

The identification of sarcoplasmic reticulum terminal cisternae proteins in platelets

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Two new proteins with apparent molecular masses of 53 kDa and 190 kDa have been identified in both sarcoplasmic reticulum and human blood platelets using a monoclonal antibody, FIIIb5. The sarcoplasmic reticulum FIIIb5 antigens were present in the terminal cisternae fraction, but were absent from light sarcoplasmic reticulum. The platelet and skeletal muscle proteins were not sensitive to digestion with endoglycosidase H under conditions that removed carbohydrate from the 53 kDa glycoprotein in sarcoplasmic reticulum or GPIIIa in platelet microsomes and did not bind ^{45}Ca in a nitrocellulose overlay calcium-binding assay. These results distinguished the FIIIb5 antigens from the 53 kDa glycoprotein and calsequestrin of sarcoplasmic reticulum. The 190 kDa platelet and sarcoplasmic reticulum proteins were extracted from membranes with high concentrations of NaCl, indicating that the high molecular mass FIIIb5 antigens are peripherally associated with the bilayers. In contrast, the platelet and muscle 53 kDa proteins remained membrane-bound in this presence of high salt concentrations, suggesting that they are integral proteins.

INTRODUCTION

Human blood platelets are contractile and motile cells which share many features with muscle cells. Platelets contain large amounts of actin and myosin as well as regulatory proteins, such as gelsolin and actin-binding protein, that are present in muscle (Fox, 1987). Also, platelets have a Ca^{2+} -transport system that is similar to that in muscle with a Ca^{2+} storage organelle called the dense tubular system, which is analogous to the sarcoplasmic reticulum of muscle (Cutler *et al.*, 1978; Menashi *et al.*, 1982), and a surface-connected canalicular system, which has been compared to the T-tubule system of muscle (Behnke, 1967; White, 1972; Robblee *et al.*, 1973).

The present study compares the protein components of the platelet and muscle Ca^{2+} -transport systems. While the proteins of the sarcoplasmic reticulum have been extensively characterized (see, for example, Campbell *et al.*, 1983, 1988), the only sarcoplasmic reticulum protein that has been identified in platelets is the Ca^{2+} -ATPase, which appears to be immunologically and functionally similar to the Ca^{2+} pump protein from the muscle cell (Javors *et al.*, 1982; Dean, 1984; Fischer *et al.*, 1985, 1987). In this paper, we present studies characterizing two new proteins in heavy sarcoplasmic reticulum (terminal cisternae) and in platelets: a 53 kDa integral membrane protein which is distinct from the sarcoplasmic reticulum 53 kDa glycoprotein and calsequestrin, and a 190 kDa peripheral protein. The presence of these two proteins in the terminal cisternae suggests that they may play a role in Ca^{2+} -signal transduction.

MATERIALS AND METHODS

Materials

All chemicals were reagent grade. Freund's adjuvant, alkaline phosphatase reagents and alkaline phosphatase-

conjugated goat anti-mouse antibody were obtained from Sigma Chemical Co. Endoglycosidase H from *Streptomyces lividans* was purchased from Boehringer Mannheim Biochemicals, and was obtained as a 1 unit/ml stock solution in 10 mM- PO_4 , pH 7.0. $^{45}\text{CaCl}_2$ (12.2 mCi/mg) was obtained from the Amersham Corporation (Arlington Heights, IL, U.S.A.). Human platelet concentrates were obtained from the American Red Cross.

Membrane isolation

Platelet microsomes were isolated as detailed elsewhere (White & Raynor, 1982). Sarcoplasmic reticulum and transverse tubule vesicles were isolated from adult rabbit skeletal muscle by the method of Campbell *et al.* (1980).

FIIIb5 monoclonal antibody production

A five-week-old Balb/c mouse was immunized subcutaneously with 100 μg of 'heavy' sarcoplasmic reticulum membrane proteins in an emulsification of 100 μl of Tris-buffered saline (150mM-NaCl/20mM-Tris/HCl, pH 7.4) and 100 μl of Freund's complete adjuvant. Two subsequent injections were performed intraperitoneally with the same amount of protein in Tris-buffered saline without adjuvant at 3-week intervals. At 4 days after the last immunization, hybridoma cells were produced by fusion with P3X63Ag8.653 myeloma cells (Kennett, 1980). The initial FIIIb5 clone was dilution-cloned twice, then antibodies were produced in medium consisting of Dulbecco's modified Eagle's medium/Hams F12 (1:1, v/v) with 10^{-4} M-hypoxanthine and 1.6×10^{-5} M-thymidine supplement and 20% (v/v) fetal calf serum. The FIIIb5 antibody was isotyped as an IgM with a Screen Type isotyping kit from Boehringer-Mannheim Biochemicals.

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SDS/polyacrylamide-gel electrophoresis

Hybridoma cell supernatants were screened by Western blotting. Polyacrylamide gels (7.5 %, w/v) were prepared using the method of Laemmli (1970), by loading 20 μ g of platelet microsomal protein and 2 μ g of sarcoplasmic reticulum protein in each lane. Protein concentrations were estimated using the method of Peterson (1977). Prestained molecular mass standards (myosin, phosphorylase B, bovine serum albumin, ovalbumin and carbonic anhydrase) from Bethesda Research Laboratories, Inc. were utilized for apparent molecular mass estimation. SDS/polyacrylamide-gel electrophoresis gels were electrotransferred to nitrocellulose and immunodetected with FII1b5 at a dilution of 100:1 as detailed elsewhere (Fischer *et al.*, 1985). Immunodetection was with an alkaline phosphatase anti-mouse second antibody used at a 1000:1 dilution and a colour development solution that contained 100 mM-NaCl, 100mM-Tris/HCl, 5 mM-MgCl₂, 0.45 mM-Nitro Blue Tetrazolium and 0.45 mM-5-bromochloro-3-indolyl phosphate, pH 9.5.

Endoglycosidase H treatment

Platelet microsomal and heavy sarcoplasmic reticulum membranes were digested with endoglycosidase H as detailed by Campbell *et al.* (1983). Briefly, platelet and heavy sarcoplasmic reticulum microsomes at protein concentrations of 1.0 mg/ml were dialysed overnight in digestion buffer [50mM-sodium citrate, 10 μ M-phenylmethanesulphonyl fluoride, 0.2% (w/v)SDS, pH 5.5] at room temperature, then incubated at 100 °C for 1 min. Endoglycosidase H was diluted 1:10 into the microsome samples for a final concentration of 100 munits/mg. The mixture was incubated for 5 h at 37 °C and then electrophoresed and immunodetected as described above.

⁴⁵Ca binding on nitrocellulose transfers

The detection of Ca²⁺-binding proteins utilized a variation of the procedure of Maruyama *et al.* (1984). After electrotransfer, nitrocellulose was washed 5 times over 30mm with phosphate-buffered saline (150mM-NaCl/20 mM-PO₄/0.05 % Tween 20, pH 7.4), and then overlaid for 10 min with overlay buffer (60mM-KCl/5 mM-MgCl₂/10 mM-imidazole/HCl/1 mCi of ⁴⁵CaCl₂/ml, pH 6.8). The nitrocellulose was then incubated for 5 min in distilled water, air-dried and used for autoradiography.

Salt extraction

Platelet and heavy sarcoplasmic reticulum microsomes were diluted to final protein concentrations of 1.0 mg/ml and 0.1 mg/ml respectively in extraction medium (94mM-KCl/5mM-MgCl₂/3.33M-NaCl/20mM-Tris/HCl, pH 7.0). After a 15 min incubation at 23 °C, the samples were centrifuged for 1 h at 150 000 g. The supernatants were withdrawn and the pellets were resuspended in the original volume of extraction medium lacking NaCl. Pellet resuspensions and supernatants were then electrophoresed and immunodetected as described above.

RESULTS

The reaction of the FII1b5 monoclonal antibody with platelet microsomal, sarcoplasmic reticulum terminal cisternae (heavy sarcoplasmic reticulum) and longitudinal cisternae (light sarcoplasmic reticulum) membranes is presented in Fig. 1. The monoclonal antibody recog-

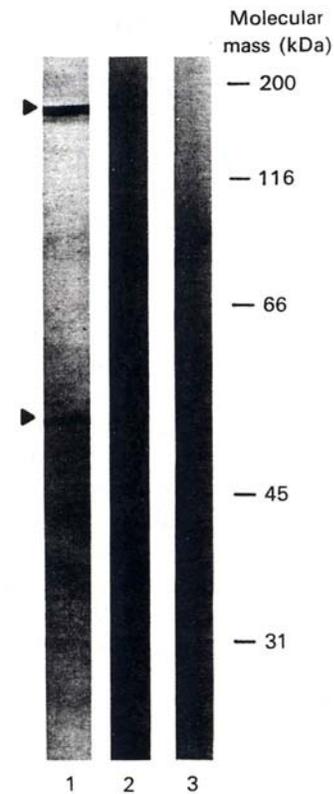


Fig. 1. Immunodetection of FII1b5 antigen

Platelet microsome (lane 1), terminal cisternae sarcoplasmic reticulum (lane 2) and longitudinal cisternae sarcoplasmic reticulum (lane 3) membranes were electrophoresed on 7.5 % (w/v) polyacrylamide gels and transferred to nitrocellulose. The gel was then overlaid with a solution containing FII1b5, washed with buffer, and developed using Nitro Blue Tetrazolium. The positions of the FII1b5 antigens (arrowheads) and molecular mass standards are indicated.

nized a pair of proteins with apparent molecular masses of 53 and 190 kDa which were present in both heavy sarcoplasmic reticulum and platelet microsomes (Fig. 1, lanes 1 and 2) but were absent in light sarcoplasmic reticulum (Fig. 1, lane 3). In transverse tubule membranes, the 190 kDa antigen was not found, and the 53 kDa antigen was only faintly detected, probably reflecting heavy sarcoplasmic reticulum impurity in this preparation (results not shown).

Skeletal muscle sarcoplasmic reticulum contains a 53 kDa glycoprotein which is glycosylated and which is sensitive to digestion with endoglycosidases such as endoglycosidase H (Campbell & MacLennan, 1981). To examine the possibility that the 53 kDa protein recognized by FII1b5 was this 53 kDa glycoprotein, sarcoplasmic reticulum and platelet microsomes were digested with endoglycosidase H, and the electrophoretic mobilities before and after digestion were compared. As shown in Fig. 2, the FII1b5 antigens were not sensitive to endoglycosidase H digestion under conditions which degraded the sarcoplasmic reticulum 53 kDa glycoprotein. An unidentified 120 kDa platelet glycoprotein, perhaps GPIIb, was also degraded.

⁴⁵Ca-binding studies with nitrocellulose were performed to ascertain if the FII1b5 antigens were Ca²⁺-

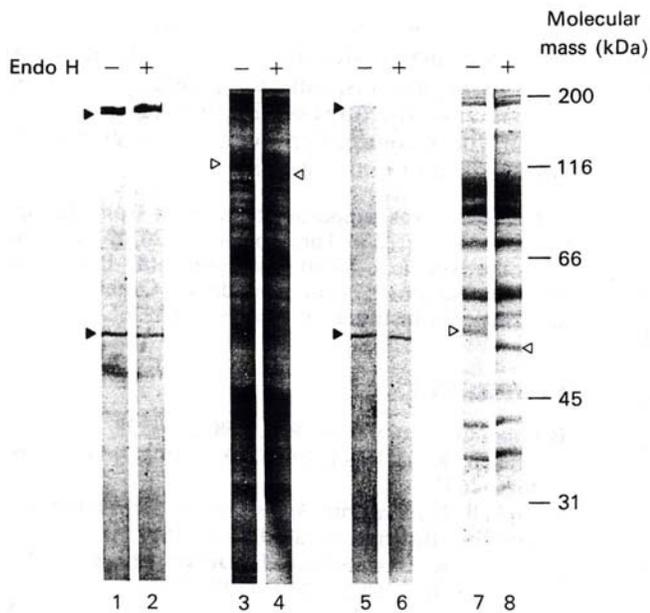


Fig. 2. Endoglycosidase H sensitivity of FII1b5 antigens

Platelet microsomes (lanes 1-4) and rabbit terminal cisternae vesicles (lanes 5-8) were incubated in the presence (lanes 2, 4, 6, 8) or absence (lanes 1, 3, 5, 7) of endoglycosidase H (endo H), and then electrophoresed on 7.5 % gels. Proteins were detected using either immunoreaction with FII1b5 (lanes 1, 2, 5, 6) or Coomassie Brilliant Blue (lanes 3, 4, 7, 8). Arrowheads show the FII1b5 antigens (lanes 1, 2, 5, 6) or endoglycosidase H digestion substrates and products (lanes 3, 4, 7, 8). Molecular mass standard positions are indicated beside lane 8.

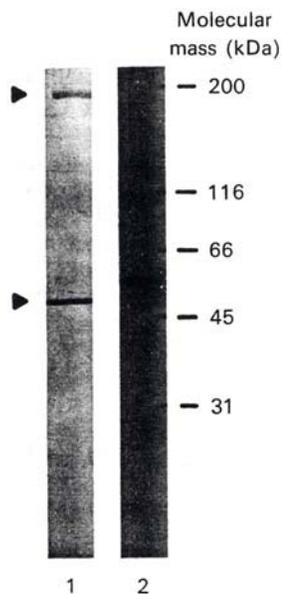


Fig. 3. Ca^{2+} -binding properties of FII1b5 antigens

Lane 1, immunodetection of terminal cisternae membranes with FII1b5; lane 2, autoradiograph of $^{45}\text{Ca}^{2+}$ overlay that corresponds to lane 1. Arrowheads show the FII1b5 antigens, and the positions of molecular mass standards are indicated beside lane 2.

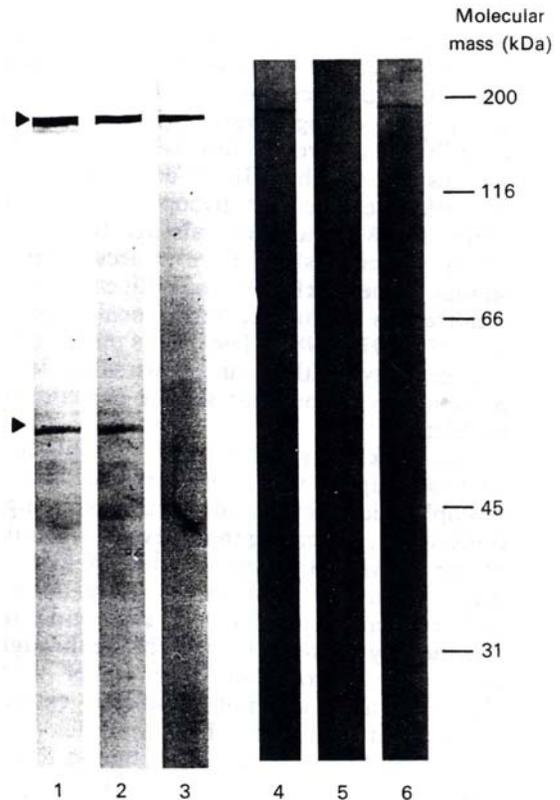


Fig. 4. Salt partitioning studies with the FII1b5 antigens

Platelet microsomes (lanes 1-3) and terminal cisternae (lanes 4-6) were incubated in 3.33 M-NaCl, and then centrifuged at 150000 g to separate membrane-bound from extracted membrane proteins. Lane 1, platelet microsomes before centrifugation; lane 2, platelet pellet resuspension; lane 3, platelet supernatant; lane 4, terminal cisternae membranes before centrifugation; lane 5, terminal cisternae pellet resuspension; lane 6, terminal cisternae supernatant. Arrowheads show the FII1b5 antigens, and the positions of molecular mass standards are indicated beside lane 6.

binding calsequestrins. The results in Fig. 3, lane 2 show that ^{45}Ca radiobands were not detected in the positions of the FII1b5 antigens, while the 63 kDa calsequestrin did bind divalent cation.

Partitioning studies in the presence of high salt concentrations were performed with platelet microsome and sarcoplasmic reticulum terminal cisternae membranes to ascertain if the FII1b5 antigens were integral or peripheral components of the bilayers. Fig. 4 shows that the platelet and sarcoplasmic reticulum 53 kDa proteins (lanes 2 and 3) remained bound to the membrane upon exposure to 3.33 M-NaCl. In contrast, the platelet and sarcoplasmic reticulum 190 kDa antigens could be dissociated into the salt supernatant (lanes 3 and 6). The salt-induced dissociation of the sarcoplasmic reticulum 190 kDa protein was complete, while some of the high molecular mass platelet antigens remained membrane-bound.

DISCUSSION

A monoclonal antibody, FII1b5, raised to terminal cisternae of rabbit skeletal sarcoplasmic reticulum, has

been shown to identify 53 kDa and 190 kDa proteins which are common to both platelet microsomes and sarcoplasmic reticulum. The FII1b5 antigens were insensitive to endoglycosidase H digestion and did not bind ^{45}Ca in a nitrocellulose overlay assay. These properties distinguish the FII1b5 antigens from the 53 kDa sarcoplasmic reticulum glycoprotein (Campbell & MacLennan, 1981) and from calsequestrin (Campbell *et al.*, 1983) proteins which have molecular masses that are similar to the 53 kDa FII1b5 antigens and which are also localized on terminal cisternae membranes (Jorgensen *et al.*, 1977, 1979, 1982). The results presented in this article thus define two hitherto unknown sarcoplasmic reticulum proteins, and show that similar proteins are present in platelets.

The 53 kDa platelet and sarcoplasmic reticulum FII1b5 antigens were not extracted from platelet and sarcoplasmic reticulum membranes with high concentrations of salt, indicating that they are integral components of the bilayers. In contrast, the 190 kDa proteins partitioned into the supernatant phase upon exposure to high concentrations of salt, suggesting that they are peripherally bound to the membrane through salt-disruptable ionic interactions. The higher molecular mass platelet antigen was not completely extracted into the supernatant with salt. This indicates that either the platelet protein is more tightly bound to the membrane with ionic bonds than the 190 kDa platelet antigen, or that there are two populations of this protein, one a peripheral and one an integral membrane component.

FII1b5 recognizes two electrophoretically distinct proteins in each tissue. Either these proteins have a common epitope or they are related in some other way. The partitioning studies, which show that the 53 kDa protein is an integral membrane protein while the 190 kDa protein is a peripheral membrane protein, provide an argument that the 53 kDa protein is not proteolytically derived from the 190 kDa protein, since one would expect in that case for the 190 kDa protein to be integral and the 53 kDa protein to be peripheral. The most likely explanation for the common reactivity is thus a shared epitope.

The sarcoplasmic reticulum proteins were found on terminal cisternae of the sarcoplasmic reticulum but were absent from light sarcoplasmic reticulum and sarcolemma membranes. The terminal cisternae region of the sarcoplasmic reticulum, being closely associated with the transverse tubule system (Kelly & Kuda, 1979; Eisenberg & Eisenberg, 1982; Saito *et al.*, 1984) and containing proteins involved in signal transduction such as the Ca^{2+} -channel protein (Campbell *et al.*, 1987), has been implicated as the part of the sarcoplasmic reticulum that mediates Ca^{2+} release (see Hille, 1984, for a general review). This raises the possibility that the FII1b5 antigens may play a role in Ca^{2+} -signal transduction. Electron microscopic studies (Behnke, 1967; White, 1972; Robblee *et al.*, 1973) have shown that the platelet dense tubular system and the surface-connected open canalicular system are associated in a manner that is analogous to the muscle triad structure. Also, upon activation with thrombin the platelet plasma membrane rapidly depolarizes (Simons & Greenberg-Sepersky, 1987), as does the sarcolemma membrane when muscle cells are stimulated

(see Hille, 1984, for a general review). These results, when considered with our finding of terminal cisternae proteins in platelets, raise the possibility that the blood cell contains structures similar to triads and has elements of the type of voltage-dependent Ca^{2+} -signal transduction system found in muscle cells.

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