Cryoelectron Microscopy Resolves FK506-Binding Protein Sites on the Skeletal Muscle Ryanodine Receptor

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ABSTRACT A 12-kDa immunophilin (FKBP12) is an integral component of the skeletal muscle ryanodine receptor (RyR). The RyR is a hetero-oligomeric complex with structural formula $(FKBP)_4(Ryr1)_4$, where Ryr1 is the 565-kDa product of the Ryr1 gene. To aid in the detection of the immunophilin's location in the receptor, we exchanged the FKBP12 present in RyR-enriched vesicles derived from sarcoplasmic reticulum with an engineered construct of FKBP12 fused to glutathione *S*-transferase and then isolated the complexes. Cryoelectron microscopy and image averaging of the complexes (in an orientation displaying the RyR's fourfold symmetry) revealed four symmetrically distributed, diffuse density regions that were located just outside the boundary defining the cytoplasmic assembly of the RyR. These regions are attributed to the glutathione transferase portion of the fusion protein because they are absent from receptors lacking the fusion protein. To more precisely define the location of FKBP12, we similarly analyzed complexes of RyR containing FKBP12 itself. Apparently some FKBP is lost during purification or storage of the RyR because, to detect the receptor-bound immunophilin, it was necessary to add FKBP12 to the purified receptor before electron microscopy. Averaged images of these complexes showed a region of density that had not been observed previously in images of isolated receptors, and its position, along the edges of the transmembrane assembly, agreed with the position of the FKBP12 deduced from the experiments with the fusion protein. The proposed locations for FKBP12 are about 10 nm from the transmembrane baseplate assembly that contains the ion channel of the RyR.

INTRODUCTION

Immunophilins are intracellular proteins that were originally identified as receptors for drugs that inhibit the immune response. Their cellular functions in the absence of immunosuppressants are unclear, but they are thought to serve as molecular chaperones that assist in protein folding and assembly (reviewed in Fruman et al., 1994). Rutherford and Zuker (1994) hypothesize that immunophilins also function in cellular signal transduction pathways by forming complexes with other macromolecules, thereby promoting structural transitions that are required for signaling. A potential example of this latter function is the stable complex formed by the immunophilin, FK506-binding protein (FKBP12), and the skeletal muscle ryanodine receptor protein (Collins, 1991; Jayaraman et al., 1992).

The ryanodine receptor (RyR) is an intracellular calcium release channel located on the junctional face membrane of the terminal cisternae regions of the sarcoplasmic reticulum in striated muscle. The receptor mediates release of Ca^{2+} from intracellular stores and hence plays a key role in excitation-contraction coupling. Several studies have documented differences in channel activity of RyRs that contain FKBP12 as compared with receptors depleted of it, but the structural basis of these effects is unknown (Timerman et al., 1993, 1995; Brillantes et al., 1994; Mayrleitner et al., 1994; Chen et al., 1994; Ma et al., 1995).

Association of FK-506 binding proteins with intracellular calcium channels appears to be of general significance as the heart isoform of the RyR binds a protein similar to FKBP12 (Timerman et al., 1994) and the inositol 1,4,5-trisphosphate receptor (the other major intracellular calcium release channel) from rat cerebellum binds FKBP12 (Cameron et al., 1995).

We have been investigating the structure of the skeletal muscle RyR by cryoelectron microscopy and three-dimensional reconstruction (Wagenknecht et al., 1989, 1994; Radermacher et al., 1992, 1994; Wagenknecht and Radermacher, 1995). This RyR comprises four copies each of a large subunit (565 kDa) encoded by the Ryr1 gene and FKBP12 (for reviews see Fleischer and Inui, 1989; Meissner, 1994; Ogawa, 1994; Coronado et al., 1994). Here we show that four symmetrically related binding sites for the FKBP12 are present on the cytoplasmic assembly of the RyR. The sites are along the edges of the square-shaped assembly, and 10

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Abbreviations used: FKBP12, the 12-kDa FK506-binding protein; GST, glutathione *S*-transferase; GST/FKBP12, fusion protein of GST and FKBP; RyR, ryanodine receptor; Ryr1, polypeptide gene product of the skeletal muscle isoform of the RyR's large subunit; CHAPS, 3-[(cholamidopropyl)dimethylammonio]-1-propane sulfate; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

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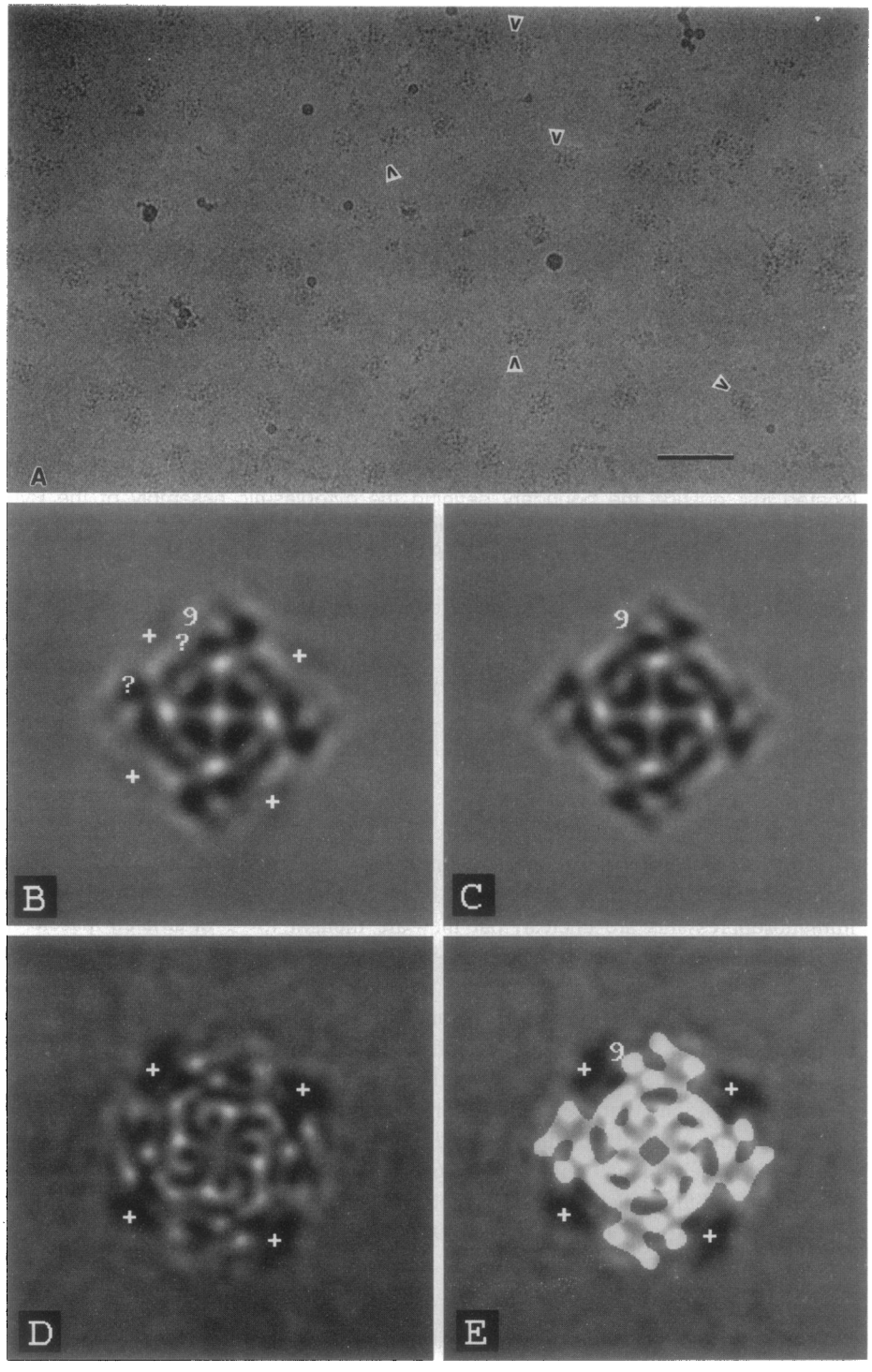


FIGURE 1 Cryoelectron microscopy and image averaging of RyRs containing GST/FKBP12. (*A*) Electron micrograph of frozen-hydrated GST/FKBP12:RyR complexes. Arrowheads point to mass densities, presumably due to the GST, that are peripherally associated with RyRs (which appear square in overall shape). Bar, 100 nm. (*B*) Averaged image (N = 371) of RyR:GST/FKBP12 complexes. +, density regions attributed to GST; ?, potential regions where GSTs are attached via FKBP12 to the RyR. The region labeled 9 corresponds to a structural domain identified previously in three-dimensional reconstructions of the RyR (Radermacher et al., 1994). (*C*) Control-averaged image of RyRs isolated by standard protocol and described previously (Radermacher et al., 1994). (*D*) Difference formed by subtracting averaged image in *C* from that in *B*. (*E*) Same as *D*, except that an outline of the control average is shown superimposed on the difference map. Width of each frame in *B–E* is 68 nm.

or more nanometers from the putative entrance of the ion-conducting channel that is located in the transmembrane assembly.

MATERIALS AND METHODS

Materials

FK590 (an analog of FK506 that is equally effective in displacing FKBP12 from the RyR), human FKBP12, and the GST/FKBP12 construct were provided by Dr. G. Wiederrecht (Merck Research Laboratories, Rahway, NJ). RyRs in which FKBP12 was replaced with GST/FKBP12 were isolated as described by Xin et al. (1995). [^{3}H]Ryanodine was from New England Nuclear. CHAPS was from Calbiochem (La Jolla, CA).

Purification of RyR from rabbit skeletal muscle

RyRs were isolated from terminal cisternae vesicles of sarcoplasmic reticulum (Saito et al., 1984) after solubilization in the presence of 4% CHAPS (Inui et al., 1987). The solubilized receptor was purified by centrifugation through sucrose density gradients (10–20% w/w) in a buffer consisting of

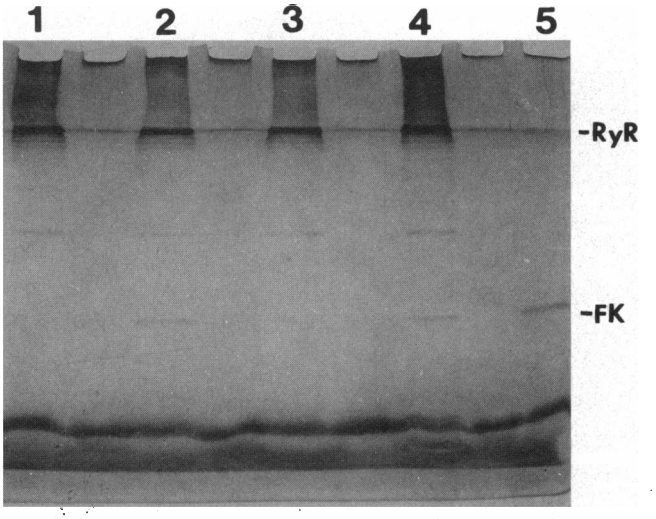


FIGURE 2 Purified RyR binds exogenously added FKBP12. SDS-PAGE of resuspended pellets resulting from centrifugation of the following (see Materials and Methods): (*Lane 1*) RyR incubated with exogenous FKBP12 in the presence of FK590. Note much reduced intensity of band corresponding to FKBP12 (labeled FK at right side). (*Lane 2*) RyR incubated with excess FKBP12. (*Lane 3*) RyR alone. (*Lane 4*) RyR before ultracentrifugation (2 μg). (*Lane 5*) FKBP12 (0.1 μg). For *Lanes 1–3*, a volume of resuspended receptor corresponding to 2 μg of protein, assuming 100% recovery in the pellets, was applied. Quantitative densitometry indicated that the amount of protein in the band corresponding to the Ryr1 gene product (RyR on right side) in each of *Lanes 1–4* was within 12% of its mean value. The band of mobility intermediate to that of Ryr1 and FKBP12 is a contaminant, most likely the sarcoplasmic Ca^{2+}-ATPase.

20 mM Tris-HCl (pH 7.4), 1.0 M NaCl, 2 mM dithiothreitol, 1 μg/ml leupeptin, 1% (w/v) CHAPS, 0.5% (w/v) soy phosphatidyl choline, essentially as described by Lai et al. (1988) and Timerman et al. (1993). The peak of ryanodine binding activity was pooled and passed through a "desalting" column (2.5 × 14 cm) containing Sephadex G25 (Pharmacia) and equilibrated with buffer (20 mM Tris-HCl (pH 7.4), 0.1 M NaCl, 2 mM dithiothreitol, 0.5% CHAPS, 0.5% phosphatidylcholine, 1 μg/ml leupeptin). The RyR was further purified and concentrated by chromatography on a heparin agarose (Bio-Rad) column (0.7 × 8 cm). The material eluted from the Sephadex G25 column was applied to the heparin-agarose column, and elution was achieved with a buffer containing 1.0 M NaCl (buffer I: 0.02 M Tris-HCl (pH 7.4), 1 M NaCl, 2 mM dithiothreitol, 0.5% CHAPS, 1 μg/ml leupeptin).

Preparation and characterization of RyR with exogenously added FKBP12 and FKBP12-depleted RyR

Purified RyR in buffer I at 0.15 μM (0.035 mg/ml) was incubated at room temperature for 60 min with FKBP12 at 4.4–4.7 μM in the absence or presence of 50 μM FK590. Aliquots were removed to prepare grids for cryoelectron microscopy (see below) or for analysis by sedimentation and electrophoresis. For the latter purpose, 21 μl of sample was loaded into a microcentrifuge tube (5 by 20 mm) containing a sucrose shelf (170 μl of 10% (w/w) sucrose in buffer I). The tubes were centrifuged for 4 h at 45,000 rpm and 4°C in a Beckman TLS-55 rotor using a Beckman Optima TLX tabletop ultracentrifuge. The supernatants were removed by aspiration, and the pellets were resuspended in sample buffer for SDS-PAGE using the Laemmli (1970) buffer system.

Cryoelectron microscopy and image processing

Five-microliter portions of specimen containing RyR at 0.035 mg/ml were applied to carbon-coated electron microscope grids and frozen by plunging into cryogen (ethane). The grids were transferred to a Gatan model 626 cryo-holder, and electron microscopy was performed using a Philips EM420 transmission electron microscope operated at 100 kV and a magnification of 36,000× or 49,000×. The procedures were essentially identical to those described previously (Wagenknecht et al., 1994).

Image processing was done using the SPIDER software package (Frank et al., 1981a) as described by Wagenknecht et al. (1994). For computing averaged images, homogeneous sets of aligned RyR images were selected by multivariate statistical analysis as described by Radermacher et al. (1994). Fourfold symmetry was enforced in all of the averages shown; no significant differences were apparent in the averages after symmetrization other than the expected improvement in signal-to-noise ratio. Reproducibility of the averaged images was estimated by the 45-degree phase residual criterion (Frank et al., 1981b). All of the averaged images shown in Figs. 1 and 3 had resolutions in the range of 3–3.5 nm. SPIDER includes programs to assess the statistical significance of differences between images (e.g., *t*-test; Frank et al., 1985). The differences that are attributed to RyR-bound FKBP12 and GST/FKBP12 in this study are highly significant (confidence limits greater than 99.9%).

RESULTS AND DISCUSSION

Our initial efforts to detect endogenous FKBP12 associated with purified, CHAPS-solubilized RyRs by electron microscopy/image analysis were unsuccessful (results not shown). In these experiments RyR was purified from terminal cisternae vesicles that had either been treated or not been treated with FK-590, a drug that has been shown to dissociate FKBP12 from the RyR (Timerman et al., 1993). Quantitative comparison of electron micrographs obtained from the two preparations failed to resolve any density differences attributable to FKBP12. Evidently, much of the FKBP12 dissociated from the receptors during purification, storage, or preparation for electron microscopy (see Fig. 2 and discussion thereof below), because RyRs in the terminal cisternae (the starting material for the purification of receptors) have 4 moles of bound FKBP per mole of receptor (Timerman et al., 1993).

Two modified experimental approaches were devised to redress the putative loss of FKBP12 from the receptor, the results of which are described in the following sections. In the first modification, RyRs were isolated from sarcoplasmic vesicles for which the endogenous FKBP12 was exchanged with a fusion protein (GST/FKBP12) consisting of FKBP12 and glutathione transferase (Xin et al., 1995). The rationale is that the larger fusion protein should be easier to detect than FKBP12, even if levels of binding are substoichiometric (i.e., less than 4 moles bound FKBP12 per mole tetrameric RyR).

In the second modified protocol, we added excess exogenous FKBP12 to purified RyRs to fill any vacant binding sites that might have been created during the isolation and preparation for electron microsocopy. Electron micrograph images of these RyRs were then quantitatively compared with images from receptors that were treated with FK590 to dissociate FKBP12.

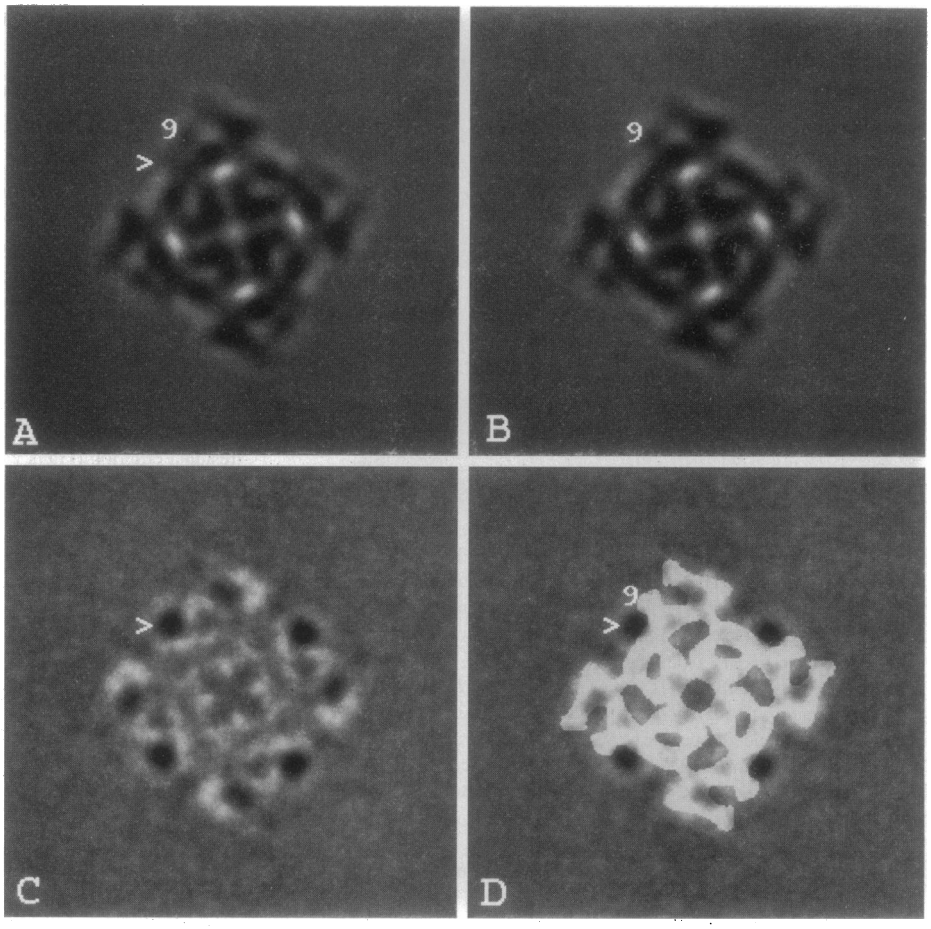
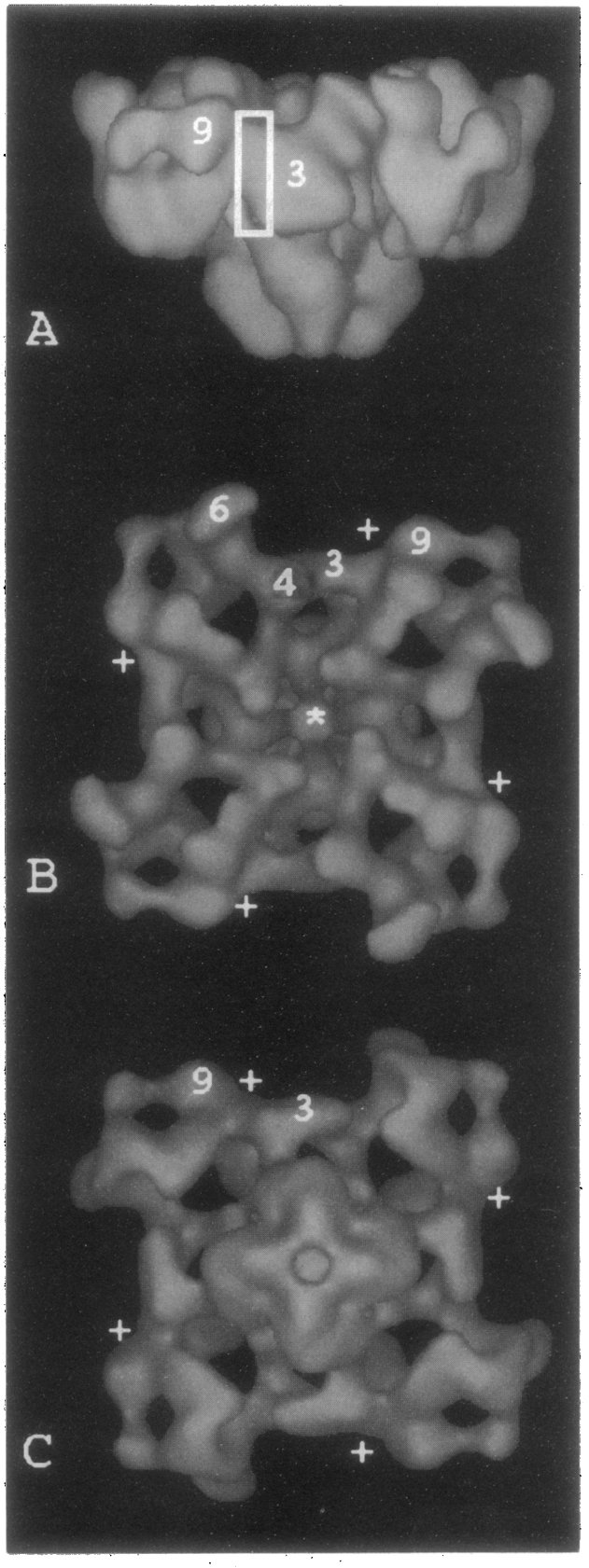


FIGURE 3 Cryoelectron microscopy and image averaging of RyRs containing exogenously added FKBP12 in the presence and absence of FK590. (*A*) Averaged image of RyR incubated with exogenous FKBP12 ($N = 632$). (*B*) Averaged image of RyR incubated with exogenous FKBP12 and FK590 ($N = 412$). (*C*) Difference image obtained by subtracting *B* from *A*. (*D*) difference image as in *C*, but with outline of control image in *B* superimposed. The four largest, symmetrically arranged peaks, one of which is marked with an arrowhead in *A*, *C*, and *D*, correspond to mass present in *A* but not in *B*. Domain 9 is also indicated in *A*, *B*, and *D*. Width of each frame, 68 nm.

Cryoelectron microscopy/image analysis of RyRs containing bound GST/FKBP12

Fig. 1 *A* shows selected images from an electron micrograph of solubilized, frozen-hydrated RyRs isolated from SR-derived vesicles containing the GST/FKBP12 fusion protein in place of FKBP12. Biochemical and functional characterization of terminal cisternae vesicles has shown that the endogenous FKBP12 readily undergoes competitive exchange with FKBP12 or various modified forms of it (Timerman et al., 1995; Xin et al., 1995). The RyRs appear to be morphologically intact for the most part and appear essentially the same as RyRs obtained without the exchange of GST/FKBP12. Upon close inspection some of the complexes appear to have some extra density outside the boundary of the receptor (see marked receptors in the electron micrograph shown in Fig. 1 *A*), but, because of the low signal-to-noise ratio in the micrographs, it is necessary to average images to establish the validity of the peripherally localized densities. An averaged image, shown in Fig. 1 *B*, clearly shows four symmetically distributed regions of density surrounding the RyR. We have never observed this peripheral density in averages of RyRs isolated in the usual manner (e.g., Fig. 1 *C*), and these regions are attributed to the glutathione transferase portion of the fusion protein. The size of the peripheral densities appears larger than expected on the basis of the known three-dimensional structures of glutathione transferases (Wilce and Parker, 1995); this could be due to flexibility of the connection between the GST and FKBP12, which would result in a smearing out of the density. Alternatively, it is possible that the fusion protein forms dimers via interactions among the GST domains (Walker et al., 1993).

From the averaged image in Fig. 1 *B* it is difficult to determine where the peripherally attached material is attached to the RyR, which should correspond to the position of the FKBP12. Weak bridges of density appear to extend to the edge of the RyR at two locations (indicated by ? in Fig. 1 *B*), with the bridge extending toward the structural feature labeled 9 (corresponding to domain 9 in the three-dimensional reconstruction described by Radermacher et al., 1994) appearing slightly more plausible because it is better defined and the distance it traverses is shorter than the other bridge. This is more clearly seen in the difference image, obtained by subtracting the control average (Fig. 1 *C*) from that of the GST/FKBP:RyR (Fig. 1 *B*), which shows, in addition to the main differences due to the GST, subsidiary regions of positive difference adjacent to domain 9 (Fig. 1, *D* and *E*). Confirmation that the region adjacent to domain 9 is indeed the location of FKBP12 was obtained from the experiments with exogenous FKBP12, described below.



Analysis of RyRs with exogenously added FKBP12

Purified RyRs were incubated with added FKBP12 at final concentrations of 0.15 and 5 μM, respectively (see Materials and Methods for details). A second incubation was done identically, except that FK-590 was also present (50 μM) to promote dissociation of FKBP12 from RyR. Evidence that substantial binding of exogenously added FKBP12 occurred in the absence of FK590 was obtained by ultracentrifugation of a portion of each incubation followed by SDS-PAGE of the pellets (Fig. 2). Comparison of Lanes 2 (RyR in presence of exogenously added FKBP12) and 1 (RyR with FK590 and FKBP12 added) shows significantly more FKBP12 in Lane 2. Thus, the drug, FK590, was effective in preventing complex formation and in dissociating endogenous FKBP12 from the RyR. Lane 3, containing isolated RyR that had not been treated with the drug or exogenous FKBP12, shows a reduced level of (bound) FKBP12 relative to that when exogenous FKBP was present (Lane 2). This indicates that isolated RyRs contain less than the maximal amount FKBP12, and is consistent with our hypothesis that some dissociation of FKBP12 occurs during purification of the receptor. Purified RyR that was not pelleted before electrophoresis (Lane 4) shows a higher level of FKBP12 than the pelleted receptor (Lane 3), indicating that some of the FKBP12 in the purified receptor is not bound or is not bound with sufficient affinity to cosediment quantitatively with the RyR.

Averaged images obtained from electron micrographs of frozen-hydrated RyRs that had been incubated with exogenous FKBP12 in the absence and presence of FK590 are shown in Fig. 3, *A* and *B*, respectively. The average of the receptors that were not treated with the drug shows an additional density protruding from each of the receptor's edges (arrowheads in the figure) that is not present in the drug-treated average. This extra density dominates the difference image (Fig. 3 *C*) formed by subtracting the average in Fig. 3 *B* from that in Fig. 3 *A*, and, therefore, likely corresponds to receptor-bound FKBP12. The positions of the differences attributed to FKBP12 are near subdomain 9 of the RyR, in agreement with the position inferred from the experiment with the GST/FKBP12 fusion protein (Fig. 1). Furthermore, its apparent diameter, ~3 nm, is consistent with the known three-dimensional structure of FKBP12 (Moore et al., 1991; van Duyne et al., 1991). It seems unlikely that the

FIGURE 4 Three-dimensional model of RyR from Radermacher et al. (1994) showing locations of FKBP12. (*A*) Solid-body representation perpendicular to fourfold symmetry axis (side view) with transmembrane assembly at bottom. The rectangle indicates range of potential positions for FKBP12. (*B*) View along fourfold axis showing face of cytoplasmic assembly that faces the transverse tubule in situ. (*C*) View along fourfold axis onto sarcoplasmic face that contains the transmembrane assembly (*center*). Asterisk indicates likely location of entrance to transmembrane ion-conducting channel; +, location of density differences as defined in Fig. 3; numerals refer to domains as assigned in Radermacher et al. (1994).

density assigned to FKBP12 in Fig. 3 is due to binding of the immunophilin to a secondary site that differs from the site occupied in isolated sarcoplasmic vesicles. First, no other differences of magnitude comparable to that assigned to FKBP12 are present in the difference map. If the exogenously added FKBP12 resulted in occupation of a secondary site, then eight peaks should have been present in the difference image instead of four. Second, the binding of exogenous FKBP12 is prevented by FK-590, which is the same behavior as for sarcoplasmic vesicles. Third, the proposed location for receptor-bound FKBP12 is consistent with the results of the experiment using GST/FKBP12 (Fig. 1). In that experiment the FKBP12 was replaced by GST/FKBP12 on RyRs that were associated with terminal cisternae, and then the receptors were isolated by affinity chromatography—no exogenous, excess GST/FKBP12 was added to the solubilized, purified receptor. Characterization of FKBP12 exchange on terminal cisternae has shown that exchange occurs by competition with endogenous FKBP12 for the same binding sites and with retention of native function (Mayrleitner et al., 1994; Timerman et al., 1995).

In this study we have localized the binding of FKBP12 in two dimensions to a position that is along the edges of the RyR's transmembrane assembly. A three-dimensional reconstruction, currently in progress, will be required to precisely define the location of the bound FKBP12 in the third dimension and to characterize any structural changes that occur when FKBP12 is removed. In the interim, we show in Fig. 4 the range of possible positions for FKBP12 on the three-dimensional reconstruction of the RyR that was described previously (Radermacher et al., 1994). Note that the uncertainty in the indeterminate ("z") direction can be no greater than 60–70 Å (Fig. 4 *A*), the thickness of the RyR in this direction. FKBP12 binds on the RyR on domain 3, which forms the sides of the cytoplasmic assembly, near domain 9. Four symmetry-related binding positions are present on the RyR, which is consistent with binding data indicating four binding sites per tetrameric RyR (Timerman et al., 1993, 1995). The FKBP12 binding sites are about 10 nm from the center of the averaged image (Fig. 3 *C*), the presumed location of the ion channel. The actual distance could be several nanometers greater than this, depending on the *z*-coordinate of the binding site.

FKBP12 is the second RyR-modulating protein ligand that has been mapped to a peripheral binding location on the cytoplasmic assembly of the receptor (Wagenknecht et al., 1994). The other ligand is calmodulin, which binds on the opposite side of domain 3 from that where FKBP12 binds, (Fig. 4 *A*) (binding studies indicate that additional calmodulin sites are present under other experimental conditions; Yang et al., 1994; Tripathy et al., 1995). Both FKBP12 and calmodulin affect the ion channel activity of the RyR, and our results argue for the involvement of long-range structural changes in the RyR because both ligands bind at large distances from the putative location of the ion-conducting channel in the transmembrane assembly.

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