Localization of an NH$_2$-terminal Disease-causing Mutation Hot Spot to the “Clamp” Region in the Three-dimensional Structure of the Cardiac Ryanodine Receptor*

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A region between residues 414 and 466 in the cardiac ryanodine receptor (RyR2) harbors more than half of the known NH$_2$-terminal mutations associated with cardiac arrhythmias and sudden death. To gain insight into the structural basis of this NH$_2$-terminal mutation hot spot, we have determined its location in the three-dimensional structure of RyR2. Green fluorescent protein (GFP), used as a structural marker, was inserted into the middle of this mutation hot spot after Ser-437 in the RyR2 sequence. The resultant GFP-RyR2 fusion protein, RyR2$_{S437}$-GFP, was expressed in HEK293 cells and characterized using Ca$^{2+}$ release, [H]ryanodine binding, and single cell Ca$^{2+}$ imaging studies. These functional analyses revealed that RyR2$_{S437}$-GFP forms a caffeine- and ryanodine-sensitive Ca$^{2+}$ release channel that possesses Ca$^{2+}$ and caffeine dependence of activation indistinguishable from that of wild type (wt) RyR2. HEK293 cells expressing RyR2$_{S437}$-GFP displayed a propensity for store overload-induced Ca$^{2+}$ release similar to that in cells expressing RyR2-wt. The three-dimensional structure of the purified RyR2$_{S437}$-GFP was reconstructed using cryo-electron microscopy and single particle image processing. Subtraction of the three-dimensional reconstructions of RyR2-wt and RyR2$_{S437}$-GFP revealed the location of the inserted GFP, and hence the NH$_2$-terminal mutation hot spot, in a region between domains 5 and 9 in the clamp-shaped structure. This location is close to a previously mapped central disease-causing mutation site located in a region between domains 5 and 6. These results, together with findings from previous studies, suggest that the proposed interactions between the NH$_2$-terminal and central regions of RyR2 are likely to take place between domains 5 and 6 and that the clamp-shaped structure, which shows substantial conformational differences between the closed and open states, is highly susceptible to disease-causing mutations.

Cardiac arrhythmia, a leading cause of sudden death, has been linked to a number of genes encoding ion channels, including the cardiac sarcoplasmic reticulum Ca$^{2+}$ release channel (ryanodine receptor (RyR) type 2) (1–4). To date, more than 40 mutations in RyR2 have been associated with at least two forms of cardiac arrhythmias, catecholaminergic polymorphic ventricular tachycardia (CPVT), and arrhythmogenic right ventricular dysplasia type 2 (ARVD2). Most of these mutations are located in the NH$_2$-terminal region (between amino acids 164 and 466), central region (between amino acids 2246 and 2504), and the COOH-terminal region (between amino acids 3778 and 4959) of RyR2 (Fig. 1). Interestingly, those mutations in the skeletal muscle RyR (RyR1) that are linked to malignant hyperthermia (MH) and central core disease (CCD) are largely clustered in the corresponding regions of RyR1; some sites even correspond exactly (2, 4) (Fig. 1). These similar distributions suggest that the molecular mechanisms by which these disease-linked RyR1 and RyR2 mutations alter the intrinsic properties of the channel are conserved.

A number of disease-linked RyR2 mutations have been expressed and functionally characterized. A common finding of these studies is that most of the RyR2 mutations enhance the activity of the channel upon stimulation (1–4). This enhancement of function activity is also a consistent feature with most of the RyR1 MH and CCD mutations (5–7). However, the precise mechanism by which channel function is altered by disease-linked mutations is not well defined. Wehrens et al. (8) showed that CPVT RyR2 mutations reduced the binding affinity of a 12.6-kDa FK506-binding protein (FKBP12.6) to RyR2, a protein believed to stabilize the RyR2 channel. When RyR2 is phosphorylated by PKA, FKBP12.6 is dissociated from RyR2, leading to faulty calcium release (8). If so, CPVT mutant channels should exhibit an enhanced activity upon PKA phosphorylation. However, George et al. (9) have demonstrated that CPVT RyR2 mutations augmented Ca$^{2+}$ release in a manner independent of both FKBP12.6 and PKA phosphorylation.

The abbreviations used are: RyR, ryanodine receptor; CCD, central core disease; MH, malignant hyperthermia; GFP, green fluorescent protein; IP$_3$, inositol 1,4,5-trisphosphate; CPVT, catecholaminergic polymorphic ventricular tachycardia; ARVD2, arrhythmogenic right ventricular dysplasia type 2; PIPES, 1,4-piperazinediethanesulfonic acid; CHAPS, 3-[[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; GST, glutathione S-transferase; PKA, cAMP-dependent protein kinase; SOICR, store-overload induced Ca$^{2+}$ release; FKBP12.6, 12.6-kDa FK506-binding protein.

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3 The abbreviations used are: RyR, ryanodine receptor; CCD, central core disease; MH, malignant hyperthermia; GFP, green fluorescent protein; IP$_3$, inositol 1,4,5-trisphosphate; CPVT, catecholaminergic polymorphic ventricular tachycardia; ARVD2, arrhythmogenic right ventricular dysplasia type 2; PIPES, 1,4-piperazinediethanesulfonic acid; CHAPS, 3-[[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; GST, glutathione S-transferase; PKA, cAMP-dependent protein kinase; SOICR, store-overload induced Ca$^{2+}$ release; FKBP12.6, 12.6-kDa FK506-binding protein.
Consistent with the latter authors, we have demonstrated that the mutations R176Q and L433P in the NH2-terminal region; S2246L, R2474S, and T2504M in the central region; and N4104K, Q4201R, R4496C, I4867M, and N4895D in the COOH-terminal region do not affect the RyR2-FKBP12.6 interaction (10). Furthermore, we have found that these mutations increase the sensitivity of RyR2 to activation by luminal Ca2+ and reduce the threshold for spontaneous Ca2+ release during store Ca2+ overload, a process that we have termed store overload-induced Ca2+ release (SOICR) (10, 11). Because SOICR can lead to delayed after-depolarizations, which in turn can produce triggered arrhythmias, a reduced threshold for SOICR as a result of enhanced luminal Ca2+ activation of RyR2 would increase the propensity for delayed after-depolarizations, cardiac arrhythmias, and sudden death. Hence, an altered luminal Ca2+ response of the channel is a common molecular defect of disease-linked RyR2 mutations. However, how mutations located in different regions of the channel similarly alter luminal Ca2+ activation is not well understood.

Ikemoto and his co-workers (4, 12, 13) have proposed that the NH2-terminal and central regions form two domains that physically interact in the three-dimensional structure of RyR1 and RyR2, and that disease-causing mutations are exposed at the interface of the two domains. They further proposed that inter-domain interactions (“zipped”) domains are involved in stabilizing the closed state of the channel, and that disease-causing mutations weaken these interactions (produce domain “unzipping”), thereby destabilizing the channel and making it more susceptible to activation by stimuli (12). Although an increasing body of biochemical and functional evidence supports this domain unzipping hypothesis, structural evidence is lacking.

To understand the structural basis of the mechanism underlying disease-causing mutations, we have recently mapped the site of a mutation in the central region to a spot between domains 5 and 6 in the three-dimensional structure of RyR2 (14). In this study, to directly test the hypothesis, we attempted to locate the site of a mutation in the NH2-terminal region in the three-dimensional structure of RyR2. To this end, an RyR2-GFP fusion protein, RyR2-S437-GFP, in which GFP was inserted into the NH2-terminal region after residue Ser-437, was constructed and expressed in HEK293 cells. Functional studies demonstrated that the insertion of GFP does not grossly alter the structure and function of RyR2. RyR2-S437-GFP protein was purified and then subjected to cryo-electron microscopy (cryo-EM) and single-particle image processing. Three-dimensional reconstruction enabled localization of the inserted GFP to a site between domains 5 and 9 that is close to the central mutation site mapped previously, consistent with the zipping/unzipping hypothesis.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases and DNA-modifying enzymes were purchased from New England Biolabs (Ipswich, MA). The anti-RyR and anti-GFP antibodies were obtained from Affinity BioReagents (Golden, CO). Soybean phosphatidylcholine was obtained from Avanti Polar Lipids, Inc (Alabaster, AL). 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 (15). HEK293 cells grown on 100-mm tissue culture dishes for 18–20 h after subculture were transfected with 12 μg of wild type RyR2 (RyR2-wt) or RyR2-S437-GFP cDNAs using Ca2+ phosphate precipitation (16).

Construction of RyR2-S437-GFP—The cloning and construction of the 15-kb full-length cDNA encoding the mouse cardiac RyR2 has been described previously (17). To generate the RyR2-S437-GFP cDNA construct, the ClaI (2350)-NotI (14,904) fragment was first removed by digesting the full-length RyR2 cDNA with ClaI and NotI, and the remaining portion of the RyR2 cDNA was then ligated with the use of a ClaI-NotI adapter to form the RyR2 (ClaI-NotI) construct. The ClaI-NotI adapter was generated by annealing two short primers, 5'-CGATCCGC-3' (forward) and 5'-GGCCGCGGATCC-3' (reverse). The cDNA encoding GFP flanked by Gly-rich spacers and an AscI site was obtained by PCR as described previously (18). The AscI site was introduced into the sequence of the RyR2 (ClaI-NotI) construct after Ser-437 by overlap extension PCR (19). The “outer” primers used were as follows: forward, 5’-GGTCGACGACGAGCTGCCCACATTGAC-3’ and reverse, 5’-GCTGTACGAGCACCAGTCGAGGGA-3’. The primers for introducing the Ascl site were as follows: forward, 5’-GATGCTCTTCAGCGGGGC-3’ and reverse, 5’-GGTGGATGTGAAATCAGCACGA-3’. The BamHI (1153)-EcoRI (1590) PCR fragment containing the Ascl site was used to replace the BamHI (1153)-EcoRI (1590) fragment in the RyR2 (ClaI-NotI) construct. The missing ClaI (2350)-NotI (14904) fragment was then added back to yield the full-length RyR2 cDNA with the inserted Ascl site. The Ascl-Ascl fragment containing GFP and the spacers was then subcloned into the full-length RyR2 cDNA at the introduced Ascl site. The sequences of all PCR fragments and the orientation of the inserted GFP cDNA were verified by DNA sequencing analysis.

Cryo-electron Microscopy and Image Processing—The expression and purification of RyR2-S437-GFP was carried out as described previously (18). The purified RyR2-S437-GFP was diluted 5–10-fold with EM dilution buffer (20 mM NaPIPES, pH 7.2, 400 mM KCl, 3 mM EGTA, 0.5% CHAPS, 2 mM dithiothreitol, and 2 mg/ml leupeptin). Grids were prepared for cryo-EM according to standard methods (20). Micrographs were recorded using low dose protocols on a Philips EM420, equipped with a low dose kit and a GATAN 626 cryotransfer holder, at 38,600 Å2/Å2. Micrographs were checked for drift, astigmatism, and the presence of Thon rings by optical diffraction. Selected electron micrographs were digitized on a Zeiss imaging scanner (Z/I Imaging Corp., Huntsville, AL) with a step size of 14 Å. Images were processed using the SPIDER/WEB software package (21), and three-dimensional reconstructions were calculated using projection matching procedure (18, 22). The final three-dimensional reconstructions of RyR2-wt and RyR2-S437-GFP were computed from 5,434 and 4,948 particles, respectively. 4-Fold symmetry was enforced in both reconstructions. The final resolution was estimated to be 34 Å by Fourier shell correlation (with a cutoff of 0.143).
were generated using the Flp-In T-Rex core kit from Invitrogen as described previously (10). Intracellular Ca\(^{2+}\) transients in HEK293 cells were measured using single-cell Ca\(^{2+}\) imaging and the fluorescent Ca\(^{2+}\) indicator dye fura-2 acetoxymethyl ester (fura-2 AM) as described previously (10).

**Ca\(^{2+}\) Release and \([^{3}H]\)Ryandine Binding Assays**—Measurements of free cytosolic Ca\(^{2+}\) concentrations in the transfected HEK293 cells using the fluorescent Ca\(^{2+}\) indicator dye fluo-3 and of equilibrium \([^{3}H]\)ryandine binding to cell lysate were done as described previously (10).

**GST-FKBP12.6 Pulldown, Immunoprecipitation, and Immunoblotting Analyses**—GST-FKBP12.6 pulldown, immunoprecipitation, and immunoblotting were carried out as described previously (18, 24).

### RESULTS

**Insertion of Green Fluorescent Protein (GFP) into the NH\(_2\)-terminal Region of RyR2 after Serine 437**—A number of mutations in the NH\(_2\)-terminal region of RyR2 have been linked to CPVT or ARVD2. Of these NH\(_2\)-terminal mutations, six are located between residues 414 and 466. We attempted to map the location of this hot spot in the three-dimensional structure of RyR2. Our approach was to use GFP, which contains 238 amino acids and is much smaller than RyR2 (~5000 amino acids), as a structural marker and to insert it into the region of interest. Three-dimensional reconstructions of the RyR2-wt and GFP-inserted RyR2 were then generated and compared. Barring major conformational rearrangements because of disruption, the primary difference between the two structures would correspond to the inserted GFP. Accordingly, we generated a RyR2-GFP fusion construct (RyR2\(_{S437\text{-GFP}}\)) in which GFP was inserted into the RyR2 sequence after residue Ser-437, near the middle of a mutation hot spot (amino acids 414–466) (Fig. 1). To minimize potential perturbations of the folding and function of RyR2, two glycine-rich spacers were added on both sides of GFP (25, 26) (Fig. 1).

**Functional Expression of RyR2\(_{S437\text{-GFP}}\)**—To determine whether the GFP-inserted RyR2 was properly expressed, we transfected HEK293 cells with the RyR2\(_{S437\text{-GFP}}\) cDNA and subsequently examined them using fluorescence microscopy. As shown in Fig. 2, green fluorescence in HEK293 cells was readily detected after transfection (Fig. 2A), whereas no fluorescence was observed in HEK293 cells transfected with RyR2-wt lacking the GFP insertion (data not shown). Because the fluorescence of GFP depends on the proper folding of its own structure and the structure of the inserted region (26, 27), the appearance of green fluorescence in transfected HEK293 cells indicates that the inserted GFP and its neighboring region are likely to have folded properly.

To assess whether the expressed RyR2\(_{S437\text{-GFP}}\) was functional, we measured intracellular Ca\(^{2+}\) release in transfected
HEK293 cells, using the fluorescent Ca$^{2+}$ indicator dye fluo-3. As shown in Fig. 2B, HEK293 cells transfected with the RyR2S437-GFP cDNA displayed multiple Ca$^{2+}$ release events in response to repeated stimulation by caffeine (Fig. 2B, panel a), an agonist of RyRs. However, transfected cells pretreated with ryanodine responded only to the first, but not the second or third, caffeine stimulation because of the modification of RyR2 by ryanodine (Fig. 2B, panel b). The same responses to caffeine and ryanodine were observed in HEK293 cells transfected with RyR2-wt (28), indicating that RyR2S437-GFP, like RyR2-wt, is able to form a caffeine- and ryanodine-sensitive Ca$^{2+}$ release channel in HEK293 cells. The immediate drop in fluorescence after the second and third caffeine stimulations (Fig. 2B, panel b) was caused by fluorescence quenching by caffeine (28). It should also be noted that no caffeine- or ryanodine-induced Ca$^{2+}$ release was detected in nontransfected HEK293 cells or in cells transfected with pCDNA3 vector DNA (data not shown).

**Responses of RyR2S437-GFP to Ca$^{2+}$, Caffeine, and Ca$^{2+}$ Overload**—We have recently shown that the CPVT RyR2 mutation L433P does not markedly alter the Ca$^{2+}$ dependence of [H]ryanodine binding, but it does increase the sensitivity of the channel to activation by caffeine, and it does enhance the propensity for spontaneous Ca$^{2+}$ release during store Ca$^{2+}$ overload or SOICR (10). The fact that GFP was inserted into the RyR2 sequence after Ser-437, a mere four residues away, raised the possibility that the GFP insertion could alter the response of the channel to activation by various stimuli. To test this, we first assessed the Ca$^{2+}$ dependence of [H]ryanodine binding to RyR2S437-GFP. [H]Ryanodine binding has been widely used as a functional assay for RyR channel activity, because ryanodine binds only to the open state of the channel (29). Fig. 3A shows [H]ryanodine binding to RyR2S437-GFP in the presence of a wide range of Ca$^{2+}$ concentrations. Analysis of the Ca$^{2+}$ dependence of [H]ryanodine binding yielded an EC$_{50}$ of 0.19 ± 0.01 µM (mean ± S.E., n = 6), a value similar to the EC$_{50}$ of RyR2-wt (0.17 ± 0.14 µM, n = 4). We then measured [H]ryanodine binding to RyR2-wt and RyR2S437-GFP in the presence of various concentrations of caffeine. As shown in Fig. 3B, RyR2-wt and RyR2S437-GFP displayed very similar responses to caffeine activation. The EC$_{50}$ values for caffeine activation of [H]ryanodine binding were 2.85 ± 0.21 mM (n = 5) for RyR2-wt and 3.32 ± 0.34 mM (n = 5) for RyR2S437-GFP. These values are not significantly different (p = 0.27, Student's t test).

Furthermore, to determine whether the insertion of GFP into the NH$_2$-terminal region of the channel altered SOICR, we generated a stable, tetracycline-inducible HEK293 cell line expressing RyR2S437-GFP. Store Ca$^{2+}$ overload was induced by elevating the external Ca$^{2+}$ concentration ([Ca$^{2+}$]$_{ex}$), and the occurrence of SOICR was monitored using single cell Ca$^{2+}$ imaging. As shown in Fig. 3C, the fraction of cells that displayed Ca$^{2+}$ oscillations at each [Ca$^{2+}$]$_{ex}$, in HEK293 cells expressing RyR2S437-GFP was very similar to that in cells expressing RyR2-wt. These observations indicate that the insertion of GFP after Ser-437 did not grossly alter the sensitivity of RyR2 to Ca$^{2+}$ or caffeine activation or the propensity for SOICR to occur.

**Purification of RyR2S437-GFP**—HEK293 cells transfected with RyR2S437-GFP cDNA were solubilized by the detergent CHAPS, and the RyR2S437-GFP protein was purified in a single step using GST-FKB12.6, which binds to RyR2 with high affinity. The purified RyR2S437-GFP protein was analyzed by SDS-PAGE and immunoblotting. A single high molecular weight band, which
migrated at a slightly slower rate than did the RyR2-wt band, was detected in the purified sample of RyR2-S437-GFP (Fig. 4A, panel a), consistent with the addition of GFP. To further confirm the insertion of GFP into RyR2, the purified samples were immunoblotted with an anti-GFP antibody. As shown in Fig. 4A, panel b, the anti-GFP antibody recognized the purified RyR2-S437-GFP sample, but not the purified RyR2-wt sample.

[3H]Ryanodine binding was then employed to assess whether the purified RyR2-S437-GFP channel is capable of binding [3H]ryanodine with a Ca^{2+} dependence of activation similar to that of RyR2-wt (18).

Three-dimensional Reconstruction of RyR2 S437-GFP—We next imaged the purified RyR2-S437-GFP proteins using cryo-EM. The purified proteins were diluted in a lipid- and sucrose-free buffer and applied to carbon-coated EM grids, which were rapidly frozen in liquid ethane. The frozen samples were then subjected to cryo-EM. Fig. 5 shows a typical electron micrograph of frozen-hydrated RyR2-S437-GFP. Individual particles with apparently intact structure and multiple orientations were readily detected. Particles that were oriented with their 4-fold symmetry axes perpendicular to the carbon support film were selected, and their images were aligned using cross-correlation methods (Fig. 6). The overall two-dimensional projection of RyR2-S437-GFP (Fig. 6A) was very similar to that of RyR2-wt reported previously (Fig. 6B). To identify subtle localized differences between these two projected structures, a difference map was generated by subtracting the two-dimensional average of RyR2-wt from that of RyR2-S437-GFP (Fig. 6C). The brightest areas in the difference map correspond to the most significant

FIGURE 4. Immunoblotting of and [3H]ryanodine binding to the purified RyR2-S437-GFP. A, the RyR2-S437-GFP protein was purified from cell lysate by affinity chromatography using GST-FKBP12.6 as the affinity ligand. The purified RyR2-S437-GFP and RyR2-wt proteins were solubilized, separated in 6% SDS-PAGE, and transferred to nitrocellulose membranes. The membrane was probed either with an anti-RyR antibody or an anti-GFP antibody for Western blotting. Note that the RyR2-S437-GFP protein migrated at a slightly slower rate in SDS-PAGE than did the RyR2-wt, because of the insertion of GFP. Also note that the anti-GFP antibody reacted only with RyR2-S437-GFP and not with RyR2-wt. B, [3H]ryanodine binding to purified RyR2-S437-GFP protein (open circles) was carried out at various concentrations of Ca^{2+} in the presence of 5 nM [3H]ryanodine. Data shown are mean ± S.E. from three separate experiments.

FIGURE 5. Cryo-electron microscopy of RyR2-S437-GFP. A portion of a cryo-EM micrograph of the purified RyR2-S437-GFP proteins embedded in a thin layer of vitreous ice is shown. Several individual RyR2-S437-GFP particles are marked with white circles. The scale bar represents 500 Å.

FIGURE 6. Two-dimensional averages of RyR2-S437-GFP and RyR2-wt. A, two-dimensional average of RyR2-S437-GFP (n = 263 particle images) “top” view; B, top view of the two-dimensional average of RyR2-wt (n = 269 particle images); C, difference map obtained by subtracting B from A. The top view represents the projection of the channel as seen from the cytoplasmic side. The largest differences shown in C, corresponding to the additional masses because of the GFP insertion, are seen as bright white areas, one of which is circled, in the clamp-shaped domains. D, map of statistically significant regions of difference obtained by t test; map is displayed at >99.9% confidence level. The width of each frame is 544 Å.
positive densities, which represent extra protein mass present in RyR2s437-GFP but absent from RyR2-wt. These areas were located within the “clamp” structures that form the corners of the square-shaped receptor (Fig. 6C) (30, 31). The differences, which are highly significant, with a confidence level of >99.9% (Fig. 6D) (32), correspond to the additional mass contributed by the GFP insertion in RyR2s437-GFP.

To obtain a more detailed structure of RyR2s437-GFP and to map more precisely the location of the inserted GFP, three-dimensional volumes were computed and compared. Fig. 7A

FIGURE 7. Three-dimensional surface representation of RyR2s437-GFP and difference map. A, three-dimensional reconstruction of RyR2s437-GFP is shown in green. B, difference map (RyR2s437-GFP-RyR2-wt) shown in green is superimposed on the three-dimensional reconstruction of the RyR2-wt (blue). The three-dimensional reconstructions are shown in three views: left, top views from the cytoplasmic surface, which in situ would face the transverse tubule; middle, views toward the bottom of the channel (i.e., as it would appear if viewed from the lumen of the sarcoplasmic reticulum); right, side views. The numbers on the cytoplasmic assembly indicate the distinguishable domains. C, detailed view of the clamp-shaped structures, showing the relative positions of the inserted GFPs at Ser-437 and Ser-2367, the inserted GST at the amino terminus (red), and the docking of a domain formed by residues 216–572 in RyR1, as indicated by a dashed circle.
shows a surface representation of the resulting three-dimensional reconstruction of RyR2\textsubscript{S437-GFP} in three orientations (top, bottom, and side views). The reconstructed structure of RyR2\textsubscript{S437-GFP} resembles a mushroom shape, which consists of the following two major components: a large square cytoplasmic assembly (290 × 290 × 130 Å) encompassing 10 distinct domains (labeled by numbers in Fig. 7), and a smaller transmembrane assembly (120 × 120 × 70Å, labeled TA). The final resolution of the reconstructed RyR2\textsubscript{S437-GFP} was estimated to be 33 Å. Although the overall three-dimensional reconstruction of RyR2\textsubscript{S437-GFP} is very similar to that of RyR2-wt, some small differences exist. The most noticeable difference was found in the region between domains 5 and 9 in RyR2\textsubscript{S437-GFP}, which appears to be larger than the corresponding region in RyR2-wt. To further analyze these differences, a three-dimensional difference map was generated by subtracting the three-dimensional volume of RyR2-wt from that of RyR2\textsubscript{S437-GFP}. The difference regions are displayed in green and superimposed on the three-dimensional reconstruction of RyR2-wt (shown in blue) (see Fig. 7B). This difference map clearly shows four significant differences located between each pair of domains 5 and 9 in the cytoplasmic assembly. It should be noted first that 4-fold symmetry was imposed, and second that these are the only significant differences that appear when the three-dimensional difference map is displayed at a density threshold similar to the one used to image the RyR2\textsubscript{S437-GFP} and RyR2-wt structures. Because the GFP tag was inserted into each RyR2 monomer, and because RyR2 is a homotetramer composed of four identical monomers, the difference would be expected to repeat four times in the three-dimensional difference map. Consistent with the notion that these four structural repeated differences are a result of GFP insertion, the calculated volume of each of these four areas showing the most difference corresponds to a molecular mass of 28 kDa, assuming a protein density of 1.37 g/cm\textsuperscript{3} (33). This value agrees well with the molecular mass of GFP. Furthermore, the locations of the major differences observed in the three-dimensional reconstruction (Fig. 7) are consistent with those observed in the averaged two-dimensional projections (Fig. 6). Based on these observations, we conclude that the NH\textsubscript{2} mutation region, as indicated by the inserted GFP at Ser-437, is located between domains 5 and 9 in each of the clamp-shaped structures of RyR2.

DISCUSSION

Localization of an NH\textsubscript{2}-terminal Mutation Hot Spot within the Clamp-shaped Structure—The NH\textsubscript{2}-terminal region is one of three major regions in the RyR sequence that harbor disease-causing mutations (2, 4). To understand the structural basis of these mutations, a considerable effort has been made to localize the NH\textsubscript{2}-terminal domain in the three-dimensional structure of RyRs. The first structural localization of the NH\textsubscript{2}-terminal region was achieved by fusing glutathione S-transferase (GST) to the NH\textsubscript{2} terminus of the type 3 ryanodine receptor (RyR3) and then comparing three-dimensional reconstructions of the RyR3-wt and the GST-tagged RyR3 (32). That study mapped the GST and thus the NH\textsubscript{2} terminus to the middle of the region defined by domains 7–10 within the clamp-shaped structures located at the corners of the cytoplasmic assembly of RyR3 (32).

The localization of the NH\textsubscript{2} terminus within the clamp-shaped structure was confirmed in subsequent bioinformatics and cryo-EM studies. Baker et al. (35) identified an oxidoreductase-like domain formed by residues 41–420 in RyR1, corresponding to residues 41–435 in RyR2, and they computationally localized this domain to a region largely defined by domain 5 within the clamp-shaped structures in the cytoplasmic region of RyR1. Similarly, using a sequence-based fold recognition method, Serysheva et al. (36) predicted a domain fold for residues 216–572 of RyR1, corresponding to residues 230–583 in RyR2, based on the crystal structure of the IP\textsubscript{3}-binding core region of the type 1 IP\textsubscript{3} receptor, and they computationally docked this domain fold to a region defined by domains 5 and 9 in the clamp structures. Consistent with this computational localization, an antibody raised against a synthetic peptide corresponding to residues 416–434 in RyR1, equivalent to 431–449 in RyR2, was mapped in two-dimensional projection to two major sites within the clamp structures and two minor sites in the central portion of the channel (35). However, the three-dimensional localization of this antibody was not done, and so the identity of the domains involved could not be unambiguously assessed.

In this study, we engineered a GFP-RyR fusion protein in which GFP was inserted into the RyR2 sequence after residue Ser-437 and then mapped the inserted GFP to a surface-exposed site between domains 5 and 9 within the clamp-shaped region in the three-dimensional structure of RyR2. As shown in Fig. 7C, the three-dimensional location of GFP\textsubscript{S437} is largely superimposed on the predicted docking of residues 216–572 of RyR1 (corresponding to 230–583 of RyR2), and it also overlaps with the predicted localization of the COOH terminus of the oxidoreductase-like domain formed by residues 41–423 of RyR1 (corresponding to residues 41–435 of RyR2). Hence, the results of this study confirm and extend those of previous studies, which consistently predicted that the mutation hot spot in the NH\textsubscript{2}-terminal region around Ser-437 would be located in the vicinity of domains 5 and 9 within the clamp-shaped structure of RyRs.

The Clamp-shaped Structure of RyR Is a Crucial Part of the Channel That Is Highly Susceptible to Disease-causing Mutations—The clamp-shaped structures at the corners of the cytoplasmic assembly are the regions in the three-dimensional structure of RyRs that display major differences in conformations between the closed and open states (30, 31). Specifically, domains 9 and 10, which are fused in the closed state, are separated in the open conformation. Similarly, domains 5 and 6 are further apart in the open state than in the closed state. Furthermore, the size and shape of each of domains 5, 6, 9, and 10 themselves exhibit some changes when RyR switches from the closed to the open state. Although no major changes were observed between domains 5 and 9, where the GFP\textsubscript{S437} was located, at the current level of resolution, this region is likely to undergo some conformational changes as a result of major changes between domains 5 and 6, between domains 9 and 10, and within domains 5 and 9. Considering its involvement in channel gating, the clamp-shaped structure is likely to be susceptible to disease-causing mutations. In support of this view, we have mapped a mutation in the central region (GFP\textsubscript{S3367}) to
a site between domains 5 and 6 in a previous study (14), and we have mapped a mutation site in the NH2-terminal region into a spot between domains 5 and 6 in the present study (Fig. 7C). Based on these findings and on the previous localizations of the NH2 terminus, GFP1366, and GFP1874, all of which are within the clamp structure (32, 37, 38), we predict that other disease-causing mutations in the NH2-terminal region and the central region will be found to lie within the clamp-shaped structure.

RyRs are regulated by a number of endogenous and exogenous ligands and protein effectors, including Ca2+, Mg2+, ATP, FKBP12.6, calmodulin, DHPR, PKA, CaMKII, caffeine, and procaaine (39, 40). The location of the FKBP12.0/FKBP12.6-binding site was mapped to a region between domains 9 and 3, adjacent to the clamp-shaped structure (41, 42), whereas the binding site for calmodulin was located in a region between domains 3 and 4 (34). The binding sites for other ligands and modulators are unknown. Given the fact that the clamp-shaped structure is associated with disease-causing mutations and conformational changes involved in channel gating, it would not be surprising to find that the clamp-shaped structure is also a target of channel modulators. In this regard, it is of interest to note that the NH2-terminal sequence of RyR exhibits a high degree of homology to the IP3-binding core of the IP3 receptor (36), suggesting that the NH2-terminal region of RyR may contain binding sites for endogenous regulators. It is also interesting that the computationally predicted location of the COOH-terminal part of a domain formed by residues 216–572 of RyR1 (corresponding to 230–583 of RyR2) is very close to the FKBP12-binding site in the three-dimensional structure of RyR1 (36), suggesting that the NH2-terminal region is involved in FKBP-RyR interaction. Consistent with this possibility, we have demonstrated that the deletion of the first 305 NH2-terminal residues abolishes FKBP12.6 binding (24).

Implication for Disease Mechanisms Associated with the NH2-terminal and Central Mutations in RyRs—We have recently shown that disease-causing mutations in the NH2-terminal, central, and COOH-terminal regions of the RyR2 sequence enhance the sensitivity of the channel to activation by luminal Ca2+ (10, 11). The COOH-terminal region is folded within the trans-membrane assembly in three-dimensional structure and encompasses the channel conduction pathway. It contains regions that are accessible to luminal Ca2+ and likely involved in the activation of the channel by luminal Ca2+. However, it is more difficult to imagine how mutations in the NH2-terminal and central regions would affect luminal Ca2+ sensing, which is presumably controlled by the COOH-terminal region. Ikemoto and Yamamoto (12) have proposed that although these are far apart in the primary sequence of RyR2, the NH2-terminal and central regions of RyR are located in close proximity in the three-dimensional structure of the channel. Those authors further hypothesize that disease-causing mutations are located on the surface of the NH2-terminal and central domains. In their view, these mutations weaken normal inter-domain interactions, thereby promoting channel opening upon stimulation via long range (>100 Å) conformational alterations that link the NH2-terminal and central regions to the COOH-terminal region (12). A test of the “zipping-unzipping” hypothesis of this domain would be to locate the NH2-terminal and central mutation regions in the three-dimensional structure. To this end, we have mapped a central mutation site to an area bridging domains 5 and 6 and an NH2-terminal mutation hot spot to a site between domains 5 and 9. The two sites are separated by about 30 Å. Hence, our data support the idea that the NH2-terminal and central mutation regions are closely neighboring in the three-dimensional structure of RyR2. However, given the moderate resolution of our three-dimensional reconstructions, it is impossible to pinpoint the areas of interaction between the NH2-terminal and central mutation regions. Because the location of GFP5437, was about 30 Å away from that of GFP52367, it is unlikely that the NH2-terminal mutations near Ser-437 directly interact with the central mutation site around Ser-2367. On the other hand, it has been proposed that the region containing another mutation Arg-614, directly interacts with the central mutation region (13). Thus, it will be of great interest to map residue Arg-614 onto the three-dimensional structure of RyR2.

Further work is needed to define how many and precisely which domains are involved in the cardiac and skeletal muscle diseases that arise from mutations in the NH2-terminal and central regions of RyRs. Based on the results discussed so far, it seems that domain 5 is certainly involved and that domains 6 and/or possibly domain 9 are as well. The simplest interpretation of our structural findings that is consistent with domain-domain interaction hypothesis of Ikemoto (12) is that the NH2-terminal mutations are located in domain 5 and the central mutations are in domain 6, and that alteration of interactions between these two domains is responsible for the observed phenotypes. However, we cannot yet exclude the other domains in the clamp-shaped structure (7, 8, 10 in Fig. 7) until the boundaries of the mutation hot spots are better defined.

An interesting observation from this study is that the insertion of GFP into an NH2-terminal mutation hot spot after residue Ser-437 did not significantly alter the functional properties of RyR2, despite the fact that mutations around the GFP insertion site (Ser-437) alter channel function. HEK293 cells expressing RyR25437-GFP displayed responses to caffeine, ryanodine, and Ca2+ overload similar to those of HEK293 cells expressing RyR2-wt. The Ca2+ - and caffeine-dependent activations of [3H]ryanodine binding to RyR2-wt and RyR25437-GFP are indistinguishable. The reason for the lack of functional impact of the inserted GFP is not clear, but it is likely related to the position of insertion and the structure of the inserted GFP. The inserted GFP was located in the middle of a small stretch of 12 amino acid residues that are highly divergent among RyR isoforms. Disease-causing RyR mutations are located on either side of this region. It is possible that this stretch of amino acids forms a surface-exposed loop, and that the insertion of GFP into this loop does not alter the nearby structures. Furthermore, the inserted GFP was flanked by 9–10 amino acid spacers containing eight glycine residues, which should provide a high degree of flexibility and minimize steric hindrance. Because the GFP-inserted region is highly divergent among RyR isoforms, the impact of the GFP insertion on the function of the RyR channels may be isoform-dependent. Investigations are currently underway to determine whether the insertion of GFP into the corresponding region of RyR1 alters the functional properties of the RyR1 channel.
In summary, despite an increasing body of biochemical and functional evidence suggesting that disease-causing RyR mutations are located along the domain interface between the NH2-terminal and central regions of RyRs and weaken inter-domain interactions (the domain unzipping hypothesis), there has been little structural evidence for these inter-domain interactions. To test this domain unzipping hypothesis, we have started to map different mutation regions in the three-dimensional structure of RyR2. Our data indicate that although they are ~2000 residues apart in the primary sequence of RyR2, the NH2-terminal and central mutation regions are closely located in the three-dimensional structure of RyR2.

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