the arrangement would have to be a hairpin with the short intermembranous segment exposed to the cytoplasm. This segment of the 53 kDa glycoprotein including the putative transmembranous domains corresponds to around residues 190–260. Since mAb L/1E12 maps to a segment of the protein from at least residue 250 and no further than residue 261 (the glycosylation site (Fig. 2) and this epitope appears to be located in the SR lumen this arrangement of the ATPase seems unlikely. These data are also consistent with the results of proteolysis experiments. The time course for the proteolysis of SR components by proteinase K (Fig. 3) shows that whereas all the (Ca^{2+}-Mg^{2+})-ATPase is digested within 10 min the 53 kDa glycoprotein is protected from proteolysis and significant amounts remain even after 2 h. There is some breakdown of the 53 kDa glycoprotein to produce a 40 kDa fragment but this only occurs after the ATPase has been completely digested. The finding that calsequestrin exhibits a similar profile to that seen for the 53 kDa glycoprotein suggests that the 53 kDa glycoprotein is intralumenal and the probable explanation for the appearance of proteolysis products following the disappearance of the ATPase from SR is that the removal of a major transmembranous component from the SR membrane has disrupted the SR membrane sufficiently to allow the proteinase access to the SR lumen. The intralumenal location is also supported by the finding that trypsin and proteinase K are unable to digest the 53 kDa glycoprotein over the initial 30 min of incubation, but the 53 kDa glycoprotein is readily proteolysed if the SR is solubilised or if the structural integrity of the SR membrane is disrupted by osmotic shock with hypotonic buffer (Fig. 4). These results are essentially in agreement with the findings of Leberer et al. [15] who showed that the 53 kDa glycoprotein was inaccessible to trypsin in intact SR. Apart from one report [12] which appeared to show that in sealed SR vesicles the 53 kDa glycoprotein reacted with a cycloheptaamylose-fluorescamine complex there is no other direct evidence for exposure of a large segment of the 53 kDa glycoprotein to the cytoplasmic environment. Even this evidence, it has been suggested, could be the result of the impermeant labelling reagent binding to a protein migrating with a similar M_r in polyacrylamide gels [15]. Indeed experiments with azidolipids indicate that a number of SR components with apparent M_r values similar to that of that of the 53 kDa glycoprotein are probably transmembranous [27]. The same study also indicates that the 53 kDa glycoprotein might have a close association with the SR membrane since it appears to be one of the SR components labelled by these azidolipids.

Kutchai and Campbell [9] reported that the simultaneous binding of monoclonal antibodies to the ATPase and the 53 kDa glycoprotein could result in an inhibition of calcium transport not seen in the presence of either antibody alone. This was interpreted as the monoclonal antibodies interfering with some interaction between these two SR components, resulting in the uncoupling of Ca^{2+} transport. In an attempt to replicate this result we used four antibodies which bind to at least three different epitopes on the ATPase [28] in combination with monoclonal antibodies against the 53 kDa glycoprotein binding to three of the epitopes identified here (Fig. 2). None of the combinations resulted in any change in the uptake of Ca^{2+} seen when in the presence of anti-ATPase antibody alone. This would be consistent with the above findings that none of the above monoclonal antibodies against 53 kDa glycoprotein were able to bind to SR. The reason for the difference between this study and that of Kutchai and Campbell [9] is unclear.

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