Structure–function relationships of the mouse inositol 1,4,5-trisphosphate receptor

(cDNA mutagenesis/NG108-15 cell/homotetrameric complex/inositol trisphosphate binding)

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ABSTRACT The homotetrameric complex of inositol 1,4,5-trisphosphate (InsP3) receptors displays a Ca²⁺ release activity in response to InsP3 molecules. Structure–function relationships of the mouse cerebellar InsP3 receptor have been studied by analyses of a series of internal deletion or C-terminal truncation mutant proteins expressed in NG108-15 cells. Within the large cytoplasmic portion of the InsP3 receptor, ≈650 N-terminal amino acids are highly conserved between mouse and Drosophila, and this region has the critical sequences for InsP3 binding that probably form the three-dimensionally restricted binding site. The N-terminal region of each InsP3 receptor subunit also binds one InsP3 molecule. Cross-linking experiments have revealed that InsP3 receptors are intermolecularly associated at the transmembrane domains and/or the successive C termini. The interaction between the receptor subunit and InsP3 may cause a conformational change in the tetrameric complex, resulting in the opening of Ca²⁺ channels.

The inositol 1,4,5-trisphosphate (InsP3) receptor directs the InsP3-induced Ca²⁺ release from intracellular stores (predominantly the endoplasmic reticulum) in a wide variety of cell types (1). The InsP3 receptor, a homotetramer, exhibits an InsP3-induced Ca²⁺ channel activity (2–6). The InsP3 receptor and the ryanodine receptor (the channel responsible for the Ca²⁺ release from the sarcoplasmic reticulum of skeletal muscle) are a type of ion channel protein present on intracellular organelles that is distinct from ion channel proteins on the plasmalemma. The structure of the InsP3 binding site in the InsP3 receptor and the mechanism of the coupling between receptor occupancy and Ca²⁺ channel opening remains to be elucidated. We obtained the InsP3 receptor cDNA from a mouse cerebellar cDNA library and determined its primary structure (7). We have shown (4, 7) that, in NG108-15 cells (mouse neuroblastoma–rat glioma hybrids) and L cells (mouse fibroblasts), the cloned cDNA directs the synthesis of a functional receptor protein with high affinity and specificity for InsP3 that is equivalent to that of the cerebellar InsP3 receptor. By using soluble mutant receptor proteins, Mignery and Südhof (8) localized the InsP3 binding site within the N-terminal fourth of the rat InsP3 receptor and demonstrated the role of the transmembrane region of the InsP3 receptor in tetramer formation but not in InsP3 binding. In the present study, by analysis of a series of membrane and soluble mutant receptor proteins expressed in NG108-15 cells, we have defined the InsP3 binding regions within the N-terminal 650 amino acids and showed that a restricted tertiary structure was required for InsP3 binding. Our cross-linking experiments demonstrated that formation of tetrameric InsP3 receptors involves the transmembrane domains and/or successive C termini and that InsP3 binding is independent of the intermolecular conformation. In addition, we have cloned a putative InsP3 receptor cDNA of Drosophila and found that both the amino acid sequences of the mouse and Drosophila InsP3 receptors share an extensive homology (S.Y., T. Tanimura, A.M., M. Nakamura, M. Yuzaki, T.F., and K.M., unpublished data) that is probably essential for the InsP3 binding and Ca²⁺ release activity.

MATERIALS AND METHODS

Construction of Mutant InsP3 Receptor cDNAs. To construct mutant cDNAs, we used a recombinant plasmid, pBactS-C1, that carries the entire protein-coding sequence of mouse cerebellar InsP3 receptor cDNA between a β-actin promoter and a simian virus 40 polyadenylylation sequence (8). We removed various portions of the cDNA from pBactS-C1 by using the combinations of restriction endonucleases as indicated in Fig. 1A. To obtain the mutant proteins in-frame, we enzymatically converted the following overhanging ends into blunt ends before the ligation: by using T4 DNA polymerase, the Pst I site of D316-352, the Kpn I site of D419-735, and the Sac I site of D650-735; by using the Klenow fragment of DNA polymerase I, the Acc I site of D316-352, the HindIII sites of D170-1252, the Ava I site of D1638-2016, the EcoRI site of D1845-2216, and the EcoRI site of T2217; by using mung bean nuclease, the Apa I site of T1079. To construct D1692-1731, we produced the Ava I (position 5237)–Nae I (position 5859) fragment that has the 120-base-pair deletion from mouse peripheral tissue mRNA by the PCR.

[³H]InsP3 Binding Assay with Membrane Fractions. Membrane protein (50 µg) was incubated with 10 nM [³H]InsP3 (NEN/DuPont) in 100 µl of binding buffer (50 mM Tris·HCl, pH 8.0/1 mM 2-mercaptoethanol/1 mM EDTA) for 10 min at 4°C. After centrifugation at 10,000 × g for 5 min at 2°C, the pellet was dissolved in Protosol (NEN/DuPont), and the radioactivity was measured in Econofluor (NEN/DuPont) by using a scintillation counter. Nonspecific binding was measured in the presence of 1 µM InsP3 (Funakoshi, Japan).

[³H]InsP3 Binding Assay with Soluble Fractions. Soluble protein (50 µg) was incubated with 10 nM [³H]InsP3 in 100 µl of binding buffer for 10 min at 4°C. The sample was then mixed with 4 µl of γ-globulin (50 mg/ml) and 100 µl of a solution containing 30% (wt/vol) PEG 6000, 1 mM 2-mercaptoethanol, and 50 mM Tris·HCl (pH 8.0). After incubation on ice for 5 min, the protein–PEG complex was collected by centrifugation at 10,000 × g for 5 min at 2°C. Under these conditions, we have confirmed by immunoblot analysis that

Abbreviations: InsP3, inositol 1,4,5-trisphosphate; mAb, monoclonal antibody; sDST, disulfosuccinimidyl tartarate.
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all sample proteins could be precipitated (data not shown). The radioactivity of the pellet was measured as described above.

Cross-Linking of Receptor Proteins. The proteins were suspended in 50 mM sodium phosphate (pH 8.0) at 1 mg/ml. Solutions (50–100 μl) were incubated with various concentrations of the cross-linker disulfosuccinimidyl tartarate (sDST, Pierce) for 30 min on ice. The samples were mixed with the same volume of agarose/PAGE loading buffer [2% (wt/vol) SDS/2 mM EDTA/10% (vol/vol) 2-mercaptoethanol/20 mM Tris·HCl, pH 8.0/20% (vol/vol) glycerol] and were heated for 3 min at 100°C. The solutions were subject to agarose/PAGE (1.75% polyacrylamide and 0.5% agarose slab gel in Tris acetate/EDTA buffer) followed by immunoblot analysis.

Other Methods. Transfection of NG108-15 cells, preparation of membrane or soluble fractions, and Western blot analysis were performed as described (4, 7, 9).

RESULTS AND DISCUSSION

Expression of Mutant InsP₃ Receptors in NG108-15 Cells. From a hydropathy profile, the InsP₃ receptor has been assumed to traverse the membrane at several hydrophobic stretches (amino acids 2272–2587) in the C terminus and to possess a large N-terminal region located in the cytoplasmic compartment (7). To locate the regions responsible for InsP₃ binding, we constructed a series of internal deletion and C-terminal truncation mutants listed in Fig. 1C. Extensive deletions were made particularly in the N-terminal region, because this cytoplasmic region seems to be involved directly in binding to InsP₃ molecule.

To confirm the production of mutant proteins, the membrane or soluble fractions from NG108-15 cells transfected with the mutant cDNAs were analyzed by immunoblot experiments using the anti-InsP₃ receptor monoclonal antibodies (mAbs) 4C11, 10A6, and 18A10. We found that mutant proteins carrying the transmembrane domain (referred to as the M series) were found in the membrane fractions; mutant proteins lacking the transmembrane domains (referred to as the S series) were mainly in the soluble fractions. Figs. 2B and 3B show immunoblot analyses of membrane fractions (for M series) and soluble fractions (for S series), respectively. As described (7), the NG108-15 cell line contains a smaller endogenous membrane protein that binds to the anti-InsP₃ receptor mAbs and is thought to be the InsP₃ receptor of NG108-15 cells. Significant amounts of mutant proteins in the M and S series were detected in membrane and soluble fractions, respectively.

[³H]InsP₃ Binding Activities of Mutant Receptors. The cerebellar-type InsP₃ receptor derived from the entire cDNA (an intact InsP₃ receptor) expressed in NG108-15 cell membrane displays a high affinity and specificity for InsP₃ with a high capacity (7). To examine the InsP₃ binding activities of the mutant proteins, we measured [³H]InsP₃ binding activity of the cell fraction containing each mutant receptor protein. Figs. 2A and 3A show the results obtained with the same fractions that were used for the immunoblot analyses shown in Figs. 2B and 3B, respectively. We compared the InsP₃ binding activity of each fraction with that of a control fraction (with no cDNA expression). InsP₃ binding to each fraction is affected by the expression efficiency of the transfected cDNA: the fractions contain different amounts of mutant InsP₃ receptors as shown in Figs. 2B and 3B. To examine the affinities for InsP₃, we performed Scatchard analyses on the fractions that showed a significant elevation in InsP₃ binding. To determine the K_d value of each of the fractions, at least two experiments with independent samples were carried out. The determined affinities for InsP₃ (K_d values) can be mostly attributed to the expressed mutant proteins, since the endog-

FIG. 1. Strategies for the construction of InsP₃ receptor mutants. (A) Restriction map of the mouse cerebellar InsP₃ receptor cDNA for mutants. A box indicates the protein coding region with the restriction endonuclease sites used for mutant construction. The regions containing epitopes for mAbs 4C11, 10A6, and 18A10 are indicated by hatched boxes, and the putative transmembrane region is indicated by a solid box. Amino acid numbers are given above the diagram. (B) Distribution of the identical amino acids in the mouse and the *Drosophila* InsP₃ receptors. Vertical lines correspond to midpoints of 3-residue spans, giving a double-matching probability that the mean score is >3.0 (20). (C) Structures of internal deletion and C-terminal truncation mutants. Horizontal lines represent the regions of the receptor carried by the mutants. Mutant names are to the left.

enous InsP₃ receptor of NG108-15 cells exhibits a very low level of binding activity. InsP₃ binding properties of the mutant proteins are summarized in Table 1.

D96-315, D169-465, and D419-735, the mutant receptors that have deletions within the N terminus, had the same level of InsP₃ binding activity as the negative control, in spite of their appreciable expression. Mignery *et al.* (11) also reported that the N-terminal 418-amino acid deletion abolishes InsP₃ binding. On the other hand, D650-735 exhibited a similar InsP₃ binding activity (K_d, 20 ± 3 nM) to the intact receptor. Recently, mAb 4C11 has been demonstrated to interact with its epitope (residues 679–727) without affecting the InsP₃ binding of the receptor (S. Nakade, N. Maeda, and K.M., unpublished data). These results suggest that the N-terminal 650-amino acid residues have critical sequences necessary for InsP₃ binding. Whether this N-terminal region is sufficient for InsP₃ binding will be discussed later.

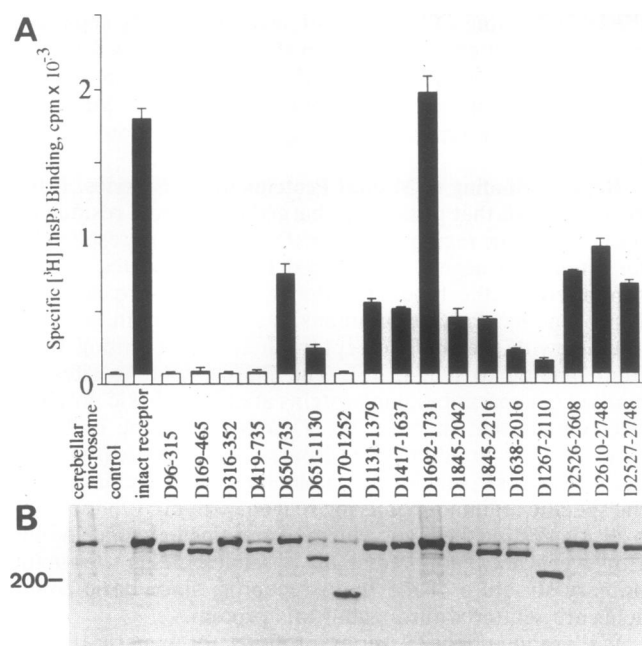Within a part of the transmembrane domains and the successive C-terminal region whose amino acid sequences

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FIG. 2. Expression and InsP₃ binding activities of M series mutants. The same membrane fractions were used in *A* and *B*. (*A*) Specific [³H]InsP₃ binding to membrane fractions, defined by subtracting the nonspecific binding, ranging from 200 to 250 cpm, from the total binding. Open bars represent the control binding activities. Results are the mean ± SD of four to eight samples. (*B*) Immunoblot analyses of M series mutants with the mAbs 10A6 and 18A10. The left-most lane contains 5 μg of mouse cerebellar microsomal fraction; other lanes contain 30 μg of membrane fraction proteins prepared from NG108-15 cells. Molecular mass at 200 kDa is shown.

FIG. 3. Expression and InsP₃ binding activities of S series mutants. The same soluble fractions were used in *A* and *B*. (*A*) Specific [³H]InsP₃ binding to soluble fractions, defined by subtracting the nonspecific binding, ranging from 580 to 620 cpm, from the total binding. Results are the mean ± SD of three to eight samples. (*B*) Immunoblot analyses of S series mutants with the mAb 4C11. The left-most lane contains 5 μg of mouse cerebellar microsomal fraction protein; other lanes contain 10 μg of soluble fraction protein prepared from NG108-15 cells. Molecular masses in kDa are shown.

Table 1. InsP₃ binding properties of mutant proteins

Mutant protein	InsP₃ binding capability	Affinity for InsP₃ (K_d), nM
D96-315	−	—
D169-465	−	—
D316-352	−	—
D419-735	−	—
D650-735	+	20 ± 3 (2)
D651-1130	+	ND
D170-1252	−	—
D1131-1379	+	20 ± 4 (3)
D1417-1637	+	23 ± 2 (3)
D1692-1731	+	20 ± 1 (5)
D1845-2042	+	47 ± 7 (3)
D1845-2216	+	35 ± 5 (2)
D1638-2016	+	ND
D1267-2110	+	ND
D2526-2608	+	25 ± 3 (2)
D2610-2748	+	20 ± 1 (2)
D2527-2748	+	21 ± 3 (2)
T736	+	ND
T1079	+	27 ± 5 (2)
T1845	+	24 ± 4 (2)
D2112-2605	+	28 ± 5 (2)
T2217	+	14 ± 2 (3)

InsP₃ binding capability was judged from the comparison of InsP₃ binding activity of each fraction to that of the control fraction. +, Capable; −, incapable. Scatchard analyses were performed in triplicate. K_d values are expressed as the mean ± SD. The number of independent samples used for Scatchard analyses is given in parentheses. ND, not determined.

share an extensive homology with the ryanodine receptor, three deletions (D2526-2608, D2527-2748, and D2610-2748) were introduced. Although D2526-2608 and D2527-2748 lacked the most hydrophobic stretch of the transmembrane domains, both the mutant proteins were found only in membrane fractions (data not shown). These three deletions did not affect the InsP₃ binding affinities (see Table 1).

The mutants with deletions along the middle part of the receptor protein retained InsP₃ binding (D1131-1379 and D1417-1637). Scatchard analyses showed that D1131-1379 and D1417-1637 receptors had similar binding affinities for InsP₃ (K_d, 20 ± 4 nM and 23 ± 2 nM, respectively) to the intact receptor.

Deletions were also introduced into the cytoplasmic region next to the transmembrane domains. D1845-2042 and D1845-2216 bound to InsP₃ with reduced binding affinities (K_d, 47 ± 7 nM and 35 ± 5 nM, respectively). When a deletion was extended, the resulting mutant proteins D1638-2016 and D1267-2110 exhibited low but reproducible InsP₃ binding.

T736, T1079, T1845, D2112-2605, and T2217 lack the C-terminal region including the complete transmembrane domains, resulting in proteins defective in membrane anchoring. Their binding activities in the soluble fractions were measured. Compared with the control soluble fraction that showed no InsP₃ binding, it was evident that all the proteins, including T736, bound to InsP₃. Thus, with the InsP₃ binding capability of D650-735, we conclude that the ≈650 N-terminal amino acids are sufficient for InsP₃ binding. This is in agreement with the report by Mignery and Südhof (8) that the N-terminal fourth of the InsP₃ receptor is sufficient for InsP₃ binding and that the C-terminal boundary of the InsP₃ binding site is located between amino acids 519 and 788. It should be noted, however, that the binding activity of T736 was quite low. Also, whereas T2217 was capable of binding to InsP₃ quite effectively (K_d, 14 ± 2 nM), T1079, T1845, and D2112-2605 exhibited lower binding affinities for InsP₃ (K_d values: T1079, 27 ± 5 nM; T1845, 24 ± 4 nM; D2112-2605, 28 ± 5
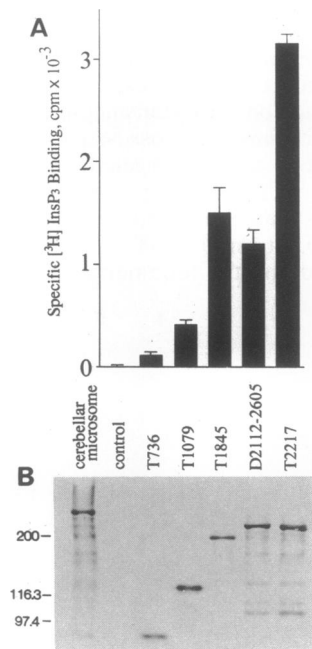
nM). We do not know whether the difference in binding affinities for InsP_3 between T2217 and the other mutant proteins in the S series is significant. Some regions in the cytoplasmic portion in addition to the N terminus might be important for full InsP_3 binding. One possibility is the region flanking the transmembrane domains, in which deletions caused a slight reduction in the binding affinity for InsP_3.

The 40-Amino Acid Deletion: A Distinct Type of the InsP_3 Receptor. We have found a mouse InsP_3 receptor subtype that has a specific 40-amino acid sequence deletion (residues 1692–1731) and is relatively enriched in peripheral tissues (T.N., H. Okano, T.F., J. Aruga, and K.M., unpublished data). The 40-amino acid deletion (D1692-1731) did not affect the affinity for InsP_3 (K_d, 20 ± 1 nM). Further studies are necessary to understand the implication of the deletion for receptor functions. It should be noted that the intact InsP_3 receptor and D1692-1731, both of which are native-form receptor proteins, were expressed more efficiently than any other mutant proteins in the M series. This might reflect a negative effect of the artificial deletions on protein stability, folding, and its integration into membrane.

Presence of Highly Conserved Regions Between the Mouse and *Drosophila* InsP_3 Receptors: Their Functional Significances. We have cloned the InsP_3 receptor cDNA of *Drosophila* and determined its primary structure (S.Y., T. Tanimura, A.M., M. Nakamura, M. Yuzaki, T.F., and K.M., unpublished data). Comparison of the mouse and *Drosophila* InsP_3 receptors has revealed 58% identity in the amino acid sequences. Fig. 1B shows the distribution of the identical amino acids of the mouse InsP_3 receptor to the *Drosophila* counterpart. The amino acid sequences of their C-terminal regions including the transmembrane domains are very similar to each other and to that of ryanodine receptor. Thus these conserved regions may be involved in forming a similar Ca^{2+} channel in the membrane. The amino acids in the N-terminal regions are also highly conserved between the two InsP_3 receptors. This N-terminal region of the mouse InsP_3 receptor (residues 1–813) can be characterized as having some segments composed of conserved amino acids: residues 1–317, 352–657, and 729–813 exhibit 76%, 67%, and 80% identity, respectively. In the present study, we demonstrated that the N-terminal 650 amino acids of the mouse InsP_3 receptor contain the essential sequences for InsP_3 binding. Within this region, amino acid residues 318–351 vary significantly between the mouse and *Drosophila* receptors. The recently detected InsP_3 receptor subtype (11) that would be capable of binding to InsP_3 has a 15-amino acid deletion (residues 318–332). We expected, therefore, that amino acid residues 318–351 are not directly responsible for InsP_3 binding. Unexpectedly, D316-352 failed to bind to InsP_3. Although the corresponding region of the *Drosophila* InsP_3 receptor differs in the number and sequence of amino acid residues from the mouse counterpart, the diverse regions of both origins probably form a β-turn structure according to the Chou–Fasman (21) rule. These data suggest that the diverse region from amino acids 316 to 352 is not directly required for InsP_3 binding but is important for taking a binding conformation. On the other hand, Ca^{2+} release experiments with various kinds of synthetic inositol phosphate analogues showed that the InsP_3 recognition site is markedly stereospecific (12): the ability of InsP_3 to release Ca^{2+} depends critically upon the positional distribution of the phosphate groups around the inositol ring. Consequently, it is concluded that the interaction between InsP_3 and the InsP_3 receptor requires the restricted higher-order structures of both.

To what extent could we define the InsP_3 binding site(s) within the N-terminal 650 amino acids? So far we have not observed any internal deletions within this N terminus that retain InsP_3 binding. D96-315, D169-465, D316-352, and D419-735 are all incapable of InsP_3 binding. It is likely that

the InsP_3 binding site consists of several distantly separated motifs, as is often the case with the binding sites for small molecules. For example, the GDP binding site in guanine nucleotide-binding proteins contains at least five conserved regions that are separated by sequences of various lengths (13).

Heparin Binding of Mutant Proteins in the S Series. It has been assumed that positively charged amino acid residues in the cytoplasmic regions of the InsP_3 receptor are responsible for binding to negatively charged InsP_3 molecules. This is supported by the blocking effects of various reagents on InsP_3 binding. Polymeric anions, such as heparin, strongly interact with the InsP_3 receptor, thereby preventing InsP_3 from binding to it (14, 15). The known binding sites for heparin in heparin-binding proteins are highly basic polypeptide regions that in most cases contain arginine or lysine residues (16). In addition, O'Rourke and Feinstein (17) reported that InsP_3 binding to platelet membranes is blocked by the specific arginine-modifying reagent p-hydroxyphenylglyoxal, suggesting the involvement of arginine in InsP_3 binding. However, we cannot predict any possible binding sites in the primary structure of the InsP_3 receptor, since basic amino acids are scattered throughout this protein.

We examined the S series proteins for heparin-binding activity by applying the soluble fractions to a column of heparin-agarose. All receptor proteins in the S series were retained on the heparin-agarose in low salt buffer (0.25 M NaCl) and were eluted in high salt buffer (0.5 M NaCl) (results not shown). The capability of T736 to bind to heparin suggests that heparin occupies some part(s) of the N-terminal 735 amino acids, thereby preventing InsP_3 from binding to the receptor, although the possibility that heparin interacts with other regions cannot be ruled out.

Tetrameric Complex Formation of the InsP_3 Receptor. Electron microscopic observations (3, 6), cross-linking (5), and sucrose-gradient centrifugation experiments (11) have revealed that the solubilized intact InsP_3 receptor is homotetrameric. To determine whether the C-terminal truncations affect such an intermolecular complex structure and whether the intermolecular structure is essential for InsP_3 binding, T2217, lacking the complete transmembrane region and the successive C terminus but possessing most of the large cytoplasmic region, was investigated and found to bind to InsP_3 with a K_d of 14 nM. Fig. 4 shows the immunoblot analyses of cross-linked products separated in an agarose/PAGE system (5). At 1 mM sDST, the intact InsP_3 receptors were cross-linked and the tetrameric form was clearly de-
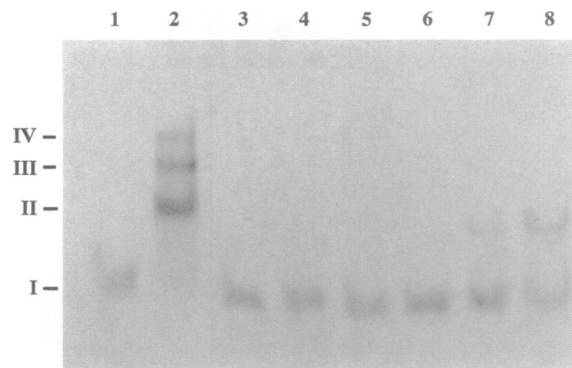

FIG. 4. Cross-linking of the receptor proteins. Fractions containing the intact receptor protein (lanes 1 and 2), T2217 (lanes 3–5), and D2112-2605 (lanes 6–8) were used. The cross-linker (sDST) concentrations were as follows. Lanes: 1, 3, and 6, 0 mM; 2, 4, and 7, 1 mM; 5 and 8, 10 mM. The cross-linked products were visualized by immunoblot analysis with the mAbs 10A6 and 18A10. I–IV indicate the positions of monomer, dimer, trimer, and tetramer of the intact InsP_3 receptor, respectively.

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2606
      *  ****          **        **  *     ***     *    *
LKTTCFICGLERDKFDNKTVTFEEHIKEEHNMWHYLCFIV

     **  **  **  ****      *          *
LVKVKDSTEYTGPESYVAEMIRERNLDWFLRMRAMSLVSS

DSEGEQNELRNLQEKLESTMKLVTNLSGQLSELKDQMTEQ

RKQKQRIGLLGHPPHMNVNPQQPA
                                        2749
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FIG. 5.   C-terminal 144-amino acid sequence of the mouse InsP₃ receptor was obtained and compared with the *Drosophila* InsP₃ receptor and the rabbit ryanodine receptor. Residues in corresponding positions that are identical are indicated by underlines (*Drosophila* InsP₃ receptor) or asterisks (rabbit ryanodine receptor). Cys-2610 and Cys-2617 are shaded.

tected (lanes 1 and 2). By contrast, T2217 proteins were not cross-linked even in the presence of 10 mM sDST (lanes 3–5), which indicates the absence of the intermolecular association in T2217 proteins. These results indicate that the tetramer structure is not necessary for InsP₃ binding of the InsP₃ receptor and support the hypothesis of Mignery and Südhof (8) that each subunit of the InsP₃ receptor tetramer contains an independent InsP₃ binding site.

D2112-2605 proteins, lacking the complete transmembrane domains but possessing the C-terminal 144 amino acids, were cross-linked to form at least a dimer by treatment of 1 or 10 mM sDST (lanes 6–8), suggesting the involvement of the C-terminal 144 amino acids (residues 2606–2749) in the intermolecular association. The amino acids in this region that are identical to those in the *Drosophila* InsP₃ receptor and the rabbit ryanodine receptor (18) are shown in Fig. 5. The conserved amino acids, including Cys-2610 and Cys-2613, are probably involved in the basic function of Ca²⁺ channels that are present on the intracellular organelles.

Among mutant proteins in the M series, D169-465, D419-735, D170-1252, D1267-2110, and D2527-2748 were used for cross-linking experiments, and all the proteins tested were cross-linked. Mignery *et al.* (11) also reported that the N-terminal 418-amino acid deletion retains the ability to form the tetramer structure. Thus it is probable that the tetramer formation of the InsP₃ receptor requires the transmembrane domains and/or the successive C termini and is responsible for the Ca²⁺ release activity. Several factors such as cAMP-dependent protein kinase (10) and ATP (19) have been reported to regulate InsP₃-mediated Ca²⁺ release activity. The regulation mechanisms may involve the large cytoplasmic region located between the N terminus and the transmembrane domains. We have combined our transient expression system with NG108-15 cells with a Ca²⁺ imaging technique, in which Ca²⁺ release caused by the application of

bradykinin can be monitored. Applying various mutant InsP₃ receptors to our system should help us to understand the molecular mechanisms of the InsP₃-mediated Ca²⁺ mobilization.

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