FK506 Binding Protein Associated with the Calcium Release Channel (Ryanodine Receptor)*

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The calcium release channel (CRC)/ryanodine receptor (RyRec) has been identified as the foot structure of the sarcoplasmic reticulum (SR) and provides the pathway for calcium efflux required for excitation-contraction coupling in skeletal muscle. The CRC has previously been reported to consist of four identical 565-kDa protomers. We now report the identification of a 12-kDa protein which is tightly associated with highly purified RyRec from rabbit skeletal muscle SR. N-terminal amino acid sequencing and cDNA cloning demonstrate that the 12-kDa protein from fast twitch skeletal muscle is the binding protein for the immunosuppressant drug FK506. In humans, FK506 binds to the 12-kDa FK506-binding protein (FKBP12) and blocks calcium-dependent T cell activation. We find that FKBP12 and the RyRec are tightly associated in skeletal muscle SR on the basis of: 1) co-purification through sequential heparin-agarose, hydroxylapatite, and size exclusion chromatography columns; 2) co-immunoprecipitation of the RyRec and FKBP12 with anti-FKBP12 antibodies; and 3) subcellular localization of both proteins to the terminal cisternae of the SR, and not in the longitudinal tubules of SR, in fast twitch skeletal muscle. The molar ratio of FKBP12 to RyRec in highly purified RyRec preparations is approximately 1.4, indicating that one FKBP12 molecule is associated with each calcium release channel/foot structure.

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In skeletal muscle, the triad junction mediates signal transfer from the plasmalemma, resulting in release of Ca2+ from the terminal cisternae of SR,1 a phenomenon referred to as excitation-contraction coupling (1). The RyRec of the SR has been purified from skeletal muscle and identified as the calcium release channel involved in excitation-contraction coupling (1-6). The RyRec has been identified morphologically as the foot structure of the triad junction, involved in the association of the transverse tubule with the terminal cisternae of SR (5-7). Molecular cloning of the cDNA encoding the RyRec has provided its primary sequence and the predicted size of its protomer (565,000 daltons) (8-10). The foot structure has been termed the junctional channel complex to indicate that it not only contains the calcium release channel but is directly involved in junctional association and senses the depolarization in the transverse tubule (8, 11). The junctional channel complex has a molecular mass of 2.3 million (12) and has 4-fold symmetry. It is, therefore, a homotetramer and the largest channel structure known (6, 7, 11).

The 12,000-Da binding protein for the immunosuppressant FK506 (FKBP12) is a cis-trans peptidyl-prolyl isomerase which has been isolated and cloned from human T cells (13, 14). The FK506-FKBP12 complex blocks IL-2 gene transcription required for T cell activation in a calcium-dependent manner (15). Little is known about the cellular role of FKBP12 protein in the absence of FK506. In the present study we examined the structure, expression, and subcellular localization of FKBP12 in skeletal muscle where we show it to be tightly associated with the calcium release channel involved in excitation-contraction coupling.

EXPERIMENTAL PROCEDURES

Co-purification of FKBP12 with the Ryanodine Receptor—Skeletal muscle RyRec was purified as described by Inui and Fleischer (16) on sequential columns: heparin-agarose, hydroxylapatite, and size exclusion chromatography using either a Pharmacia LKB Biotechnology Inc. Sepharose 6B or a Toso Haas TSK-gel G 4000 SW high pressure liquid chromatography column. Four separate preparations of purified RyRec were electrophoresed in reducing buffer through 12% gels in Tris-Tricine SDS-polyacrylamide (17) or through 5-15% gradient gels in Tris-glycine buffers (18). Each purified RyRec contained a low molecular weight polypeptide of relative molecular mass (M_r) 12 kDa. This polypeptide was observed by Coomassie Blue staining of SDS-polyacrylamide gel electrophoresis gels in four out of four different RyRec preparations (data not shown). The amount of FKBP12 in the purified RyRec was determined by gel densitometry using BSA (0.1-1 μg) as the protein standard with an automated gel analyzer and image processing system (Technology Resources, Nashville, TN). FKBP12 protein content for each RyRec preparation was calculated as molar ratios compared with the RyRec. Molar ratio calculations assume that the extinction coefficient of Coomassie Blue staining is the same for the FKBP12 and the RyRec protomer.

Obtaining Amino Acid Sequence and Isolation of Rabbit Skeletal Muscle FKBP12 cDNA—Amino acid sequence was obtained from the 12-kDa protein, which co-purified with the RyRec, for the purpose of designing synthetic oligonucleotides to be used as probes for cDNA cloning. Approximately 30 μg of rabbit skeletal muscle RyRec purified as described previously (16) were electrophoresed on a 15% polyacrylamide gel in 50 μl of Laemmli sample buffer (18) after heating at 95 °C for 2 min. Cathode and anode buffers were as described (17).

1 The abbreviations used are: SR, sarcoplasmic reticulum; FKBP12, FK506-binding protein; RyRec, ryanodine receptor; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; kb, kilobase(s).
The gel was electroblotted onto polyvinylidene difluoride, and two bands were visualized on the blot after staining with Coomassie Brilliant Blue (data not shown). The RyRec had barely entered the 15% separating gel and was clearly visible at the uppermost part of the gel; a faint 12-kDa band, estimated by comparison with standards to be less than 100 ng of protein, was observed and used for direct sequencing. The same Northern blot, which had been hybridized with the cDNA probe encoding the rabbit FKBP12; the RyRec cDNA probe was a 700-base pair rabbit skeletal muscle cDNA described previously (8). Hybridization was at 42 °C overnight and washing at 65 °C in 0.2 × SSC. Films were autoradiographed with a single intensifying screen at −70 °C for 24 h. To ensure that equivalent amounts of RNA were present in each lane, the same Northern blot was also hybridized with a cDNA encoding the glyceraldehyde-3-phosphate dehydrogenase from chicken muscle (data not shown).

RESULTS

To date, the calcium release channel has been considered to consist solely of four 565-kDa proteomers. We now report that an additional protein, FKBP12, has been found in four different samples of highly purified (>90% purity) RyRec prepared in our laboratory.

We have previously reported the extensive proteolytic mapping and microsequencing of highly purified RyRec from rabbit fast twitch skeletal muscle (8, 27). Thirty out of thirty-one peptides mapped to the 5,037-amino acid sequence of the RyRec proteome (8, 27). The only peptide which did not map to the deduced amino acid sequence of the RyRec was subsequently mapped to the NH2 terminus of the FKBP12 (28). Thus, our earlier results provided the initial indication that FKBP12 is tightly associated with the RyRec (8, 27).

The RyRec was purified using three sequential columns (heparin-agarose, hydroxyapatite, and size exclusion chromatography with either Sepharose 6B or TSK-gel G-4000 SW) essentially as described previously (16). High molecular weight bands other than the intact RyRec, seen in heavily loaded protein gels (Fig. 1), appear to be proteolytic breakdown products of the RyRec because: 1) addition of protease inhibitors greatly reduces their abundance (5); and 2) they react with polyclonal anti-RyRec antibodies.2 In the low molecular weight range, the only prominent band seen by Coomassie staining was a 12-kDa protein (Fig. 1). The NH2-terminal amino acid sequence (amino acids 3–18) and the complete deduced amino acid sequence of the 12-kDa protein determined by cDNA cloning from skeletal muscle (Fig. 2) are identical to that reported for the human T cell FKBP12 (14). The evolutionary conservation of sequence found in the FKBP12 (100% identity over the 108 amino acid residues between the rabbit skeletal muscle and the human Jurkat T cell forms) suggests that it performs a fundamental function in diverse tissues.

Having identified the 12-kDa protein as FKBP12, we sought to demonstrate that it was associated directly with the RyRec. To do so we used an anti-FKBP12 antibody to immunoprecipitate the RyRec. This anti-FKBP12 antibody was incubated with highly purified RyRec, and the resulting immunoprecipitate was electrophoresed on a 6% SDS-polyacrylamide gel (Fig. 3a). A 565-kDa band which co-migrates with purified RyRec was seen after electrophoresis followed by staining with Coomassie Brilliant Blue (Fig. 3a). Immunoblotting showed that this 565-kDa band was recognized by a polyclonal anti-RyRec antibody but not by the FKBP12 antibody (data not shown). The anti-FKBP12 antibody was not able to immunoprecipitate RyRec which had been electrophoresed from a Coomassie-stained gel (and thus separated from FKBP12). Taken together these data indicate a tight association between the FKBP12 and the RyRec.

To determine whether the skeletal muscle FKBP12 co-localized with the RyRec in the terminal cisterna of the SR, sucrose gradient fractions of SR longitudinal tubules (23), SR terminal cisternae (24), and highly purified preparations of RyRec were immunoblotted with anti-FKBP12 antibody (Fig. 3b).

A. Timerman and S. Fleischer, unpublished studies.
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Fig. 1. FKBP12 is present in a highly purified RyRec preparation. Electrophoresis on a 5–15% polyacrylamide gradient gel of bovine serum albumin (BSA, 1 µg) is shown in nonreducing conditions (lane 1), molecular mass markers (lanes 2), and 10 µg of purified RyRec (lane 3, both in reducing conditions). The position of the 12-kDa FKBP12 is indicated by the arrow on the right. Four different RyRec preparations were analyzed (data not shown). The molar ratio of FKBP12 is indicated by the standard curve used for densitometric estimation of the FKBP12 concentration. Molecular separation. Electrophoresis on a 5–15% polyacrylamide gradient gel of bovine carbonic anhydrase (31 kDa), ovalbumin (42.7 kDa), rabbit muscle phosphorylase b (97.4 kDa). The BSA in reducing conditions, include (from bottom to top): hen egg white lysozyme (14.4 kDa), soybean trypsin inhibitor (21.5 kDa), bovine carbonic anhydrase (31 kDa), ovalbumin (42.7 kDa), and rabbit muscle phosphorylase b (97.4 kDa). The BSA in lane 1 represents the high range of the standard curve used for densitometric estimation of the FKBP12 quantity. Note that BSA mobility differs from that under reducing conditions (lane 2).

Fig. 2. Comparison of the nucleotide sequence for the human (Hum, first line) and rabbit (Rab, second line) FKBP12 (top two lines) and the deduced amino acid sequence (third line) of the rabbit fast twitch skeletal muscle FKBP12. The deduced amino acid sequence of the rabbit skeletal muscle FKBP12 is identical to that reported for the human T cell FKBP12 (14). The underlined sequence indicates the region near the amino terminus for which amino acid sequence was obtained by the microsequencing of the 12-kDa protein which co-purified with the RyRec (as shown in Fig. 1). Vertical lines (!) indicate sequence identity. Lower case letters in the top line represent base pair mismatches between the human (lower case) and rabbit (upper case) sequences. The final TGA is the stop codon.

3b). These immunoblots showed that FKBP12 was present in the purified RyRec and the terminal cisternae but not in longitudinal tubules (Fig. 3b). These results indicate that FKBP12 is associated specifically with the terminal cisternae of SR.

Northern blot analysis, using a rabbit skeletal muscle FKBP12 cDNA probe, showed that the 1.5-kb FKBP12 mRNA was found in all forms of muscle examined, including smooth, cardiac, and fast (Fig. 4) and slow twitch skeletal muscles (data not shown).

Discussion

One function of the FK506-FKBP12 complex is to block T cell activation. The FK506-FKBP12 complex blocks T cell activation by inhibiting the antigen-induced transcription of the IL-2 gene. It has been shown that the FK506-FKBP12 complex inhibits the calcium-induced translocation of the transacting factor NF-AT (nuclear factor of activated cells)
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which binds to the transcriptional regulatory region of the IL-2 gene (15). Independently, it has been shown that the FK506-FKBP12 complex inhibits the calcium-activated phosphatase calcineurin (29). However, the cellular function of FKBP12 in the absence of FK506 remains to be elucidated. FKBP12 had previously been regarded as a cytosolic protein; the present study demonstrates the tight association between FKBP12 and the ryanodine receptor, and the localization of FKBP12 to the terminal cisternae of the sarcoplasmic reticulum.

FKBP12 which we have purified and cloned from rabbit skeletal muscle is identical in primary structure to the major cytoplasmic form of FKBP12 in human T cells (14) (Fig. 2). The FKBP12 associated with the RyRec in skeletal muscle is identical in primary structure to the major cytosolic form of FKBP12 in human T cells (14) (Fig. 2). It remains to be elucidated. FKBP12 in the absence of FK506 remains to be elucidated. FKBP12 and the ryanodine receptor, and the localization of FKBP12 to the terminal cisternae of the sarcoplasmic reticulum.

FKBP12 is a member of a family of proteins known as immunophilins. This family of immunophilins includes cyclophilin, the binding protein for cyclosporin A (30, 31). FKBP12 and cyclophilin are both cis-trans peptidyl-prolyl isomerases whose primary structures are unrelated. FK506 and cyclosporin A have been reported to inhibit T cell activation pathways under conditions in which activation is associated with increased cytoplasmic calcium (32, 33). On the basis of this observation it has been proposed that FKBP12 may regulate the conformation of ion channels involved in the signal transduction pathway for T cell activation (34). Furthermore, a recent study reported that FK506, cyclosporin A, and rapamycin all significantly decreased the magnitude of intracellular Ca\(^{2+}\) release typically seen after activation of the T cell receptor in human peripheral blood T lymphocytes (35). The present study is the first to report a direct association between FKBP12 and a calcium channel. It remains to be determined whether FKBP12 is associated with calcium channels in T cells.

The cis-trans peptidyl-prolyl isomerase activity of the FKBP12 raises the question of whether it might isomerize a peptidyl-proline bond in the RyRec. Proline residues are found in or near the transmembrane regions of transport proteins (36). The putative M2 transmembrane region near the carboxyl terminus of the skeletal muscle RyRec contains a proline residue (residue 4641). The finding that FKBP12 is present in the terminal cisternae of the SR and is tightly associated with the RyRec suggests that one of its cellular functions may be to alter channel conformation.

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