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Negatively Charged Amino Acids within the Intraluminal Loop of Ryanodine Receptor Are Involved in the Interaction with Triadin*

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In mammalian striated muscles, ryanodine receptor (RyR), triadin, junctin, and calsequestrin form a quaternary complex in the lumen of sarcoplasmic reticulum. Such intermolecular interactions contribute not only to the passive buffering of sarcoplasmic reticulum luminal Ca^{2+}, but also to the active Ca^{2+} release process during excitation-contraction coupling. Here we tested the hypothesis that specific charged amino acids within the luminal portion of RyR mediate its direct interaction with triadin. Using *in vitro* binding assay and site-directed mutagenesis, we found that the second intraluminal loop of the skeletal muscle RyR1 (amino acids 4860–4917), but not the first intraluminal loop of RyR1 (amino acids 4581–4640) could bind triadin. Specifically, three negatively charged residues Asp^{4878}, Asp^{4907}, and Glu^{4908} appear to be critical for the association with triadin. Using deletional approaches, we showed that a KEKE motif of triadin (amino acids 200–232) is essential for the binding to RyR1. Because the second intraluminal loop of RyR has been previously shown to contain the ion-conducting pore as well as the selectivity filter of the Ca^{2+} release channel, and Asp^{4878}, Asp^{4907}, and Glu^{4908} residues are predicted to locate at the periphery of the pore assembly of the channel, our data suggest that a physical interaction between RyR1 and triadin could play an active role in the overall Ca^{2+} release process of excitation-contraction coupling in muscle cells.

Ultrastructural and biochemical evidence suggests that a protein complex exists at the junctional SR[1] membrane in cardiac and skeletal muscle to facilitate the Ca^{2+} release that occurs during muscle contraction (1–4). These include the ryanodine receptor (RyR), triadin, junctin, and calsequestrin, which may associate into a stable complex at the junctional membrane (5). The RyR contains a large cytoplasmic foot region plus a transmembrane domain at the carboxyl-terminal end. The transmembrane domain of RyR has been shown to contain the conduction pore of the Ca^{2+} release channel (6). According to the hydropathy plot of Takeshima *et al.* (7), four putative transmembrane segments (TM1 to TM4) are predicted to span the SR membrane, with residues connecting TM1 and TM2 forming the first intraluminal loop (aa 4581–4640) and residues connecting TM3 and TM4 contributing to the second intraluminal loop (aa 4860–4917). Elegant studies from other investigators have shown that the second intraluminal loop of RyR participates in the overall ion conduction and selectivity process of the Ca^{2+} release channel (8–10).

Calsequestrin is the high capacity Ca^{2+} storage protein located in the lumen of the SR (11). In striated muscles, calsequestrins are largely tethered to the terminal cisternae of SR, leading to the suggestion that they could sequester Ca^{2+} to sites of Ca^{2+} release (12). Triadin and junctin are structurally similar integral membrane proteins having a short amino-terminal cytoplasmic domain and a long stretch exposed to the luminal side of the SR (13, 14). The luminal region is especially enriched in multiple clusters of alternating lysine and glutamic acid residues, named as the KEKE motif (14–16).

Previous studies show that triadin could directly modulate the activity of the RyR1 channel in a reconstitutional system (17, 18). Calsequestrin has been proposed to indirectly regulate the RyR function presumably through its interaction with triadin and junctin (5, 19). Recently, the binding regions of calsequestrin and the histidine-rich Ca^{2+}-binding protein to triadin were identified in our group (20, 21). However, the nature of interaction between triadin and RyR, and the amino acids involved in such interaction have not been elucidated.

In the present study, we tested the hypothesis that the highly conserved and charged residues within the intraluminal loop of RyR1 contain the binding sites for triadin. Using *in vitro* binding assay, we showed that the second intraluminal loop, but not the first intraluminal loop of RyR1 could interact with triadin. Furthermore, we identified specific amino acid residues within the second intraluminal loop that are critical for the binding to triadin. This identification of interaction sites between RyR1 and triadin will help to understand the contribution of the quaternary complex formation in the process of Ca^{2+} release.

EXPERIMENTAL PROCEDURES

Isolation of Heavy SR Vesicles—Heavy SR vesicles were isolated from skeletal muscle of male New Zealand White rabbits according to the procedure described previously (22). These heavy SR vesicles were solubilized at a protein concentration of 5 mg/ml in a buffer containing 2% Triton X-100, 1 M NaCl, 1 mM dithiothreitol, 20 mM Tris-Cl (pH 7.4), and protease inhibitor mixture (Roche Applied Science) for 4 h at 4 °C.

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[1] The abbreviations used are: SR, sarcoplasmic reticulum; RyR, ryanodine receptor; aa, amino acid(s); TM, transmembrane; GST, glutathione *S*-transferase.

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TABLE I
Primer sets for RT-PCR, PCR, and site-directed mutagenesis

The changed nucleotide is underlined.

Primer name	Sequence
Tri-A	5′-TAAGAATTCAAAAACTTTTCAGCAAGC-3′, 5′-TAACTCGAGCTGTTCTTGTTTTGAAACAGC-3
Tri-B	5′-TAAGAATTCAAAAACTTTTCAGCAAGC-3′, 5′-TAACTCGAGTTTTGAAACAGCAGCAGT-3′
Tri-C	5′-AGTCGAATTCCAGGAAAAACCTGAGAGG-3′, 5′-TTAGCTCGAGGTTTTGAAACAGCAGCAG-3′
Tri-D	5′-AGTCGAATTCCAGGAAAAACCTGAGAGG-3′, 5′-TCTTCTCGAGTTTTGTCACTGTCTTTGTTTCTGG-3′
Tri-E	5′-AGTCGAATTCGAGGAGAAGAAAGCTCG-3′, 5′-TTAGCTCGAGGTTTTGAAACAGCAGCAG-3′
Tri-F	5′-ATAGAATTCAAGACAGTGACAAAAGAG-3′, 5′-ATACTCGAGTTTCACTTTCTCCTGTTT-3′
Tri-G	5′-ATAGAATTCCAAACGGTTGCAAAGGCA-3′, 5′-ATACTCGAGTTTTGAAACAGCAGCAGT-3′
Loop I	5′-TAAGAATTCAAGGTCTCAGACTCTCCA-3′, 5′-TAACTCGAGCTCCATGTAGCCCGTGCT-3′
Loop II	5′-TAAGAATTCCGCAAGTTCTACAACAAG-3′, 5′-TAACTCGAGGTCGAAGACCACCCGGTA-3′
P-Loop II	5′-TAAGAATTCCGCAAGTTCTACAACAAG-3′, 5′-TAACTCGAGCACGTACATGTGGAACAG-3′
D-Loop II	5′-TAAGAATTCGGCGTCCGGGCTGGCGGA-3′, 5′-TAACTCGAGGTCGAAGACCACCCGGTA-3′
D4877A	5′-GAGCCGGACATGAAGTGCGCTGACATGATGACGTGCTAC-3′, 5′-GTAGCACGTCATCATGTCAGCGCACTTCATGTCCGGCTC-3′
D4878A	5′-CCGGACATGAAGTGCGATGCCATGATGACGTGCTACCTG-3′, 5′-CAGGTAGCACGTCATCATGGCATGGCACTTCATGTCCGG-3′
D4907A	5′-ATCGAGGACCCAGCGGGCGCTGAATACGAGCTCTACCGG-3′, 5′-CCGGTAGAGCTCGTATTCAGCGCCCGCTGGGTCCTCGAT-3′
E4908A	5′-GAGGACCCAGCGGGCGATGCATACGAGCTCTACCGGGTG-3′, 5′-CACCCGGTAGAGCTCGTATGCATCGCCCGCTGGGTCCTC-3′

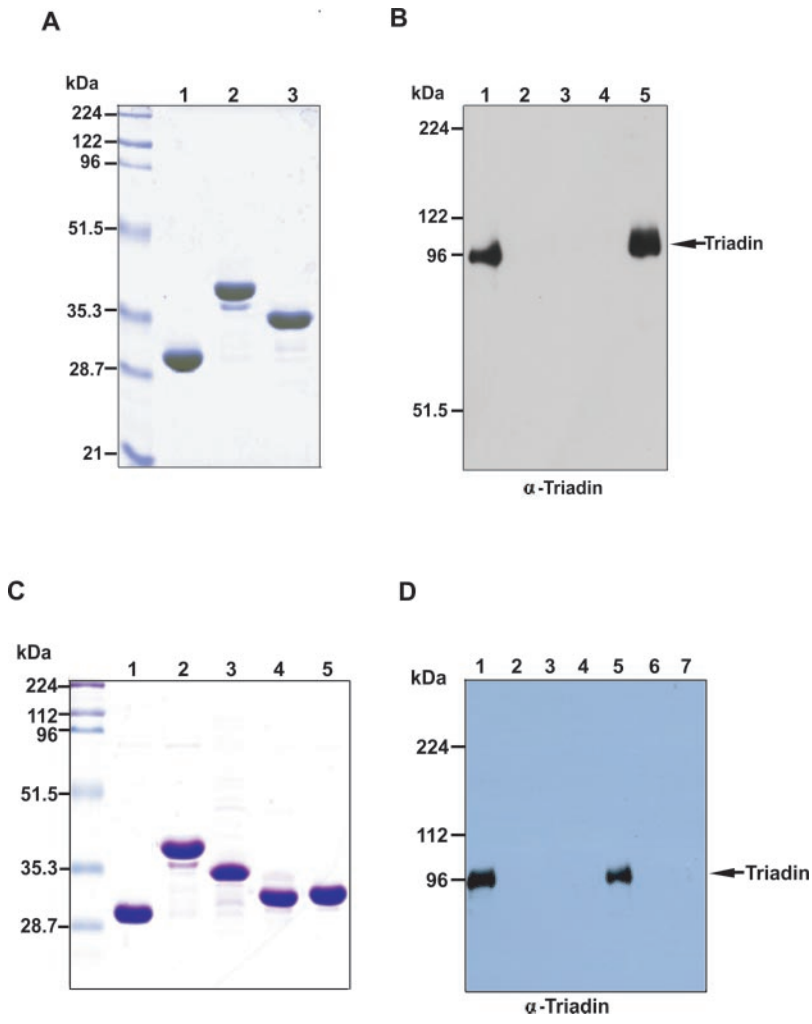

FIG. 1. **Triadin binds to the second intraluminal loop of RyR1.** *A*, purified GST control (*lane 1*), GST-Loop I (*lane 2*), and GST-Loop II (*lane 3*) fusion proteins expressed in *E. coli* (DH5α) were separated in a 10% SDS-PAGE gel (10 μg each), and stained with Coomassie Blue. *B*, the purified GST control, GST-Loop I, and GST-Loop II Sepharose affinity beads (250 μg each) were incubated with Triton X-100-solubilized heavy SR vesicle. The bound proteins were eluted by SDS sample buffer, separated by 6% SDS-PAGE, and immunoblotting was conducted with anti-triadin antibody. *Lane 1*, heavy SR (2.5 μg); *lane 2*, affinity beads alone; *lane 3*, GST control; *lane 4*, GST-Loop I affinity beads; *lane 5*, GST-Loop II affinity beads. *C*, Coomassie Blue staining of GST control (*lane 1*), GST-Loop I (*lane 2*), GST-Loop II (*lane 3*), GST-P-Loop II (*lane 4*), and GST-D-Loop II (*lane 5*) proteins expressed in *E. coli* (10 μg each lane). *D*, *in vitro* binding assays of the GST control, GST-Loop I, GST-Loop II, GST-P-Loop II, and GST-D-Loop II with triadin in solubilized heavy SR were performed as in *B*. *Lane 1*, heavy SR (2.5 μg); *lane 2*, affinity beads alone; *lane 3*, GST control; *lane 4*, GST-Loop I; *lane 5*, GST-Loop II; *lane 6*, GST-P-Loop II; *lane 7*, GST-D-Loop II.

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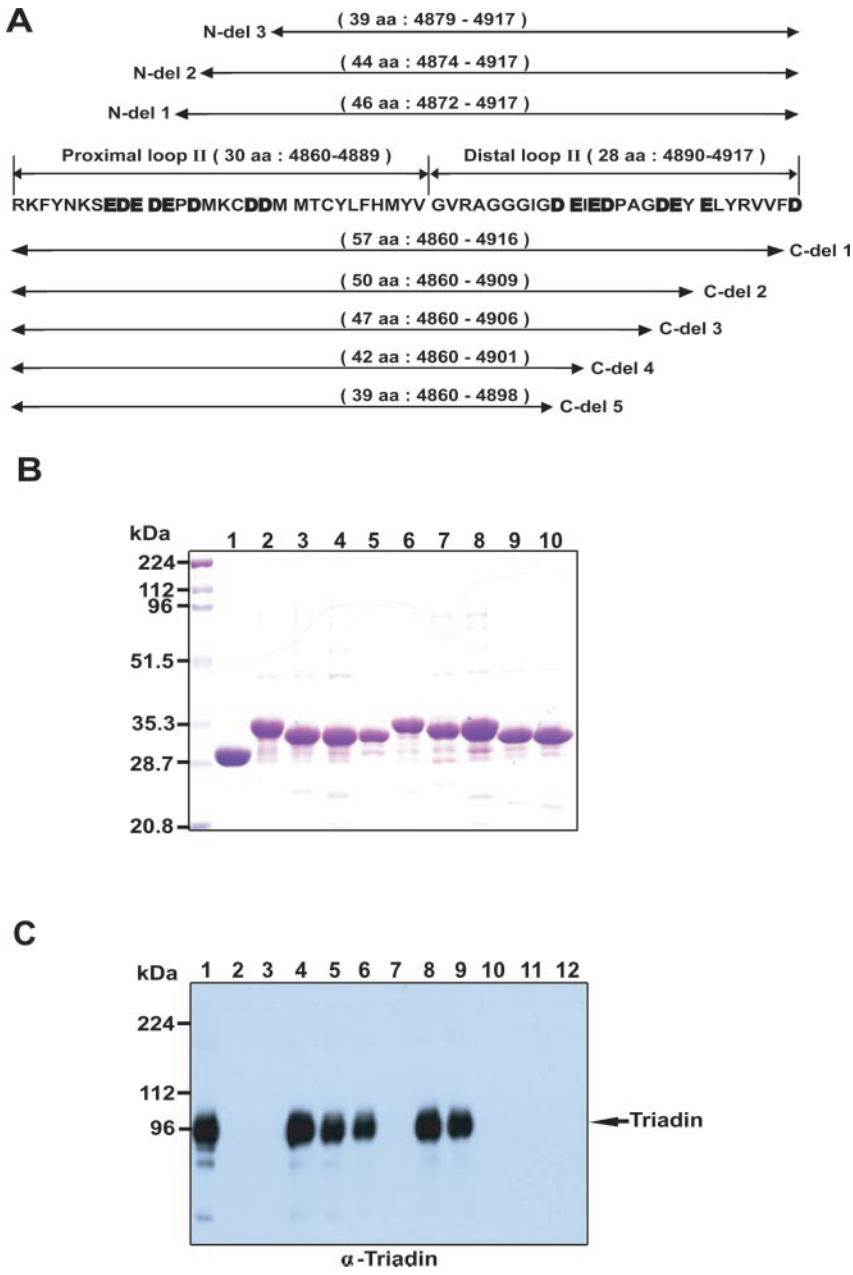

FIG. 2. **Interaction of triadin with deletion mutants of the second intraluminal loop of RyR1.** *A*, amino acid sequence of Loop II of rabbit RyR1 was divided into the proximal and distal portions (*middle*). Serial deletions from the amino (*N*)-terminal end (*upper 3 constructs*) or the carboxyl (*C*)-terminal end were introduced through the PCR method. *Numbers in parentheses* correspond to the labeling of aa residues in rabbit skeletal RyR (7). Negatively charged aa residues are indicated by the *bold letters*. *B*, purified GST control (*lane 1*), GST-Loop II (*lane 2*), GST-N-del 1 to 3 (*lane 3–5*), and GST-C-del 1 to 5 (*lane 6–10*) fusion proteins expressed in *E. coli* (DH5α) were separated in a 10% SDS-PAGE (10 μg each), and stained with Coomassie Blue. *C*, interaction of triadin with the GST fusion protein containing the different deletion mutants of Loop II peptide assayed with the GST-Sepharose affinity column (as in Fig. 1). The bound proteins were eluted by SDS sample buffer, and separated by 6% SDS-PAGE, and immunoblotting was done with anti-triadin antibody. *Lanes 1–4* correspond to loading with, heavy SR, affinity beads alone, GST control, and GST-Loop II affinity beads, respectively. *Lanes 5–7* represent assay with GST-N-del 1, N-del 2, and N-del 3, respectively. *Lanes 8–11* represent assay with GST-C-del 1 to C-del 5, respectively.

Solubilized proteins were obtained by centrifugation (a Beckman TLA-100.3 rotor) at 60,000 rpm for 45 min at 4 °C.

Reverse Transcriptase-PCR of Triadin from Skeletal Muscle—Total RNA was isolated from the rabbit skeletal muscle using the Invitek-Invisorb Spin Tissue RNA Mini Kit according to the manufacturer's manual. The cDNA encoding triadin was obtained through reverse transcriptase-PCR using the Qiagen-Omniscript reverse transcriptase kit. Oligo(dT) primer and random hexamer primer were purchased from Promega. The forward and reverse primers including EcoRI or XhoI site, respectively, were designed according to the published rabbit skeletal triadin gene sequence (23). Specific gene primer sets used for the different deletion mutants of triadin are indicated in Table I.

Construction of GST Fusion Vectors—cDNA sequences corresponding to the intraluminal loop I (4581–4640 residues), intraluminal loop II (4860–4917 residues), proximal loop II (4860–4889 residues), and distal loop II (4890–4917 residues) of RyR1 were amplified by PCR using the DNA template of the COOH-terminal region of rabbit skeletal RyR (7). The PCR products were digested with EcoRI and XhoI, and subcloned into the EcoRI-XhoI sites of pGEX 4T-1, which contain the sequence for GST at the 5′ end (Amersham Biosciences). Reverse transcriptase-PCR products of triadin deletion mutants (Triadin-A to G) were also cloned behind the GST sequence in the pGEX 4T-1 plasmid. See Table I for details for the primer design.

Site-directed Mutagenesis—Point mutations (D4877A, D4878A, D4907A, and E4908A) within intraluminal loop II of RyR1 were generated using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol. The oligonucleotide primer sets for each mutant constructs are listed in Table I. The presence of all mutations was confirmed by direct sequencing (Macrogen, Seoul, Korea).

Fusion Proteins-Sepharose Affinity Binding Assay—GST fusion constructs were induced in *Escherichia coli* DH5α (A_{600} = 0.3) with 1.0 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 37 °C. Cells were harvested, washed with phosphate-buffered saline, resuspended in STE buffer, incubated in 10% lysozyme (10 mg/ml) for 30 min, and then lysed by sonication on ice. The soluble fraction was obtained by centrifugation at 13,000 rpm for 15 min at 4 °C. The fusion proteins were immobilized by incubating 10 ml of the soluble *E. coli* fraction with a 250-μl bead volume of glutathione-Sepharose 4B (Amersham Biosciences) for 1 h at room temperature and washed 5 times with 30 ml of phosphate-buffered saline. The solubilized heavy SR proteins obtained by centrifugation were diluted 1:10 with a buffer containing 20 mM Tris-Cl (pH 7.4), 1 mM dithiothreitol, and protein inhibitor mixture, and incubated with GST fusion protein affinity beads for 4 h at 4 °C. After the incubation, the fusion protein-Sepharose complexes were washed 3 times with 20 mM Tris-Cl (pH 7.4), 0.15 M NaCl, and 0.2% Triton X-100. Bound proteins were eluted by boiling in SDS-PAGE sample buffer, and subjected to SDS-PAGE (24).

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SDS-PAGE and Western Blot Analysis—Proteins separated by 6 or 10% SDS-PAGE were electophoretically transferred onto nitrocellulose membrane in transfer buffer (130 mM glycine, 48 mM Tris base, 0.037% SDS). The blots were rinsed briefly with TBS-T (20 mM Tris-Cl, pH 7.6, 137 mM NaCl, and 0.1% Tween 20), blocked with 5% skim milk in TBS-T and incubated in primary antibody containing 0.5% skim milk in TBS-T for 1 h at room temperature with gentle agitation. The blots were washed with 3 changes of TBS-T for 15 min each. Secondary antibody (horseradish peroxidase-conjugated) containing 0.5% skim milk in TBS-T was treated for 1 h at room temperature with gentle shaking. The blots were washed 3–4 times with TBS-T for 10 min each, and detected using the ECL system (Amersham Biosciences). Mouse monoclonal antibodies against rabbit skeletal triadin and ryanodine receptor were purchased from Affinity BioReagents.

RESULTS

Localization of the Triadin Binding Region in RyR1—To examine the potential intraluminal triadin binding sites in RyR1, the most outstanding two intraluminal loops, Loop I (4581–4640 residues) and Loop II (4860–4917 residues) (7) were generated by PCR using the DNA template of the carboxyl-terminal region of rabbit skeletal RyR. As shown in Fig. 1*A*, abundant amounts of GST fusion protein containing Loop I and Loop II of RyR1 could be expressed in *E. coli*. The GST-Loop I and GST-Loop II proteins exhibit differential interaction with the endogenous triadin in skeletal muscle. As shown in Fig. 1*B*, GST-Loop II coupled to an affinity column can specifically pull-down triadin from the detergent-solubilized SR vesicle obtained from rabbit skeletal muscle (Fig. 1*B*, *lane 5*), whereas GST-Loop I was ineffective (Fig. 1*B*, *lane 4*).

Loop II of RyR1 has two clusters of negatively charged residues that could potentially participate in the binding to triadin. To test the contribution of these negatively charged residues to the interaction with triadin, GST fusion proteins containing the proximal (aa 4860–4889, GST-P-Loop II) and the distal regions (aa 4890–4917, GST-D-Loop II) of Loop II of RyR1 were generated (Fig. 1*C*). Surprisingly, neither GST-P-Loop II nor GST-D-Loop II could pull-down triadin from the SR vesicle (see Fig. 1*D*). This result suggests that both parts of the negatively charged residues are required for the binding to triadin.

To further identify the triadin-binding amino acid residues within the Loop II region, two additional approaches were used. First, serial deletions from either amino- or carboxyl-terminal ends were introduced in the Loop II sequence (Fig. 2*A*). These various fragments of Loop II peptides were expressed as GST fusion proteins (Fig. 2*B*), and used for the *in vitro* binding assay. Immunoblot analysis showed that the intrinsic association with triadin disappeared when residue Asp^{4877}-Asp^{4878} at the NH_2 terminus and residues Asp^{4907}-Glu^{4908} at the COOH terminus were deleted from the GST-Loop II fusion protein (Fig. 2*C*). Such results demonstrate that the specific negatively charged residues Asp^{4877}-Asp^{4878} at the proximal Loop II, and Asp^{4907}-Glu^{4908} at the distal Loop II are critical for binding to triadin.

Second, to investigate which of these negatively charged residues (Asp^{4877}, Asp^{4878}, Asp^{4907}, and Glu^{4908}) constitute the putative binding site for triadin, site-specific mutagenesis was used to convert them into alanines (Fig. 3*A*). The GST-Loop II protein containing the specific mutations, D4877A, D4878A, D4907A, and E4908A, were expressed in *E. coli* (Fig. 3*B*), and assayed for their ability to specifically pull-down triadin from the skeletal muscle SR membrane. As shown in Fig. 3*C*, the specific triadin-Loop II interaction was maintained in the D4877A mutant (*lanes 5* and *6*). However, mutation of the other three residues, D4878A, D4907A, or E4908A, led to a remarkable loss of association with triadin (*lanes 7*-9). These results provide evidence that the three negatively charged residues Asp^{4878}, Asp^{4907}, and Glu^{4908} likely constitute the critical binding sites for interaction with triadin.

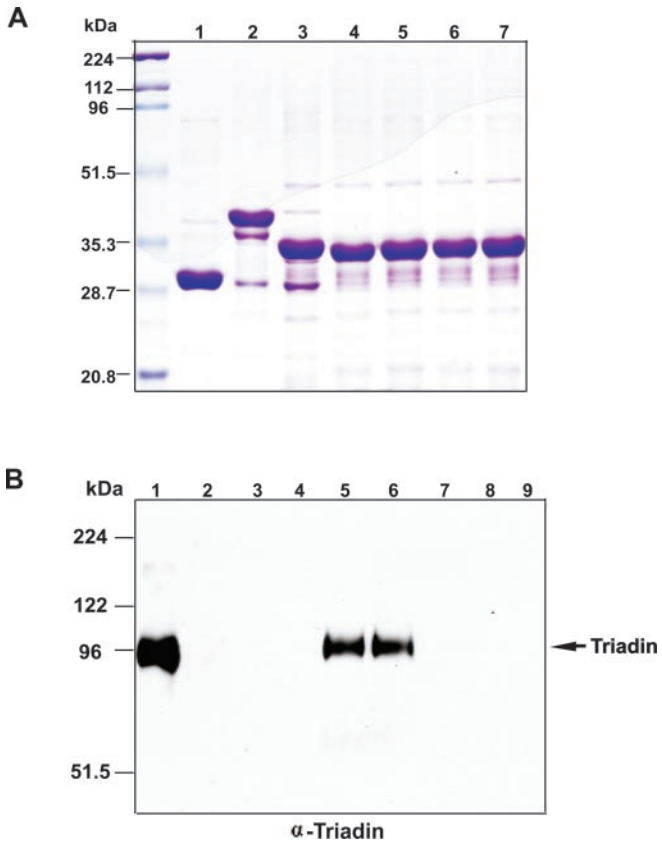

FIG. 3. **Role of negatively charged amino acids in the second intraluminal loop of RyR1 in mediating the interaction of RyR1 with triadin.** *A*, Coomassie Blue staining of the purified GST control (*lane 1*), GST-Loop I (*lane 2*), GST-Loop II (*lane 3*), GST-Loop II (D4877A, *lane 4*), GST-Loop II (D4878A, *lane 5*), GST-Loop II (D4907A, *lane 6*), and GST-Loop II (E4908A, *lane 7*) fusion proteins expressed in *E. coli* (10 μg each lane). *B*, interaction of the various GST-Loop II fusion proteins with the endogenous triadin in skeletal muscle was assayed as described in the legend to Fig. 1. *Lane 1*, heavy SR; *lane 2*, affinity beads alone; *lane 3*, GST control; *lane 4*, GST-Loop I affinity beads; *lane 5*, GST-Loop II affinity beads; *lane 6*, GST-Loop II (D4877A) affinity beads; *lane 7*, GST-Loop II (D4878A) affinity beads; *lane 8*, GST-Loop II (D4907A) affinity beads; *lane 9*, GST-Loop II (E4908A) affinity beads.

Identification of the RyR1 Binding Region in Triadin—All triadin isoforms in a given species have identical amino acid sequences over the first 260 residues (23, 25, 26). This common region contains a short amino-terminal cytoplasmic domain, a membrane-spanning region, and negatively charged luminal domains. The luminal domain (aa 68–264) of triadin in skeletal muscle is highly conserved among different species. Within the luminal domain of triadin, there exists a stretch of KEKE motifs that have been speculated to participate in the interaction with calsequestrin, junctin, or RyR (5, 19, 25).

To probe the domains within triadin that are involved in association with RyR1, several GST-triadin fusion constructs representing the different deletion mutants of triadin were generated (see Fig. 4*A*, *Tri-A*: aa 75–263; *Tri-B*: aa 75–160; *Tri-C*: aa 152–260; *Tri-D*: aa 152–199; *Tri-E*: aa 200–260; *Tri-F*: aa 200–232; *Tri-G*: aa 233–260), and expressed in *E. coli*. (Fig. 4*B*). The purified GST control and GST-triadin fusion protein affinity beads were incubated with the detergent-solubilized SR vesicle to probe their differential interactions with RyR1 (Fig. 4*C*). Western blot analysis showed that triadin-A, triadin-C, triadin-E, and triadin-F GST fusion proteins were able to pull-down the endogenous RyR1 (*lanes 4*, *6*, *8*, and *9*), whereas triadin-B, triadin-D, and triadin-G did not appear to interact with RyR1 (*lane 5*, *7*, and *10*). Interestingly, the GST-

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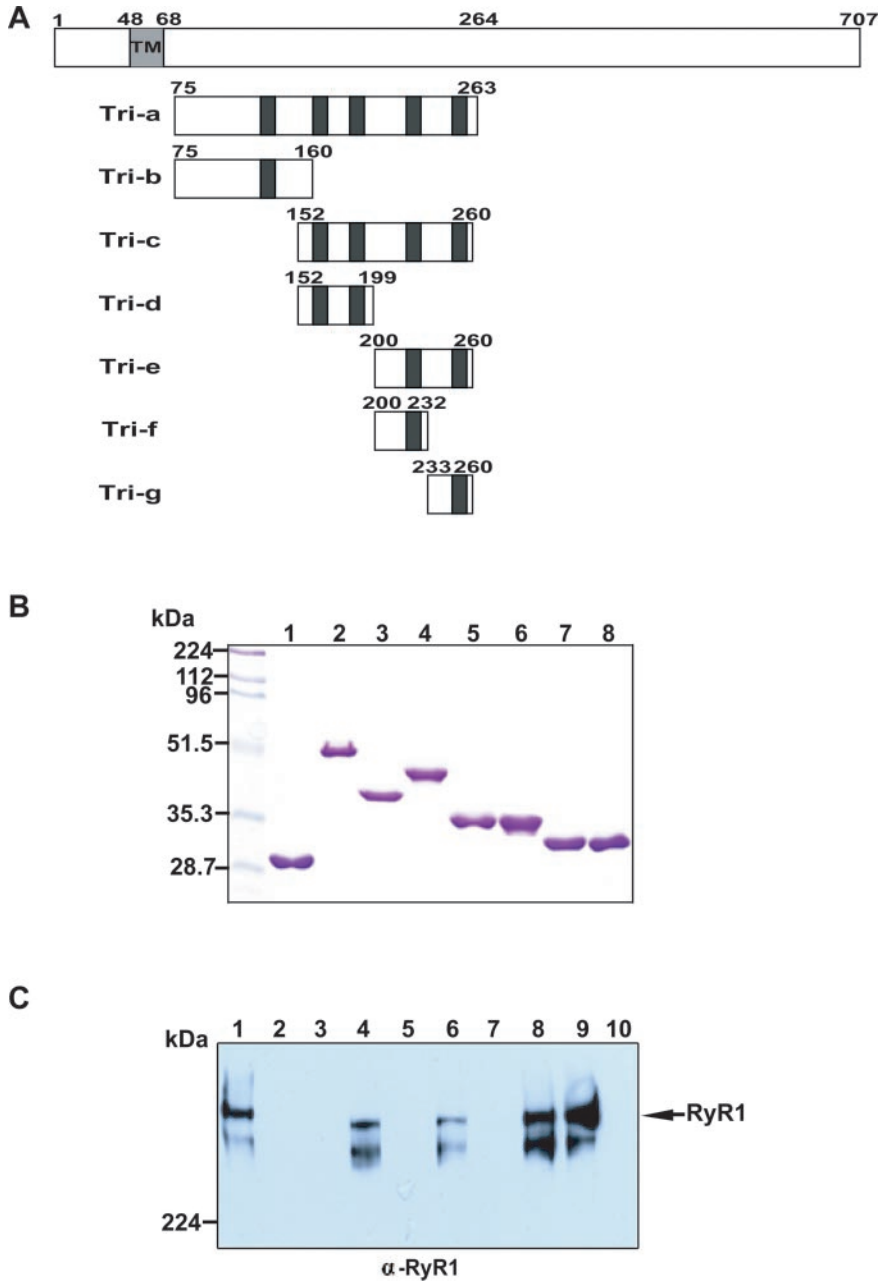

FIG. 4. **Role of the KEKE motif of triadin in mediating the interaction with RyR1.** *A*, schematic diagram of the primary structure of triadin, and the various deletion mutants of triadin. The *black filled boxes* indicate the KEKE motifs. The *star* denotes the KEKE motif that interacts with calsequestrin (16). Amino acids corresponding to Tri-E are known as a binding site for the histidine-rich Ca^{2+}-binding protein (21). The *numbers* correspond to aa residues of rabbit skeletal triadin (24). *B*, purified GST control (*lane 1*), GST-Tri-A to G (*lanes 2–8*) fusion proteins expressed in *E. coli* (DH5α) were separated in a 10% SDS-PAGE gel (10 μg each) and stained with Coomassie Blue. *In vitro* binding assays of the purified GST control and GST-triadin fusion protein affinity beads (250 μg each) were performed with Triton X-100-solubilized heavy SR. *C*, the bound proteins eluted by SDS sample buffer were separated by 6% SDS-PAGE, and the immunoblotting was carried out with anti-RyR1 antibody. *Lane 1*, heavy SR (25 μg); *lane 2*, control affinity beads; *lane 3*, GST affinity beads; *lane 4*, GST-Tri-A affinity beads; *lane 5*, GST-Tri-B affinity beads; *lane 6*, GST-Tri-C affinity beads; *lane 7*, GST-Tri-D affinity beads; *lane 8*, GST-Tri-E affinity beads; *lane 9*, GST-Tri-F affinity beads; *lane 10*, GST-Tri-G affinity beads.

triadin fusion proteins that exhibit positive interaction with RyR1 all share a common KEKE motif, especially with the triadin-F construct (as indicated by the star in Fig. 4*A*). Based on this, we speculate that the KEKE motif of triadin (aa 200–232) likely participates in the interaction with RyR1.

DISCUSSION

It is now generally accepted that RyR forms a stable quaternary structure with calsequestrin, triadin, and junctin at the luminal surface of the junctional SR membrane (5). Growing evidence supports that calsequestrin may actively participate in regulating the amounts of Ca^{2+} release through the RyR (12, 27, 28). The regulatory effect of calsequestrin has been suggested to involve calsequestrin-anchoring proteins such as triadin and junctin (5, 19), which are integral membrane proteins with a short amino-terminal segment located in the cytoplasm and a largely charged carboxyl-terminal segment in the luminal side of the SR (13, 14). The luminal domains of triadin and junctin mainly serve as scaffold structures that bring calsequestrin into the proximity of the RyR Ca^{2+} release channel. This anchoring mechanism presumably results in concentration of Ca^{2+} near the inner surface of the junctional SR membrane, and thus would facilitate the rapid Ca^{2+} release process in excitation-contraction coupling. On the other hand, triadin and several other accessory proteins of RyR (such as FKBP12) have been shown to have direct inhibitory effects on the Ca^{2+} release channel (17, 18, 29). Therefore understanding the nature of protein-protein interaction within the quaternary complex of RyR should provide important insights into the cellular mechanism of Ca^{2+} signaling in muscle cells.

In the present study, we demonstrated a direct physical interaction between triadin and RyR1, consistent with previous studies of Guo and Campbell (19). Using *in vitro* binding assay and mutagenesis analyses, we showed that the association between RyR1 and triadin: (*a*) involves the negatively charged residues located in the second intraluminal loop of RyR; and (*b*) requires the presence of the KEKE motif in triadin. Our data suggest that charge-charge interactions probably play a major role in determining the affinity of interaction between triadin and RyR1.

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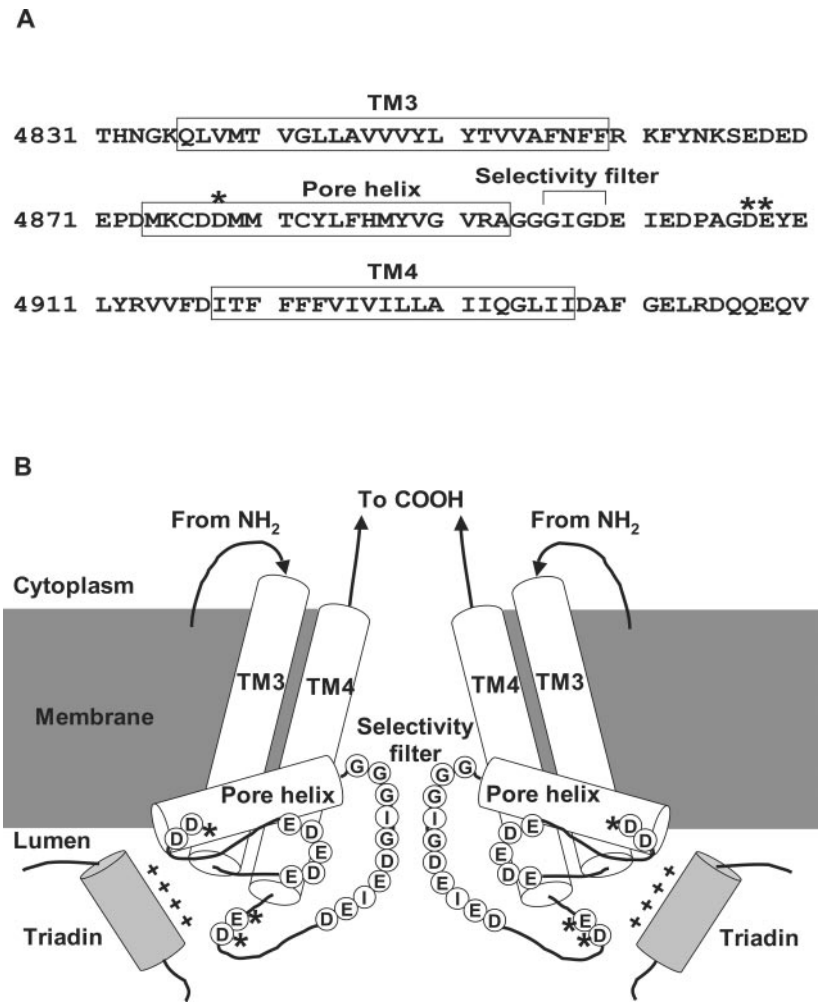

FIG. 5. **A hypothetical model to depict interactions between Loop II and triadin.** *A*, the structural elements such as the transmembrane helices 3 (*TM3*), 4 (*TM4*), and the pore helix were assigned onto the rabbit RyR1 sequence based on the RyR2 model proposed by Chen *et al.* (34). Asp^{4878}, Asp^{4907}, and Glu^{4908} are indicated by *asterisks*. *B*, cartoon illustrating the triadin-RyR1 interactions. Only the putative pore-forming parts from two RyR1 monomers are shown. The model has a topology similar to that found in the KcsA channel. The residues in the selectivity filter segment and the acidic residues on the loop regions are indicated. Note that Asp^{4878}, Asp^{4907}, and Glu^{4908} are located at the periphery of the ion conduction pathway and could interact with the positively charged residues in the KEKE motif (200–232 residues) of triadin.

Previous structure-function studies of RyR have shown that the second intraluminal loop of RyR plays a major role in the overall function of the Ca^{2+} release channel (8–10, 30). In RyR1, the removal of a negative charge by mutating Asp^{4878}, Asp^{4907}, or Glu^{4908} to alanine disrupted the interaction with triadin. It is intriguing that, among 15 acidic residues on the loop between transmembrane helix TM3 and TM4 (TM8 and TM10 according to the model of Zorzato *et al.*, Ref. 31), only three are found to be important for the binding to triadin. Tu *et al.* (32) showed that the RyR channel likely contains a negatively charged vestibule in the luminal side of the Ca^{2+} release channel, which serves to concentrate the permeating Ca^{2+} ions near the conduction pore of the channel. Recent studies with the recombinant RyRs have provided clues to the nature of the ion conduction pore as well as the vestibular structure of the Ca^{2+} release channel. According to the models of Williams *et al.* (33) and Chen *et al.* (34), the amino acid sequence extending from TM3, intraluminal loop II, to TM4 would form part of the backbone for the pore structure and the selectivity filter of the Ca^{2+} release channel (see Fig. 5). This arrangement is similar to the known crystal structure of the voltage-gated KcsA channel, with motifs corresponding to the outer helix, the pore helix, the signature sequence, and the inner helix of the KcsA channel (35). Interestingly, the predicted pore helix of RyR1 is longer than that of the KcsA structure by two helix turns, and this extra structure might constitute the putative binding pockets for RyR accessory proteins. Because Asp^{4878} is located at the amino-terminal end of the pore helix, the extended pore helix in RyR1 will bring the location of Asp^{4878} away from the ion conduction pathway. Also Asp^{4907} and Glu^{4908} are located right before the amino-terminal end of transmembrane helix TM4 that is apparently at the periphery of the pore assembly. Thus Asp^{4878}, Asp^{4907}, and Glu^{4908} are easily accessible by triadin, whereas the other acidic residues are not. Presumably, the interaction of triadin with RyR1 would alter the dielectric property near the pore region of the Ca^{2+} release channel, leading to the inhibition of the channel activity as observed in previous studies (17, 18).

Another important consequence of the RyR1-triadin interaction is the anchoring of calsequestrin. Our previous study has shown that the *aspartate*-rich region of calsequestrin, also known as the Ca^{2+} binding motif, is directly involved in the association of calsequestrin with triadin. Our data presented here show that the KEKE motif of triadin (aa 200–232) consisting of a cluster of positively and negatively charged residues is critical for the interaction of triadin with RyR1. It is interesting to note that the KEKE motif has been suggested to promote association of various membrane proteins (36, 37) and previous results have shown that the KEKE motif of triadin could also be involved in the association with the *aspartate*-rich region of calsequestrin (16, 17) and the *His*-rich acidic repeats of the histidine-rich Ca^{2+}-binding protein (21). Because of the competitive nature of the KEKE motif of triadin in the dual interaction with RyR1 and calsequestrin, and because of the close proximity of these interaction sites to the pore region of the Ca^{2+} release channel, a dynamic association and dissociation of triadin and calsequestrin from RyR1 would have significant impact on the overall Ca^{2+} release process in muscle cells. When muscle is at the resting state, the majority of calsequestrin will be occupied with luminal Ca^{2+} forming oligomer or

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polymer (38–40). Triadin is free to interact with RyR that could have an inhibitory role on the Ca^{2+} release channel activity (17, 18). In response to an excitation signal from the transverse-tubule membrane, opening of the RyR/Ca^{2+} release channels might cause a dissociation of triadin from RyR. Opening of RyR leads to efflux of Ca^{2+} from the SR and dissociation of Ca^{2+} ions from calsequestrin (20), resulting in a temporal decrease of the local Ca^{2+} concentration in the junctional region. This transient decrease in local Ca^{2+} might cause association of calsequestrin with triadin, which could trigger more Ca^{2+} ions to be dissociated from calsequestrin. In principle, this cascade of interaction could serve as a positive feedback signal enabling rapid and efficient release of Ca^{2+} into the myoplasm in the event of excitation-contraction coupling.

REFERENCES

1. Johnson, E. A., and Sommer, J. R. (1967) *J. Cell Biol.* **33,** 103–129
2. Fleischer, S., and Inui, M. (1989) *Annu. Rev. Biophys. Biophys. Chem.* **18,** 333–364
3. Meissner, G. (1994) *Annu. Rev. Physiol.* **56,** 485–508
4. Franzini-Armstrong, C., and Jorgensen, A. O. (1994) *Annu. Rev. Physiol.* **56,** 509–534
5. Zhang, L., Kelley, J., Schmeisser, G., Kobayashi, Y. M., and Jones, L. R. (1997) *J. Biol. Chem.* **272,** 23389–23397
6. Bhat, M. B., Zhao, J., Takeshima, H., and Ma, J. (1997) *Biophys. J.* **73,** 1329–1336
7. Takeshima, H., Nishimura, S., Matsumoto, T., Ishida, H., Kangawa, K., Minamino, N., Matsuo, H., Ueda, M., Hanaoka, M., Hirose, T., and Numa, S. (1989) *Nature* **339,** 439–445
8. Lynch, P. J., Tong, J., Lehane, M., Mallet, A., Giblin, L., Heffron, J. J., Vaughan, P., Zafra, G., MacLennan, D. H., and McCarthy, T. V. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 4164–4169
9. Balshaw, D., Gao, L., and Meissner, G. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 3345–3347
10. Gao, L., Balshaw, D., Xu, L., Tripathy, A., Xin, C., and Meissner, G. (2000) *Biophys. J.* **79,** 828–840
11. MacLennan, D. H., Campbell, K. P., and Reithmeier, R. A. F. (1983) *Calcium and Cell Function* (Cheung, W. Y., ed) pp. 151–173, Academic Press, New York
12. Jones, L. R., Suzuki, Y. J., Wang, W., Kobayashi, Y. M., Ramesh, V., Franzini-Armstrong, C., Cleemann, L., and Morad, M. (1998) *J. Clin. Invest.* **101,** 1385–1393
13. Guo, W., Jorgensen, A. O., and Campbell, K. P. (1994) *J. Biol. Chem.* **269,** 28359–28365
14. Jones, L. R., Zhang, L., Sanborn, K., Jorgensen, A. O., and Kelley, J. (1995) *J. Biol. Chem.* **270,** 30787–30796
15. Realini, C., Rogers, S. W., and Rechsteiner, M. (1994) *FEBS Lett.* **348,** 109–113
16. Kobayashi, Y. M., Alseikhan, B. A., and Jones, L. R. (2000) *J. Biol. Chem.* **275,** 17639–17646
17. Ohkura, M., Furukawa, K., Fujimori, H., Kuruma, A., Kawano, S., Hiraoka, M., Kuniyasu, A., Nakayama, H., and Ohizumi, Y. (1998) *Biochemistry* **37,** 12987–12993
18. Groh, S., Marty, I., Ottolia, M., Prestipino, G., Chapel, A., Villaz, M., and Ronjat, M. (1999) *J. Biol. Chem.* **274,** 12278–12283
19. Guo, W., and Campbell, K. P. (1995) *J. Biol. Chem.* **270,** 9027–9030
20. Shin, D. W., Ma, J., and Kim, D. H. (2000) *FEBS Lett.* **486,** 178–183
21. Lee, H. G., Kang, H., Kim, D. H., and Park, W. J. (2001) *J. Biol. Chem.* **276,** 39533–39538
22. Kim, D. H., Sreter, F. A., Ohnishi, S. T., Ryan, J. F., Roberts, J., Allen, P. D., Meszaros, L. G., Antoniu, B., and Ikemoto, N. (1984) *Biochim. Biophys. Acta* **775,** 320–327
23. Knudson, C. M., Stang, K. K., Moomaw, C. R., Slaughter, C. A., and Campbell, K. P. (1993) *J. Biol. Chem.* **268,** 12646–12654
24. Laemmli, U. K. (1970) *Nature* **227,** 680–685
25. Guo, W., Jorgensen, A. O., Jones, L. R., and Campbell, K. P. (1996) *J. Biol. Chem.* **271,** 458–465
26. Kobayashi, Y. M., and Jones, L. R. (1999) *J. Biol. Chem.* **274,** 28660–28668
27. Ikemoto, N., Ronjat, M., Meszaros, L. G., and Koshita, M. (1989) *Biochemistry* **28,** 6764–6771
28. Kawasaki, T., and Kasai, M. (1994) *Biochem. Biophys. Res. Commun.* **199,** 1120–1127
29. Gaburjakova, M., Gaburjakova, J., Reiken, S., Huang, F., Marx, S. O., Rosemblit, N., and Marks, A. R. (2001) *J. Biol. Chem.* **276,** 16931–16935
30. Zhao, M., Li, P., Li, X., Zhang, L., Winkfein, R. J., and Chen, S. R. (1999) *J. Biol. Chem.* **274,** 25971–25974
31. Zorzato, F., Fujii, J., Otsu, K., Phillips, M., Green, N. M., Lai, F. A., Meissner, G., and MacLennan, D. H. (1990) *J. Biol. Chem.* **265,** 2244–2256
32. Tu, Q., Velez, P., Brodwick, M., and Fill, M. (1994) *Biophys. J.* **67,** 2280–2285
33. Williams, A. J., West, D. J., and Sitsapesan, R. (2001) *Q. Rev. Biophys.* **34,** 61–104
34. Chen, S. R., Li, P., Zhao, M., Li, X., and Zhang, L. (2002) *Biophys. J.* **82,** 2436–2447
35. Doyle, D. A., Morais, C. J., Pfuetzner, R. A., Kuo, A., Gulbis, J. M., Cohen, S. L., Chait, B. T., and MacKinnon, R. (1998) *Science* **280,** 69–77
36. Perutz, M. (1994) *Protein Sci.* **3,** 1629–1637
37. West, R. W., Jr., Kruger, B., Thomas, S., Ma, J., and Milgrom, E. (2000) *Gene* (*Amst.*) **243,** 195–205
38. He, Z., Dunker, A. K., Wesson, C. R., and Trumble, W. R. (1993) *J. Biol. Chem.* **268,** 24635–24641
39. Maguire, P. B., Briggs, F. N., Lennon, N. J., and Ohlendieck, K. (1997) *Biochem. Biophys. Res. Commun.* **240,** 721–727
40. Wang, S., Trumble, W. R., Liao, H., Wesson, C. R., Dunker, A. K., and Kang, C. H. (1998) *Nat. Struct. Biol.* **5,** 476–483

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