Interaction of FKBP12.6 with the Cardiac Ryanodine Receptor C-terminal Domain*

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The ryanodine receptor-calcium release channel complex (RyR) plays a pivotal role in excitation-contraction coupling in skeletal and cardiac muscle. RyR channel activity is modulated by interaction with FK506-binding protein (FKBP), and disruption of the RyR-FKBP association has been implicated in cardiomyopathy, cardiac hypertrophy, and heart failure. Evidence for an interaction between RyR and FKBP is well documented, both in skeletal muscle (RyR1-FKBP12) and in cardiac muscle (RyR2-FKBP12.6), however definition of the FKBP-binding site remains elusive. Early reports proposed interaction of a short RyR central domain with FKBP12/12.6, however this site has been questioned, and recently an alternative FKBP12.6 interaction site has been identified within the N-terminal half of RyR2. In this study, we report evidence for the human RyR2 C-terminal domain as a novel FKBP12.6-binding site. Using competition binding assays, we find that short C-terminal RyR2 fragments can displace bound FKBP12.6 from the native RyR2, although they are unable to exclusively support interaction with FKBP12.6. However, expression of a large RyR2 C-terminal construct in mammalian cells encompassing the pore-forming transmembrane domains exhibits rapamycin-sensitive binding specifically to FKBP12.6 but not to FKBP12. We also obtained some evidence for involvement of the RyR2 N-terminal, but not the central domain, in FKBP12.6 interaction. Our studies suggest that a novel interaction site for FKBP12.6 may be present at the RyR2 C terminus, proximal to the channel pore, a sterically appropriate location that would enable this protein to play a central role in the modulation of this critical ion channel.

Ryanodine receptors (RyRs) are intracellular Ca\(^{2+}\) channels located in the sarco(endo)plasmic reticulum of muscle and non-muscle cells. They govern the release of Ca\(^{2+}\) from intracellular stores and play an essential role in various cellular processes, including muscle contraction, neurotransmission, secretion, and apoptosis. Three genes coding for mammalian RyRs have been identified; ryr1, the major form expressed in skeletal muscle, ryr2, the major form expressed in heart and brain, and ryr3, which is found at low levels in a number of tissues.

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‡ The abbreviations used are: RyR, ryanodine receptor; FKBP, FK506-binding protein; SR, sarcomplasmic reticulum; IP, immunoprecipitation; GST, glutathione S-transferase; CHAPS, 3-(3-cholamido
dimethylammonio)-1-propanesulfonic acid; Ab, antibody; PBS, phosphate-buffered saline; Pipes, 1,4-piperazinediethanesulfonic acid.

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† The abbreviations used are: RyR, ryanodine receptor; FKBP, FK506-binding protein; SR, sarcomplasmic reticulum; IP, immunoprecipitation; GST, glutathione S-transferase; CHAPS, 3-(3-cholamidodimethylammonio)-1-propanesulfonic acid; Ab, antibody; PBS, phosphate-buffered saline; Pipes, 1,4-piperazinediethanesulfonic acid.

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FIG. 1. Schematic diagram of RyR2 overlapping fragments. The complete human RyR2 protein coding sequence of 4967 amino acids is depicted schematically by the top bar with the three main divergent regions (D1, D2, and D3) shown in gray. The ten putative membrane-spanning domains at the C terminus are numbered below the full-length sequence. The series of overlapping RyR2 protein constructs prepared, and the epitope sites of the antibodies used in this study, are illustrated schematically below the full-length RyR2.

dues 2358–2530, or mutations within the central domain, did not prevent GST-FKBP12.6 from binding to RyR2 mutant channels expressed in HEK293 cells (30). In agreement with the latter report, we were also unable to demonstrate an interaction between fragments containing the previously suggested FKBP-binding site in any of the three mammalian RyR isoforms, with either FKBP12 or FKBP12.6, using two different techniques, the yeast two-hybrid and co-immunoprecipitation experiments (31). Recently, the N-terminal region of the RyR2 has been implicated in FKBP12.6 binding. Using co-immunoprecipitation assays and GST-FKBP affinity chromatography, the first 1855 N-terminal residues of RyR2 was shown to interact with FKBP12.6 (30, 32).

Our studies indicated that there is no interaction between FKBP12.6 and a series of overlapping fragments encompassing the entire RyR2 (31), suggesting that short peptide fragments (<900 residues) containing the central region or other domains of the cardiac RyR are not sufficient to support the interaction with FKBP12.6 (31). Due to the controversy with regard to the precise identity of the FKBP-binding site on the RyR, we undertook a strategy to indirectly identify the FKBP12.6 interaction site(s) on RyR2 using the aforementioned RyR2 overlapping fragments. Using solubilized cardiac heavy sarcoplasmic reticulum (SR) vesicles and competition binding experiments, we report here that C-terminal fragments (spanning RyR2 residues 3788–4765) displace bound FKBP12.6 from the native channel. RyR2 N-terminal or central domain constructs displayed no effect on the RyR2-FKBP12.6 interaction. Furthermore, an RyR2 C-terminal construct encompassing the pore-forming transmembrane domains (amino acids 3788–4967), was shown to interact with FKBP12.6 in a rapamycin-sensitive way. Under identical conditions, FKBP12 failed to interact with the RyR2 C terminus. In addition, we found that a large RyR2 N-terminal fragment (amino acids 1–2064) was also capable of FKBP12.6 binding, however protein expression differences prevented a direct comparison of the FKBP12.6-binding affinities of these two RyR2 domains.

EXPERIMENTAL PROCEDURES

Materials—Cell culture reagents were from Invitrogen. Radioactive sulfur was obtained from Amersham Biosciences in the form of Pro-Mix containing ~70% L-[^35]S)methionine and 30% L-[^35]S)cysteine. Pfu DNA polymerase was obtained from Promega, and oligonucleotides were from MWG Biotech or Sigma-Genosys. DNA restriction endonucleases and other DNA-modifying enzymes were from Amersham Biosciences. CHAPS and rapamycin were obtained from Calbiochem. Electrophoresis reagents were from Bio-Rad. All other reagents were from Sigma. Antibodies—Ab[^2149], a mouse monoclonal to c-Myc antigen (9E10, Santa Cruz Biotechnology), was used at 1:500 dilution for Western blotting and 1:100 dilution for immunoprecipitation (IP). Ab[^2160], a goat polyclonal to FKBP (C-19, Santa Cruz Biotechnology), was used at 1:200 dilution for Western blotting.

The RyR antibodies, 33, 1093, 129, 2149, and 2160, were used at 1:500 dilution for Western blotting and 1:40 dilution for IP. The RyR2-specific Ab[^33] (epitope at residues 4454–4474) (33) and Ab[^2160] (epitope at residues 4673–4697) (34), and the RyR consensus Ab[^2896] (epitope at residues 4950–4967) (35) have been described previously. The RyR consensus Ab[^33] was produced in rabbits against a synthetic peptide, TLTAKEKAKDREKAQDILKFL, which corresponds to residues 2876–2896. The RyR consensus Ab[^2896] was produced in rabbits against a synthetic peptide, TQEQSYWKMYQER, which corresponds to residues 4933–4948.

Plasmid Construction—Preparation of the human RyR2-overlapping fragments (BT constructs, Fig. 1), human FKBP12 and FKBP12.6 have been recently described (31).

A large RyR2 C-terminal construct (pc-MycRyR2C), encoding amino acids 3788–4967 with an N-terminal c-Myc tag, was generated as follows: the BT71 cDNA insert together with the c-Myc tag-encoding sequence was PCR-amplified using primers annealing on the pGBK7 vector, and cloned in the mammalian expression vector pCR3 (Invitrogen) using the HindIII-Xho1 sites. This construct was extended to the end of the hRyR2 coding sequence by transferring the FseI11505 to Xho1-15335 cDNA fragment from the full-length hRyR2.

A large RyR2 N-terminal construct (pc-Myc1–2064), encoding amino acids 1–2064 with an N-terminal c-Myc tag, was generated as follows: the BT4 cDNA insert (nucleotides 122–2398) was subcloned in the c-Myc-modified pCR3 vector using the NdeI-SalI sites. This construct was extended to nucleotide 4987 by transferring the BT3 cDNA sequence (nucleotides 2153–4987) using the BglII-2398 and SalI (vector) sites. The latter construct was extended to nucleotide 6315 by transferring the BamHI-4987 to NcoI-6315 cDNA fragment from BT5 (nucleotides 4811–7645); the NcoI site at the 3′-end was blunted using T4 DNA polymerase and ligated into the SalI site of the vector.

Cell-free Protein Expression—In vitro cell-free protein expression was carried out using the TnT T7 Quick-coupled transcription and translation system (Promega). Reactions were carried out in 10-μl volumes by adding the TnT mix with 1 μl of phosphatidyl DNA together with 1 μl (0.53 MBq or 14 μCi) of [35S]labeled methionine (Promo-Mix, Amersham Biosciences) or 1 μl of 1 mCi unlabeled methionine. Where necessary, 1.5 μl of canine pancreatic microsomes (Promega) was included.
Reagent samples were incubated in a 30 °C water bath for 90 min and terminated by placing on ice.

**Cell Culture, Transfection, and Immunolocalization—**HEK293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, in a humidified atmosphere of 5% CO2 at 37 °C. Cells at 80–90% confluence were transiently transfected with the pc-MycRyR2C or pc-Myc-2064 plasmids using either Lipofectamine2000 (Invitrogen) or the calcium phosphate precipitation method, depending on the nature of the experiment (see below). For microscopy preparation and use in co-immunoprecipitation experiments, eight 100-mm Petri dishes were transfected with 24 μg of pc-MycRyR2C plasmid DNA per dish using Lipofectamine2000, according to the manufacturer's instructions, at a 1:2 plasmid DNA:transfection reagent ratio. Cells were harvested 24 h post-transfection, and crude microsomes were prepared as described below. For cell lysate preparation and use in co-immunoprecipitation experiments, six 100-mm Petri dishes were transfected with 24 μg of either pc-Myc1–2064 or pc-MycRyR2C plasmid DNA per dish, using the calcium phosphate precipitation method according to procedures described elsewhere (36). Cells were harvested 24 h post-transfection, and cell lysates were prepared as described below.

Transfected cells grown on glass coverslips were washed in PBS (157 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4), trypsinized on ice by 20 passages through a needle (23-gauge, 0.6 × 25 mm) and dispersing the cell suspension through a half volume of glass beads (425–600 μm, Sigma). Cell nuclei and glass beads were removed by centrifugation at 1,500 × g for 10 min at 4 °C, and the supernatant was subjected to centrifugation at ~120,000 × g (30,000 rpm, Beckman 45-Ti rotor) for 45 min at 4 °C. The pellet representing the crude microsomal fraction was resuspended in IP buffer (20 mM Tris, 150 mM NaCl, 0.4% CHAPS, 2 mM dithiothreitol, pH 7.4) by incubation for 1 h at 4 °C. The crude microsomes were re-suspended in IP buffer supplemented with Complete protease inhibitors (Roche Applied Science) at one freeze-thaw cycle and automated sonication (Leica Ultrasonic 2064 using the Lipofectamine 2000 transfection reagent).

**Cell Lysate and Microsome Preparation—**Crude microsomes were prepared as follows: HEK293 cells were resuspended in homogenization buffer (0.3 M sucrose, 5 mM Hepes, pH 7.4), (~ 15 × 106 cells/ml buffer, supplemented with Complete protease inhibitors (Roche Applied Science) at one freeze-thaw cycle and automated sonication (Leica Ultrasonic 2064). The homogenate was divided in two aliquots, and samples were incubated overnight at 4 °C. Microsomal membranes were sedimented at ~120,000 × g (55,000 rpm, Beckman TLA100.4 rotor) for 1 h at 4 °C, resuspended in SDS-PAGE loading buffer (8 M urea, 3% SDS, 10% glycerol, 5 mM EDTA, 2% β-mercaptoethanol, 0.01% bromphenol blue, pH 6.8), heated at 85 °C for 5 min, and analyzed by SDS-PAGE and Western blotting using Ab21 derivative, an antibody that recognizes both FKBP12/12.6.

**Preparation of Cardiac Heavy SR Vesicles—**Cardiac heavy SR vesicles from pig hearts were prepared as described by Meissner and Henderson (37) and modified by Sitsapesan and Williams (38). Briefly, ventricle muscle was homogenized in four volumes of homogenization buffer (10 mM Pipes, 0.3 M sucrose, pH 7.4, and protease inhibitors, 2 mM benzamidine, 0.2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, and 10 μM leupeptin) with the use of a blender, and the homogenate was centrifuged at 8,000 × g for 20 min at 4 °C. The pellet was re-homogenized in one volume of homogenization buffer, and the centrifugation step was repeated. The two supernatants were combined, filtered through four sheets of cheesecloth, and centrifuged at ~120,000 × g (30,000 rpm, Beckman 45-Ti rotor) for 45 min at 4 °C. The pellet representing the crude cardiac microsomal fraction was resuspended in gradient buffer (10 mM Pipes, 0.4 M KCl, 0.5 mM MgCl2, 0.5 mM EGTA, 0.55 M KCl, pH 7.0) layered on a discontinuous sucrose gradient consisting of three layers of 40%, 30%, and 20% sucrose (in the salt solution used for the gradient buffer). The gradients were centrifuged at ~70,000 × g (20,000 rpm, Beckman SW28.1 rotor) for 14 h at 4 °C, and the 30–40% interface containing the heavy SR vesicles was collected and diluted with an equal volume of 10 mM Pipes, pH 7.4. Cardiac heavy SR vesicles were pelleted at ~125,000 × g (30,000 rpm, Beckman 50.2 Ti rotor) for 1 h at 4 °C, resuspended in homogenization buffer at 20 μg/ml protein concentration, and stored at ~80 °C.

**Co-immunoprecipitation Assays—**In vitro expressed proteins, produced in individual TNT reactions (Promega), were mixed and solubilized in 200 μl of IP buffer (20 mM Tris, 150 mM NaCl, 0.4% CHAPS, 2 mM dithiothreitol, pH 7.4) by incubation for 1 h at 4 °C. A specific anti-mouse Alexa Fluor488-conjugated antibody (Molecular Probes) was added at the appropriate dilution, and the sample was incubated for 2 h at 4 °C with continuous mixing, followed by addition of 20 μl of protein G Dynabeads (Dynal) and incubation for a further 2 h. Protein immunocomplexes were isolated with the use of a magnetic particle concentrator (MPC-S, Dynal), and beads were washed three times with IP buffer. Immunoprecipitated proteins were resuspended in SDS-PAGE loading buffer, heated at 85 °C for 5 min, and analyzed by SDS-PAGE and Western blotting. Where more than one sample of the same protein were to be processed, the individual TNT reactions (of the same protein) were mixed and an equal aliquot was distributed to the IP samples, to minimize variations in protein expression.

For the experiments with native RyR2, cardiac heavy SR vesicles (1 mg) were solubilized in 200 μl of IP buffer by overnight incubation at 4 °C, with continuous mixing. The insoluble material was pelleted at 125,000 × g (30,000 rpm, Beckman 45-Ti rotor) for 45 min at 4 °C, and the supernatant was withdrawn, mixed with the in vitro synthesized, radiolabeled FKBP protein for 1 h at room temperature, and processed as described above.

For the expression studies in HEK293 cells and use of crude microsomes, the supernatants (~ 1.5 mg) were solubilized in 200 μl of IP buffer by overnight incubation at 4 °C with continuous mixing. The insoluble material was pelleted at 125,000 × g (30,000 rpm, Beckman 45-Ti rotor) for 45 min at 4 °C, and the supernatant was withdrawn, mixed overnight with the in vitro synthesized, radiolabeled FKBP protein for 4 h, and processed as described above.

For the expression studies in HEK293 cells and use of whole cell lysates, the cell lysates (~ 8 mg) were incubated with Ab21 derivative (2 μg) and 50 μl of protein G Dynabeads (Dynal) for 8 h at 4 °C with continuous mixing to isolate the c-Myc-tagged RyR2 fragments. Protein immunocomplexes were isolated, the beads were washed three times with IP buffer, and immunoprecipitated proteins were resuspended in SDS-PAGE loading buffer, heated at 85 °C for 5 min, and analyzed by SDS-PAGE and autoradiography.

**Western Blot Analysis—**Proteins from SDS-PAGE gels were transferred to a polyvinylidene difluoride membrane (Immobilon, Millipore) using a semi-dry transfer system (Trans-Blot SD, Bio-Rad) in transfer buffer (48 mM Tris, 39 mM glycine, 0.0375% SDS, 20% methanol) at 20 V for 30 min to 2 h, depending on protein size. The membrane was blocked with 5% nonfat milk protein (Marvel) in TBS-T buffer (20 mM Tris, 150 mM NaCl, pH 7.4) for 1 h at room temperature, and processed as described above.

**Autoradiography—**Radiolabeled proteins were separated by SDS-PAGE, and the gel was fixed (40% methanol, 10% acetic acid) for 30 min. The fixing solution was removed, and the gel was incubated with...
a fluorographic enhancer (Amplify, Amersham Biosciences) for a further 30 min and subsequently dried. The dried gel was exposed to an x-ray film (Kodak) at −80 °C for variable periods of time. Densitometric analysis was performed using a GS-700 scanner (Bio-Rad) and Quantity One software (Bio-Rad).

RESULTS

RyR2 C-terminal Fragments Compete for FKBP12.6 Binding with the Native Channel—We recently demonstrated that the native RyR2 interacts with FKBP12.6 in a rapamycin-sensitive way but not with FKBP12; in contrast, the native RyR1 was found to interact with both the FKBP12s, 12 and 12.6 (31). We further investigated the association of RyR2 with FKBP12.6 by co-immunoprecipitation using a panel of RyR-specific antibodies that recognize distinct sequence epitopes. Solubilized pig cardiac heavy SR vesicles were incubated with in vitro synthesized, radiolabeled FKBP12.6, the RyR2 was immunoprecipitated by co-precipitation with the extreme RyR C-terminal tail antibodies Ab2160 and Ab2149, whereas lower levels were recovered with Ab2160 and Ab2149, which were also precipitated by co-precipitation with the extreme C-terminal tail. The lower recovery by the C-terminal RyR antibodies is most likely due to a steric effect on immunoprecipitation efficiency due to the reduced accessibility of the native RyR2 C terminus (39).

The FKBP co-IP experiments using RyR-specific antibodies establish a reliable system to monitor the interaction of FKBP12.6 with native RyR2, which was then extended to examine the effects of overlapping RyR2 fragments on this association. Our hypothesis was that, if a determinant of the FKBP-binding site is contained within the in vitro synthesized RyR2 constructs, then this specific construct would be able to compete for, and displace the binding of FKBP12.6 protein on the native RyR2. Thus, we performed co-IP experiments with RyR2 from solubilized cardiac heavy SR vesicles incubated with in vitro synthesized, radiolabeled FKBP12.6, in the presence of a 5-fold excess of various non-radiolabeled RyR2 fragments encoding disparate regions of the RyR2 protein. The results obtained for the RyR2 fragments were normalized against BT71, a region of the RyR2 (residues 3791–4204) that previously had not been implicated in FKBP binding.

Co-IPs following incubation with a series of RyR2 constructs containing the previously suggested, central domain FKBP-binding site (i.e. BT5, BT6, and BT2R2), and a variety of N-terminal constructs (BT4, BT3, and BT7), which together comprise the large cytoplasmic portion of the RyR2 molecule (~80%; residues 1–3940), did not show any effect on FKBP12.6 binding to the native RyR2 (Fig. 3). In contrast, a series of C-terminal constructs BT71, BT1A2, BT1D2, and BTIB2 encompassing residues 3788–4765, were able to displace bound FKBP12.6 from the native RyR2. Densitometric analysis indicates that presence of the C-terminal constructs result in a >70% reduction in FKBP12.6 recovery in the RyR2 immunoprecipitate compared with N-terminal and central domain fragments (Fig. 3C).

Association of the RyR2 C-terminal Portion with FKBP12.6—Displacement of bound FKBP12.6 from the native channel by C-terminal RyR2 constructs suggests that this region of the molecule contains determinants for the FKBP-binding site. We therefore generated a large RyR2 C-terminal construct (pc-MycRyR2C) encoding amino acids 3788–4967, to examine whether it displayed specific FKBP-binding properties. In previous studies using yeast two-hybrid interaction assays with expressed, short RyR2 fragments, reconstitution of robust FKBP interaction was not detected with any region of the RyR2 molecule (31).

The pc-MycRyR2C construct expressed in the cell-free TNT system as a doublet with peptides of ~140 and 110 kDa, irrespective of the presence of pancreatic microsomal membranes in the TNT system (Fig. 4A). The lower 110-kDa band corresponds to a C-terminal truncated peptide product, because IP with the extreme RyR C-terminal tail antibodies Ab2160 and Ab2149 precipitated only the upper 140-kDa band, whereas antibodies Ab1093 and Ab-Myc precipitated both bands. In addition, Western blot analysis using either Ab2160 or Ab2149 detected only the upper 140-kDa band, whereas Ab1093 detected both bands (not shown).

The pc-MycRyR2C construct, expressed as an unlabeled protein in the TNT system in the presence of pancreatic microsomal membranes, was incubated with radiolabeled FKBP, followed by IP with Ab-Myc, and the presence of co-precipitated FKBP was analyzed by autoradiography. As shown in Fig. 4C, we found that the pc-MycRyR2C fragment specifically interacts with FKBP12.6, but not with FKBP12, and FKBP12.6 binding was abolished by rapamycin (20 μM). Under identical conditions, BT7 (containing the previously suggested, central domain FKBP-binding site) failed to interact with FKBP12.6, in agreement with our previous report (31). The interaction of the RyR2 C-terminal fragment with FKBP12.6 was detected only when the pc-MycRyR2C construct was expressed in the presence of pancreatic microsomal membranes in the TNT system (not shown). In these experiments the RyR2 fragments were expressed as unlabeled proteins, because some of their degradation products obscured the radiolabeled FKBP band due to the prolonged exposure of the autoradiograms. Thus, their expression was separately verified by Western blot analysis (Fig. 4B).

The FKBP interaction assay was also applied to the pc-MycRyR2C fragment expressed in mammalian HEK293 cells. HEK293 cells are devoid of RyR (40–42) and are the most widely used mammalian cell line for expression studies of these proteins. The pc-MycRyR2C construct is expressed as a single band of about 140 kDa in HEK cells, and subcellular fractionation indicates that the protein is exclusively found in the crude microsomal fraction (Fig. 5A). Consistent with this, a single band of ~140 kDa was also detected by the RyR-specific antibodies, Ab1093 and Ab2149 (not shown). Immunocytochemical analysis further demonstrated a reticular distribution of the expressed pc-MycRyR2C (Fig. 5B), in accord with the subcellular fractionation data (Fig. 5A). Immuno precipitation of the pc-MycRyR2C-encoded protein from solubilized microsomes prepared from transiently transfected HEK293 cells, after incubation with exogenous, in vitro synthesized, radiola-
beled FKBP, resulted in the specific co-precipitation of FKBP12.6, but not FKBP12 (Fig. 5C). Importantly, in control experiments, there was no FKBP12.6 recovered in the presence of rapamycin (20 μM), or when solubilized microsomes prepared from untransfected cells were used.

Interaction of the FKBP proteins with the RyR2 C-terminal fragment was further examined under more physiological conditions. In these experiments, the FKBP and pc-MycRyR2C plasmids were co-expressed in HEK293 cells, and evidence for binding was monitored by assaying the amount of FKBP co-sedimenting with the microsomal membrane fraction. Centrifugation-based binding assays are often used in situations of rapid ligand dissociation and low affinity, because the receptor and ligand remain in equilibrium throughout the separation period, and they have been used for the study of the RyR-FKBP association (27, 29, 43, 44), as well as the RyR-calmodulin interaction (45). In addition, the assay takes place in a detergent-free environment where the RyR2 C-terminal fragment is most likely to retain a native conformation. Cells singly transfected with the FKBP plasmid only, or co-transfected with both the FKBP and pc-MycRyR2C plasmids, express similar levels of the FKBP protein (Fig. 6, lanes 1 and 2). Endogenous FKBP12/12.6 was not detected using our Western blot assay system (Fig. 6, lane 3). We found that a small fraction of FKBP12.6 is associated with microsomal membranes in a rapamycin-sensitive manner, even in untransfected cells (Fig. 6B). Importantly, the amount of FKBP12.6 co-sedimenting with microsomal membranes is greatly increased in pc-MycRyR2C-expressing cells suggesting a direct interaction of FKBP12.6 with the RyR2 C-terminal construct. In contrast, no interaction was observed between the pc-MycRyR2C construct and FKBP12 (Fig. 6A).

**Association of the RyR2 N-terminal Portion with FKBP12.6**—Recently, it was reported that a large N-terminal fragment of the cardiac ryanodine receptor is sufficient for the interaction with FKBP12.6 (30). The minimal peptide sequence retaining FKBP12.6 binding was further defined to be present within residues 1–1855 (32, 46).

We attempted to compare the FKBP12.6-binding affinity of the RyR2 N-terminal portion relative to that of the C-terminal portion. We therefore generated a large RyR2 N-terminal construct (pc-Myc1–2064) encoding the first 2064 N-terminal res-
Fig. 4. Expression of the RyR2 C terminus in a cell-free system and interaction with FKBP12.6. A, characterization of in vitro synthesized pc-MycRyR2C. Radiolabeled pc-MycRyR2C expressed in the cell-free TNT system in the presence of canine pancreatic microsomal membranes was immunoprecipitated as indicated and analyzed by SDS-PAGE (8% gel) and autoradiography. An aliquot of the TNT reaction, 10% of the volume processed in co-IP, was separated by SDS-PAGE (8% gel) and the blot was probed with Abc-Myc. C, FKBP12/12.6 were tested for an interaction with pc-MycRyR2C and BT6 constructs. Co-IP experiments of in vitro synthesized, unlabeled pc-MycRyR2C or BT6 with radiolabeled FKBP proteins was analyzed by autoradiography. An aliquot of the TNT reaction, 10% of the volume processed in co-IP, was included in the first two lanes of the autoradiogram.

**DISCUSSION**

It is now widely accepted that the FKBP is an important regulator of the RyR, a phenomenon underscored by evidence that a defective regulation of the RyR-FKBP association has been implicated in cardiomypathy (47), cardiac hypertrophy (48), heart failure (16, 49), and exercise-induced sudden cardiac death (50). This has recently led to the RyR-FKBP association being the target of a therapeutic approach for the treatment of heart failure with the use of the drug JTV519 (51). Despite its predicted vital importance in elucidating the physiology and pathology of heart function, the structural basis of the RyR-FKBP interaction remains unresolved. Evidence for two putative interaction sites involved in FKBP binding have been reported, one at the RyR central domain (27, 28) another one at the N-terminal domain (30). The present report now provides strong evidence for a third site of interaction with FKBP, at the RyR2 C terminus.

HEK293 cells incubated with in vitro synthesized, radiolabeled FKBP12.6. As shown in Fig. 7C, IP of the pc-Myc1–2064 construct with c-Myc antibody resulted in co-precipitation of FKBP12.6 in a rapamycin-sensitive way. However, this result was not reproducible as the interaction between the pc-Myc1–2064 fragment and FKBP12.6 was detected in only two out of six such experiments. In parallel experiments, an interaction between the RyR2 C-terminal fragment and FKBP12.6 was detected in ~70% of the experiments (n = 6). These observations may be due to the limited protein expression levels achieved for the RyR2 C- and N-terminal constructs, using HEK cell transfection with calcium phosphate.
FIG. 5. Expression of the RyR2 C terminus in HEK293 cells and interaction with FKBP12.6. A, subcellular fractionation of HEK293 cells expressing pc-MycRyR2C. HEK293 cells were transfected with pc-MycRyR2C using the cationic lipid-mediated method and harvested 24 h post transfection. Subcellular fractions were prepared from transfected or untransfected cells as indicated, and 10 μg of protein per fraction was analyzed by SDS-PAGE (8% gel) and Western blotting using Abc-Myc. B, immunocytochemistry of pc-MycRyR2C-expressing cells. HEK293 cells were transfected with pc-MycRyR2C using the cationic lipid-mediated method and the protein was immunolocalized 24-h post transfection using Abc-Myc. C, FKBP12/12.6 were tested for an interaction with pc-MycRyR2C expressed in HEK293 cells by co-IP experiments and detection by autoradiography (15% SDS-PAGE gel). Solubilized crude microsomes (1.5 mg) prepared from untransfected HEK293 cells or cells expressing the pc-MycRyR2C construct were incubated with in vitro synthesized, radiolabeled FKBP12/12.6 in the presence or absence of 20 μM rapamycin (RAP) as indicated; proteins were immunoprecipitated by Abc-Myc, and presence of co-precipitated FKBP was analyzed by autoradiography. An aliquot of the TNT reaction, 1% of the volume processed in co-IP, was included in the first two lanes of the autoradiogram.

FIG. 6. Increased FKBP12.6 content of crude microsomal membranes in HEK293 cells expressing the RyR2 C terminus. FKBP12/12.6 was tested for an interaction with pc-MycRyR2C expressed in HEK293 cells by co-sedimentation experiments and detection by Western blotting (15% SDS-PAGE gels). HEK293 cells transfected with FKBP12 (A) or FKBP12.6 (B) were subsequently transfected with pc-MycRyR2C and subcellular fractions were prepared. Cell homogenates (1.5 mg) prepared from cells expressing FKBP only, cells co-expressing FKBP and pc-MycRyR2C, or from untransfected cells were incubated with or without 20 μM rapamycin (RAP) as indicated, crude microsomes were pelleted at 120,000 × g for 1 h, and pellets were analyzed by Western blotting using an antibody that recognizes both FKBP12/12.6. The first three lanes include 100 μg of homogenate prepared from cells expressing FKBP only, cells co-expressing FKBP and pc-MycRyR2C, or from untransfected cells.
Our recent yeast two-hybrid assay and IP study (31) concurs with the result of Masumiya and co-workers (30) using GST pull-down methods indicating that a short, central domain RyR2 fragment is insufficient to fully constitute the interaction site of RyR2 with FKBP12.6. This view is discordant with previous studies on RyR1 and RyR3, which led to the proposal that the central domain residue Val-2461 in RyR1, and the corresponding Val-2322 in RyR3, are indispensable for FKBP12/12.6 binding (27, 28). Possible explanations for the discrepant conclusions are that the RyR2 central domain, specifically, is not involved in the interaction with FKBP12.6, in contrast to the other two RyR isoforms, or that the central domain site of RyR2 is of very low affinity compared with other candidate sites that may also play a role in FKBP12.6 interaction. An alternative explanation is that the RyR2 central domain does not assume the correct conformation when expressed as a short peptide fragment, and needs to be in the context of the full-length RyR to constitute the FKBP12.6 site.

In the present study, we adopted a novel strategy to indirectly identify potential candidate RyR2 sites for the interaction with FKBP12.6, employing a robust co-IP methodology that worked reliably with a variety of RyR antibodies targeted to distinct epitopes (Fig. 2). By using this co-IP method in competition binding assays, we found that a number of small C-terminal RyR2 fragments were able to reduce the level of FKBP12.6 binding to the native cardiac muscle receptor by ~70% (Fig. 3), although previously we found these fragments were not able to individually bind to FKBP12.6 (31). This result prompted us to construct a large RyR2 C-terminal fragment (containing the extreme C-terminal 1179 residues) and examine the ability of this RyR2 domain, comprising one quarter of the entire RyR2, to directly bind to FKBP12.6. The C-terminal construct, either synthesized in a cell-free system or expressed in mammalian HEK293 cells, was found to consistently co-IP with FKBP12.6 in a rapamycin-sensitive manner (Figs. 4 and 5). In contrast, no interaction with the highly homologous FKBP12 isoform was detected under identical conditions, indicating a stringent isoform-specificity between RyR2 and FKBP12.6, a property not shared with RyR1, which can bind to both FKBP isoforms. In the in vitro synthesis reaction for the C terminus, there was a need for pancreatic microsomal membranes to be
present, indicating a requirement for membrane incorporation of the hydrophobic C terminus to assume the correct conformation to provide the FKBP12.6-binding site. Consistent with this observation, when expressed in HEK293 cells, the RyR2 C terminus was localized to internal membranes and showed no appreciable degradation, suggesting correct subcellular membrane targeting and protein folding. In addition, the positive RyR2 C terminus interaction with FKBP12.6 was demonstrated under more “physiological” conditions, where the two proteins were co-expressed in HEK293 cells and then evidence for binding was tested by co-sedimentation assays. We found that co-expression of the RyR2 C-terminal fragment specifically induced an enrichment in the FKBP12.6-content, but not the FKBP12-content, of microsomal membranes (Fig. 6). These findings are complementary with data for RyR2 C-terminal binding to FKBP12.6 obtained using the co-IP and competition binding assays. We could not further localize the FKBP12.6-binding site within the RyR2 C terminus, because smaller fragments did not retain the interaction (31), suggesting that the intact RyR2 C-terminal, predicted transmembrane portion, comprising 24% of the RyR2, is minimally required. This may not be surprising, because, in the case of other interacting proteins, there is a strict requirement for FKBP12 association upon the presence of either the intact protein, e.g. aspartokinase (52), FAP48 (53), or a large, well defined structural domain of a protein, e.g. the cytoplasmic domain of the type I transforming growth factor-β receptor (54, 55).

In disparity with our results, Masumiya and co-workers (30) did not detect an interaction between FKBP12.6 and the C-terminal portion of the RyR2, whereas an interaction with the N terminus was readily detected. A lack of association between the RyR2 C terminus and FKBP12.6 could be due to the GST-FKBP12.6 affinity assay employed. The GST fusion moiety might afford a degree of steric hindrance and prevent FKBP12.6 binding to the RyR2 C-terminal fragment. In this case, an unattached FKBP12.6 N terminus may be required for interaction with the RyR2 C-terminal portion when the latter is in the context of the full-length RyR2, due to further stabilization via additional contact sites. Requirement for a free N terminus for FKBP would suggest that this specific region of the molecule represents the major interaction site within FKBP12.6, with some low affinity binding via the FKBP12.6 C terminus. Alternatively, the GST pull-down assay, in which FKBP12.6-interacting RyR2 fragments were identified by Coomassie staining of proteins after SDS-PAGE, may not provide sufficiently sensitive detection of interactions that occur via the C terminus (30). In our assays using radiolabeled FKBPbs, the RyR2 N terminus interaction with FKBP12.6 was detected, but was not reproducibly observed (33%), suggesting this region may provide a low affinity or transient interaction (Fig. 7). This lower detection rate could also be due to the relatively low levels of the RyR2 N-terminal fragment being expressed in HEK293 cells (see “Results”). Notably, in these studies we determined the interactions with RyR2 constructs exhibited rapamycin-sensitive FKBP12.6 binding, in contrast to the previous report (30).

We confirmed that FKBP12.6 interaction with the RyR2 C-terminal portion is prevented by rapamycin treatment, indicating that it involves the FK506- and rapamycin-binding pocket. Interestingly, there is a Val-Pro-Leu-Val motif (amino acids 4594–4597 for human RyR2) within putative transmembrane segment M6 (nomenclature of the 10TM model (56)), which could constitute part of the FKBP-binding core. Notably, the specific residue Pro-4595 was initially proposed to be involved in FKBP binding, based on the fact that FKBP has a peptidyl-prolyl cis-trans isomerizing activity and that its dissociation destabilizes the channel and results in appearance of sub-conductance states (14, 23, 24). Consistent with this, it has also been suggested that proline residues contained within α-helical transmembrane segments are involved in conformational changes within transmembrane domains of ion channels, transport proteins and G-protein-coupled receptors (57, 58). Notably, this tetrapeptide motif is extremely well conserved during RyR evolution, from Caenorhabditis elegans to human, whereas the previously suggested, central domain FKBP-binding core is relatively less well conserved, with a notable substitution of the essential proline by serine in C. elegans RyR (Fig. 8), which would suggest that this isoform does not bind to FKBP. The Pro to Ser sequence substitution in the central RyR domain of C. elegans is unexpected, because FKBP proteins are an integral part of the ryanodine receptor-calcium release channel complex, however, further studies of this interaction site within the C. elegans RyR would be required to resolve this issue.

The three-dimensional structure of the cytoplasmic domain of type I transforming growth factor-β receptor in complex with FKBP12 has revealed that receptor residues Leu-193 and Pro-194 are not directly involved in FKBP12 binding, and they appear to stabilize the interface between the molecules. Instead, FKBP12 binds directly to residues Leu-195 and Leu-196 and accommodates the two aliphatic side chains in the same hydrophobic binding pocket used to engage the macrodiles FK506 and rapamycin (59). To our knowledge, the only other FKBP12-binding protein whose FKBP binding core has been investigated in detail is FAP48, for which a mutational analysis has identified Pro-219 of FAP48 as the critical proline for the FKBP12 interaction (53). Importantly, the FKBP12 binding core of FAP48 is Cys-Pro-Leu-Leu (residues 218–221) where the two critical leucine residues are preserved. Thus, on the basis of a requirement for two leucine residues (or similar) following proline, the putative FKBP binding core within RyR2 transmembrane domain M6 is a better candidate for binding the catalytic site of the FKBP proteins than the previously suggested one in the RyR2 central domain (Fig. 8). The crystal structure of the type I transforming growth factor-β receptor-FKBP12 complex has also revealed that additional contact sites outside the FK506- and rapamycin-binding pocket exist between the two proteins (59). Thus, it is plausible that there are multiple FKBP-binding sites in the RyR with different binding properties, as proposed by Masumiya and co-workers (30). It is interesting to note that, in the latter study, N-terminal fragments combined with overlapping C-terminal fragments encompassing the pore-forming transmembrane domains were observed to form functional channels. Thus, there is a possibility of a physical interaction between the RyR N- and C-terminal domains, as indicated by partial digestion studies of the native RyR (60) and demonstrated for the closely related intracellular calcium channel, inositol 1,4,5-triphosphate receptor (61–63). FKBP12.6 binding to both the N- and C-terminal domains could facilitate, or conversely inhibit, the transduction of cytoplasmic signals to the RyR channel pore. To distinguish between these two possibilities, functional studies between N-terminal and overlapping C-terminal fragments should be performed in the presence and absence of FKBP12.6.

Binding of FKBP12.6 to the RyR2 C terminus should involve its catalytic site, because it is rapamycin-sensitive, whereas binding to the central and/or N-terminal region may be mediated by different FKBP12.6 residues. Indeed, Gaburjakova and co-workers (27) have speculated that it may not be the catalytic site involved in the binding of FKBP12.6 to the RyR central region. This in turn would imply that FKBP does not necessar-
ily bind to its protein targets on proline-containing motifs. Interestingly, there is evidence that the peptidyl-prolyl cis-trans isomerase domain of the Escherichia coli trigger factor, a domain with homology to FKBP12, binds preferentially to a stretch of eight amino acids enriched in basic and aromatic residues and with a positive net charge, independently of proline residues (64). A model where the RyR C terminus binds the FKBP catalytic site while the N-terminal and/or central domain bind to disparate FKBP surface residues, is compatible with the three-dimensional image reconstruction of the RyR1-FKBP12 complex, which indicates that the FKBP12-binding site lies at the periphery of the cytoplasmic portion about 11 nm away from the putative channel pore (65). This scenario could also explain the apparent 1:1 stoichiometry of RyR protomer to FKBP (10, 12). Thus, although there are up to three FKBP-binding sites per subunit, it is feasible that they are all involved in binding a single FKBP molecule. Multiple protein alignment was produced using software contained within the Genetic Computing Group package (GGC, University of Wisconsin) accessed via the Medical Research Council website (www.hgmp.mrc.ac.uk).

An important finding of the present study is that the RyR2 C terminus does not interact with the FKBP12 isoform, which is in agreement with the concept that the intact, native RyR2 associates specifically with FKBP12.6. In contrast, the other two RyR subtypes, 1 and 3, are known to bind both FKBP isoforms with similar affinities. All three mammalian RyRs exhibit their highest percentage sequence identity at the C terminus, with the exception of a highly divergent region termed D1 (residues 4210–4562 in RyR2) (69). It will be of interest to examine whether the RyR1 C terminus interacts with FKBP12.6 and/or FKBP12, and further test the involvement of the D1 variable region in FKBP binding with the use of RyR2/RyR1 chimaeras.

In conclusion, we provide several lines of evidence for an FKBP12.6-binding site at the C terminus of the human cardiac muscle RyR. We also find supporting evidence for an interaction site at the N-terminal but not for the central RyR2 domain. Additional studies are required to test the specific contribution of each of the three proposed interaction sites in the context of the intact, native RyR.
