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IDENTIFICATION OF A REGION OF CALSEQUESTRIN THAT BINDS TO THE JUNCTIONAL FACE MEMBRANE OF SARCOPLASMIC RETICULUM

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SUMMARY: The interaction of calsequestrin (CSQ) with the channel-containing region of the sarcoplasmic reticulum (junctional face membrane, jfm) is involved in Ca^{2+} release, and it seemed of interest to identify the jfm-binding region of the CSQ molecule. For this purpose, CSQ was digested with trypsin, and peptides were screened for jfm binding. Partial amino acid sequencing of selected peptides allowed us to localize a critical site for jfm binding in the stretch encompassing residues 86-191.

CSQ is a high capacity Ca^{2+}-binding protein present in both skeletal and cardiac muscles serving as an intravesicular calcium sink for transported Ca^{2+} (reviews; refs. 1,2). Our recent studies (3,4) suggest that CSQ also plays an important role in Ca^{2+} release. Electron microscopic (5,6) as well as biochemical (4,7) evidence suggests that CSQ is linked with the junctional face membrane (jfm), the region of the terminal cisternal SR where the channel-containing foot protein (8,9,10) is localized. Thus, an identification of the jfm-binding region of the CSQ molecule is important not only for the eventual elucidation of the molecular structure of CSQ, but also for understanding the mechanism by which the Ca^{2+} release channel is regulated by CSQ. The present results localize the critical binding region within the segment of CSQ encompassing residues 86-191.

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Abbreviations

ConA, concanavalin A; CSQ, calsequestrin; EGTA, ethylene glycol bis (β-aminoethyl ether) - N,N,N',N'-tetraacetic acid; HPLC, high performance liquid chromatography; jfm, junctional face membrane; MES, 2-(N-morpholino) ethanesulfonic acid; PEI, proteolytic enzyme inhibitors; PMSF, phenylmethyl sufonyl fluoride; SR, sarcoplasmic reticulum; TFP, trifluoperazine.

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EXPERIMENTAL PROCEDURES

CSQ was purified from rabbit skeletal muscle as described previously (11). CSQ-depleted jfm was prepared by treating the heavy fraction of SR with Triton X-100 followed by dissociation of the attached CSQ with 0.5 M NaCl as described elsewhere (3).

For tryptic digestion, CSQ (1 mg ml^{-1}) was incubated in a solution containing 0.02 mg ml^{-1} trypsin, 0.5 M NaCl, 1 mM EGTA, 50 mM Tris-HCl, pH 8.0 at 22^0 for 10 min, and the reaction was stopped by 2 mM PMSF. Fractionation of ConA-binding peptides: 0.5 mg of the digested CSQ was applied to a 0.5 ml volume of ConA-sepharose 4B (Sigma) affinity column equilibrated with a solution containing 0.5 M NaCl, 1 mM $CaCl_2$, PEI (10 ug ml^{-1} aprotinin, 0.8 ug ml^{-1} antipain, 2 ug ml^{-1} trypsin inhibitor), 50 mM Tris-HCl, pH 8.0, the column was washed with 2 x 1.0 ml of the above solution, and the peptides that had been bound to the column were eluted with 1.0 ml of 0.1 M 1-0-methyl-alpha-D-glucopyranoside in the same solution. Fractionation of jfm-binding peptides: 0.5 mg of digested CSQ was incubated with 1 mg of CSQ-depleted jfm in 0.6 ml of sucrose-PEI solution (0.3 M sucrose, the above proteolytic enzyme inhibitors, 20 mM MES, pH 6.8) containing 0.5 M NaCl for 2 min; (NaCl) was then reduced to 0.1 M by dilution up to 3 ml with the sucrose-PEI solution to reconstitute a mixture of jfm-CSQ peptide complexes. The reconstituted material was centrifuged in a Beckman benchtop ultracentrifuge (TL-100.3 rotor at 90,000 rpm for 15 min), the precipitate was washed with the sucrose-PEI solution, and treated with 0.5 M NaCl to dissociate peptides that had been bound to the jfm. After sedimentation of the jfm the supernatnat fraction was used for peptide analysis. Peptide sequencing: HPLC of tryptic fragments was performed as described previously (12). Sequencing was performed on an Applied Biosystems Model 477A Sequencer, equipped with an on-line PTH-Analyzer, using standard protocols provided by the manufacturer.

RESULTS AND DISCUSSION

We found that tryptic cleavage of the purified CSQ proceeds much more slowly and more reproducibly at higher concentrations of monovalent cations (e.g 0.5 M NaCl) than in salt-free solutions. Figure 1 illustrates the Coomassie blue staining pattern of the fragments of CSQ obtained by tryptic digestion (total: figure 1). The tryptic fragments important for the following discussion are labeled as A through D as indicated in figure 1.

The digested CSQ (total: figure 1) was passed through a ConA-affinity column, and the fragments bound to the column were collected (ConA binding fraction: figure 1). Fragments A, B, D and some smaller M_r fragments were bound to the ConA column. We assumed that the carbohydrate moiety of CSQ plays a role in the binding to the ConA column. the sequence Asn-Val-Thr (residues 316-318) has been suggested as the glycosylation site (13), and since there is no tryptic cleavage site in the region between Lys 301 and the C-terminal Glu 360, corresponding to the 59-residue C-terminal peptide isolated and sequenced by Ohnishi and Reithmeier (14), we may assume that all ConA column-bound peptides contain the C-terminus of CSQ (cf. figure 2).

As shown in our recent report (3), CSQ is spontaneously associated with CSQ-depleted jfm by lowering the salt concentration of the mixture. In order to screen for the jfm-binding peptides, the total tryptic digest (cf. figure 1) was incubated with the CSQ-depleted jfm to reconstitute the jfm-CSQ fragment complexes, unbound peptides were removed, and the tryptic fragments that had been bound to the jfm were collected (JFM-binding fraction: figure 1). Fragments A, B, and C are identified as the jfm-binding peptides.

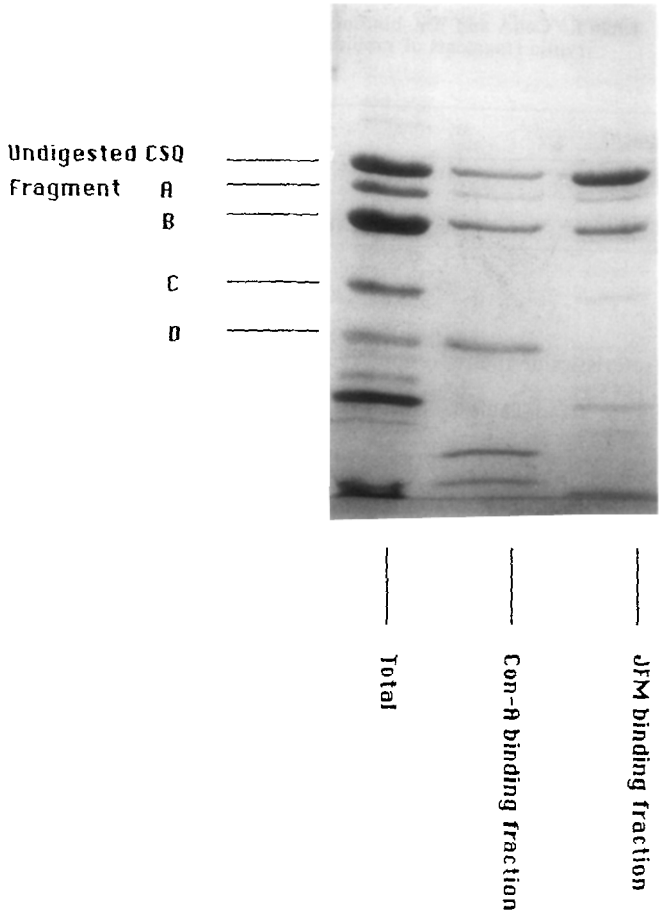

Figure 1: Coomassie blue-staining pattern of total fraction (lane 1), ConA binding fraction (lane 2), and jfm-binding fraction (lane 3) of tryptic fragments of CSQ. These fractions were prepared as described in Materials and Methods, and 30 ug of each fraction was electrophoresed in a 5-15% polyacrylamide gradient slab gel.

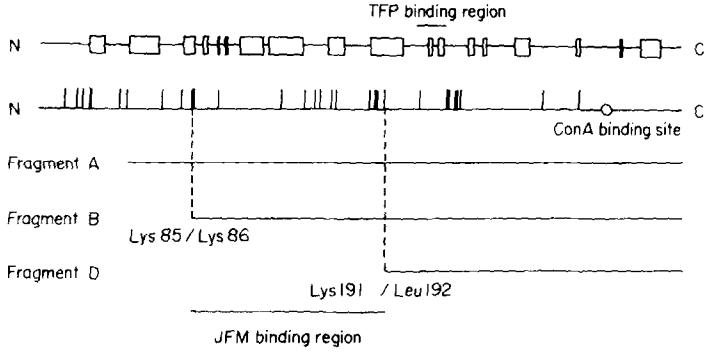

Figure 2: Schematic illustration of helical portions (blocks, top), potential trypsin cleavable sites (vertical lines, second), and localization of the major tryptic fragments. Localization of N-termini of Fragments B and D are based on the amino acid sequence data described in the text; while, that of Fragment A is tentative.

Table I. ConA and jfm-binding capabilities of the representative tryptic fragments of rabbit skeletal muscle calsequestrin

Fragment	A	B	C	D
ConA binding	+	+	-	+
jfm binding	+	+	+	-

The characteristics of the representative tryptic fragments described above are summarized in figure 2 and Table I. Note that both Fragments B and D retain the C-terminal region of the parent molecule (of figure 2), but the jfm-binding capability was lost upon cleavage of Fragment B resulting in Fragment D. Thus, the critical region for the jfm-binding must be located within the stretch between the N-termini of Fragments B and D.

The digested CSQ was subjected to micropreparative HPLC to purify individual fragments. HPLC fractions containing Fragments B and D were identified by coelectrophoresis of the isolated fractions with the fragments. The N-terminal sequences of these HPLC fractions were determined and localized within the known amino acid sequence of rabbit skeletal muscle CSQ (13). Fragment D gave the sequence Leu-Asn-Glu-Ile-Asp, corresponding to residues 192-196. Heterogeneity was observed at the N-terminus of Fragment B, due to partial tryptic cleavage occurring at lysine residues 85, 86 and 99. Three major sequences were identified: Lys-Leu-Gly-Leu-Thr-Glu-Glu-Asp-Ser-Ile (residues 86-95), Leu-Gly-Leu-Thr-Glu-Glu-Asp-Ser-Ile-Tyr (residues 87-96), and Glu-Asp-Glu-Val-Ile-Glu-Tyr-Asp-Gly-Glu (residues 100-109). These results suggest that the site(s) essential for the CSQ-jfm interaction is located in the region encompassing residues 86-191.

We investigated the effects of TFP, which binds to CSQ (13), and ConA on the association of CSQ with the jfm. Neither TFP nor ConA had an appreciable effect even at higher concentrations (100 uM TFP; 500 uM ConA). Thus, it appears that other binding regions, such as those for TFP and ConA (cf. figure 2, reference 13), do not interact with the jfm.

It is interesting that the jfm-binding region revealed in this study represents the portion of the CSQ molecule that is most highly enriched in α-helix (13) (cf. figure 2). In view of the fact that the Ca^{2+}-induced conformational change of CSQ involves a considerable increase of the helix content (11, 15), we propose that the coil-helix transition in the particular region plays an important role in the mechanism by which CSQ regulates the jfm proteins.

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