Identification of a novel 45 kDa protein (JP-45) from rabbit sarcoplasmic-reticulum junctional-face membrane

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Using a biochemical/immunological approach to analyse the protein constituents of skeletal-muscle junctional-face membrane (JFM), we identified a 45 kDa protein. Its N-terminal amino acid was blocked, but the amino acid sequence obtained from several peptides after proteolytic treatment did not significantly match that of any protein present in the SwissProt and NCBI (National Center for Biotechnology Information) databases. We synthesized a peptide whose sequence matched that of one of the peptides obtained after CNBr cleavage of the 45 kDa protein; the peptide was conjugated to a carrier and used to raise antibodies. The antiserum was used to study in more detail the biochemical characteristics of the novel 45 kDa protein. Analysis of the proteins present in different subcellular membrane fractions show that the novel 45 kDa polypeptide: (i) is an integral membrane constituent present both in neonatal and adult skeletal-muscle sarcoplasmic reticulum; (ii) is selectively localized in the JFM; (iii) is not present in microsomes obtained from rabbit heart, liver or kidney. Immunoprecitation with anti-(45 kDa protein) antibody indicates that the 45 kDa protein is part of a complex which can be phosphorylated *in vitro* by the catalytic subunit of protein kinase A.

Key words: phosphorylated protein, skeletal muscle, transmembrane.

INTRODUCTION

During the past few years an enormous effort has been directed towards the identification of novel proteins present in human tissues. The main approach has been through direct sequencing of the human chromosome and identification of probable protein-coding sequences; the identified human gene transcripts are then classified on the basis of their tissue distribution and disease linkage. The identification of all the protein constituents of a membrane fraction/subcellular organelle is of fundamental importance, not only to understand the fine mechanisms underlying a particular function, but also to understand in greater detail phenotypic characteristics of genetic diseases.

Malignant hyperthermia is an autosomal dominant muscle disorder of skeletal-muscle excitation–contraction (EC) coupling [1]. Genetic studies have clearly indicated that this disease is heterogeneous, and mutations in genes encoding at least six distinct proteins have so far been reported (for reviews, see [2,3]). Such a result implies that the molecular machinery responsible for EC coupling comprises several protein components whose identification and functional role has yet to be defined. The anatomical site of skeletal-muscle EC coupling is the triad (TR), a unique intracellular synapse formed by the association of two membrane compartments: transverse tubules, which are an invagination of the sarcolemma and the sarcoplasmic-reticulum (SR) terminal cisternae (TC) [4,5]. The portion of TC facing the transverse tubules is called junctional-face membrane (JFM) SR [5]. Ordered arrays of junctional feet, referable to as 'ryanodine-receptor Ca^{2+}-release channel' (RYR), bridge the gap of 9–12 nm (90–120 Å) which separates the membrane of the transverse tubules from the JFM [6]. The dihydropyridine-sensitive Ca^{2+} channel of the transverse tubules acts as the voltage sensor for EC coupling and plays a crucial role in the regulation of the RYR calcium channel [7,8]. In addition to the RYR, the junctional face membrane contains several proteins, including the histidine-rich Ca^{2+}-binding protein, triadin, calsequestrin and junctin [9,10]. In the present study we have used a biochemical approach to study the protein constituents of rabbit SR and have identified a novel 45 kDa transmembrane protein which is present only in skeletal muscle, is enriched in the JFM and is found in both fast- and slow-twitch fibres.

EXPERIMENTAL

Materials

Maleimide-activated, keyhole-limpet haemocyanin (KLH) conjugation kit, MPL (monophospholipid A from *Salmonella minnesota*) adjuvant, heparin–agarose, trypsin, soybean trypsin inhibitor and protein kinase A were from Sigma (St. Louis, MO, U.S.A.); Protein A–Sepharose and nitrocellulose was from Amersham-Pharmacia Biotech; monoclonal antibodies [$VIIID1_2$ to fast-skeletal-muscle calsequestrin, IIG12 to triadin, and VF1c to the 90 kDa junctional face protein (JFP)] were from Affinity Bioreagents (Golden, CO, U.S.A.); polyclonal anti-RYR1 antibodies were from Upstate Biotechnologies (Lake Placid, NY, U.S.A.); peroxidase-conjugated secondary anti-

Abbreviations used: EDL, extensor digitorum longus; H(SR), (heavy) sarcoplasmic reticulum; JFM, junctional-face membrane; JFP, junctional face protein; RYR, ryanodine-receptor Ca^{2+}-release channel; SOL, soleus; TA, tibialis anterior; TC, terminal cisternae; EC, excitation–contraction; DTT, dithiothreitol; NCBI, National Center for Biotechnology Information; KLH, keyhole-limpet haemocyanin; TR, triad.

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bodies and chemicals for enhanced chemiluminescence were from Roche Molecular Biochemicals. Protein molecular-mass standards were from Bio-Rad or Gibco BRL Life Technologies. All other chemicals were of reagent grade.

Methods

Preparation of rabbit tissue subcellular fractions

Total microsomes were isolated from New Zealand White rabbit hearts, livers, kidneys and neonatal (14-day-old) skeletal muscle as previously described [11], except that a high-salt wash with 0.6 M KCl was added to remove proteins loosely associated with the membranes. Integral membrane proteins were separated from their soluble counterpart by extraction with Na_2CO_3 at alkaline pH as described by Cala and Jones [12]. TC were obtained from the white skeletal muscle of New Zealand White rabbits as described by Saito et al. [13]. Fractions enriched in light (LSR) and heavy SR (HSR) were prepared as described by Campbell et al. [14], JFM was prepared as described by Costello et al. [5], TRs were prepared as described by Rosenblatt et al. [15] and the surface membrane was prepared as described by Ohlendieck et al. [16]. Microsomes were also prepared from isolated soleus (SOL), tibialis anterior (TA) and extensor digitorum longus (EDL) muscles in the presence of protease-inhibitor cocktail as previously described [17]. Protein concentration was determined as described by Bradford [18], using either myofibrillar proteins or BSA as standards.

Heparin–agarose chromatography

Vesicles obtained from rabbit skeletal-muscle TC were resuspended at a final concentration of 1 mg/ml in 1 % Triton X-100/200 mM NaCl/50 mM Tris/HCl (pH 8.5)/1 mM dithiothreitol (DTT)/1 mM EDTA, containing 1 μg/ml leupeptin, 100 μM PMSF and benzamidine and 1 μM pepstatin as antiproteolytic agents. The vesicles were solubilized for 30 min at room temperature under gentle agitation and centrifuged at 100000 $\boldsymbol{g}_{max}$ for 30 min at 4 °C. The supernatant was incubated with heparin–agarose (1 ml of resin/mg of protein) previously equilibrated in 50 mM Tris/HCl (pH 8.5)/200 mM NaCl/1 mM DTT/1 mM EDTA (Buffer A) for 60 min at room temperature under gentle agitation. The column was washed with 10 bed vol. of Buffer A containing 0.1 % Triton X-100 and proteins were eluted with a step gradient of NaCl (300 mM, 500 mM, 750 mM and 1 M) in buffer A.

Protein microsequencing

The fraction eluted from the heparin–agarose column at 500 mM NaCl was concentrated by addition of 3 vol. of cold acetone. The insoluble protein fraction was collected as a pellet after centrifugation for 30 min at 3000 $\boldsymbol{g}_{max}$ in an ALC 5400 centrifuge. The pellet was resuspended in 50 mM Tris/HCl (pH 6.8)/2 % (w/v) SDS, 0.02 % Bromophenol Blue and the proteins were separated by SDS/PAGE in a 10 % slab gel. The proteins were revealed by imidazole/$ZnSO_4$. To concentrate the protein, gel slices containing the 45 kDa protein bands were transferred to a rod gel in a Pasteur pipette. The glass of the Pasteur-pipette tube was treated with dichloromethylsilane/chloroform (5:95, v/v), dried, and filled with a solution containing 5 % (w/v) acrylamide, 0.13 % bisacrylamide and 125 mM Tris/HCl, pH 6.7. Polymerization was initiated by addition of ammonium persulphate and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine. The gel slices were loaded on the rod gel in the presence of 5 μl of 50 mM Tris/HCl (pH 6.8)/2 % SDS/0.1 % Bromophenol Blue. Electrophoresis was carried out at 150–200 mV until the Bromophenol Blue reached the narrow part of the Pasteur pipette. The protein band was revealed by Coomassie Brillant Blue staining. The slice of gel containing the protein was washed three times with 1 ml of Milli Q water and then incubated for 1 h in 1 ml of Milli Q water at room temperature. The water was discarded and the gel slice was incubated in 80 μl of a solution containing 50 mM Tris/HCl (pH 8.5)/0.1 % SDS in a 1.5 ml Eppendorf tube with vortex stirring. After 2 h, 20 μl of 20 % (v/v) acetonitrile, followed by 1 μg of trypsin, were added and the incubation was continued overnight at room temperature with vortex stirring. The supernatant was concentrated to 100 μl in a Speed-Vac apparatus. The peptides derived from the 45 kDa protein were purified by HPLC using a 250 mm × 1.0 mm column packed with 5 μm RP18 from Vydac (The Separation Group, Hesperia, CA, U.S.A.). Mobile phases A and B contained 0.12 % trifluoroacetic acid in water and 0.10 % trifluoroacetic acid/70 % acetonitrile in water respectively. Elution of the polypeptides derived from the 45 kDa protein was carried out by a linear gradient from 5 % B to 60 % B at flow rate of 40 μl/min over 100 min. Peaks eluted at approx. 66, 71 and 92 min were collected. To obtain CNBr peptides, the slice of gel containing the 45 kDa protein was incubated in 140 μl of CNBr/formic acid (58.1 mg of CNBr/1.162 ml of formic acid) plus 60 μl of water overnight at room temperature with vortex stirring. The next day the supernatant was transferred to another microvial and dried in a Speed-Vac concentrator. The peptides derived from the 45 kDa protein were separated by SDS/PAGE and blotted on to a PVDF membrane. The bands were revealed by Coomassie Blue Staining. Amino acid sequences of tryptic and CNBr-derived peptides were determined using an Applied Biosystem 476A protein sequencer.

Electrophoresis and Western blotting

SDS/PAGE was carried out as described by Laemmli [19]; gels were run at room temperature at a constant 150 V. Proteins were revealed by Coomassie Brilliant Blue staining. Electrophoretic transfer on to nitrocellulose or Immobilon membranes was performed at 4 °C as described by Towbin et al. [20].

Polyclonal-antibody production, immunoblotting and immunoprecipitation

A peptide derived from protein-microsequencing data was synthesized as described by Guerrini et al. [21] and conjugated to maleimide-activated KLH, as described in the conjugation-kit instructions. The carrier protein–peptide conjugate was purified by gel filtration and a 100 μg portion was used to intraperitoneally immunize Balb/c mice at weekly intervals in the presence of MPL as adjuvant. After 6 weeks, a small amount of blood was collected from the tail and the serum tested for the presence of antibodies against the 45 kDa protein by Western blotting. Approx. 1×10^6 tissue-culture-grown myeloma P3X63 cells were washed with PBS and injected into the peritoneal cavity of each positive mouse in order to induce production of ascitic fluid. Ascites was collected and IgGs purified by precipitation with octanoic acid [22].

For immunoblots, after saturating nitrocellulose or Immobilon membranes with 10 % (w/v) fat-free milk, blots were incubated with 1:1000-diluted primary antibodies, followed by washes and incubation with peroxidase-conjugated secondary antibodies. Immunodecorated protein bands were revealed by the enhanced-chemiluminescence method.

Immunoprecipitation was carried out as described in [22]. Briefly, after carrying out phosphorylation of the heparin–agarose fraction (see under the subsubsection 'Heparin–agarose chromatography'), approx. 20 μg of protein was incubated for

60 min with (or without) 10 μg of purified anti-(45 kDa peptide) IgGs. At the end of the incubation, 40 μl of Protein A–Sepharose was added to immunoprecipitate the 45 kDa protein; the precipitate was washed three times with 50 mM Tris/HCl/0.5 M NaCl, pH 8.0, and the proteins were loaded on a 10 %-polyacrylamide gel. After electrophoresis, phosphorylated proteins were revealed by autoradiography.

Trypsin proteolysis

Trypsin proteolysis was essentially as described by Chu et al. [23], except that porcine pancreatic trypsin (13000–20000 units/mg of protein) was used and its concentration adjusted to give the desired TC protein/trypsin (w/w) ratios. The digestion was carried out for 2 min at 25 °C, after which a 5-fold molar excess of soybean trypsin inhibitor was added. The samples were then placed on ice, processed for SDS/PAGE within 60 min and blotted on to nitrocellulose.

Protein phosphorylation

Protein phosphorylation was carried out as previously described using the catalytic subunit of cAMP-dependent protein kinase [24]. Approx. 20 μg of protein eluted from the heparin–agarose column was incubated at 37 °C for 10 min in a solution containing 50 mM Tris/HCl, pH 8.0, 250 μM ATP (1000–1500 c.p.m./pmol of [γ-^{32}P]ATP, 250 μM $MgCl_2$, 0.07 unit of cAMP-dependent protein kinase, with or without 0.5 mM $CaCl_2$. The reaction was stopped by the addition of 5-fold-concentrated SDS/PAGE loading buffer, the proteins were separated by SDS/10 %-PAGE and the gel was subsequently processed for autoradiography.

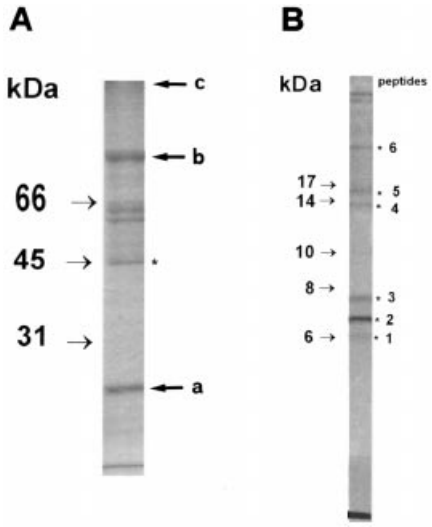

Figure 1 Protein composition of the fraction eluted at 500 mM NaCl from the heparin–agarose column and proteolysis of the 45 kDa protein using CNBr

(**A**) Fractions eluted from the heparin–agarose column at 500 mM NaCl were pooled, and the proteins concentrated and resuspended in 100 μl of Laemmli buffer. A 10 μl portion (approx. 25 μg of protein) of resuspended protein was loaded on to a SDS/10 %-polyacrylamide gel. After electrophoresis the proteins were stained with Coomassie Brilliant Blue. '*' Indicates the 45 kDa protein band; a, junctin; b, triadin; c, RYR. (**B**) The 45 kDa band was cut from the SDS/10 %-polyacrylamide gel and digested with CNBr. The proteolytic fragments were separated by SDS/15 %-PAGE and blotted on to PVDF membrane. The proteins were stained with Coomassie Brilliant Blue**.** Figures were processed using Corel Photopaint.

RESULTS AND DISCUSSION

The biochemical characterization of the molecular components present in TC is an important issue for understanding the fine mechanisms underlying Ca^{2+} homoeostasis in skeletal muscle under normal and pathological conditions. Rabbit TC vesicles were solubilized with 1 % Triton X-100 at low ionic strength, and the soluble fraction was then passed through a heparin–agarose column. Proteins were eluted with a step gradient of 0.3, 0.5, 0.75 and 1 M NaCl. In addition to the RYR, the fraction eluted at 500 mM NaCl contains other components with molecular masses of 200, 110, 80, 65, 63, 45 and 27 kDa (Figure 1A). The identity of some polypeptides was determined by immunoblotting using antisera against a variety of SR proteins [triadin determined by using anti-triadin antibody described in Figure 2(D) (Figure 1, band b); RYR determined by using anti-RYR antibody described in Figure 2(B) (Figure 1, band c)], whereas other components were identified by N-terminal amino acid sequencing [junctin determined by amino acid sequencing (Figure 1, band a)]. The N-terminal residue of the 45 kDa protein was blocked, so we proceeded to determine internal amino acid sequences. Figure 1(B) shows the peptides that were generated from the 45 kDa polypeptide after proteolytic degradation with CNBr, whereas the amino acid sequence obtained from peptide bands 2 and 3 is given in Table 1. CNBr-derived band 1 was blocked, and thus most probably derives from the N-terminus. The 45 kDa protein was also subjected to tryptic digestion and the sequence information generated is also given in Table 1. It should be mentioned that: (i) proteolysis with CNBr and trypsin yielded some fragments which exhibited overlapping sequences [sequences 952 and 931 show an overlap of nine residues, whereas sequences 954 and 930 show an overlap six residues (the sequence numbers are for reference only and have no added meaning)]; and (ii) the indicated sequences were generated from at least two separate proteolytic digestions of different preparations of the 45 kDa protein. The polypeptide sequencing data that we obtained did not significantly match that of any protein present in the Swiss Prot or NCBI databases and thus this represents a novel protein.

In order to gain more insight into the functional characteristics and subcellular distribution of this polypeptide we prepared a polyclonal antibody by immunizing mice with an *in-vitro*-synthesized peptide (corresponding to band 2 after CNBr treatment) coupled to the KLH carrier. The antibody reacted with a single polypeptide band of 45 kDa which was present in rabbit skeletal-muscle HSR (Figure 2A); the immunological reactivity is specific, since pre-incubation of the peptide with the antibody abolished the reactivity (results not shown). The immunoreactive band was enriched in the JFM, but clearly absent from LSR and surface membrane, and slightly visible in the TRs (Figure 2A). Since the 45 kDa protein is present in fractions enriched in TC and absent from longitudinal tubules, we compared its distribution with that of other well-characterized JFM proteins such as the RYR (Figure 2B), calsequestrin (Figure 2C), triadin (Figure 2D) and the 90 kDa JFP (Figure 2E). Our results clearly demonstrate that the 45 kDa component has a subcellular membrane distribution which is unique to known JFM proteins such as RYR, triadin, calsequestrin and 90 kDa JFP. Henceforth this novel protein will be referred to as JP-45.

To demonstrate further that the novel protein, JP-45, is an integral membrane constituent, two additional experimental approaches were used. TC were treated with Na_2CO_3 at alkaline pH, followed by washing with 0.6 M KCl to remove loosely associated proteins. The vesicles were then centrifuged for 30 min

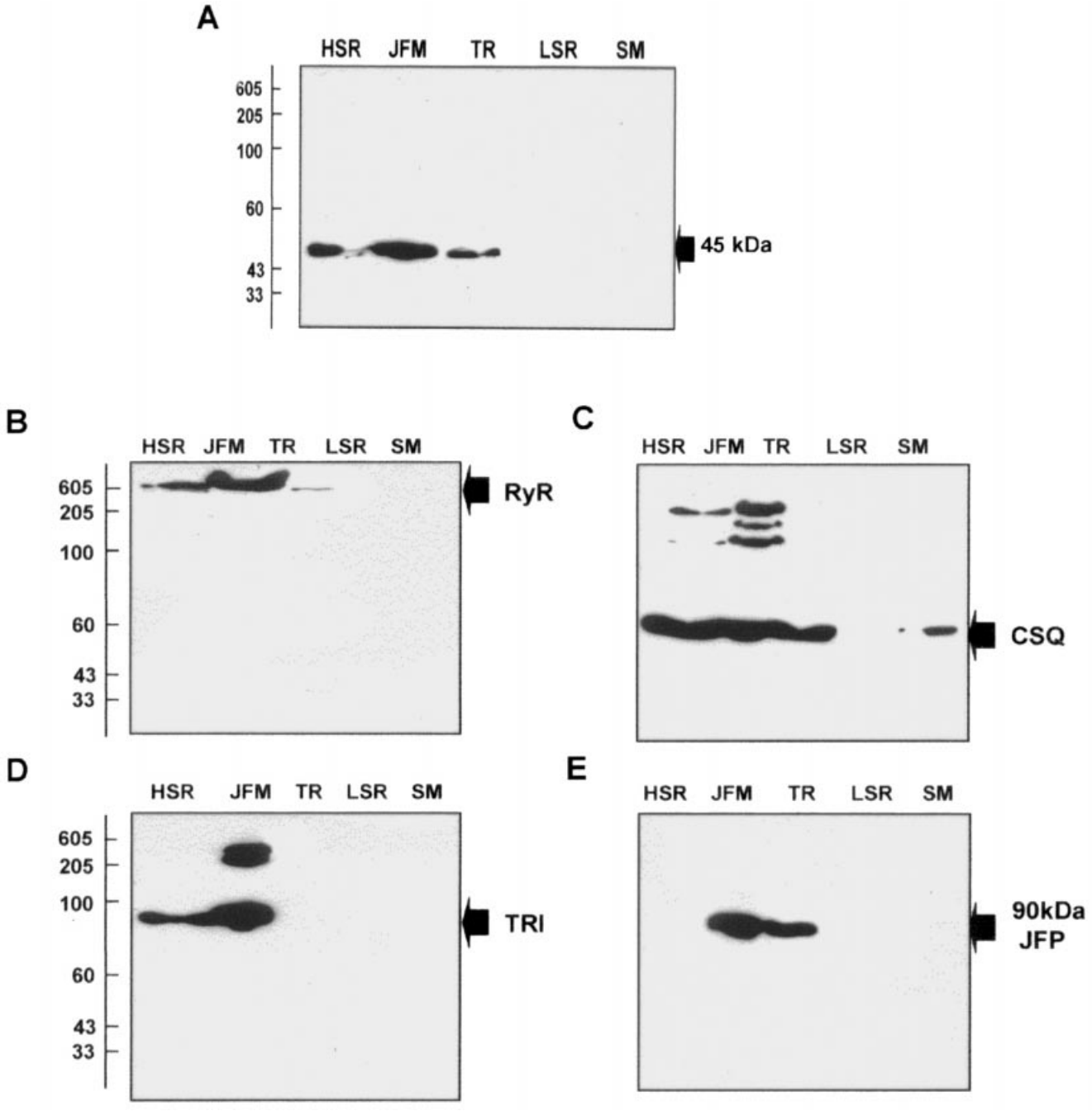

Figure 2 Immunoblotting showing the subcellular localization of the 45 kDa protein in rabbit skeletal muscle

(**A**) Polyclonal antibody raised against the 45 kDa protein; (**B**) polyclonal anti-RYR antibody; (**C**) monoclonal anti-calsequestrin (CSQ) antibody; (**D**) monoclonal anti-triadin (TR1) antibody; (**E**) monoclonal anti-90 kDa JFP antibody. Portions (15 μg) of protein obtained from membrane preparations enriched in HSR, JFM, LSR and TR were loaded in each lane. Arrows indicate the immunodecorated bands; sizes of molecular-mass standards (in kDa), as deduced from rat myofibrillar proteins, are indicated on the left. Figures were processed using Corel Photopaint.

at 100000 g_{max}, and the proteins present in the resulting soluble and insoluble fractions were separated by SDS/10%-PAGE. Figure 3 shows that calsequestrin, a luminal protein, is, as expected, in the supernatant (Figure 3A, lane 'Sup', band labelled '*'). Western blotting using the anti-peptide antibodies demonstrated that the JP-45 is exclusively present in the insoluble fraction (Figure 3B, lane 'Pt') and is thus an integral membrane component. The membrane topology of the 45 kDa protein was further characterized by trypsin digestion of TC proteins, followed by immunoblotting of the proteolysed polypeptides. Figure 4 shows that, after mild proteolysis of TC with trypsin, the high-molecular-mass RYR band (*) was degraded (note that the high-molecular-mass band is present in Figure 4, lane 1, but disappears thereafter), whereas more drastic conditions, i.e. higher trypsin/TC ratios, were required to degrade the Ca^{2+}-ATPase (Figure 4, **). Figure 4 also shows that calsequestrin (***) is not

Table 1 Amino acid sequence of peptides obtained after proteolytic treatment of JP-45

Proteolytic treatment	Sequence number*	Amino acid sequence
Trypsin	952	EGGRGPWAR
CNBr (band no. 3)	931	MXXREGGRGPXARD
Trypsin	954	RHDREDRLPGR
CNBr (band no. 2)	930	MXRDSRDPGRSKRQARA(S)PRRHDRED
Trypsin	955	KEPVAPL(P)Q(P)XVLAPA
Trypsin	957	XXL(P)(P)GLA

* The sequence number is for reference only and has no added meaning.

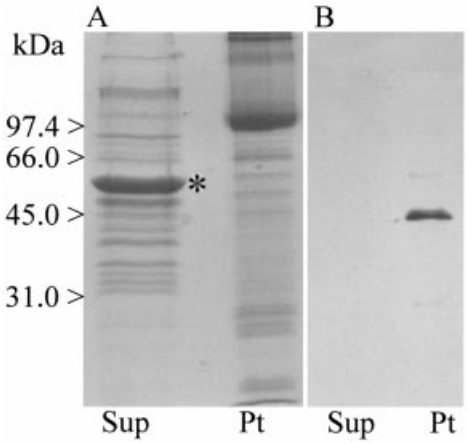

Figure 3 SDS/PAGE of carbonate-solubilized rabbit skeletal-muscle TC vesicles

Vesicles were treated with 100 mM Na_2CO_3 as described in the Experimental section to obtain a pellet ('Pt') and a supernatant ('Sup'). The pellet was resuspended in 0.3 M sucrose/10 mM Hepes, pH 7.2, and 30 μg of each fraction was subjected to SDS/10%-PAGE. Proteins were revealed by Coomassie Brilliant Blue staining (**A**) or blotted on to nitrocellulose and subjected to immunoblotting using the polyclonal antibody raised against the 45 kDa protein (**B**). '*' indicates calsequestrin. Figures were processed using Photoshop Adobe.

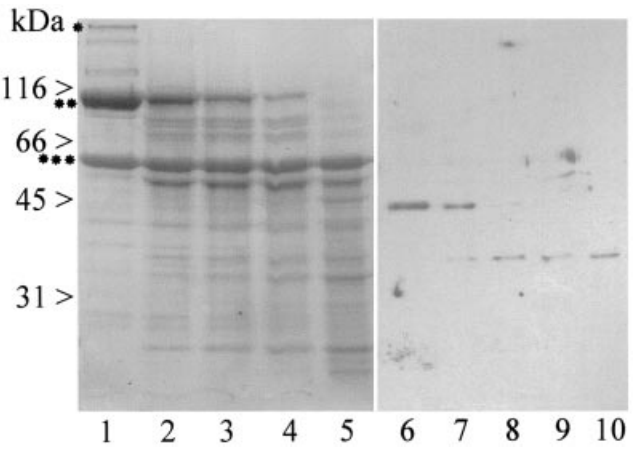

Figure 4 SDS/PAGE of TC vesicles after digestion with increasing trypsin/TC ratios

Conditions were as described in the Experimental section. Lanes 1 and 6, 2 and 7, 3 and 8, 4 and 9, 5 and 10 show samples digested with protein/trypsin ratios of 4000:1, 2000:1, 1000:1, 500:1 and 250:1 respectively. Proteins present in lanes 1–5 were stained with Coomassie Brilliant Blue, whereas those in lanes 6–10 were blotted on to nitrocellulose and subjected to immunoblotting using the polyclonal antibody raised against the 45 kDa protein (30 μg of protein/lane). *, RYR; **, CaATPase; ***, calsequestrin. Figures were processed using Photoshop Adobe.

degraded under these conditions. On the other hand, the 45 kDa protein is degraded to an immunopositive peptide of approx. 35 kDa (Figure 4, lanes 7–10). These results indicate that a small portion of the 45 kDa protein is exposed to the myoplasm, whereas most of the protein, including the epitope recognized by the antibodies, is luminal and thus not accessible to trypsin.

The level of expression of the 45 kDa protein in three types of skeletal muscles was also determined: SOL, which contains slow type fibres, TA, which contains a mixture of fast- and slow-type fibres, and EDL, a predominantly fast-type fibre.

Western blots of microsomes from the three muscles show that the 45 kDa immunoreactive band is present in the three muscle types, but is apparently enriched in the microsomes obtained from muscles containing fast-type fibres (Figure 5). The content of the immunoreactive 45 kDa protein in the three types of muscles was also quantified by performing densitometric analysis of several immunoblots. The immunoreactivity of the EDL was normalized to $100 \pm 5.5\,\%$. TA and SOL displayed an immunoreactivity of $81 \pm 13\,\%$ (mean + S.D., $n = 7$) and $71.7\,\% \pm 25$ (mean + S.D., $n = 7$). However, the apparent differences in the pattern of expression of the novel 45 kDa protein between predominantly fast- and slow-twitch muscles were not statistically significant (Student's t-test for EDL versus TA, $P < 0.2$; EDL versus SOL, $P < 0.29$).

We also investigated whether the 45 kDa protein is only present in skeletal muscle or whether it is a protein constituent of other tissues as well. The microsomal fractions of heart, liver, kidney, as well as neonatal skeletal muscle, were treated with Na_2CO_3/KCl as described in the Experimental section. The extracted proteins were separated by SDS/10%-PAGE, transferred on to nitrocellulose and subjected to immunoblotting; Figure 6 shows that the immunoreactive band having a molecular mass of 45 kDa is present in skeletal-muscle TA (lane 6) as well as in the membrane-associated proteins of neonatal skeletal muscle (lane 10), but no immunoreactive polypeptide is present in any of the other tissues investigated. An identical result was obtained using a different, affinity-purified, polyclonal antibody raised by immunizing mice with the native 45 kDa protein present in the heparin–agarose fraction (results not shown).

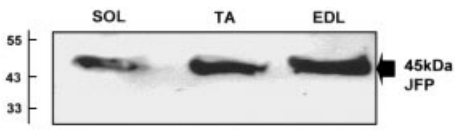

Figure 5 Immunoblotting showing the distribution of the 45 kDa protein in muscles containing predominantly slow- or fast-type fibres

The anti-(45 kDa protein) polyclonal antibody was tested on Western blots of microsomal membrane preparations from isolated SOL, TA and EDL muscles (25 μg of protein/lane). The arrow indicates the immunodecorated bands; sizes of molecular-mass standards (in kDa), as deduced from rat myofibrillar proteins, are indicated on the left. Figures were processed using Corel Photopaint.

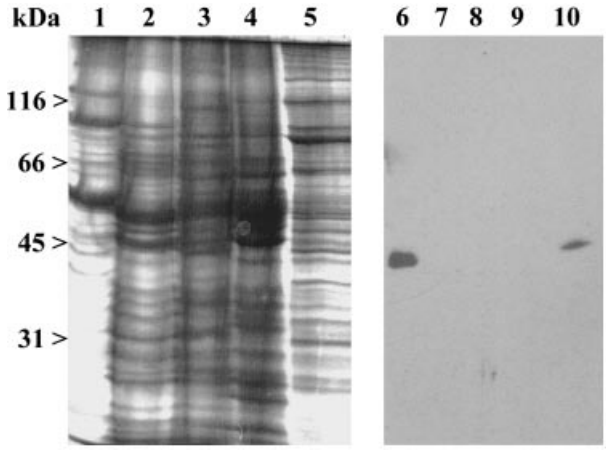

Figure 6 The 45 kDa protein is present in skeletal muscle, but absent from the heart, liver and kidney

Integral membrane proteins from heart SR (lanes 2 and 7), kidney (lanes 3 and 8), liver (lanes 4 and 9), neonatal rabbit (14-day-old) SR (lanes 5 and 10) (40 μg of protein each) or TA (25 μg of protein, lanes 1 and 6) were separated by SDS/10%-PAGE and stained with Coomassie Brilliant Blue (lanes 1–5) or transferred on to nitrocellulose and subjected to immunoblotting using the polyclonal antibody raised against the 45 kDa protein (lanes 6–10). Figures were processed using Photoshop Adobe.

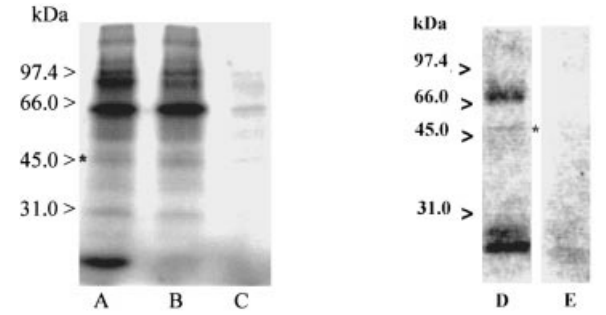

Figure 7 Phosphorylation of proteins present in the fraction eluted at 500 mM NaCl from the heparin–agarose column by cAMP-dependent protein kinase

Conditions were as described in the Experimental section. Lane A, phosphorylation was performed in the absence of $CaCl_2$; lane B, phosphorylation was performed in the presence of 0.5 mM $CaCl_2$; lane C, conditions as in lane A, except that the reaction was stopped at zero time; lanes D and E, after carrying out the phosphorylation reaction, the 45 kDa protein was immunoprecipitated with the anti-(45 kDa protein) antibody (lane D) or pre-immune serum (lane E). Proteins were then separated by SDS/10%-PAGE and the gel was then processed for autoradiography at -70 °C using an intensifier screen. '*' Indicates the 45 kDa protein. Figures were processed using Photoshop Adobe.

To gain an insight into a possible functional role for the 45 kDa protein, we performed *in vitro* phosphorylation using the catalytic subunit of the cAMP-dependent protein kinase.

Figure 7 shows that a number of proteins present in the fraction eluted at 500 mM NaCl from the heparin–agarose column are phosphorylated. A 45 kDa protein present in the heparin–agarose column fraction (Figure 7, *) can act as a weak substrate for the cAMP-dependent protein kinase, and its phosphorylation is independent of the presence or absence of millimolar [Ca^{2+}] (Figure 7, lanes A and B). In order to confirm the identity of the 45 kDa phosphorylated polypeptide, we performed an immunoprecipitation reaction of the phosphorylated proteins present in the heparin–agarose fraction (Figure 7, lanes D and E). The antibody that we used for immunoprecipitation was raised against a synthetic peptide and did not show any cross-reactivity with other proteins present in skeletal-muscle SR, liver, kidney or heart microsomes (see Figures 3B and 6). Our results show that the anti-45 kDa antibody immunoprecipitates a complex made up of at least four phosphorylated proteins of 66 kDa, 45 kDa and a doublet of 27 kDa. Thus the 45 kDa protein appears to be part of a composite structure; some polypeptides present in such a complex can be phosphorylated *in vitro* by the catalytic subunit of protein kinase A. The nature of the other proteins present in such a complex was not investigated further.

Identification of all the molecular components present in the JFM is helpful in order to understand the fine mechanisms underlying EC coupling. A number of studies has tackled this issue, and, so far, several protein components have been clearly located to this subcellular membrane: junctin is a transmembrane protein which has been demonstrated to interact with calsequestrin (present in the SR lumen) [10,25] and triadin, another JFM integral membrane protein [26,27]. As to JP-45, the novel protein we describe in the present paper, its presence in the same heparin–agarose fraction in which junctin, triadin and the RYR are found may indicate that the proteins interact with one other. Unlike triadin and junctin, however, JP-45 does not appear to be present in the heart and may exert a role specific for skeletal-muscle function. Alternatively, different isoforms of this protein may exist, but our antibodies may be isoform-specific and thus unable to recognize other forms of the protein. As to its exact role, the production of an animal model having the gene encoding the 45 kDa protein 'knocked out' is required. The observation that the novel protein can be phosphorylated *in vitro* by cAMP-dependent protein kinase may indicate that its function, regardless of what that may be, is regulated by its state of phosphorylation. Thus, by analogy with other transmembrane phosphorylated proteins, it may be part of an intracellular signalling pathway and its state of phosphorylation may act as a molecular switch. On the other hand, its targeting to a particular membrane domain depends on this post-translational modification.

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