Preparation and characterization of monoclonal antibody specific for FKBP12.6*

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＜Abstract＞

FK506 binding proteins (FKBPs) are a protein family that is the intracellular target of a potent immunosuppressive drug, FK506. An isoform termed FKBP12.6 has been found to be bound with the ryanodine receptor 2 (RYR2). The RYR2 is the major calcium release channel (CRC) of the sarcoplasmic reticulum of cardiac muscle, and is also expressed in a variety of cell types. However, the function of the FKBP12.6 bound to the RYR2 is controversial. To use as a probe to study the role of FKBP12.6 bound to the RYR2, we prepared monoclonal antibodies that are genuinely specific for FKBP12.6. Lymph node cells from mice immunized with recombinant His-FKBP12.6 were fused with a myeloma cell line, and the resulting hybridomas were screened by ELISA for production of a relevant antibody. Two monoclonal hybridomas (1B4/G3 and 1C2/G10) that produce antibodies that react only with FKBP12.6 but not FKBP12, a closely related isoform, were established. The monoclonal 1B4/G3 antibody was applicable to detect native FKBP12.6 by Western blotting in cardiac sarcoplasmic reticulum fraction enriched in the RYR2-FKBP12.6 complex. These results suggested that our monoclonal antibodies are candidates to be useful probes to elucidate the physiological role of FKBP12.6.

Keywords: FKBP12.6, ryanodine receptor, monoclonal antibody

Introduction

FK506 binding proteins (FKBPs) are a protein family, most of whose members bind to a powerful immunosuppressive drug, FK506, and show peptidyl-prolyl *cis/trans* isomerase (PPIase) activity. Among numbers of FKBPs, the most abundant isoform termed FKBP12 is the sole isoform responsible for mediating the immunosuppressive actions of the drug *in vivo*. The PPIase activity of FKBPs is inhibited by FK506[1]. However, inhibition of the PPIase activity of FKBP12 is unrelated to the immunosuppressive action of FK506. Instead, the immunosuppressive action of the drug results from the inhibition of calcineurin, a calmodulin-dependent/Ca^{2+}-activated protein phosphatase. During T cell activation, calcineurin dephosphorylates the cytosolic component of NF-AT that leads this key transcription factor to be translocated to the nucleus, which in turn triggers IL-2 gene expression. The FKBP12·FK506 complex inhibits calcineurin to block T cell activation, which then results in immunosuppression. However, neither FKBP12 nor FK506 alone interact with calcineurin; it is not until forming a complex with FK506 that FKBP12 interacts

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with and inhibits calcineurin[2]. Furthermore, FKBP12 is expressed, not only in T cells, but in most, if not all, cell types. Thus, FKBP12 is a mere bystander protein during T cell activation that becomes involved in preventing activation after it binds FK506. To date, the most well understood physiological function of FKBPs is their interaction with the ryanodine receptors.

The ryanodine receptors (RYRs) are a protein family that function as an intracellular calcium release channel (CRC) located on the endo(sarco)plasmic reticulum membrane. The RYRs are by far the largest ion channel protein complexes that exist as homotetramers thereby exhibiting 4-fold symmetry. There are three types, RYR1, RYR2 and RYR3. Although the RYRs are expressed in many cell types, they are expressed at a very high level, especially in skeletal and cardiac muscles. The RYR1 and RYR2 of the sarcoplasmic reticulum (SR) of skeletal and cardiac muscle, respectively, play a crucial role in excitation-contraction coupling of these striated muscle[3].

The FKBP isoforms that have been shown to interact with the RYRs are FKBP12 and FKBP12.6, an isoform structurally closely related to FKBP12. FKBP12 is tightly bound to[4] and modulates[5)-10)] the CRC/RYR1 of skeletal muscle sarcoplasmic reticulum (SR) ***in vivo***, although ***in vitro*** experiments have shown that the RYR1 is capable of binding with both FKBP12 and FKBP12.6[11), 12)]. It was first found that the CRC isolated from rabbit skeletal muscle terminal cisternae (TC) of SR is tightly associated with FKBP12[4] in a stoichiometry of four FKBP12 per one RYR[15]. Subsequently, the tight association between the RYR1 and FKBP12 in skeletal muscle TC was found to be common to each of the five classes of vertebrates, ***i.e.*** mammals, birds, reptiles, fish, and amphibians[13]. Thus, the native CRC of skeletal muscle SR is a heterooligomer with a structural formula of $(RYR1\ protomer)_4(FKBP12)_4$. In contrast to the RYR1, the RYR2 has been shown to have a binding preference to FKBP12.6 over FKBP12[11), 14)]. Particularly, canine cardiac RYR2 showed a 500~1,000 fold higher affinity for FKBP12.6 compared to FKBP12[11]. Likewise the RYR1, one mole of the RYR2 channel also has four moles of the binding sites for FKBP.

FKBP bound to the RYR is gently dissociated when the SR fraction or purified channel is treated with FK506[5),11)]. Extensive ***in vitro*** studies to elucidate the function(s) of FKBP12 and/or FKBP12.6 bound to the RYR1 or RYR2 have been carried out using FKBP-stripped RYRs. In studies comparing the function of the native RYR1 with FKBP-stripped RYR1, it has been shown that FKBP12 bound to the RYR1 modulates the channel activity by stabilizing the closed conformation of the channel[5),7),10)]. It has also been proposed that FKBP12 synchronizes the gating of neighboring RYR1 channels (coupled gating)[15), 16)]. In an ***in situ*** study employing skinned skeletal muscle fiber, E-C coupling was impaired upon depletion of bound FKBP from the RYR1 by treatment of FK506 or rapamycin[17]. Thus, FKBP12 seems to have established its status as an integral component of the RYR1 that modulates the channel function. In contrast to the FKBP12 bound to the RYR1, the physiological role of the FKBP12.6 bound to the RYR2, at present, remains unclear and controversial. In ***in vitro*** studies comparing the function of the native RYR2 with FKBP-stripped RYR2, some research groups have reported similar effects of FKBP12.6 on the RYR2 as FKBP12 on the RYR1[18), 19)], while some have shown there are no similar effects[11), 20)]. Interestingly, FKBP12.6-knockout mice show abnormalities in heart function[21), 22)], although results from the two laboratories differ in detail. Moreover, although controversial, Marks and colleagues have proposed that removal of FKBP12.6 from the RYR2 is involved in the mechanism for the progression of heart failure[23]. They raised a hypothesis that in the hyperadrenergic state in heart failure, protein kinase A is overactivated,

resulting in hyperphosphorylation of the RYR2, causing FKBP12.6 to dissociate from the channel. Marks's group insists that this is due to the fact that FKBP12.6-depleted RYR2s exhibit an abnormally high open probability during diastole that in turn allows leakage of Ca^{2+} from the SR; this subsequently reduces the Ca^{2+} that is required to induce contraction during systole, and might also trigger ventricular tachycardia. It is, at present, controversial if this hypothesis for heart failure is legitimate since both evidence for[24), 25)] and against it has been reported. Nevertheless, in light of these results, FKBP12.6 must be implicated in the modulation of the channel function of the RYR2.

To elucidate the role of FKBP12.6 bound to the RYR2, probes such as antibodies specific for FKBP12.6 would be a powerful tool. For that, in this study, we developed monoclonal antibodies that specifically recognize FKBP12.6, but not FKBP12.

Materials and methods

Recombinant FK506 binding proteins

Recombinant rabbit FKBP12.6 and human FKBP12 tagged with six consecutive His residues at the N-terminus (His-FKBP12.6 and His-FKBP12) were kindly gifted by Dr. Yoshiro Chuman, Division of Chemistry, Graduate School of Science, Hokkaido University, Sapporo, 060-0810.

Immunization and cell fusion

To obtain monoclonal antibodies (mAbs) against FKBP12.6, recombinant His-FKBP12.6 protein was used as the immunogen. It was dissolved in phosphate buffered saline (1 mg/mℓ) and emulsified with an equal volume of Freund's complete adjuvant (Difco, BD, Franklin Lakes, NJ). Two female BALB/c mice were injected with the emulsion (50 μℓ/mouse) in the footpad. Three weeks after immunization, the inguinal lymph node cells (4×10^7 cells) were fused with P3X63Ag8U.1 myeloma cells (1×10^7 cells) using polyethylene glycol 1500 (Roche Diagnostics). Fused cells were cultured in 96-well plates at 2×10^5 cell/well in HAT selection medium. After the HAT-resistant cells had grown sufficiently, the supernatants were assayed for anti-FKBP12.6 antibody titer by an enzyme-linked immunosorbent assay (ELISA) using recombinant His-FKBP12.6, and His-FKBP12 proteins.

Enzyme Linked Immunosorbent Assay (ELISA)

All the procedures were carried out at room temperature. 96-well ELISA plates were inoculated with 50 μℓ/well of His-FKBP12.6 or His-FKBP12 diluted in 50 mM Na_2CO_3 at 1 mg/mℓ, and incubated overnight to immobilize the protein. After three washings with Tris buffered saline (TBS, 20 mM Tris-Cl, pH 7.5, 150 mM NaCl), the plates were incubated with 0.5% defatted milk in TBS (200 μℓ/well) for 1 hour at room temperature to block non-specific binding of immunoglobulin. After blocking, the culture supernatant of hybridomas were added to the wells (50 μℓ/well) and incubated for 1 hour, then washed six times with TBS containing 0.1% Tween 20 (TBST). The wells were incubated with 50 μℓ of a alkaline phosphatase-conjugated donkey anti-mouse Ig G (Jackson ImmunoResearch) at a dilution of 1: 2,500 in TBST containing 0.1% defatted milk for 1 hour. After six washings with TBST, immunodetection was performed by color development using p-nitro phenyl phosphate (1mg/mℓ in 100mM Tris-Cl, pH 9.5, 100mM NaCl, 5mg/mℓ $MgCl_2$) as substrate reagents. Optical density at 405 nm was measured by an automated microtiter plate reader (Model 550, Bio-Rad).

Isotyping of the monoclonal antibodies

The isotypes of monoclonal antibodies 1B4/G3 and 1C2/G10 were determined by use of the Monoclonal Antibody Isotyping Kit (PIERCE) following the manufacturer's instructions.

Western blot analysis

All the procedures were carried out at room temperature. SDS-PAGE was performed according to Laemmli[26] using 12.5% polyacrylamide gel. Separated proteins were transferred onto PVDF membranes (Immobilon-P; Millipore) using a semi-dry electrotransblotter (BioCraft) with the buffer containing 48 mM Tris, 39 mM glycine, 0.0375% SDS, and 10% methanol. The membrane was blocked for 1 hr with TBST containing 5% (w/v) defatted milk. The blocked membrane was probed with the anti-FKBP12.6 mAb. After washing with TBST, the membrane was incubated with goat anti-mouse Ig G antibody conjugated with alkaline phosphatase. The membrane was washed with TBST and immunodetection was performed by color development using 5 bromo-4-chloro-3-indolyl phosphate-toluidine salt/p-nitro-blue tetrazolium chloride as substrate reagents.

Results

Establishment of monoclonal hybridomas producing antibody specific for FKBP12.6

Lymph node cells (4 x 10^7 cells) of mice immunized with His-FKBP12.6 were fused with myeloma cell line, and cultured till the hybridomas propagated sufficiently. Then the supernatants were screened for their immunoreactivity by ELISA. Supernatants of four wells displayed strong immunoreactivity against His-FKBP12.6 (Fig. 1, empty bars). These supernatants were further tested for their immunoreactivity against His-FKBP12 (Fig. 1, filled bars). Three of the four supernatants (1A10, 1B4 and 1C2) did not recognize His-FKBP12, while one did (4A11). The supernatants that reacted with His-FKBP12.6, but not His-FKBP12, should have contained the antibody specific for FKBP12.6. Cells in two wells (1B4 and 1C2) showed good growth, in addition to the specificity for FKBP12.6, therefore hybridomas in these wells were cloned by limiting dilution. Finally, two independent relevant clones, 1B4/G3 and 1C2/G10, that retained both good antibody productivity and growth were established. These two hybridomas were adapted to propagate in a serum-free culture medium. Hereafter, the antibodies from these two hybridomas are referred to as 1B4/G3 and 1C2/G10 respectively.

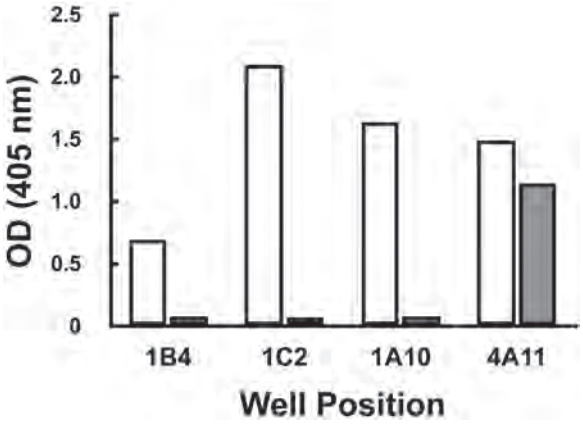

Fig. 1 Screening for anti-FKBP12.6 producing wells by ELISA. 96 well ELISA plates coated with His-FKBP12.6 (empty bars) or His-FKBP12 (filled bars) were probed with culture supernatants of wells of 96 well culture plates (50 μℓ/well). Representative data obtained from the wells whose supernatants reacted with at least His-FKBP12.6 or His-FKBP12.

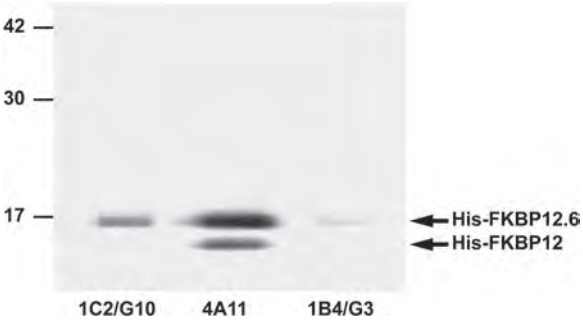

Fig. 2 Specific detection of FKBP12.6 by monoclonal anti-FKBP12.6 antibodies. Western blot analysis probed with monoclonal anti-FKBP12.6 mAbs, 1C2/G10 and 1B4/G3, and antibody that recognizes both His-FKBP12 and FKBP12.6, 4A11. 50 ng of His-FKBP12.6 and His-FKBP12 were separated in each lane.

Antigen specificity of the mAbs, 1B4/G3 and 1C2/G10 were further examined by Western blot analysis (Fig. 2). Equal amounts of His-FKBP12.6 and His-FKBP12 proteins

were separated in all the lanes. Bands of His-FKBP12.6 and His-FKBP12 were both detected in the lane probed with 4A11 (Fig. 2), the antibody that had been shown to recognize both isoforms of FKBP (Fig. 1). In contrast, 1B4/G3 and 1C2/G10 mAb detected only the band of His-FKBP12.6, but not His-FKBP12. The results from Western blot analysis (Fig. 2) were consistent with those from ELISA (Fig. 1).

Isotyping of the monoclonal antibody specific for FKBP12.6

Isotypes of mAbs 1B4/G3 and 1C2/G10 were determined by an ELISA using a series of isotype-specific antibodies (goat anti-mouse Ig G1, Ig G2a, Ig G2b, Ig G3, Ig M, and Ig A). As shown in Fig. 3, 1B4/G3 (empty bars) reacted most prominently with anti-Ig G3, whereas 1C2/G10 (filled bars) reacted exclusively with anti-Ig G2b.

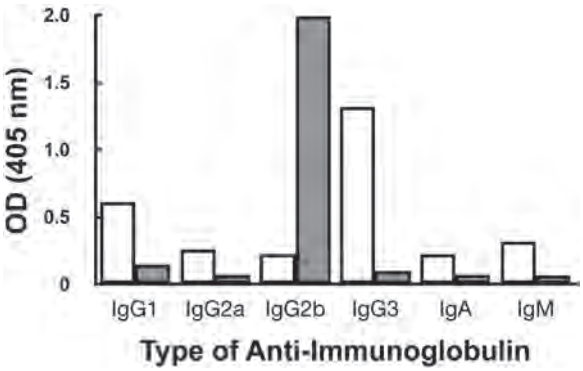

Fig. 3 Isotyping of monoclonal anti FKBP12.6 antibodies,1B4/G3 (empty bars), and 1C2/G10 (filled bars). Experimental procedures were analogous to that in Fig. 1, with the exception of secondary antibodies that are highly specific for mouse Ig G1, Ig G2a, Ig G2b, Ig G3, Ig A or Ig M were used instead of the antibody used in Fig 1 which cannot distinguish precisely the isotypes of the immunoglobulins.

Detection of native FKBP12.6 by monoclonal antibody

Cardiac and skeletal muscle SR fractions are known to contain detectable amounts of FKBP12.6 and FKBP12 respectively by Western blot. To examine if or not the monoclonal antibody raised against recombinant His-FKBP12.6 recognizes native FKBP12.6 as well, Western blot against dog cardiac SR was carried out. To see if the band of FKBP12.6 could be detected in ER enriched sample from other tissues such as cerebellum, cerebrum, liver, and skeletal muscle were also examined. Membranes were probed with 1B4/G3 mAb. As shown in Fig. 4, 1B4/G3 detected a band of FKBP12.6 in cardiac SR (upper panel), as well as the band of His-FKBP12.6 (lower panel). FKBP12 in skeletal muscle SR was not detected by 1B4/G3. No FKBP bands were detected in cerebellum, cerebrum, and liver samples. A similar result was obtained from an experiment using 1C2/G10 mAb (data not shown).

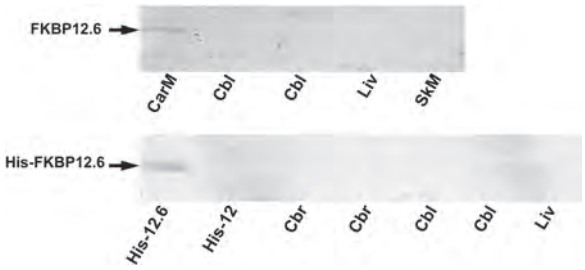

Fig. 4 Detection of native FKBP12.6 by 1B4/G3 mAb. SR or ER fractions (5 μg protein/lane) were separated and Western blot analysis probed with 1B4/G3 mAb was carried out. CarM, cardiac muscle; Cbl, cerebellum; Liv, Liver; SkM, skeletal muscle; Cbr, cerebrum; His-12.6, His-FKBP12.6; His-12, His-FKBP12.

Discussion

The goal of the present study was to obtain an antibody specific for FKBP12.6, which can distinguish FKBP12.6 from another closely related isoform, FKBP12. Both FKBP12.6 and FKBP12 consist of 108 amino acids. Since homology between their primary structures is 85%, it was expected that if an animal is immunized with either FKBP12.6 or FKBP12, some B cell clone would produce an antibody that is truly specific for the immunized isoform, while some would produce a general antibody that would not only react with the immunized isoform, but also crossreact with the other one. Thus, a polyclonal antiserum would be a mixture of the isoform-specific and general

antibodies that, as a result, recognizes both FKBP12.6 and FKBP12. In fact, a polyclonal antiserum raised against a peptide with a partial sequence that is specific to FKBP12 but also 90% identical to the corresponding region of FKBP12.6, reacted with both isoform (Onoue H., unpublished data). Therefore, we decided to raise the anti-FKBP12.6 antibody by monoclonal strategy and select the relevant clone. In the course of screening utilizing ELISA, three wells were shown to contain antibodies that react with His-FKBP12.6, but not with His-FKBP12, suggesting the hybridomas in these wells (1A10, 1B4 and 1C2) were producing antibodies truly specific for FKBP12.6 (Fig. 1). A hybridoma (4A11) that produces an antibody that reacts with both His-FKBP12.6 and His-FKBP12 was also obtained. Although the reason for this is at present unclear since we have not investigated further, there seem to be two explanations for the fact that 4A11 reacted with His-FKBP12 in addition to the immunized antigen, His-FKBP12.6, 1) the antibody produced by 4A11 reacts with both FKBP12.6 and FKBP12,or; 2) reacts with the His-tag moiety of the recombinant FKBPs. 1B4 and 1C2 were cloned, and two independent monoclonal hybridomas 1B4/G3 and 1C2/G10 were established. The isotypes of 1B4/G3 mAb and 1C2/G10 mAb were Ig G3 and Ig G2b, respectively (Fig. 3).

His-FKBP12.6 used as the immunizing antigen, differs from native FKBP12.6 in that it has six His residues recombinantly added to the N-terminus of the native sequence of FKBP12.6. Moreover, recombinant His-FKBP12.6 has not received post-translational modification(s), such as phosphoryration, which might occur in native-FKBP12.6. The primary purpose that we prepare mAbs specific for FKBP12.6 is to use them as probes to elucidate the role of FKBP12.6-RYR2 interaction. For this, it is particularly important that the mAbs react with native FKBP12.6 contained in biological specimens such as cardiac SR fraction. To examine if or not the mAbs prepared in the present study recognize native FKBP12.6, Western blot against cardiac SR fraction was carried out and probed with 1B4/G3 or 1C2/G10 mAb. 1B4/G3 mAb successfully detected the band of FKBP12.6 in cardiac SR sample (Fig. 4) but did not detect FKBP12, which is abundant in skeletal muscle SR. 1C2/G10 mAb showed essentially the same immunoreactivity as 1B4/G3 (data not shown).

There would be a number of situations that mAb specific for FKBP12.6 serves as a powerful probe. The RYR2 is expressed in many tissue/cell types, including cardiac muscle, cerebellum smooth muscle cells, and pancreatic β-cells, although expression levels are low except for cardiac muscle. In addition to its crucial role in the E-C coupling in the heart, the RYR2 is also involved in other cellular functions related to intracellular calcium signaling, such as fertilization, secretion, and apoptosis. It is of great interest whether or not the RYR2 expressed in cells other than cardiac muscle also interacts with FKBP12.6. For this, it is important to compare the localizations of FKBP12.6 with that of the RYR2 in such cell types. FKBP12 is expressed in most, if not all, cell types, while FKBP12.6 is less ubiquitous. Therefore it is necessary to use an antibody that can distinguish FKBP12.6 from FKBP12. Our monoclonal anti-FKBP12.6 antibodies are suitable for this purpose since they recognized FKBP12.6, but not FKB12 (Figs. 1, 2, and 4). Although they detected FKBP12.6 in cardiac SR, in which the RYR2 is highly enriched, they could not detect FKBP12.6 in the cerebellum sample where the RYR2 is expressed in an extremely low level compared to the heart. The reason for this could be that; 1) the affinity of our mAb for FKBP12.6 was not sufficiently high, or 2) the amount of FKBP12.6 was too low in cerebellum microsome fraction. Condensation of the antibody and/or purification of FKBP12.6 by immunoprecipitation might be solutions for the problems mentioned above. Isotypes of the two mAbs, 1B4/G3 and 1C2/G10, were Ig G3

and Ig G2b respectively that are convenient for immunoprecipitation. Moreover, Ig G is an antibody class that is also suitable for immunohistochemistry, which might be an effective method to study protein localization in situ. These facts suggest that our mABs specific for FKBP12.6 could be assets for clarifying the physiological role of the interaction between RYR2 and FKBP12.6 in future studies.

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原　著

FKBP12.6に特異的なモノクローナル抗体の作製と特徴づけ

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＜要　旨＞

FK506結合タンパク質（FKBP）は、強力な免疫抑制剤であるFK506の細胞内標的となるタンパク質のファミリーである。これらの中で、FKBP12.6と呼称されるアイソフォームは、リアノジン受容体2（RYR2）と結合することが知られている。RYR2は、心筋小胞体の主要な細胞内Ca^{2+}放出チャネルである。また、RYR2は、その他にも種々の細胞腫で発現している。しかしながら、RYR2にFKBP12.6が結合していることの生理的役割は、明らかでない。今回我々は、RYR2に結合しているFKBP12.6の生理的役割を明らかにしていく研究にプローブとして用いる目的で、FKBP12.6に真に特異的なモノクローナル抗体を作製した。His-FKBP12.6で免役したマウスのリンパ節細胞をミエローマ細胞株と融合して作製したハイブリドーマ群の中からFKBP12.6に特異的な抗体を産生する細胞をELISA法にて選別した。その結果、FKBP12.6は認識し、かつ、FKBP12.6と近縁のアイソフォームであるFKBP12を認識しないクローン2株（1B4/G3、1C2/G10）樹立した。モノクローナル1B4/G3抗体を用いたWestern blot法により、nativeなFKBP12.6をRYR2-FKBP12.6複合体を高濃度で含む画分である心筋小胞体から検出することができた。我々のモノクローナル抗体がFKBP12.6の生理的役割を解明していく上でのプローブとして有望であることが、今回の結果から示唆された。

キーワード：　FKBP12.6、リアノジン受容体、モノクローナル抗体

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