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Antibodies against the non-muscle isoform of the endoplasmic reticulum Ca^{2+}-transport ATPase

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We report here the production of a polyclonal antiserum which specifically recognizes an epitope confined to the ultimate 12-residue-long *C*-terminus of an alternatively spliced transcript of gene 2 encoding the sarcoplasmic reticulum Ca^{2+} pump in slow skeletal and cardiac muscle. This alternatively spliced transcript was shown to be mainly represented in non-muscle tissues. These antibodies have enabled us to show the presence of the unique *C*-terminus of this type of Ca^{2+} pump, as predicted from the cDNA sequence, in the endoplasmic reticulum of vascular and gastric smooth muscle, liver and kidney.

INTRODUCTION

cDNA cloning and sequencing have shown that the Ca^{2+} pumps of the mammalian sarco(endo)plasmic reticulum are represented by at least two different, but closely related, genes [1–11]. Gene 1 encodes a sarcoplasmic reticulum Ca^{2+} pump that is only expressed in fast-twitch skeletal muscle [2,4]. Gene 2 is believed to be expressed in most other tissues [1,3,5–11]. Tissue-dependent alternative splicing of the primary transcript of gene 2 results in either transcript 2.1, coding for a 997-residue-long protein, or in transcript 2.2, coding for a 1042-residue-long protein. The 2.1 transcript has been detected in cardiac muscle [3], slow-twitch skeletal muscle [1,3], smooth muscle [7,11], and also in renal tissue [5]. The alternatively spliced 2.2 transcript on the other hand is found in non-muscle tissues [5,6], but it is also present in smooth muscle [7,11]. There are some indications for the existence of a third gene, but this question deserves further attention [5,6].

As deduced from their cDNA sequences, the 2.2 isoforms of the gene 2 Ca^{2+} pump differ only in their *C*-terminus. The first 993 amino acids are common to both isoforms, but the four ultimate amino acids of the 2.1 isoform (total 997 amino acids) are replaced in the 2.2 protein (total 1042 amino acids) by an additional tail of 49 amino acids [5,6,7,11]. This small difference in primary structure explains why it has not been possible hitherto to differentiate between the 2.1 and the 2.2 protein isoforms. Using a polyclonal antibody directed against the unique *C*-terminus of the 2.2 isoform as predicted from cDNA sequencing, we have been able to identify the corresponding Ca^{2+} pump and to study its expression in both muscle and non-muscle tissues.

MATERIALS AND METHODS

Preparation of the immunogen

A 12-residue-long peptide of the following composition: STDTNFSDMFWS was custom-synthetized (Eurosequence, b.v. Gronigen, The Netherlands). This peptide corresponds to the 12 *C*-terminal amino acids of the 2.2 endoplasmic reticulum Ca^{2+} pump [11]. The peptide was conjugated to a carrier protein, either bovine serum albumin or bovine thyroglobulin, via glutaraldehyde cross-linking as described by Harlow & Lane [12]. In short, 2 mg of peptide and 12.5 mg of carrier protein were dissolved in 1.25 ml of 0.1 M-sodium phosphate buffer, pH 7.6, and an equal volume of 0.2% glutaraldehyde in the same buffer was added slowly (i.e. dropwise, spread over a time period of 5 min) at room temperature with constant stirring. The mixture was stirred further for 1 h. Remaining unreacted aldehyde groups were quenched by addition of 0.5 ml of 1 M-glycine followed by 1 h of stirring at room temperature. The reaction mixture was then dialysed overnight against phosphate-buffered saline (PBS; 0.9% NaCl/10 mM-sodium phosphate, pH 7.6).

Immunization and preparation of antisera

Rabbits were immunized with an emulsion of 0.5 ml of the peptide–serum albumin conjugate or the peptide–thryoglobulin conjugate (2 mg of carrier protein/0.33 mg of peptide) in PBS and 0.5 ml of complete Freund's adjuvant. Booster injections of the same immunogens with incomplete Freund's adjuvant were given at 3 week intervals. Blood was taken and serum was prepared after the second booster.

Antibodies directed against the carrier component were removed from the antiserum by running 5 ml of the antiserum, 10-fold diluted in TBS/Tween (0.9% NaCl/10 mM-Tris/HCl, pH 7.5 and 0.05% Tween-20), over a 1 ml gel column of the corresponding Sepharose 4B–carrier protein conjugate.

These Sepharose gels were prepared by binding 10 mg of bovine serum albumin or 10 mg of bovine thyroglobulin to 2 ml of CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) according to the manufacturers' instructions.

Immunodetection and phosphoprotein isotyping

Electrophoresis, immunoblotting and phosphoprotein isotyping of the Ca^{2+}-transport ATPases were done as described earlier [13].

Abbreviation used: PBS, phosphate-buffered saline.
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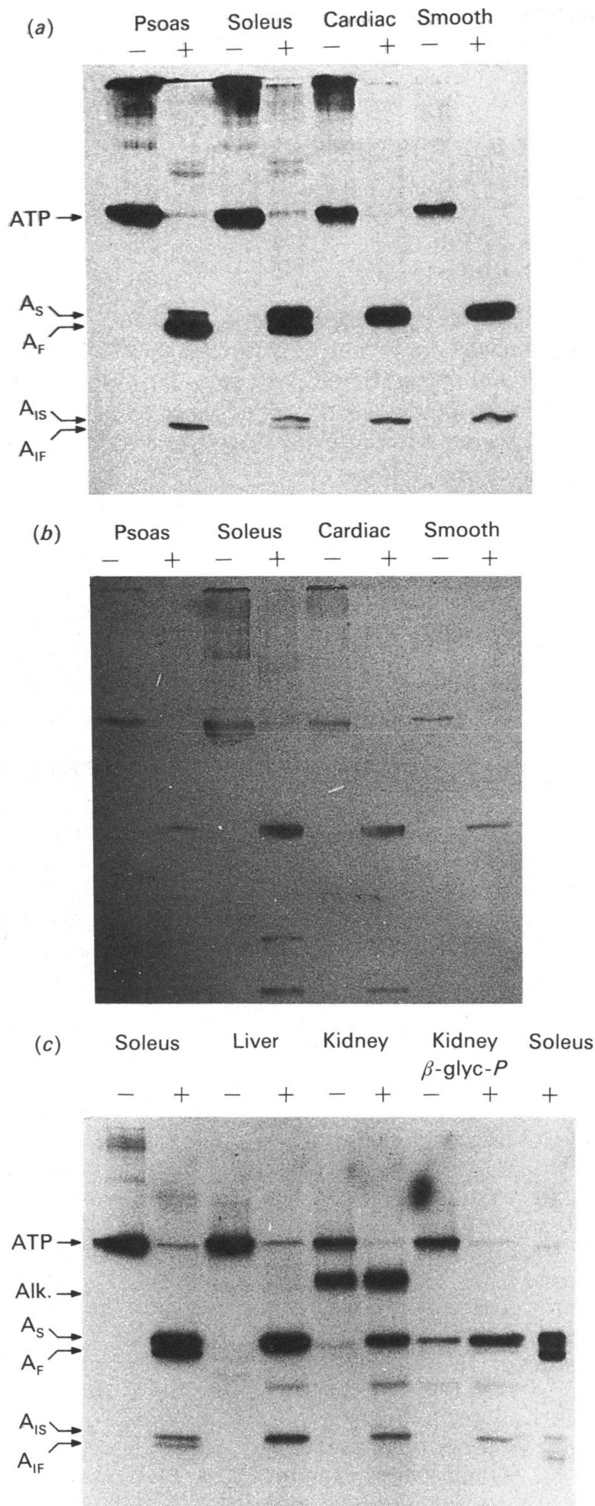

Fig. 1. Distinguishing between gene 1 and gene 2 related Ca^{2+} pumps in muscle and non-muscle tissues by means of their tryptic phosphoprotein fragments

(*a*) Autoradiogram of a blot showing the phosphoprotein intermediates of the Ca^{2+} pump of pig psoas, soleus, cardiac ventricle and antrum smooth muscle without (−) or with (+) short trypsin treatment before phosphorylation [13]. (*b*) The same blot stained with monoclonal antibody IID8. This antibody is specifically directed against an epitope in the A fragment of the cardiac sarcoplasmic reticulum Ca^{2+} pump. A 50 μg portion of membrane protein was applied in each lane. (*c*) Autoradiogram from a similar experiment using pig liver and kidney smooth endoplasmic reticulum and for comparison, pig soleus sarcoplasmic reticulum. Alk., alkaline phophatase. This enzyme, present in kidney microsomes, is also phosphorylated in the conditions used in these experiments. In the lanes labelled kidney β-glyc-*P*, 5 mM-β-glycerophosphate was applied during phosphorylation to suppress the phosphorylation of alkaline phosphate [18]. A 50 μg portion of membrane proteins was applied to each lane, except for soleus membranes where only 5 μg was used. ATP indicates the position of the unproteolysed ATPase. A_S, A_{1S}, A_F and A_{1F} indicate the positions of the tryptic phosphoprotein fragments A and A_1 of the slow and fast skeletal-muscle isoforms.

Preparation of microsomes

Mixed microsomes from pig stomach smooth muscle and from the sarcoplasmic reticulum of pig cardiac ventricle, pig fast skeletal muscle (psoas), and a pig mixed-type skeletal muscle (soleus) were prepared as in [13]. Density-gradient separation of pig antrum smooth-muscle membranes, and preparation of endoplasmic reticulum vesicles from bovine main pulmonary artery was done according to [14].

Rough and smooth microsomal fractions from pig liver and kidney were obtained by density-gradient centrifugation [15]. The density-gradient fractions were diluted 3-fold with ice-cold water, pelleted at 204000 $g_{max.}$ for 30 min in a Beckman Ti 60 rotor and resuspended in 0.25 M-sucrose/1.5 mM-dithiothreitol.

Proteins were assayed by the enhanced protocol of the bicinchoninic acid method of Pierce (Rockford, IL, U.S.A.) using bovine serum albumin as standard [16].

RESULTS AND DISCUSSION

Partial isotyping of Ca^{2+} pumps by means of phosphoprotein fragments

It is possible to discriminate between the gene products of gene 1 (i.e. the gene for the Ca^{2+} pump in the sarcoplasmic reticulum of fast-twitch skeletal muscle) and those of the gene 2 (the gene encoding the Ca^{2+}-pump enzymes present in other muscle types and in non-muscle cells) by comparing the relative mobilities of their phosphorylated tryptic A and A_1 fragments in SDS/polyacrylamide gels [13]. The tryptic A and A_1 fragments of the gene 1 and the gene 2 Ca^{2+} pumps contain the same number of amino acids (A, 505 amino acids; A_1, 307 amino acids) [1,2] but they differ in their amino acid compositions. Fig. 1(*a*) shows the phosphoprotein intermediates of the Ca^{2+} pumps and their tryptic A and A_1 fragments for four representative muscle types: psoas (a fast skeletal muscle), soleus (a muscle containing both slow and fast fibres), cardiac muscle and smooth muscle. Although there is no discernable mobility difference for the phosphoproteins in the unproteolysed pumps, it is clear that the A and A_1 tryptic phosphoprotein fragments of fast skeletal muscle gene 1 product (A_F and A_{1F}) show a higher electrophoretic mobility relative to the corresponding A_S and A_{1S} fragments of cardiac and smooth muscle (gene 2 products). The soleus muscle contains both gene 1 and gene 2 type Ca^{2+}-pumps. Fig. 1(*c*) shows that non-muscle tissues like liver and kidney contain phosphorylated A and A_1 fragments which migrate more slowly than the corresponding A-fragment in fast-twitch

skeletal muscle, but which cannot be discriminated from the slow skeletal, cardiac and smooth-muscle A fragment. However, whereas this method for isotyping can discriminate between the gene 1 and gene 2 type Ca^{2+} pumps, it cannot differentiate between the 2.1 and the 2.2 Ca^{2+} pumps which result from alternative splicing of the gene 2 transcript. Indeed, these isoforms differ only in their *C*-terminus, and as the A fragment is localized in the common part of the sequence, they generate identical A fragments.

Characterization of the antibodies

To distinguish between the 2.1 and the 2.2 Ca^{2+} pump isoforms, we took advantage of the *C*-terminal diversity. A synthetic peptide corresponding to the 12 *C*-terminal amino acids of the 2.2 Ca^{2+} pump was used as an antigen to raise polyclonal antibodies (see the Materials and methods section) specifically reacting with the 2.2 isoform.

The antibodies gave a positive reaction with the synthetic peptide spotted on PVDF Immobilon (Millipore Corporation, Bedford, MA, U.S.A.) membranes (results not shown). On Western blots, they recognized an M_r-100000 polypeptide in the endoplasmic reticulum of pig stomach smooth muscle (Fig. 2). However, they failed to react with any peptide present in the cardiac sarcoplasmic reticulum (Fig. 2, blot 3). That the antibodies elicited against the predicted unique *C*-terminus of the 2.2 endoplasmic-reticulum Ca^{2+} pump indeed recognized the 2.2 isoform is supported by the following observations. (1) The M_r (100000) and the subcellular

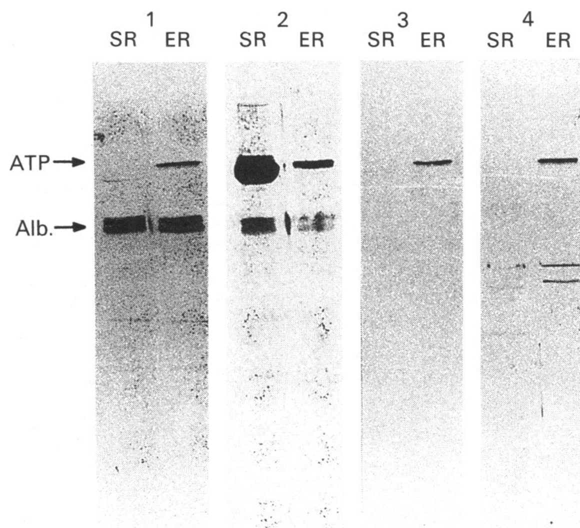

Fig. 2. Characterizing the antibodies against the peptide

Four identical Western blots of SDS/7.5%-polyacrylamide gels. SR, pig cardiac muscle sarcoplasmic reticulum; ER, pig stomach smooth muscle endoplasmic reticulum. ATP, Ca^{2+}-transport ATPase (M_r approx. 104000); Alb., muscle albumin (M_r approx. 70000) [19]. Blot 1, immunoreaction with antibodies against the peptide–BSA conjugate; blot 2, same as blot 1 but subsequently reacted with antibody IID8 against the cardiac muscle Ca^{2+} pump; blot 3, reaction with the same antiserum as blot 1 but after adsorption of anti-BSA antibodies; blot 4 reaction with antibodies against the peptide–thyroglobulin conjugate.

distribution (endoplasmic reticulum) of the immunoreactive peptide correspond to those of the endoplasmic-reticulum Ca^{2+} pump [14] (results not shown). (2) The antibodies do not react with the cardiac sarcoplasmic-reticulum Ca^{2+} pump. S_1 nuclease mapping studies with probes specific for the 2.1 and 2.2 transcripts have demonstrated that in cardiac muscle, more than 95% of the RNAs coding for the sarcoplasmic-reticulum Ca^{2+} pump are represented by the 2.1 transcript [7]. Therefore the 2.2 isoform can be expected to be present only at very low levels in cardiac muscle, if at all. (3) The immunoreactive M_r-100000 peptide can be further stained with a monoclonal antibody IID8 directed against an epitope located in the A_1 fragment of the cardiac sarcoplasmic reticulum Ca^{2+} pump (2.1 isoform) [17]. As the A_1 fragment and hence the IID8 epitope is common to both isoforms, the IID8 monoclonal antibody should react with both the 2.1 and the 2.2 isoforms.

cDNA transcripts coding for both the 2.1 isoform and the 2.2 isoform have been found in smooth-muscle cDNA libraries. We have therefore explored whether we could detect proteins corresponding to both isoforms in smooth muscle. On probing Western blots of smooth-muscle endoplasmic reticulum fractions with the non-discriminatory antibody IID8, only one band was detected which coincided with the band reacting with the 2.2-specific antibodies. Even in low percentage (5%) polyacrylamide gels, there was no indication for the presence of a peptide reacting with monoclonal antibody IID8 that did not react with the 2.2-specific antibodies. Yet this does not mean that the 2.1 isoform is not present in smooth muscle, because even in a 5% polyacrylamide gel, both isoforms may co-migrate. Therefore only antibodies specific for the 2.1 isoform will answer this question. However, because a stretch of four amino acids at the ultimate *C*-terminus is the only epitope that is specific for this isoform [11], it might not be easy to obtain such antibodies.

Tissue-dependent expression of the 2.2-type Ca^{2+}-pump

Western blot analysis with our polyclonal anti-peptide antibodies convincingly demonstrated the presence of the 2.2 isoform ATPase in the endoplasmic reticulum of pig stomach or bovine pulmonary artery smooth muscle (Fig. 3*a*), but it was not possible to demonstrate its presence in the sarcoplasmic reticulum of pig cardiac or soleus muscle, not even if this fraction was heavily overloaded on the gel (Fig. 3*a*). This indicates that neither the 2.1 isoform of the cardiac Ca^{2+} pump nor the fast skeletal muscle Ca^{2+} pump contains the epitope used for immunization, and also that the relative amount of 2.2 isoform to the 2.1 isoform must be very low in cardiac and slow skeletal muscle. In contrast, the 2.2 isoform of the endoplasmic reticulum Ca^{2+} pump could be demonstrated in membranes enriched in smooth and rough endoplasmic reticulum of liver and kidney (Fig. 3*c*). In a crude microsomal fraction of liver or kidney, very little reaction was observed, suggesting a relatively low level of expression in the latter tissues compared with smooth muscle (Fig. 3*a*). A reaction with low-M_r (< 20000) proteins was observed in the rough microsomal fractions from both tissues, but was absent from the smooth microsomes. This latter reaction was however unrelated to the ATPase because it also occurred in controls where non-immune rabbit antiserum was used instead of the anti-peptide antiserum.

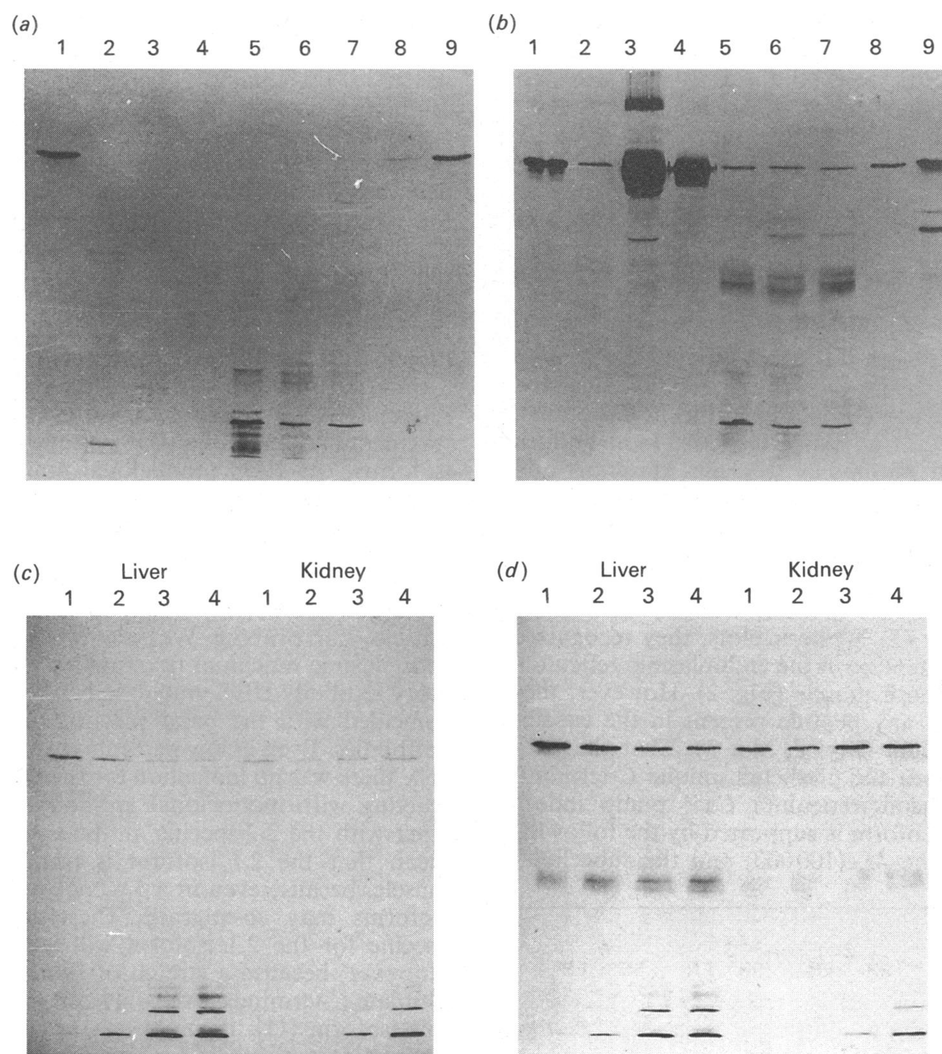

Fig. 3. Tissue-specific expression of the 2.2 Ca^{2+} pump isoform

(*a*) and (*b*) are two immunoblots of different membrane fractions. (*a*) Reaction with polyclonal antibodies against the 2.2 Ca^{2+} pump (adsorbed antiserum against the peptide–BSA conjugate); (*b*) reaction with monoclonal IID8 against the cardiac Ca^{2+} pump. Lane 1, endoplasmic reticulum of bovine main pulmonary artery; 2, pig platelets; 3, pig soleus sarcoplasmic reticulum; 4, pig cardiac sarcoplasmic reticulum; 5, pig spleen microsomes; 6, pig liver microsomes; 7, pig kidney microsomes; 8, pig mixed microsomes from stomach smooth muscle; 9, endoplasmic reticulum subfraction of 8. Portions of 40 μg of membrane proteins were applied to all lanes except for lanes 3 and 4, where 18 μg was applied. (*c*) and (*d*) are two immunoblots of pig liver and kidney microsomes (60 μg of protein). (*c*) Reaction with polyclonal antibodies against the type 2.2 Ca^{2+} pump (adsorbed antiserum against the peptide–BSA conjugate); (*d*) reaction as in (*c*), but subsequently reacted with monoclonal IID8 against the cardiac Ca^{2+} pump. Lanes 1 and 2, light and heavy subfractions of smooth microsomes; lanes 3 and 4, light and heavy subfractions of rough microsomes.

In conclusion, using a polyclonal antiserum against the unique *C*-terminus predicted for the alternatively spliced gene 2 (i.e. 2.2 isoform type) endoplasmic reticulum Ca^{2+} pump, we have been able to demonstrate the corresponding isoform in smooth muscle, liver and kidney, but not in cardiac or skeletal muscle.

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