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Monoclonal Antibodies to the Ca^{2+} + Mg^{2+}-Dependent ATPase of Sarcoplasmic Reticulum Identify Polymorphic Forms of the Enzyme and Indicate the Presence in the Enzyme of a Classical High-Affinity Ca^{2+} Binding Site[1]

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Abstract

In order to determine whether polymorphic forms of the Ca^{2+} + Mg^{2+}-dependent ATPase exist, we have examined the cross-reactivity of five monoclonal antibodies prepared against the rabbit skeletal muscle sarcoplasmic reticulum enzyme with proteins from microsomal fractions isolated from a variety of muscle and nonmuscle tissues. All of the monoclonal antibodies cross-reacted in immunoblots against rat skeletal muscle Ca^{2+} + Mg^{2+}-dependent ATPase but they cross-reacted differentially with the enzyme from chicken skeletal muscle. No cross-reactivity was observed with the Ca^{2+} + Mg^{2+}-dependent ATPase of lobster skeletal muscle. The pattern of antibody cross-reactivity with a 100,000 dalton protein from sarcoplasmic reticulum and microsomes isolated from various muscle and nonmuscle tissues of rabbit demonstrated the presence of common epitopes in multiple polymorphic forms of the Ca^{2+} + Mg^{2+}-dependent ATPase. One of the monoclonal antibodies prepared against the purified Ca^{2+} + Mg^{2+}-dependent ATPase of rabbit skeletal muscle sarcoplasmic reticulum was found to cross-react with calsequestrin and with a series of other Ca^{2+}-binding proteins and their proteolytic fragments. Its cross-reactivity was enhanced in the presence of EGTA and diminished in the presence of Ca^{2+}. Its lack of cross-reactivity with proteins that do not bind Ca^{2+} suggests that it has specificity for antigenic determinants that make up the Ca^{2+}-binding sites in several Ca^{2+}-binding proteins including the Ca^{2+} + Mg^{2+}-dependent ATPase.

Key Words: ATPase monoclonal antibodies; Ca^{2+}-ATPase; Ca^{2+}-binding sites; ATPase polymorphism.

[1]This paper is dedicated to the memory of Dr. David E. Green.

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Introduction

The Ca^{2+} + Mg^{2+}-dependent ATPase is a major intrinsic membrane protein in fast-twitch muscle sarcoplasmic reticulum (MacLennan and Holland, 1975), and a form of the enzyme is also found in sarcoplasmic reticulum vesicles from differentiating skeletal muscle, slow-twitch muscle, and heart muscle (Boland *et al.*, 1974; Zubrzycka *et al.*, 1979; Heilmann *et al.*, 1977; Zubrzycka-Gaarn *et al.*, 1982; van Winkle *et al.*, 1978). Nonmuscle tissues such as platelets and liver contain membrane systems capable of regulation of Ca^{2+} concentrations in the cytoplasm (Moore *et al.*, 1975; Bygrave, 1978; Dawson, 1982; Kaser-Glanzman *et al.*, 1977). Polypeptides of 100,000 daltons have been observed in crude microsomes and in partially purified preparations of the Ca^{2+} + Mg^{2+}-dependent ATPase obtained from these nonmuscle tissues (Dean and Sullivan, 1982; Moore and Kraus-Friedmann, 1983). On the basis of their M_r, specific enzymatic activities, and cross-reactivity with antisera prepared to the Ca^{2+} + Mg^{2+}-dependent ATPase of sarcoplasmic reticulum, these polypeptides have been identified as Ca^{2+} + Mg^{2+}-dependent ATPases (Dean and Sullivan, 1982; Moore and Kraus-Friedmann, 1983). In contrast to the Ca^{2+} + Mg^{2+}-dependent ATPase of fast-twitch muscle sarcoplasmic reticulum, the structure, localization, and sequence of these enzymes is not known and very little is known about the homology among these enzymes in different tissues.

In this report we show that monoclonal antibodies to the rabbit fast-twitch muscle Ca^{2+} + Mg^{2+}-dependent ATPase differentially cross-react with polypeptides of M_r 100,000 in various muscle and nonmuscle tissues. These observations suggest that the structure of the ATPase is not highly conserved and that polymorphic forms of the Ca^{2+} + Mg^{2+}-dependent ATPase are present in different species and tissues.

One of our monoclonal antibodies has been found to cross-react with the ATPase and calsequestrin. Both the Ca^{2+} + Mg^{2+}-dependent ATPase and calsequestrin are Ca^{2+}-binding proteins (MacLennan and Holland, 1975). While the ATPase contains two high-affinity Ca^{2+}-binding sites per mole, or about 1 site per 500 amino acid residues (MacLennan and Holland, 1975), calsequestrin contains about 50 low-affinity Ca^{2+}-binding sites per mole, or about 1 site per 9 amino acid residues (MacLennan *et al.*, 1983). Attempts to rationalize the cross-reactivity of the antibody with these two proteins has led us to the finding that the antibody cross-reacts with a series of Ca^{2+}-binding proteins and their proteolytic fragments. These results suggest that the antibody recognizes a common structure in these proteins. Since it did not react with proteins which do not bind Ca^{2+}, it is probable that it recognizes antigenic determinants located in Ca^{2+}-binding sites. This monoclonal anti-

body is of potential use in identification, purification, and characterization of a variety of Ca^{2+}-binding proteins.

Materials and Methods

Sources

BALB/c female mice were obtained from Jackson Laboratories, Bar Harbor, Maine; pristane, from British Drug House; Immulon II microelisa plates, from Dynatech; goat anti-mouse IgG and IgM, from Boehringer–Mannhein Biochemicals; disodium *p*-nitrophenyl phosphate and *o*-dianisidine from Sigma Chemicals; rabbit anti-mouse immunoglobulin subclass-specific antibodies and goat anti-rabbit immunoglobulin conjugated to alkaline phosphatase, from Bionetics Laboratories; horseradish peroxidase conjugated to rabbit anti-mouse immunoglobulin and rabbit anti-mouse immunoglobulin antibodies, from Cedarlane Laboratories, Hornby, Ontario; goat anti-mouse immunoglobulin conjugated to fluorescein, from Miles-Yeda.

Preparation of Sarcoplasmic Reticulum and Microsomal Vesicles from Different Tissues

Sarcoplasmic reticulum vesicles from rabbit fast-twitch muscle, rat skeletal muscle, chicken skeletal muscle, and lobster muscle were prepared according to the method of MacLennan (1970) as modified by Campbell and MacLennan (1981). Microsomal membranes from rabbit slow-twitch muscle (Zubrzycka-Gaarn *et al.,* 1982), 3-day old rabbit skeletal muscle (Zubrzycka-Gaarn, E., Cornell, R., and MacLennan, D. H., in preparation), rabbit heart muscle (Jones *et al.,* 1979), rabbit stomach smooth muscle (Raeymekers *et al.,* 1980), and human platelets (Menashi *et al.,* 1981) were prepared as previously described. Rabbit liver microsomes were obtained from liver homogenates by differential centrifugation. The pellet sedimenting between 10,000 and 80,000 $\times$ *g* was used in these studies.

Purification of Ca^{2+} + Mg^{2+}-Dependent ATPase

The Ca^{2+} + Mg^{2+}-dependent ATPase from rabbit fast-twitch muscle sarcoplasmic reticulum was purified as described by MacLennan (1970) except that the ammonium acetate fractionation was carried out at pH 8.35.

Treatment of Sarcoplasmic Reticulum Vesicles with Trypsin

Sarcoplasmic reticulum vesicles isolated from rabbit fast-twitch skeletal muscle were digested with trypsin according to the method of Stewart *et al.*

(1976). A suspension of digested sarcoplasmic reticulum vesicles was also extracted with potassium deoxycholate (0.25 mg/mg protein) as described by Stewart *et al.* (1976).

Immunization and Monoclonal Antibody Preparation

Two 12-week-old BALB/c female mice were immunized once, subcutaneously, with 130 μg of the purified Ca^{2+} + Mg^{2+}-dependent ATPase, emulsified in complete Freund's adjuvant, and three additional times at two-week intervals with the same amount of antigen emulsified in incomplete adjuvant. One week after the fourth injection, the mice were injected intravenously daily for three days with 30 μg of antigen in phosphate-buffered saline (PBS).[4] On the fifth day, splenic lymphocytes from the hyperimmunized animals were mixed with the myeloma nonproducing cell line SP2/0-Ag14 (Shulman *et al.*, 1978) at a ratio of 3:1, respectively, and hybrid cell fusion was promoted with 50% polyethylene glycol 1500 (Kohler, 1980). After fusion, the cell pellet was suspended in 50 ml of Dulbecco's modified Eagle's medium supplemented with 20% heat-inactivated fetal calf serum, 50 μM 2-mercapthoethanol, and 10 mM HEPES (complete medium), and 1.5 ml per well of this suspension was plated in 24-well Linbro plates containing a peritoneal macrophage feeder layer at 1×10^5 cells per well. Viable hybrid cells were selected in complete medium supplemented with 50 μM hypoxanthine, 0.42 μM aminopterin, and 15 μM thymidine (HAT medium). HAT medium was changed on the third and sixth day after fusion, and on the ninth day it was replaced with complete medium. Twelve days after fusion, cell culture supernatants were screened for the production of specific antibodies. Colonies secreting antibodies to the Ca^{2+} + Mg^{2+}-dependent ATPase were stabilized by repeated subcloning at limiting dilution in 96-well Linbro plates containing a syngeneic red blood cell feeder layer at 1×10^5 cells per well. Antibody-rich ascites fluids were prepared by injecting 1×10^7 cells of selected hybrid clones in 0.2 ml of PBS into the peritoneal cavity of pristane-primed mice and harvesting the resultant fluids 7–10 days later. Monoclonal antibodies were purified from the ascites fluids by precipitation with 50% ammonium sulfate.

Immunological Assays

a. Enzyme-Linked Immunosorbent Assay (ELISA). The enzyme-linked immunosorbent assay described by Engvall and Perlmann (1971) was

[4]Abbreviations: PBS, phosphate-buffered saline; EGTA, Ethyleneglycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; SDS, sodium dodecyl sulfate; ELISA, enzyme-linked immunosorbent assay; HAT medium, culture medium supplemented with hypoxanthine, aminopterin, and thymidine.

used to screen for clones secreting antibodies to the Ca^{2+} + Mg^{2+}-dependent ATPase, for the determination of immunoglobulin classes, and for the titration of monoclonal antibodies against the ATPase. In each case, Immulon II microelisa plates were coated overnight at 4°C with the purified Ca^{2+} + Mg^{2+}-dependent ATPase at 10 μg protein/ml in a buffer containing 15 mM Na_2CO_2, 35 mM $NaHCO_2$, pH 9.6, and 3 mM NaN_3. In the direct assay, supernatants or ascites fluids were added first, followed by alkaline phosphatase conjugated to goat anti-mouse IgG and IgM and then by disodium *p*-nitrophenyl phosphate at appropriate dilution and at appropriate time intervals. In the indirect assay, antibodies were added first, followed by rabbit anti-mouse immunoglobulin subclass-specific antibodies and goat anti-rabbit immunoglobulin conjugated to alkaline phosphatase. The end point of this colorimetric assay was quantitated spectrophotometrically at 405 nm with a Multiskan Plate Reader.

b. Immunoprecipitation on Protein A–Sepharose Beads. Sarcoplasmic reticulum proteins (200 μg) were radioiodinated using Bio-Rad Enzymobeads with covalently attached lactoperoxidase and glucose oxidase according to the method of Habener *et al.* (1979).

Radioiodinated sarcoplasmic reticulum proteins were solubilized with 1 ml of a mixture containing 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 7.2, 150 mM NaCl, and 100 K.I.U. Trasylol. After centrifugation for 1 h at 100,000 × *g*, 10-μl samples of the supernatant were used for immunoprecipitation with monoclonal antibodies. Immunoprecipitation on protein A–Sepharose beads and subsequent washing procedures were carried out as described by Zubrzycka-Gaarn *et al.* (1983), except that 100 μl of hybridoma supernatant was added in each case instead of polyclonal serum. Immunoprecipitates were extracted from the beads as described by Zubrzycka-Gaarn *et al.* (1983) and the proteins of the extracts were separated in slab gels (Laemmli, 1970). Gels were stained with Coomassie blue, destained, and, after drying, exposed to Kodak X-Omat R film at −70°C.

c. Electrophoretic Blotting and Immunodetection of the Antigen on Nitrocellulose Blots. Protein samples separated in 8.5% slab gels (Laemmli, 1970) or 7.5% slab gels (Weber and Osborn, 1969) were transferred electrophoretically from the gel to a nitrocellulose sheet as described by Towbin *et al.* (1979). The electrophoretic blots were incubated for 1 h at 37°C with 3% bovine serum albumin in a saline buffer (150 mM NaCl and 10 mM Tris-HCl, pH 7.2) as described by Towbin *et al.* (1979). They were rinsed in saline buffer and incubated for 2 h at room temperature with ascites fluids appropriately diluted in 3% bovine serum albumin and 10% fetal calf serum in saline buffer. The blots were washed in saline buffer and incubated for 1 hr at room temperature with horseradish peroxidase conjugated to rabbit anti-

mouse immunoglobulin diluted 1:500 in saline buffer and containing 3% bovine serum albumin and 10% fetal calf serum. For the color reaction, blots were incubated with *o*-dianisidine as described by Towbin *et al.* (1979). The reaction was terminated by washing the blots with water.

d. Immunoprecipitation of Sarcoplasmic Reticulum Vesicles. Sarcoplasmic reticulum vesicles (200 μg) suspended in 200 mM Tris-HCl, pH 7.5, 500 mM KCl, 88 mM sucrose, and 1 mM PMSF (total volume 0.6 ml) were incubated with purified monoclonal antibodies in ratios derived from their binding curves in the ELISA. The concentration of antibody which gave 50% of maximal binding to the antigen was used in each case.

Control samples were incubated in parallel in the absence of monoclonal antibodies. After 90-min incubation at room temperature, the samples were centrifuged at 150,000 $\times$ *g* for 45 min through a layer of 300 mM sucrose in 10 mM Tris-HCl, pH 7.5. The pellets were resuspended in 0.5 ml of 10 mM Tris-HCl, pH 7.5, 150 mM KCl, and 1 mM PMSF. Rabbit anti-mouse immunoglobulin antibodies (10 μl) were added to each sample as a second antibody to precipitate vesicles which had the first antibody bound. Samples were centrifuged at 200 rpm for 1 min and the optical density of the supernatants was measured at 500 nm. In some experiments, antibodies A52, A25, and A22 were added to the vesicle preparation at a higher concentration than was indicated by the antibody titers. Photographs of these samples were taken 90 min after incubation of these samples at room temperature with the second antibody.

Proteins

Calsequestrin (MacLennan and Wong, 1971) was purified from rabbit skeletal muscle sarcoplasmic reticulum. Ca^{2+}-binding proteins were generously provided as gifts by the following scientists: rabbit skeletal muscle tropomyosin (Bailey, 1948), Dr. Renata Dabrowska, Nencki Institute, Warsaw, Poland; bovine brain calcineurin (Klee *et al.*, 1979) and calmodulin fragments (Newton *et al.*, 1984), Dr. Claude Klee, N.I.H., Bethesda, Maryland; bovine brain calmodulin (Teo *et al.*, 1973), Dr. Balwant Tuana, University of Toronto; bovine brain S-100 protein (Labourdette and Marks, 1975), Dr. Alexander Marks, University of Toronto; pig intestinal Ca^{2+}-binding protein (O'Neill *et al.*, 1982), Dr. T. Hoffman, University of Toronto; Human salivary Ca^{2+}-binding proteins (Bennick, 1975, 1977), Dr. A. Bennick, University of Toronto; rabbit skeletal muscle troponin C (McCubbin and Kay, 1973), bovine cardiac muscle troponin C (Byers and Kay, 1982), and pike parvalbumin (Haiech *et al.*, 1979), Dr. Cyril Kay, University of Alberta; rabbit skeletal muscle troponin C fragments (Grabarek *et al.*, 1981), Dr. Paul Leavis, Boston Biomedical Research Institute; Vitamin D-dependent, chick intestinal Ca^{2+}-binding protein (Friedlander and Norman,

1980), Dr. Anthony Norman, University of California; the 140,000-dalton Ca^{2+} + Mg^{2+}-dependent ATPase from erythrocyte plasma membrane (Niggli *et al.*, 1979), Dr. John Penniston, Mayo Clinic, Rochester, Minnesota.

Preparation of Human Platelet Homogenate

Human platelets (Menashi *et al.*, 1981) (1 g wet weight) were sonicated for 10 sec at 4°C in 4 ml of 10 mM HEPES, pH 7.2, and 250 mM sucrose and then subjected to SDS–polyacrylamide gel electrophoresis.

Fixation, Sectioning, and Immunofluorescence Labeling of Muscle Tissues

Cryostat sections (4 μm) were cut from tissues of adult rat gracilis muscle, adult rat ventricular muscle, and embryonic rat skeletal and heart muscle (15th day of gestation) which were either unfixed, fixed in 2% paraformaldehyde, or fixed in a mixture of 2% paraformaldehyde and 0.3% glutaraldehyde as previously described (Jorgensen *et al.*, 1979, 1982a, 1982b).

Indirect immunofluorescence labeling of sections of muscle tissue was carried out with monoclonal antibodies A3, A22, A25, and A52 as previously described (Jorgensen *et al.*, 1979). The monoclonal antibodies were used at 1/10 dilution in PBS. Goat anti-mouse immunoglobulin conjugated to fluorescein was used as the secondary reagent (dilution 1/20).

Analytical Methods

Protein concentration was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Results

Characterization of Monoclonal Antibodies to the Ca^{2+} + Mg^{2+}-Dependent ATPase

When mouse myeloma cells were fused with splenic lymphocytes from mice immunized with the purified Ca^{2+} + Mg^{2+}-dependent ATPase of rabbit fast-twitch muscle sarcoplasmic reticulum, six hybridoma cell lines secreting antibodies specific for the injected antigen were obtained. The designation and immunoglobulin class as determined by indirect ELISA and cross-reactivity with different antigens on immunoblots of five of the antibodies are summarized in Table I. Antibody A9 belonged to the immunoglobulin class IgG_{2b}.

a. Antibody Specificity. Specificity of binding of the monoclonal antibodies to the Ca^{2+} + Mg^{2+}-dependent ATPase antigen is shown in Fig. 1.

Table I. Characterization of Reactivity of Monoclonal Antibodies with Various Antigens[a]

	Monoclonal antibody				
	A52	A25	A20	A3	A22
	Immunoglobulin class				
	IgG_{2b}	IgG_{2b}	IgG_1	IgG_1	IgM
	Reactivity with tryptic fragments of the ATPase				
	45,000	55,000, 30,000	45,000	45,000	45,000
Vesicle precipitation	+	+	+	+	−
Cross-reactivity with microsomes from:					
rat skeletal muscle	+	+	+/−	+	+
chicken skeletal muscle	+	+/−	—	+	+
lobster skeletal muscle	—	—	—	—	—
rabbit slow-twitch muscle	+	+	+	+	+
rabbit 3-day old skeletal muscle	+	+	+	+	+
rabbit heart muscle	—	+/−	—	—	—
rabbit smooth muscle	+	+	+	—	+/−
rabbit liver	—	+	—	—	+
human platelet	—	+	+	—	—

[a] +, −, +/− indicate reactivity, lack of reactivity, and weak reactivity on immunoblots, respectively.

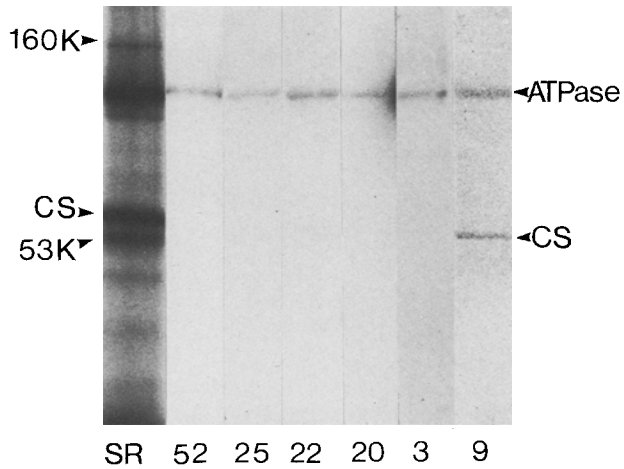

Fig. 1. Specificity of binding of monoclonal antibodies to the Ca^{2+} + Mg^{2+}-dependent ATPase. Sarcoplasmic reticulum proteins were radioiodinated and precipitated with different monoclonal antibodies on protein A–Sepharose beads as described under Materials and Methods except that rabbit anti-mouse IgM was added to the mixture when the proteins were incubated with A22. Immunoprecipitates were eluted from the beads, separated in 8.5% Laemmli gel, and exposed to X-ray film. An autoradiogram of each gel is presented. The numbers indicate different monoclonal antibodies; SR, radioiodinated sarcoplasmic reticulum proteins; CS, calsequestrin; 53K and 160K, 53,000- and 160,000-dalton glycoproteins.

When radioiodinated sarcoplasmic reticulum proteins from rabbit fast-twitch muscle were reacted with the positive hybridoma culture supernatants and immunoprecipitates were collected on protein A–Sepharose beads, binding of all six monoclonal antibodies to the 100,000-dalton Ca^{2+} + Mg^{2+}-dependent ATPase was observed. While five of the antibodies were specific, antibody A9 cross-reacted with calsequestrin (M_r 63,000).

b. Titration of Monoclonal Antibodies Against the ATPase. The effect of dilution of the six ascites fluids on the binding of monoclonal antibodies to the Ca^{2+} + Mg^{2+}-dependent ATPase is shown in Fig. 2. The antibody titers, taken as the dilution of ascites fluid at which 50% of maximal binding occurred, were $1:10^6$ for A25 and A52, $1:10^5$ for A22 and A3, and $1:10^4$ for A20. A lower affinity for the antigen was observed with A9, which had a titer between $1:10^3$ and $1:10^4$. When the monoclonal antibodies purified from ascites fluids were titrated against the ATPase, 50% of maximal binding occurred when 37 μg of antibody was added for A9, when 7 μg of antibody was added for A3 and A20, and when 6, 1.5, and 0.75 ng of the antibody were added for A22, A52, and A25, respectively (not shown).

c. Binding of Monoclonal Antibodies to Tryptic Fragments of the Ca^{2+} + Mg^{2+}-Dependent ATPase. The reactivity of different tryptic fragments of the Ca^{2+} + Mg^{2+}-dependent ATPase with various monoclonal antibodies was determined by electrophoretic blotting and immunodetection

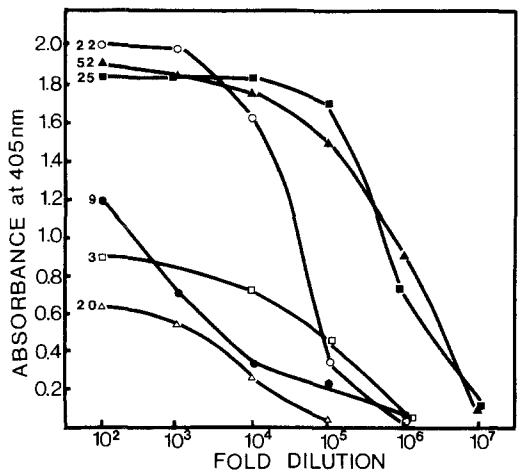

Fig. 2. Titers of monoclonal antibodies against the Ca^{2+} + Mg^{2+}-dependent ATPase. The Ca^{2+} + Mg^{2+}-dependent ATPase, immobilized on micro-ELISA plates, was incubated with serial dilutions of ascites fluid containing monoclonal antibodies. Binding of the monoclonal antibodies to the antigen was detected using the ELISA assay as described under Materials and Methods.

of the antigen on a nitrocellulose blot. The proteins of rabbit fast-twitch muscle sarcoplasmic reticulum vesicles, digested with trypsin and washed with deoxycholate, were separated in Weber and Osborn slab gels and transferred electrophoretically to a sheet of nitrocellulose. Under these conditions, four large tryptic fragments of 55,000, 45,000, 30,000, and 25,000 daltons were transferred to nitrocellulose as shown in Fig. 3A. The reactivity of various monoclonal antibodies with the tryptic fragments is shown in Fig. 3B. Six monoclonal antibodies reacted with the undigested ATPase. Four antibodies (A52, A22, A20, and A3) reacted with the 45,000-dalton tryptic fragment and one (A25) reacted with the 55,000- and 30,000-dalton tryptic fragments. Antibody A9 did not react with any of the trypsinized, denatured fragments of the ATPase.

d. Precipitation of Intact Sarcoplasmic Reticulum Vesicles with Monoclonal Antibodies. Experiments on precipitation of intact sarcoplasmic reticulum vesicles with various monoclonal antibodies to the Ca^{2+} + Mg^{2+}-dependent ATPase were designed to determine the location of antigenic

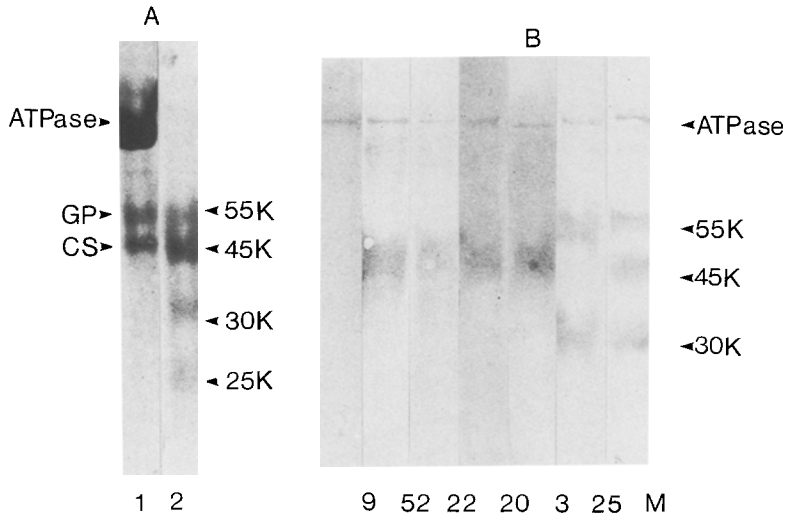

Fig. 3. Reactivity of monoclonal antibodies with tryptic fragments of the Ca^{2+} + Mg^{2+}-dependent ATPase. (A) Sarcoplasmic reticulum proteins were digested with trypsin and washed with deoxycholate solution (Stewart *et al.*, 1976). Proteins were separated in 7.5% Weber and Osborn slab gel, transferred to a nitrocellulose sheet, and stained with amido black. (B) Reactivity of different monoclonal antibodies with tryptic fragments of the ATPase was determined on nitrocellulose sheets as described under Materials and Methods. Each sample contained 12 μg of digested sarcoplasmic reticulum proteins. Ascites fluids containing monoclonal antibodies A52, A25, and A22 were diluted 1:1000 and those containing A20 and A3 were diluted 1:100. (1) Undigested sarcoplasmic reticulum proteins; (2) tryptic fragments of the ATPase. M, nitrocellulose blot incubated with a mixture of A52 and A25, both at a dilution of 1:1000.

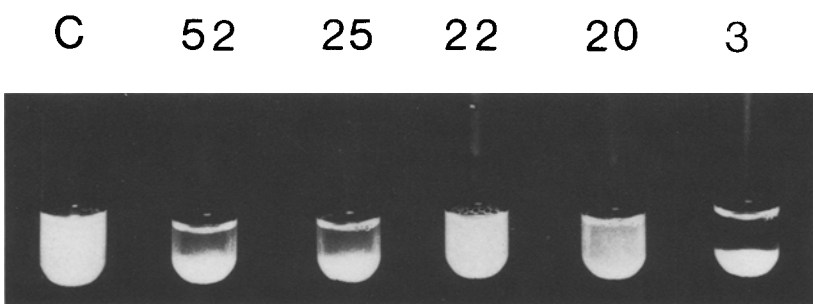

Fig. 4. Immunoprecipitation of sarcoplasmic reticulum vesicles with monoclonal antibodies. Sarcoplasmic reticulum vesicles were immunoprecipitated with different monoclonal antibodies (as indicated by numbers) in the presence of a second antibody as described under Materials and Methods. In each case the ratio of sarcoplasmic reticulum protein to purified antibody was 1:4. Photographs were taken after 90 min of incubation at room temperature. C, control sample incubated in the absence of monoclonal antibodies.

determinants on the ATPase molecule. Since primary antibody reactions did not induce precipitation (Stewart *et al.*, 1976), a second, anti-IgG or IgM antibody was added to cross-link vesicles and bring about precipitation. When the vesicles were incubated with the purified antibodies in a ratio of 1:4, three of the monoclonal antibodies, A3, A25, and A52, precipitated sarcoplasmic reticulum vesicles in the presence of a second antibody during 90 min of incubation at room temperature (Fig. 4). The reaction was strongest with A3 and was also evident at an antibody-to-sarcoplasmic reticulum protein ratio of 1:1. The monoclonal antibodies A20 and A22, even at the highest concentration, did not cause precipitation of sarcoplasmic reticulum vesicles after incubation at room temperature for 90 min. When antibodies were added to the sarcoplasmic reticulum vesicles according to their titers, A3 precipitated 16% of the sarcoplasmic reticulum vesicles during the first 60 min but A20, A22, A25, and A52 did not cause any precipitation (Table II). After incubation for 16 h in ice, approximately 45% of the vesicles were precipitated with A3 and A20 (Table II) and 10 and 14% of the vesicles were precipitated with A25 and A52, respectively. No precipitation was observed with A22 (Table II). From this experiment we conclude that the antigenic determinants of A3 and A20 are localized in a part of the ATPase molecule which is exposed on the exterior surface of the sarcoplasmic reticulum vesicles, whereas the antigenic determinants of A22 are situated in a part which is not exposed to the exterior (MacLennan and Reithmeier, 1982). The antigenic determinants of A25 and A52 are probably partially exposed to the membrane surface or become exposed after prolonged incubation.

e. Immunocytochemical Analysis. To evaluate the usefulness of these monoclonal antibodies as reagents for immunocytochemical localization of the Ca^{2+} + Mg^{2+}-dependent ATPase in various muscle tissues, cryostat

Table II. Precipitation of Intact Sarcoplasmic Reticulum Vesicles with Monoclonal Antibodies[a]

Monoclonal antibody	Percent of initial density remaining in the supernatant		
	0.5 h	1 h	16 h
A3	88	84	55
A20	100	100	58
A22	100	100	100
A25	100	100	90
A52	100	100	86

[a] Vesicles were precipitated with the monoclonal antibodies as described under Materials and Methods. Optical density of the samples at 500 nm was measured spectrophotometrically at different time intervals. Values presented are the averages of two determinations after subtraction of the percentage of the initial density in the control samples which were removed due to nonspecific precipitation.

sections from fixed and unfixed tissues were labeled with the monoclonal antibodies A3, A22, A25, and A52 by indirect immunofluorescence labeling. The fixation procedures used were similar to those used previously for the immunolocalization of the Ca^{2+} + Mg^{2+}-dependent ATPase in adult skeletal and cardiac muscle with polyclonal antibodies (Jorgensen *et al.*, 1979, 1982a,b). The results obtained are summarized in Table III.

In general, the avidity of the antibodies for the antigens was found to be acceptable for immunolocalization when unfixed or paraformaldehyde-fixed tissue was used. The avidity of the antibodies for the antigens, following fixation with a low concentration of glutaraldehyde, was greatly reduced and,

Table III. Characterization of Monoclonal Antibodies by Immunofluorescence Labeling of Sections from Various Muscle Tissues[a]

Tissue	Fixation	Monoclonal antibody			
		A22	A25	A52	A3
Adult rat skeletal muscle	Unfixed	+++	++	+++	+++
	2% paraformaldehyde	++	+	+	+/−
	2% paraformaldehyde + 0.3% glutaraldehyde	+	—	—	—
Embryonic rat skeletal muscle	2% paraformaldehyde + 0.3% glutaraldehyde	+	—	+/−	—
Adult rat heart Muscle	Unfixed	−	+/−	—	+/−
	2% paraformaldehyde + 0.3% glutaraldehyde	—	—	—	—
Embryonic rat heart muscle	2% paraformaldehyde + 0.3% glutaraldehyde	—	—	—	—

[a] +++, high intensity; ++, medium intensity; +, low intensity; +/−, borderline intensity; −, background intensity. All reactions were carried out with ascites fluids diluted 1/10.

except for antibody A22, the intensity of labeling was indistinguishable from that of the background. None of the four antibodies labeled the Ca^{2+} + Mg^{2+}-dependent ATPase of adult rat ventricular muscle above the background level.

Analysis of Structural Conservation of the Ca^{2+} + Mg^{2+}-Dependent ATPase

a. Cross-reactivity of Monoclonal Antibodies with the Ca^{2+} + Mg^{2+}-Dependent ATPase of Various Species. Cross-reactivity of the five monoclonal antibodies with sarcoplasmic reticulum vesicles isolated from rat, chicken, and lobster muscle was determined by the immunoblotting technique. Rabbit fast-twitch muscle sarcoplasmic reticulum vesicles were used as a control. All of the monoclonal antibodies cross-reacted with the ATPase present in rat skeletal muscle (Fig. 5), but A20 had a substantially lower avidity for the enzyme than the other monoclonal antibodies. Cross-reactivity with chicken sarcoplasmic reticulum ATPase was observed in the case of A52, A25, A22, and A3, but the avidity for the antigen was very low in the case of A25 (Fig. 5). The monoclonal antibody A20 did not react with chicken ATPase on the immunoblot. None of the five monoclonal antibodies reacted with the Ca^{2+} + Mg^{2+}-dependent ATPase of lobster sarcoplasmic reticulum. These results suggest that the primary structure of the Ca^{2+} + Mg^{2+}-dependent ATPase is not highly conserved among various species. As the species were further separated phylogenetically, less immunologic similarity between ATPase molecules was observed.

b. Cross-reactivity of Monoclonal Antibodies with the Ca^{2+} + Mg^{2+}-dependent ATPase from Different Tissues. Sarcoplasmic reticulum and microsomal vesicles were prepared from various types of muscle and nonmuscle tissues which contain membrane systems capable of regulating Ca^{2+} concentrations within the cell. Cross-reactivity of various monoclonal antibodies with the antigen present in different tissues is shown in Fig. 6. All five monoclonal antibodies cross-reacted with the Ca^{2+} + Mg^{2+}-dependent ATPase of sarcoplasmic reticulum isolated from rabbit adult slow-twitch muscle and 3-day-old rabbit skeletal muscle. Only A25 cross-reacted on the immunoblot with the ATPase of sarcoplasmic reticulum isolated from rabbit heart muscle, but the avidity of the antibody for the enzyme was very low (Fig. 6). Four of the five monoclonal antibodies, A52, A25, A20, and A22, showed a positive reaction with the ATPase of rabbit stomach smooth muscle, but the avidity for the antigen was substantially lower in the case of A22.

When nonmuscle cell microsomes were tested, the monoclonal antibody A22 recognized an antigenic determinant on a protein of M_r 100,000 in rabbit liver microsomes. A20 cross-reacted with a 100,000-dalton protein of human platelet microsomes, and A25 showed cross-reactivity with a 100,000-dalton

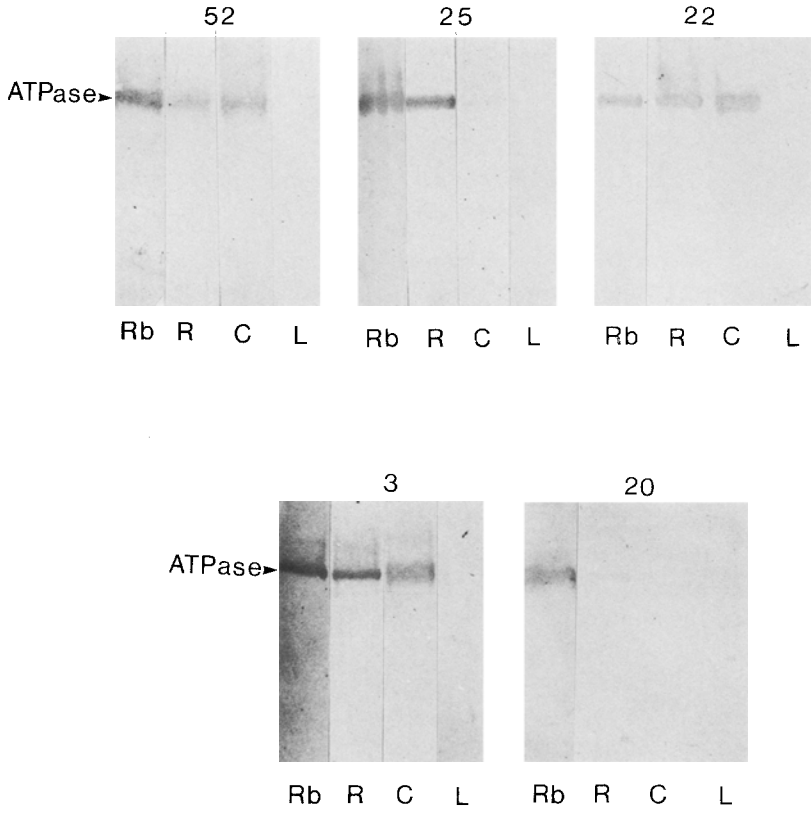

Fig. 5. Analysis of the cross-reactivity of monoclonal antibodies with the Ca^{2+} + Mg^{2+}-dependent ATPase from different species. Sarcoplasmic reticulum proteins from skeletal muscle of rabbit (Rb), rat (R), chicken (C), and lobster (L), separated in 8.5% Laemmli slab gel, were transferred electrophoretically to nitrocellulose sheets. Cross-reactivity with different monoclonal antibodies was determined as described under Materials and Methods. Ascites fluids were used at the same dilution as in the legend to Fig. 3. Rb and R contained 2.5 μg protein, and C and L contained 8 μg protein.

protein of both liver and platelet microsomes. In some experiments, A25 also cross-reacted with a polypeptide of M_r 70,000 (not shown), which probably represented a degradation product of the 100,000-dalton protein since it was not consistently observed. The results of these experiments suggest that, in spite of some common epitopic sites, polymorphic forms of the ATPase are present in various tissues even in the same species.

Cross-reactivity of Antibody A9 with Various Ca^{2+}-Binding Proteins

The cross-reactivity of A9 with calsequestrin suggested that the antibody recognized a common epitopic site present on the ATPase and calsequestrin

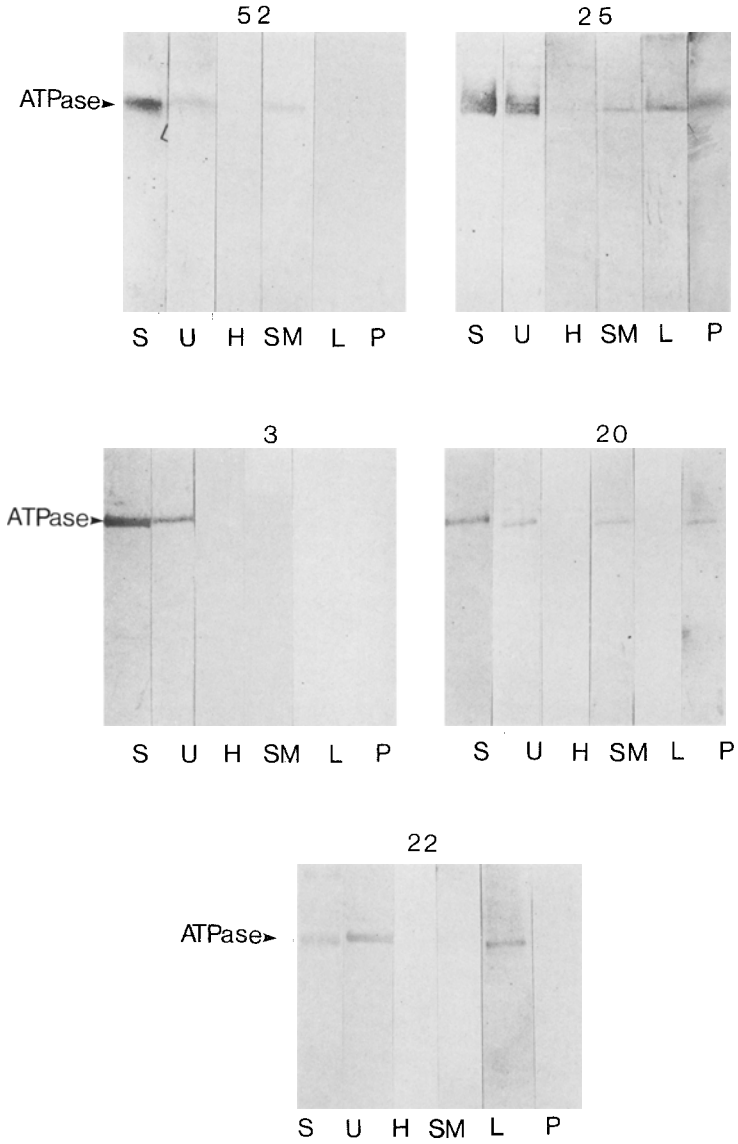

Fig. 6. Analysis of the cross-reactivity of monoclonal antibodies with the Ca^{2} + Mg^{2+}-dependent ATPase from different tissues. Samples of rabbit slow-twitch muscle (S), 3-day-old skeletal muscle (U), heart muscle (H) and smooth muscle (SM) sarcoplasmic reticulum, rabbit liver microsomes (L), and human platelet microsomes (P), separated in 8.5% Laemmli slab gels, were transferred to nitrocellulose sheets. Cross-reactivity of the proteins with different monoclonal antibodies was examined as described under Material and Methods. Ascites fluids were used at the same dilution as in the legend to Fig. 3. S and U contained 8 μg; H,50, μg; and SM, L, and P, 30 μg of protein.

Table IV. Cross-reactivity[a] of Monoclonal Antibody A9 with Ca^{2+}-Binding Proteins in the Absence and Presence of 1 mM EGTA

	O.D. 405 nm	
	(−) EGTA	(+) EGTA
S-100 protein	0.722 ± 0.052	1.000 ± 0.158
Calsequestrin	0.650 ± 0.037	0.958 ± 0.054
Calmodulin	0.523 ± 0.026	0.725 ± 0.032
Ca^{2+} + Mg^{2+}-ATPase	0.458 ± 0.115	0.856 ± 0.084
Cardiac troponin C	0.450 ± 0.018	0.797 ± 0.028
Skeletal troponin C	0.270 ± 0.024	0.285 ± 0.056
Calcineurin	0.259 ± 0.016	0.704 ± 0.049
CaBP (chick intestine)	0.183 ± 0.007	—
CaBP (pig intestine)	0.141 ± 0.013	0.180 ± 0.022
Ca^{2+} + Mg^{2+}-ATPase (erythrocyte)	0.167 ± 0.010	0.363 ± 0.021
Parvalbumin	0.122 ± 0.010	0.036 ± 0.021
P_A (saliva)	0.000 ± 0.000	0.043 ± 0.012
P_C (saliva)	0.000 ± 0.000	0.036 ± 0.005
Reagent blank	0.001 ± 0.001	0.004 ± 0.001

[a]The cross-reactivity of monoclonal antibody A9 with various Ca^{2+}-binding proteins was analyzed in the presence and absence of EGTA using ELISA with alkaline phosphatase conjugated to sheep anti-mouse IgG + IgM. EGTA was present in all solutions during all of the steps of the ELISA. Coating protein concentration was 10 μg/ml. Ascites fluid containing the monoclonal antibody was diluted 1:100.

molecules. Since both calsequestrin and the ATPase bind Ca^{2+} (MacLennan and Holland, 1975), we used ELISA to analyze the cross-reactivity of antibody A9 with a series of other Ca^{2+}-binding proteins: S-100 protein, calmodulin, cardiac muscle troponin C, skeletal muscle troponin C, calcineurin, chick intestinal vitamin D-dependent Ca^{2+}-binding protein, pig intestinal Ca^{2+}-binding protein, erythrocyte plasma membrane Ca^{2+} + Mg^{2+}-ATPase (M_r 140,000), and parvalbumin. The antibody recognized all of these antigens but did not cross-react with the two Ca^{2+}-binding proteins of saliva or with tropomyosin from skeletal muscle (Table IV). Therefore, tropomyosin and salivary Ca^{2+}-binding proteins were used in further studies as negative controls.

When 1 mM EGTA was present in solutions during all of the steps of the ELISA, the cross-reactivity of A9 with the Ca^{2+}-binding proteins was increased (Table IV). The presence of 2 mM Ca^{2+} inhibited the degree of reactivity of the antibody with many of the proteins (Table V). Neither EGTA nor Ca^{2+} affected the antibody cross-reactivity with parvalbumin (Tables IV and V). The effect of Ca^{2+} and EGTA on the cross-reactivity of A9 with the various Ca^{2+}-binding proteins may be explained on the basis of conformational changes which take place in these proteins upon binding and releasing Ca^{2+}.

The monoclonal antibody A9 cross-reacted with all of the proteolytic

Table V. Cross-Reactivity[a] of Monoclonal Antibody A9 with Ca^{2+}-Binding Proteins in the Absence and Presence of 2 mM Ca^{2+}

	O.D. 405 nm	
	(−) 2 mM Ca^{2+}	(+) 2 mM Ca^{2+}
Calsequestrin	0.977 ± 0.079	0.732 ± 0.074
Ca^{2+} + Mg^{2+}-ATPase	0.869 ± 0.032	0.851 ± 0.017
S-100 protein	0.763 ± 0.059	0.669 ± 0.053
Calmodulin	0.414 ± 0.011	0.403 ± 0.046
Cardiac troponin C	0.365 ± 0.033	0.276 ± 0.039
Skeletal troponin C	0.153 ± 0.003	0.131 ± 0.004
Parvalbumin	0.106 ± 0.014	0.107 ± 0.009
P_C (saliva)	0.066 ± 0.003	0.055 ± 0.013

[a]The cross-reactivity of monoclonal antibody A9 with Ca^{2+}-binding proteins in the presence or absence of Ca^{2+} was analyzed using ELISA as described in the legend to Table IV, except that Ca^{2+} was present only in the coating buffer.

fragments of troponin C and of calmodulin (Table VI and VII), and no obvious selectivity of the antibody for any of the fragments was observed. This would suggest that the epitopic site for antibody A9 is located in all Ca^{2+}-binding domains in these proteins.

Antibody Cross-reactivity with Platelet Cellular Proteins

When antibody A9 was incubated with human platelet proteins, separated electrophoretically and transferred to a nitrocellulose blot, it cross-reacted specifically with five proteins (Fig. 7). Two of these had M_r of 140,000 and 100,000 and probably represented the plasma membrane Ca^{2+} + Mg^{2+}-ATPase and the endoplasmic reticulum Ca^{2+} + Mg^{2+}-ATPase, respectively. The three other unknown proteins had M_r lower than 40,000. None of

Table VI. Cross-reactivity[a] of Monoclonal Antibody A9 with Calmodulin Fragments

	Ca^{2+}-binding sites	O.D. 405 nm
Calmodulin	I, II, III, IV	0.464 ± 0.061
Calmodulin fragments:		
78–148	III, IV	0.147 ± 0.015
1–77	I, II	0.164 ± 0.006
107–148	IV	0.226 ± 0.034
1–90	I, II	0.153 ± 0.016
1–106	I, II, III	0.186 ± 0.007
Tropomyosin		0.065 ± 0.025

[a]The cross-reactivity of monoclonal antibody A9 with calmodulin fragments (Newton *et al.*, 1984) was analyzed in the absence of EGTA using ELISA as described in the legend to Table IV except that purified immunoglobulin was used instead of ascites fluid. Antibody dilution was 1:100.

Table VII. Cross-reactivity of Monoclonal Antibody A9 with Rabbit Skeletal Muscle Troponin C Fragments[a]

	Amino acid residues	Ca^{2+} binding sites	O.D. 405 nm
Troponin C	1–159	I, II, III, IV	0.172 ± 0.007
Troponin C fragments:			
E1	1–100	I, II	0.135 ± 0.006
C	9–84	I, II	0.180 ± 0.007
E2	101–153	III, IV	0.189 ± 0.015
P_C (saliva)			0.002 ± 0.002
Reagent blank			0.001 ± 0.001

[a]The cross-reactivity of monoclonal antibody A9 with troponin C fragments (Grabarek *et al.*, 1981) was analyzed using ELISA as described in the legend to Table VI.

the proteins which gave a positive reaction represented a major component of the homogenate. These results suggest that the antibody specifically recognized its antigenic determinant among a group of platelet cellular proteins.

Discussion

Polymorphic Forms of the ATPase

Five monoclonal antibodies raised against the purified Ca^{2+} + Mg^{2+}-dependent ATPase of rabbit fast-twitch muscle sarcoplasmic reticulum were

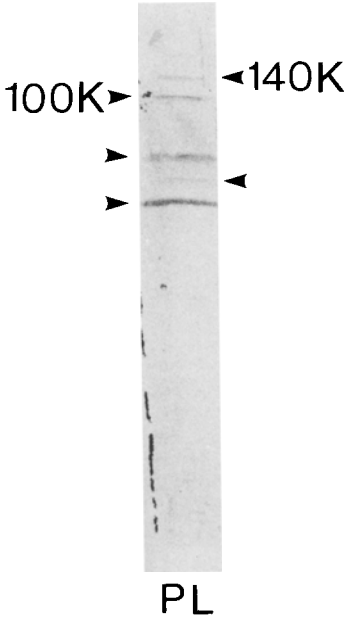

Fig. 7. Cross-reactivity of monoclonal antibody A9 with platelet cellular proteins. A human platelet homogenate (100 μg), prepared as described under Materials and Methods, was separated in 12.5% Laemmli gel. The immunoblotting technique described by Towbin *et al.* (1979) was used to detect antibody cross-reactivity with platelet proteins. Pure antibody was diluted 1:100.

found to bind specifically to this enzyme when tested by ELISA, immunoprecipitation on protein A–Sepharose beads with radioiodinated sarcoplasmic reticulum proteins, and immunoblotting. Tests of the ability of the antibodies to immunoprecipitate intact sarcoplasmic reticulum vesicles and to bind to tryptic fragments of the Ca^{2+} + Mg^{2+}-dependent ATPase indicated that the various monoclonal antibodies were raised against different antigenic determinants along the polypeptide chain of the Ca^{2+} + Mg^{2+}-dependent ATPase. Four of the monoclonal antibodies recognized epitopic sites of the ATPase which are located on the outer surface of intact membranes. We have not noted inhibition of Ca^{2+} uptake by any of these antibodies, however. Four of the antibodies reacted with the 45,000-dalton tryptic fragment of the ATPase and one reacted with the 55,000- and 30,000-dalton fragments.

Studies of tryptic peptide maps, C-terminal sequences, and partial proteolysis patterns of the Ca^{2+} + Mg^{2+}-dependent ATPase by Ohnoki and Martonosi (1980) suggested the existence of structural differences between the Ca^{2+} + Mg^{2+}-dependent ATPases present in the skeletal muscle of rabbit, chicken, and lobster. The results of the present study, obtained with monoclonal antibodies to the Ca^{2+} + Mg^{2+}-dependent ATPase, show that polymorphic forms of the ATPase exist in rabbit, chicken, human, and lobster. Moreover, there is less apparent immunologic homology among ATPase molecules which exist in species not closely related phylogenetically. This supports the view that the Ca^{2+} + Mg^{2+}-dependent ATPase of sarcoplasmic reticulum is not as well conserved during evolution as some other proteins, such as actin (Korn, 1982) or the S100 protein which is distributed in the nervous system of vertebrates (Marks *et al.*, 1983).

The identification of polymorphic forms of the Ca^{2+} + Mg^{2+}-dependent ATPase in skeletal and cardiac muscle has been reported in studies with polyclonal antisera (DeFoor *et al.*, 1980). The fact that four of our five monoclonal antibodies did not show any cross-reactivity with the Ca^{2+} + Mg^{2+}-dependent ATPase of rabbit cardiac muscle confirms that immunological differences exist between the Ca^{2+} + Mg^{2+}-dependent ATPase of cardiac and skeletal muscle. In a comparable study, Fambrough and Bayne (1983) reported the existence of polymorphic variants of the Na^{+} + K^{+}-dependent ATPase in different tissues of chicken.

It has been shown that a microsomal fraction capable of transporting Ca^{2+} is present in smooth muscles of different organs (Raeymekers *et al.*, 1980; Wuytack *et al.*, 1978; Carsten and Miller, 1980) and that a partially purified preparation of the Ca^{2+} + Mg^{2+}-dependent ATPase from smooth muscle microsomes contains a major polypeptide of 140,000 daltons (Wuytack *et al.*, 1981). In this study we have used monoclonal antibodies to identify the Ca^{2+} + Mg^{2+}-dependent ATPase in calcium oxalate-loaded sarcoplasmic reticulum vesicles of rabbit stomach smooth muscle. Four of the five monoclonal antibodies reacted with a 100,000-dalton polypeptide present in the

calcium-loaded vesicles, showing that the enzymes present in skeletal and smooth muscle are polymorphic forms of the same protein. Moreover, these data suggest that the Ca^{2+} + Mg^{2+}-dependent ATPase of smooth muscle sarcoplasmic reticulum has a M_r similar to that of skeletal muscle microsomes rather than to the M_r of 140,000 which seems to be typical of the Ca^{2+}-ATPase of plasma membranes (Niggli *et al.*, 1979).

It has been reported that several nonmuscle tissues contain membrane systems able to transport Ca^{2+} (Moore *et al.*, 1975; Bygrave, 1978; Dawson, 1982; Kaser-Glanzmann *et al.*, 1977; Blitz *et al.*, 1977; Kato and Tonomura, 1977; Zubrzycka-Gaarn *et al.*, 1979; Heilmann *et al.*, 1983). In some of these systems the presence of a Ca^{2+} + Mg^{2+}-dependent ATPase was demonstrated by determination of its specific activity, phosphoenzyme formation, mobility in SDS–polyacrylamide gels, and cross-reactivity with polyclonal antisera prepared against the Ca^{2+} + Mg^{2+}-dependent ATPase of skeletal muscle sarcoplasmic reticulum (Dean and Sullivan, 1982; Moore and Kraus-Friedmann, 1983; Blitz *et al.*, 1977; Kato and Tonomura, 1977; Zubrzycka-Gaarn *et al.*, 1979; Heilmann *et al.*, 1983). In this study we have shown that antibodies A25, A22, and A20 cross-reacted on immunoblots with a 100,000-dalton polypeptide from human platelet microsomes and with a polypeptide of the same molecular weight from liver microsomes. However, A3 and A52 did not cross-react with these polypeptides. These results provide evidence that the Ca^{2+} + Mg^{2+}-dependent ATPases in the microsomal fractions of various tissues have a similar M_r and exist in polymorphic forms which are probably characterized by different epitopic sites which react with different monoclonal antibodies.

We anticipate that these monoclonal antibodies will be useful for further biochemical and morphological characterization of Ca^{2+} transport ATPases from various tissues. Our morphological studies show the feasibility of using the antibodies for immunolabeling at the light microscope level which requires only mild fixation. One of the antibodies (A22) is potentially useful for immunolabeling at the ultrastructural level where cross-linking reagents are usually required for fixation.

Antibody Cross-reactivity Against Ca^{2+}-Binding Sites

The monoclonal antibody A9, prepared against the Ca^{2+} + Mg^{2+}-dependent ATPase of sarcoplasmic reticulum, cross-reacts with a series of known Ca^{2+}-binding proteins and, selectively, with unknown proteins which may be Ca^{2+}-binding proteins in platelet homogenates. We have not yet observed cross-reactivity with proteins that are known not to bind Ca^{2+}. This antibody may be particularly valuable in the identification of unknown Ca^{2+} binding proteins in tissue fractions. In this respect, the antibody might serve a

similar function to Stains-All (Campbell *et al.*, 1983), or supplement its use in the identification of Ca^{2+}-binding proteins in gels or transfers of electrophoretically separated mixtures of proteins from various sources.

Both calmodulin and troponin C contain four Ca^{2+}-binding sites per mole (Haiech *et al.*, 1981; Keller *et al.*, 1982; Leavis *et al.*, 1978). These sites are all of the classical *EF* hand conformation described by Kretsinger and Nockolds (1973). Calmodulin contains all sites of similar affinity (Haiech *et al.*, 1981), whereas troponin C contains two low (I and II) and two higher (III and IV) affinity Ca^{2+}-binding sites (Leavis *et al.*, 1978). We do not know the form of the antigenic site recognized by the antibody. The antibody cross-reacts more or less equally with all tryptic peptides containing different Ca^{2+}-binding sites in calmodulin and troponin (Tables VI and VII). The antibody also reacts with calsequestrin which has lower-affinity Ca^{2+}-binding sites (MacLennan and Wong, 1971). These sites have not been defined in terms of amino acid sequence as yet, although a sequence homologous to about 1/2 of a Ca^{2+}-binding site was observed in the NH_2 terminal sequence of calsequestrin (MacLennan *et al.*, 1983). Thus the antibody might be reacting with a few Ca^{2+}-binding sites of the *EF* hand conformation in calsequestrin. The salivary Ca^{2+}-binding proteins did not react with the monoclonal antibody. Since these proteins have very low affinity for Ca^{2+} (Bennick *et al.*, 1981), it is probable that they do not have any *EF* hand type of Ca^{2+}-binding sites. Thus it is not surprising that they did not display the same epitopic site as the high-affinity Ca^{2+}-binding proteins.

The effect of Ca^{2+} and EGTA on the antibody binding affinity is of interest. We believe that this is due to changes in protein conformation induced by Ca^{2+} binding. Conformational changes induced by Ca^{2+} in Ca^{2+}-binding proteins have been measured by various physicochemical techniques (Klee *et al.*, 1980; Potter *et al.*, 1976; Leavis *et al.*, 1978). It has been estimated, for example, that as a result of Ca^{2+}-induced conformational changes, the helical content of calmodulin increases from 40 to 50% (Walsh *et al.*, 1979) and that a hydrophobic region, which may be important for interaction with other enzymes, becomes exposed or ordered when Ca^{2+} is bound (LaPorte *et al.*, 1980; Tanaka and Hidaka, 1980). Binding of Ca^{2+} to the high-affinity Ca^{2+}-binding sites of troponin C produces a large enhancement of intrinsic fluorescence and of circular dichroism in the 200–240 nm region (Van Eerd and Kawasaki, 1972; Kawasaki and Van Eerd, 1972). This observation indicates that the helical content increases from approximately 30 to 50%. The removal of Ca^{2+} by EGTA from the Ca^{2+}-binding proteins may also cause conformational changes in the Ca^{2+}-binding domains. These Ca^{2+}-free antigenic determinants may have a higher degree of cross-reactivity for the monoclonal antibody A9.

The potential use of the monoclonal antibody in definition of the

Ca^{2+}-binding site in the Ca^{2+}-ATPase is of great interest. Although antibody A9 cross-reacted on immunoblots with the denaturated, undigested Ca^{2+} + Mg^{2+}-dependent ATPase, we did not observe antibody binding on immunoblots to any of the denatured tryptic fragments of the enzyme. These results suggest that the conformations of the fragments do not re-form after denaturation by boiling in SDS, so that the antigenic site is no longer recognized by the antibody. It is also possible, however, that trypsin digests the ATPase molecule within the site of the antigenic determinant, leaving parts of it attached to two different fragments. The affinity of the antibody for incomplete antigenic determinants could be very low, making binding unlikely. A third possibility is that the antigenic determinant is split between two or more fragments after tryptic digestion of the ATPase molecule. The antigenic determinant could be composed of two groups of amino acids which are distant in the linear polypeptide chain, but which are closely apposed in the tertiary structure of the protein. Tryptic cleavage between these two groups of amino acids might separate the components which collectively compose the antigenic determinant. We are currently exploring the possibility that the antibody will be useful in defining the Ca^{2+}-binding site in the Ca^{2+} + Mg^{2+}-dependent ATPase.

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