Localization of the 12.6-kDa FK506-binding Protein (FKBP12.6) Binding Site to the NH2-terminal Domain of the Cardiac Ca2+ Release Channel (Ryanodine Receptor)*

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The 12.6-kDa FK506-binding protein (FKBP12.6) interacts with the cardiac ryanodine receptor (RyR2) and modulates its channel function. However, the molecular basis of FKBP12.6-RyR2 interaction is poorly understood. To investigate the significance of the isoleucine-proline (residues 2427–2428) dipeptide epitope, which is thought to form an essential part of the FKBP12.6 binding site in RyR2, we generated single and double mutants, P2428Q, I2427E/P2428A, and P2428A/I2429E, expressed them in IHEK293 cells, and assessed their ability to bind GST-FKBP12.6. None of these mutations abolished GST-FKBP12.6 binding, indicating that this isoleucine-proline motif is unlikely to form the core of the FKBP12.6 binding site in RyR2. To systematically define the molecular determinants of FKBP12.6 binding, we constructed a series of internal and NH2- and COOH-terminal deletion mutants of RyR2 and examined the effect of these deletions on GST-FKBP12.6 binding. These deletion analyses revealed that the first 305 NH2-terminal residues and COOH-terminal residues 1937–4967 are not essential for GST-FKBP12.6 binding, whereas multiple sequences within a large region between residues 305 and 1937 are required for GST-FKBP12.6 binding. Furthermore, an NH2-terminal fragment containing the first 1937 residues is sufficient for GST-FKBP12.6 binding. Co-expression of overlapping NH2- and COOH-terminal fragments covering the entire sequence of RyR2 produced functional channels but did not restore GST-FKBP12.6 binding. These data suggest that FKBP12.6 binding is likely to be conformation-dependent. Binding of FKBP12.6 to the NH2-terminal domain may play a role in stabilizing the conformation of this region.

Ryanodine receptors (RyRs)† are intracellular Ca2+ channels located in the sarco(endo)plasmic reticulum of muscle and non-muscle cells. They govern the release of Ca2+ from intracellular stores and play an essential role in various cellular processes including muscle contraction, fertilization, secretion, and apoptosis (1). These channels are regulated by a number of protein modulators, such as the 12- and 12.6-kDa FK506-binding proteins (FKBP12 and FKBP12.6) (2–6). FKBP12 is tightly associated with the type 1 ryanodine receptor (RyR1) predominantly expressed in skeletal muscle (7), whereas FKBP12.6 is selectively associated with the type 2 ryanodine receptor (RyR2) mainly expressed in cardiac muscle and in the brain (8, 9). The type 3 ryanodine receptor (RyR3), which is expressed at relatively low levels in a variety of tissues, has also been shown to be capable of interacting with both FKBP12 and FKBP12.6 (10). The interactions between FKBP and RyR are believed to be involved in the stabilization of the full conductance state (11), channel gating (12), and modulating the sensitivity to Ca2+ activation of RyR (13, 14). Alterations in these interactions have been implicated in cardiomyopathy (15), cardiac hypertrophy (16), and heart failure (17, 18).

Given the important roles of FKBPbs in RyR regulation, a number of studies have focused on the structural basis of FKBP-RyR interactions, and some insights into the molecular determinants of FKBP binding have recently been revealed (10, 12, 14, 19–21). Using the yeast two-hybrid technique, it has been shown that a 114-amino acid region containing residues 2497–2520 of RyR1 interacts with FKBP12. An analogous region in the inositol 1,4,5-trisphosphate receptor (IP3R) has also been shown to bind FKBP12 (19). This 114-amino acid fragment contains a valine-proline (residues 2461 and 2462) dipeptide epitope thought to be the FKBP12 binding motif in RyR1 (19), and its significance in FKBP12 binding to the intact full-length RyR1 protein has been confirmed. Mutations of valine 2461 to glycine, glutamate, or isoleucine eliminate FKBP12 binding to RyR1 (12). This valine-proline dipeptide motif is conserved in RyR3, where mutation of the corresponding valine to aspartate also diminishes FKBP12 binding (10). These observations demonstrate that the valine-proline motif is essential for FKBP12 interaction with RyR1 and RyR3.

In the corresponding location of RyR2, the valine-proline motif is replaced with isoleucine-proline. The role of this motif in FKBP12.6 binding to RyR2 has not yet been defined. Using the yeast two-hybrid method, Marx et al. (14) reported that a 136-amino acid fragment of the human RyR2 containing residues 2361–2496 interacted with FKBP12.6 and that this interaction was inhibited by rapamycin, a drug known to dissociate FKBP12.6 from RyR2. Based on these observations, Marx et al. (14) proposed that the FKBP12.6 binding site is defined by isoleucine 2427 and proline 2428, analogous to the FKBP12 binding motif in IP3Rs, RyR1, and RyR3. However, contradictory to this proposal and the observations by Marx et al. (14),...
recent studies by Zissimopoulos and Lai (21) using the yeast two-hybrid and immunoprecipitation assays demonstrated that the 10 overlapping fragments covering the entire sequence of human RyR2, none interacted with FKBP12.6. Using the same assay, they were, however, able to observe interactions between the cytoplasmic domain of the type 1 tumor growth factor-β receptor and FKBP12.6 and FKBP12.12. Zissimopoulos and Lai (21) also found that a large COOH-terminal fragment of human RyR2 encompassing all 10 transmembrane domains interacted with FKBP12.6, which suggests that the FKBP12.6 binding site is located at the COOH terminus of RyR2 (21). Therefore, the location of the FKBP12.6 binding site in RyR2 remains controversial and has yet to be defined.

In the present study, we investigated the significance of the isoleucine-proline motif in FKBP12.6 interaction with RyR2. Using a GST-FKBP12.6 pull-down assay, we found that mutations of this motif did not prevent GST-FKBP12.6 from binding to RyR2, in contrast to observations with RyR1 and RyR3. This prompted us to conduct systematic studies to define the FKBP12.6 binding site in RyR2. Through internal, NH₂-terminal, and COOH-terminal deletion analysis, we showed that the first 1937 NH₂-terminal residues are sufficient for GST-FKBP12.6 binding, whereas the last −3000 COOH-terminal residues encompassing the proposed FKBP12.6 binding motif are neither required nor sufficient for GST-FKBP12.6 binding. In addition, we demonstrated that multiple regions within the NH₂-terminal domain are required for GST-FKBP12.6 interaction. Furthermore, co-expression of overlapping NH₂ and COOH-terminal fragments led to the formation of functional RyR channels but did not reconstitute the GST-FKBP12.6 COOH-terminal fragments. We also confirmed that GST-FKBP12.6 binding site in RyR2 remains controversial and that of the 10 overlapping fragments covering the entire sequence of this motif did not prevent GST-FKBP12.6 from binding to RyR2.

**Identification of the FKBP12.6 Binding Site in RyR2**

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**EXPERIMENTAL PROCEDURES**

**Materials—**Restriction endonucleases and DNA-modifying enzymes were purchased from New England Biolabs Inc. The anti-c-Myc antibody was kindly provided by the Immunology Core Facilities at the Wadsworth Center of the New York State Department of Health. Soybean phosphatidylcholine was obtained from Avanti Polar Lipid. CHAPS and other reagents were purchased from Sigma.

**Cell Culture and DNA Transfection—**HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium as described previously (22). HEK293 cells grown on 100-mm tissue culture dishes for 18–24 h after subculture were transfected with 6 μg of wild type or mutant RyR cDNAs using Ca²⁺ phosphate precipitation (23).

**Site-directed Mutagenesis—**The point mutation, RyR2 (I4827T), was introduced in D1636—the 10 overlapping fragments covering the entire sequence of this motif did not prevent GST-FKBP12.6 from binding to RyR2.

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stop linker, 5′-TACTGATCATA3′, was inserted into the SalI site (induced at 11,231) after being cut and blunted by Klenow treatment to form the 1–3940 COOH-terminal deletion mutant. The 1–2958 COOH-terminal deletion mutant was constructed by inserting a stop linker, 5′-TACTGATCATA3′, into the BsiWI (8864) site after being cut and blunted by Klenow treatment in the full-length RyR2 cDNA. An NheI (vector)-BsiWI (8864) fragment in pCDNA was digested with HpaII and ligated with a stop linker, 5′-TACTGATCATA3′, yielding the 1–2531 COOH-terminal deletion mutant. Similarly, a stop linker, 5′-TACTGATCATA3′, was inserted into the EcoRV (6439) site, forming the 1–2150 COOH-terminal deletion mutant. PCR was used to introduce a stop codon after residue 1937. The forward primer used is 5′-GATCTCTCCGATGCTGCTCCCTC-3′. The reverse primer containing a stop codon followed by an EcoRV site was 5′-GATACCTCTATTGGAATTTAGCTAA-3′ (1957-stop). The Bsu361 (4900)-EcoRV (introduced after the stop codon) PCR fragment was used to replace the Bsu361 (4900)-EcoRV (13,873) fragment in the full-length RyR2 cDNA to yield the 1–1937 COOH-terminal deletion mutant. In order to produce the 1–1636 COOH-terminal deletion mutant, a stop linker, 5′-GATACCTCT-3′, was inserted into the Bsu361 (4900) site after being cut and blunted by Klenow treatment. To construct the 1–1072 and 1–531 COOH-terminal deletion mutants, a stop linker, 5′-AGTGATCATA3′, was inserted into the XhoI (3216) and EcoRI (1590) sites, respectively, after being cut and blunted by Klenow treatment. All point mutations and deletions were confirmed by DNA sequencing.

**RESULTS**

**Identification of the FKBP12.6 Binding Site in RyR2**

A. *Proposed FKBP12.0/12.6 binding motif*

| Construct | Amino Acid Range | Binding Motif |
|-----------|------------------|---------------|
| RyR2      | 2414-2419        | A1R1R3I3R5TP  |
| RyR1      | 2453-2459        | A1R1R3I3R5TP  |
| RyR3      | 2314-2319        | A1R1R3I3R5TP  |

**B. GST-FKBP12 pull-down**

**Fig. 1. Significance of the proposed FKBP12.6 binding motif in GST-FKBP12.6 interaction with RyR2.**

1. The amino acid sequences encompassing the proposed FKBP12/FKBP12.6 binding motif, indicated in *boldface* letters, in three RyR isoforms (RyR1, RyR2, and RyR3) and in three IP3R isoforms (IP3R1, IP3R2, and IP3R3) are shown. B. HEK293 cells were transfected with wild type RyR2 (RyR2 (wt)) (lane 1), a single point mutant, P2428Q (lane 2), or the double mutants I2427E/P2428A (lane 3) and P2428A/L2429E (lane 4) cDNA. The expressed wild type and mutant RyR2 proteins were precipitated from cell lysates by GST-FKBP12.6 glutathione-Sepharose. The precipitates were then solubilized and separated in SDS-PAGE and stained with Coomassie Brilliant Blue. C. HEK293 cells were transfected with 12 µg of wild type RyR2 (RyR2 (wt)) (a), P2428Q (b), I2427E/P2428A (c), or P2428A/L2429E (d) cDNA. Fluorescence intensity of the fluo-3-loaded transfected cells was monitored continuously before and after the addition of 2.5 mM caffeine at a point indicated by the letter C. Similar results were obtained from three separate experiments.

**Mutations in the Proposed FKBP12.6 Binding Motif Do Not Abolish GST-FKBP12.6-RyR2 Interaction**—It has been shown that mutating the proline at position 1401 in IP3R into glutamine abolishes FKBP12 binding (19). To determine the significance of the equivalent proline in RyR2 to FKBP12.6 binding, we mutated proline 2428 to glutamine (P2428Q). As shown in Fig. 1, both the wild type RyR2 (RyR2 (wt)) and the P2428Q mutant were pulled down by GST-FKBP12.6 (lanes 1 and 2) but not by GST (not shown), indicating that the P2428Q mutation does not abolish FKBP12.6 binding to RyR2, in contrast to observations with IP3R. It has also been shown that mutations of valine 2461 in RyR1 or valine 2327 in RyR3 abolish FKBP12 binding and that leucine 195 after the leucine-proline motif in the tumor growth factor-β receptor is critical for FKBP12 binding (10, 12, 30). To assess the role of the corresponding RyR2 residues in FKBP12.6 interaction, we generated two double mutations, I2427E/P2428A and P2428A/L2429E. Both mutants were pulled down by GST-FKBP12.6 (Fig. 1, lanes 3 and 4) but not by GST (not shown). Like the wild type RyR2-transfected cells, HEK293 cells transfected with mutants P2428Q, I2427E/P2428A, and P2428A/L2429E exhibited caffeine-induced Ca2+ release (Fig. 1C). These data demonstrate that the isoenceine-proline motif is not essential for the interaction of RyR2 with FKBP12.6, although the corresponding motif is critical for FKBP12 binding to RyR1, RyR3, IP3Rs, and the tumor growth factor-β receptor.

**Multiple Regions Within Residues 305–1937 in RyR2 Are Required for GST-FKBP12.6 Binding**—To identify regions in RyR2 that are essential for FKBP12.6 binding, we carried out a systematic deletion analysis. As shown in Fig. 2, deletion of amino acid residues 2530–4770 (construct 2) did not remove GST-FKBP12.6 binding, whereas deletion of residues 1636–4414 (construct 3) did, suggesting that a region between 1636–2530 is required for FKBP12.6 binding. In line with this suggestion, deletion of this region (1636–2530, construct 4) also abolished GST-FKBP12.6 binding (Fig. 2B).

The region between residues 1636–2530 was divided into four subregions. Deletion of subregions 2358–2530 (construct 5), 2150–2358 (construct 6), or 1937–2150 (construct 7) did not
eliminate FKBP12.6 binding. It should be noted that subregion 2358–2530 encompasses the isoleucine-proline motif, further indicating that this motif and its flanking regions are not required for FKBP12.6 binding, which is consistent with the results of point mutations (Fig. 1). On the other hand, deletion of subregion 1636–1937 (construct 11) did not abolish FKBP12.6 binding. As shown in Fig. 4, deletion of the first 305 NH2-terminal residues abolishes FKBP12.6 binding. Thus, a large region between residues 305 and 1937 is required for FKBP12.6 binding to RyR2.

**GST-FKBP12.6 Binding-deficient Mutants Are Capable of Forming Functional Heteromeric Channels**—The effects of these internal deletions on RyR2 channel function were assessed by measuring the caffeine response of HEK293 cells transfected with each deletion mutant. All deletions completely abolished caffeine-induced Ca2+ release in transfected HEK293 cells (Fig. 3, A, C, E, G, I, K, M, O, Q, S, and U), indicating that a large portion of the RyR2 sequence is required for activation by caffeine.

To assess their ability to form heteromeric channels, we co-expressed deletion mutants with an RyR2 mutant, RyR2 (I4827T), in HEK293 cells and determined their caffeine responses. We have shown previously that HEK293 cells transfected with the RyR2 (I4827T) mutant exhibited no caffeine-induced Ca2+ release (24). Reasoning that if a deletion mutant is capable of forming a heteromeric channel with RyR2 (I4827T), co-transfection of HEK293 cells with a deletion mutant and RyR2 (I4827T) may rescue their caffeine response, since the mutants may complement each other’s defects through the formation of heteromeric channel complexes. As shown in Fig. 3, HEK293 cells co-transfected with RyR2 (I4827T) and all internal deletion mutants except for mutant D2530–4770 (Fig. 3B) displayed caffeine-induced Ca2+ release. Hence, most deletion mutants retain the ability to form functional heteromeric channels, suggesting that these deletions did not grossly alter the channel structure.

**The First 305 NH2-terminal Amino Acid Residues Are Not Essential for GST-FKBP12.6 Binding and Caffeine Activation**—To investigate the role of the NH2 terminus in FKBP12.6 binding, we constructed a series of NH2-terminal deletion mutants and examined the effect of these deletions on GST-FKBP12.6 binding. As shown in Fig. 4, deletion of the first 305 NH2-terminal residues (D305) did not eliminate GST-FKBP12.6 binding (Fig. 4B), indicating that the first 305 NH2-terminal residues are not essential for FKBP12.6 binding. On the other hand, deletion of the first 378 residues (D378) abolished GST-FKBP12.6 binding (Fig. 4A). This indicates that a NH2-terminal region after residue 305 is critical for FKBP12.6 binding, which is consistent with the results of internal deletion studies (Fig. 1). These data also indicate that a >4000-amino acid COOH-terminal fragment of RyR2 encompassing the proposed FKBP12.6 binding motif is insufficient for GST-FKBP12.6 binding.

The effect of NH2-terminal deletions on channel function is shown in Fig. 5. HEK293 cells transfected with deletion mutant D305 remained sensitive to caffeine (Fig. 5A), indicating that the first 305 NH2-terminal amino acid residues are not essential for caffeine activation of RyR2. On the other hand,
deletion of the first 784, 1072, 1636, or 2150 NH$_2$-terminal amino acid residues abolished caffeine-induced Ca$^{2+}$ release in transfected HEK293 cells (Fig. 7A, B, D, F, and H). Co-expression of these deletion mutants with RyR2 (I4827T) restored their response to caffeine activation (Fig. 5, C, E, G, and I), indicating that these GST-FKBP12.6 binding-deficient NH$_2$-terminal deletion mutants remain capable of forming functional heteromeric channels.

The NH$_2$-terminal Fragment Including the First 1937 Amino Acid Residues Is Sufficient for GST-FKBP12.6 Binding and Is Capable of Restoring the Function of NH$_2$-terminal Deletion Mutants—The significance of the COOH-terminal region of RyR2 in FKBP12.6 interaction was assessed by constructing a series of COOH-terminal deletions and determining the effect of these deletions on GST-FKBP12.6 binding. Deletion of up to ~3000 COOH-terminal amino acid residues (Fig. 6, constructs 2–8) did not abolish GST-FKBP12.6 binding, whereas further deletion of the COOH terminus to residue 1636 led to a complete loss of GST-FKBP12.6 binding (Fig. 6B). These observations demonstrate that the COOH-terminal region starting at residue 1937 is not required for GST-FKBP12.6 binding and that the first 1937 NH$_2$-terminal residues are sufficient for GST-FKBP12.6 binding.

These findings together with the observation that multiple deletions within the NH$_2$-terminal region abolish GST-FKBP12.6 binding and channel function (Figs. 2 and 3) raise the possibility that the NH$_2$-terminal region may constitute a unique domain structure. To test this possibility, we co-expressed this NH$_2$-terminal fragment (amino acids 1–1937) with the NH$_2$-terminal deletion mutants, D1072, D1636, and D2150, in HEK293 cells (Fig. 7A) and examined the caffeine response of the transfected cells. As shown in Fig. 7B, caffeine-induced Ca$^{2+}$ release was observed in HEK293 cells co-transfected with the 1–1937 and D1072 fragments (Fig. 7Ba) and with the 1–1937 and D1636 fragments (Fig. 7Bb). On the other hand, cells co-transfected with 1–1937 and D2150 fragments did not exhibit caffeine-induced Ca$^{2+}$ release (Fig. 7Bc), which is consistent with the results of internal deletion studies in which residues between 1937–2150 are essential for caffeine activation (Fig. 3K).

Co-expression of Overlapping NH$_2$ and COOH-terminal Fragments Produces Functional RyR2 Channels but Does Not Restore GST-FKBP12.6 Interaction—We next examined...
whether a GST-FKBP12.6 binding-deficient NH₂-terminal fragment was able to form functional channels with an overlapping COOH-terminal fragment. We co-expressed the NH₂-terminal fragment containing residues 1-1937 and COOH-terminal fragments missing the first 1072, 1636, or 2150 NH₂-terminal residues are shown by open and filled boxes, respectively, in A, B, HEK293 cells were co-transfected with the NH₂-terminal fragment (residues 1-1937) plus COOH-terminal fragments D1072 (a), D1636 (b), or D2150 (c). Fluorescence intensity of the fluo-3-loaded transfected cells was monitored continuously before and after the addition of 2.5 mM caffeine indicated by the letter C. A transient increase in fluorescence was detected in cells co-transfected with mutants 1-1937 and D1072 and mutants 1-1937 and D1636, indicating caffeine-induced Ca²⁺ release from intracellular stores. Similar results were obtained from three separate experiments. IP, isoleucine-proline.

**DISCUSSION**

It is widely believed that the isoleucine-proline (residues 2427 and 2428) motif constitutes an essential part of the FKBP12.6 binding site in RyR2. However, we found that RyR2 fragments containing residues 1-1937 and COOH-terminal fragments missing the first 1072, 1636, or 2150 NH₂-terminal residues are shown by filled boxes. All of these fragments are incapable of binding GST-FKBP12.6. B, HEK293 cells were co-transfected with the NH₂-terminal fragment (residues 1-1636) plus the COOH-terminal fragment D1072 (a), D1636 (b), or D2150 (c). Fluorescence intensity of the fluo-3-loaded transfected cells was monitored continuously before and after the addition of 2.5 mM caffeine (letter C). Similar results were obtained from three separate experiments. C, HEK293 cells were co-transfected with the NH₂-terminal fragment (residues 1-1636) plus the COOH-terminal fragment D1072, D1636, or D2150. The c-Myc-tagged RH₂ and COOH-terminal fragments were precipitated from cell lysates by anti-c-Myc antibody and by GST-FKBP12.6 glutathione-Sepharose. The precipitates were solubilized and separated in SDS-PAGE and were stained with Coomassie Brilliant Blue (CBB). A similar SDS-PAGE gel was transferred to nitrocellulose membrane. The membrane was probed with the anti-c-Myc antibody in Western blotting (WB). The smear staining around 200 kDa probably represents aggregates of the anti-c-Myc antibodies from the immunoprecipitates.

In the present study, we directly examined the role of this isoleucine-proline motif and regions containing this motif in the interaction of FKBP12.6 with the full-length RyR2. Site-directed mutagenesis and deletion analysis revealed that mutations of this motif do not abolish GST-FKBP12.6 binding to RyR2. In addition, the removal of large fragments containing the motif does not eliminate the ability of the mutant RyR2 to bind GST-FKBP12.6. These results provide the first biochemical evidence that the isoleucine-proline motif and regions flanking the motif are unlikely to form the core of the FKBP12.6 binding site in RyR2.

These findings differ from those observed with RyR1 and RyR3, in which mutations in the FKBP12 binding motif abolish FKBP12 and FKBP12.6 interactions (10, 12), raising a question as to whether the FKBP12.6 binding site in RyR2 is structurally different from the FKBP12.6 binding site in RyR1 and RyR3. It has been shown that a 114-amino acid fragment containing residues 2497-2520 of RyR1 is sufficient for FKBP12 binding (19). However, we found that RyR2 fragments containing the corresponding region do not bind FKBP12.6 (Fig. 4), whereas an NH₂-terminal fragment of RyR2 contain-
We have shown that an NH2-terminal fragment is able to form functional channels with overlapping COOH-terminal fragments (Fig. 7), suggesting that the NH2-terminal region contains one or more functional domains capable of interacting with the COOH-terminal portion of the channel protein. It is possible that binding of FKBP12.6 to the NH2-terminal region may stabilize intradomain interactions either within the NH2-terminal region or between the NH2-terminal and COOH-terminal regions. Further identification of the residues involved in direct interaction with FKBP12.6 should provide important insight into its roles in RyR function and regulation.

Acknowledgments—We thank the Immunology Core Facilities at the Wadsworth Center of the New York State Department of Health for providing the anti-c-Myc antibody, Dr. Wayne R. Giles for continued support, Dr. Paul Schnetkamp for the use of the luminescence spectrometer, Pin Li and Cindy Brown for excellent technical assistance, and Jeff Bolstad for critical reading of the manuscript.

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