Identification of the Regions Conferring Calmodulin-like Properties to Troponin C*

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The structural and functional correlations between troponin C (TnC) and calmodulin (CaM) were investigated by mutating a synthetic cDNA coding rabbit skeletal muscle TnC. Compared with TnC, calmodulin lacks the N-terminal α-helical arm (N-helix), and its central helix is shorter due to the absence of "KRGK" residues. Deleting both regions concomitantly (∆NtAKGK) elicited CaM-like regulation as tested (i) by smooth muscle contractility (maximal tension = 80 ± 5% of control) and (ii) by the activation of phosphodiesterase (Vmax = 75 ± 2% of control). The Ca2+-binding capacity of the mutant and the effect of the mutation on maximally Ca2+-activated tension of skinned rabbit psoas muscle fibers were both conserved. Furthermore, in the linker region of the central helix, replacing the TnCcharacteristic "EDAKGK" successive residues with CaM-specific DTD residues generated a highly effective CaM mimic (Vmax = 96 ± 27%), whether or not the N-helix was also retained. Apparent Kd values (i.e. concentrations for half-maximal response) for the successful mutants were similar to each other but about 200-fold higher than that for CaM. A part of the helical linker region in CaM may unfold and bend to promote multiplicity of target interaction using all four hands (Ikura, M., Clore, G. M., Gronenborn, A. M., Zhu, G., Klee, C. B., and Bax, A. (1992) Science 256, 632-638; Meador, W. E., Means, A. R., and Quiocio, F. A. (1992) Science 257, 1251-1265). In contrast, our results suggest that the TnCcentral helix evolved to be less pliable by the combined influences of "EDAKGK" residues and the α-helical extension in N terminus, thereby keeping the N-terminal hands well separated from their C-terminal counterparts.

Calmodulin and skeletal fast muscle TnC are canonical dumbbell shaped Ca2+-binding proteins and are prominent members of an extensive EF-hand (helix-loop-helix) family (see Strynadka and James (1989)). Neither protein has intrinsic enzymatic activity; however, both mediate the activities of specific enzymes by Ca2+. Calmodulin is ubiquitous and multifunctional, regulating phosphodiesterase, many protein kinases, Ca2+ pump and channels, among others (Cohen and Klee, 1988). TnC, on the other hand, is found exclusively in cardiac and skeletal muscles and also is function-specific, singularly involved in the Ca2+-induced regulation of tension development. Experimentally, calmodulin can even partially substitute for TnC in tension regulation by skeletal fibers (Babu et al., 1988a). In fact, calmodulin, not TnC, is the regulator of smooth muscle contractility (Gulati et al. (1990); also see Davis (1992)). The structural basis of such striking disparity between CaM and TnC remains obscure. To pursue this, in this study, we have engineered TnC derivatives by mutating specific regions to identify which mimic the functional diversity of calmodulin. The skinned smooth muscle contractility and an in vitro phosphodiesterase activity were assayed as two representative probes. The TnC variants were also tested for tension regulation in fast-twitch fibers to inform whether the chief TnC function was retained as well. A salient structural feature of both calmodulin and TnC dumbbell structures is a long central helical stem linking the two lobes (hereafter termed the N-domain and C-domain, respectively). However, the amino acid sequences indicate a "KSKGK" triplex in TnC, which extends the linker (by about 4.5 Å) compared with calmodulin. We have deleted the 3 residues, and alternatively also deleted the same number of adjacent residues "SEE" to investigate their functional consequences.

Moreover, it is now known that the solution and crystal structures of CaM in the presence of target peptides markedly deviate from the dumbbell such that the N and C EF-hands come nearly in contact (Ikura et al., 1992; Meador et al., 1992). This striking transformation entails unwinding and bending of part or all of the linker domain residues 75-83 in calmodulin. The linker is similar in TnC, except that the region containing "DTDS" in CaM corresponds in TnC to the sequence "EDAKGKS". We have examined the effect of "EDAKGK"-DTD exchange in TnC to establish the importance of this region in TnC and calmodulin.

Another noteworthy structural distinction between CaM and TnC concerns the N terminus itself. In TnC, it embodies a 10-residue three-turn α-helical extension preceding the helix-loop-helix motif of site 1. This N-helix is highly truncated in calmodulin. Consequently, we have also investigated the influence of eliminating the 10-residue region in TnC.

The findings identify the regions where specific alterations conferred CaM-like functional multiplicity to TnC without evident forfeiture of the cross-bridge switching mechanism in skeletal muscle. The pliancy of the central helix may differ.

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The abbreviations used are: TnC, troponin C; STnC, skeletal fast muscle troponin C; Tnl, troponin I; CaM, calmodulin; PAGE, polyacrylamide gel electrophoresis; IPTG, isopropyl-1-thio-β-D-galactopyranoside; MLCK, myosin light chain kinase; PDE, phosphodiesterase; Nt, N-terminal α-helical arm; Pmax, maximal tension of the skinned fiber in pCa4.

1 For clarity we have used the residue numbering from the CaM sequence as denoted by Strynadka and James (1989). However, rabbit TnC residues indicated can be converted to the turkey sequence in Strynadka and James by adding 3 to our numbers for rabbit.
between unmodified TnC and CaM to account for part of their functional disparities.

**MATERIALS AND METHODS**

**Genetic Engineering of TnC with Cassette Mutagenesis and Polymerase Chain Reaction**

A rabbit skeletal TnC-encoding cDNA was synthesized from 11 pairs of oligonucleotides as described earlier (Babu et al., 1992), except that Asp-2 was reinserted to closely match the tissue TnC. The gene was assembled in pBluescript II SK+ containing recognition sequences for 22 restriction enzymes. The gene was cloned in pt7-7 plasmid (gift of Dr. S. Tabor, Harvard University) between the NdeI and ClaI sites; the insertion was designed to abolish the resident ClaI site, and another such sequence was embodied in the synthetic cDNA for added flexibility. The above steps were accomplished conjointly with Dr. C. Miller (HHMI, Brandeis University).

The mutants of TnC deleting the N-terminal α-helical arm were made with polymerase chain reaction, by using a 5'-primer that contained 15 nucleotide bases complementing the plasmid upstream of the TnC NdeI site. These were followed by codons for a methionine and alanine sequentially to retain them as the initial 2 residues consistent with the "wild-type" TnC; the primer omitted codons for the next 10 residues corresponding to the tissue TnC (TDQQAEPARSY) and included the subsequent 15 bases. The 4KGR residues, as well as other modifications of the central helix linker, were more conveniently made by cassette replacements (Babu et al., 1992). Table I denotes a comprehensive list of TnC derivatives. All mutations were confirmed by DNA sequencing (Sequence 2.0, United States Biochemical Corp.). The protein expression was in the DE3-plyS cells (containing the gene for T7 RNA polymerase) utilizing the IPTG induction protocol (Novagen). IPTG induces T7 RNA polymerase, which in turn transcribes the target DNA in the plasmid.

The protein purification was accomplished with column chromatography, as described recently (Babu et al., 1992). The typical purity of the proteins in the present study is indicated on SDS-PAGE gels (Fig. 1). The bacterially generated proteins depicted in Fig. 1 are defined in the top portion of Table I. Bovine brain calmodulin (Sigma) was included for comparison. The protein concentrations were estimated using a Bio-Rad assay. The TnC standard used in this assay was prepared with UV spectroscopy (Babu et al., 1992).

**Skinned Fibers**

Smooth Muscle Strips—Taenia coli from guinea pig was used as described earlier (Reisin and Gulati, 1972; Gulati et al., 1986). The tissue immersed in vigorously bubbling (5% CO, 95% O) Kreb's solution was cleaned of adventitia (room temperature) and transferred to a skinning solution (containing 5 mM EGTA, 50 mM KCl, 1% Triton X-100) for 3 h. The samples were then stored at -20 °C in the storage solution containing: 150 mM potassium propionate, 20 imidazole, 5 ATP, 5 EGTA, 20 phosphocreatine, 6.06 MgCl, pH 7 and ionic strength 190-200 mM. For activation, a calculated amount of EGTA was replaced with Ca2+. EGTA to make pCa4. Activations were in pCa4 solution unless indicated otherwise. The strip length was adjusted in the relaxing solution to remove the slack (under 32X magnification).

Typically, skinned taenia coli strips were first depleted of endogenous CaM. This required between 3 and 10 activation-relaxation cycles at 20 °C without added CaM in pCa4 solution. Endogenous CaM was considered depleted when the force level was less than 10% of that in the very first activation. Following this, the preparation was reactivated in the presence of 0.5 μM calmodulin with pCa4 solution for a control P, measurement. The strip was multiply recycled in zero CaM to assure the removal of the residual CaM before a test contraction was performed. Presumably, in this assay, the added CaM activates the intrinsic MLCK; no MLCK was added externally in these experiments.

For test contractions, the desired TnC variant was used instead of calmodulin. All test contractions were again followed by CaM-induced contractions. The data were normalized to the mean of maximal tension levels (Po) achieved with 0.5 μM calmodulin before and after each test. Each contraction-relaxation cycle typically lasted 5-15 min, and due to such protracted activations the preparation was usually discarded after one completed cycle with a protein (ie: two contraction-relaxations with added CaM and one with test protein). Because only a single reliable data point was possible on each preparation in this protocol, a dose-response curve could not be reproducibly generated on smooth muscle. Corresponding dose-response curves for characterizing the mutants were instead developed more conveniently in a second independent assay (PDE activity) described below (see "Phosphodiesterase Assay").

**Fast-twitch Skeletal Muscle Fibers and TnC Depletion—Isolated single-fiber segments from psoas muscle of the rabbit were used at 22-25 μm sarcomere length (Babu et al., 1987; Gulati et al., 1991). The sarcomere length was adjusted with laser diffraction. Typically, the maximal force (Po) of the normal fiber was measured in pCa4 following which the endogenous TnC was depleted as described earlier (Babu et al., 1988b). The TnC-depleted fiber was then reconstituted with purified TnC or with the selected derivative. The maximal tension response was measured again; activation solution was devoid of free protein. Tension response at 5 °C was normalized to Po for the analysis.

**Phosphodiesterase Assay**

The CaM-dependent phosphodiesterase (PDE) activity catalyzing the hydrolysis of cAMP to AMP was determined in a cascade reaction as described by Schiefer (1986). Briefly, nanomolar to micromolar concentrations of CaM or TnC derivatives were incubated in the

<table>
<thead>
<tr>
<th>Deletion mutants</th>
<th>Bacterially synthesized (wild-type) STnC</th>
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<tbody>
<tr>
<td>ΔNt</td>
<td>N-terminal α-helical arm deleted</td>
</tr>
<tr>
<td>ΔKGR</td>
<td>4KGR residues in the central helix deleted</td>
</tr>
<tr>
<td>ΔNtΔKGR</td>
<td>Double mutant: N-terminal α-helical arm (Nt) and the 4KGR residues deleted concurrently</td>
</tr>
<tr>
<td>ΔNtΔSEE</td>
<td>Double mutant: Nt-arm and ΔSEE residues deleted concurrently</td>
</tr>
<tr>
<td>Supplemental central helix mutants</td>
<td>Double mutant: ΔEDA residues converted to DTD in STnC4</td>
</tr>
<tr>
<td>EDA→DTD-S4</td>
<td>4EDA residues converted to DTD conversion plus Nt-arm deletion</td>
</tr>
<tr>
<td>EDA→DTDΔNt</td>
<td>4EDA to DTD conversion plus 4KGR residues deletion</td>
</tr>
<tr>
<td>EDA→DTDΔAKGR</td>
<td>Triple mutant: 4EDA to DTD conversion plus concurrent deletion of Nt-arm and 4KGR residues</td>
</tr>
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</table>

**TABLE I**

**Description of TnC mutants generated**
Calmodulin Mimicry by TnC in Smooth Muscle and PDE

**RESULTS**

To delineate the mechanisms for functional diversity between calmodulin and TnC, we scrutinized initially two specific regions of rabbit skeletal fast-twitch muscle TnC. (a) The influence of the N-terminal α-helical arm (putative N-helix) was investigated by deletion. As depicted in Fig. 2, the N-helix in TnC atomic structure is 10 residues long (\textsuperscript{1}TDQQAERSY\textsuperscript{10}), but calmodulin has no N-helix and instead only 3 residues are present (ADQ in bovine brain CaM). (b) The influence of KGG triplex (residues 88-90 in TnC) was examined, also by deletion. This triplex is found in TnC in the linker region of the central helix, and is missing in the equivalent region in calmodulin (Fig. 2).

These initial aims were approached by generating four distinct TnC variants (listed in Table I as deletion mutants):

1. ANt, in which all 10 residues of the N-helix were deleted;
2. ANtAKG, in which the N-helix was deleted as well but the \textsuperscript{88}KGK\textsuperscript{90} triplex was retained; instead, the \textsuperscript{91}SEE\textsuperscript{88-90} residues adjacent to \textsuperscript{86}KGK\textsuperscript{90} were deleted in this case. The functional performances of each of these constructs were evaluated on fast-twitch skeletal muscle fibers to check whether the original TnC function was retained. Finally, the maximal Ca\textsuperscript{2+}-binding capacity was measured on four mutants as well to gain an assessment of the global conformation.

**Contractility of Smooth Muscle and Skeletal Muscle**—Fig. 3A shows the results with wild-type TnC and all four variants on tension development in smooth muscle. The skinned smooth muscle strips of the guinea pig taenia coli normally produced maximal (100%) tension with 0.1-0.5 μM free calmodulin externally added to the Ca\textsuperscript{2+} activation medium. The CaM in the presence of Ca\textsuperscript{2+} activates the intrinsic MLCK to phosphorylate the light chains of myosin, which is a critical step in the release of smooth muscle cross bridge mechanism above but the \textsuperscript{88}KGK\textsuperscript{90} triplex was retained; instead, the \textsuperscript{91}SEE\textsuperscript{88-90} residues adjacent to \textsuperscript{86}KGK\textsuperscript{90} were deleted in this case. The functional performances of each of these constructs were evaluated on smooth muscle for contractility, and in an in vitro enzymatic assay for isolated phosphodiesterase (obtained from Boehringer Mannheim; see "Materials and Methods") activity. The tension response was also evaluated on fast-twitch skeletal muscle fibers to check whether the original TnC function was retained. Finally, the maximal Ca\textsuperscript{2+}-binding capacity was measured on four mutants as well to gain an assessment of the global conformation.
Calmodulin Mimicry by TnC in Smooth Muscle and PDE

(see, e.g., Stull (1989)). With holo-STnC4, in lieu of calmodulin, there was no significant tension development. This was tested with STnC4 at 0.5 and 5 μM concentrations, in pcA4 medium. In one case we used STnC4 at 500 μM concentration (i.e., over 1000-fold of the effective calmodulin) and the force response was still negligible.

With either the ΔNt (0.5 and 5.0 μM) mutant or the ΔKGK-deletion mutant (also 0.5 and 5.0 μM), the force response was also negligible. However, in total contrast, with the addition of 0.5 μM ΔNtΔKGK mutant in the activation medium, replacing 0.5 μM calmodulin, the taenia coli smooth muscle strips produced 80% P0 tension on activation with pcA4. The alternative double mutant (ΔNtΔSSEE) was unable to restore activation of smooth muscle, indicating that the KGK triplex per se rather than the shortened linker in the central helix was instrumental in the CaM-like transformation of TnC, as assayed by the smooth muscle contractility response.

The ability to regulate contractility in skeletal fibers was also confirmed with these four TnC derivatives. As detailed under "Materials and Methods," psosas muscle fibers were depleted of endogenous TnC and subsequently replenished individually with each derivative. Unlike in the tests with smooth muscle, TnC-depleted skinned skeletal muscle single fiber (tension 0–15% P0 following TnC depletion) typically was reloaded with protein in relaxing solution, following which the fiber was extensively rinsed in protein-free relaxing solution to wash away any untethered protein. The tension response with each of the proteins is summarized in Table II. Except with the ΔNt derivative, the maximal force was similar to P0 (P0: maximal force of the skinned fiber in pcA4 with native TnC), indicating that all mutants (excepting only the ΔNt-TnC variant) were equally adept in switching on the cross-bridge mechanism in skeletal muscle.

The results in Table II also indicate that close to 4 mol of Ca2+ were bound/mol of protein in each case. This value denotes the saturation level, and this similarity in the Ca2+-binding capacities of the mutants with that of the wild type is a good indication that global structure of the derivatives was relatively normal.

Phosphodiesterase (PDE) Activation with TnC Derivatives—As an additional probe for CaM mimicry by the TnC derivatives, we assayed for the competencies of the mutants with that of the wild type. Except with the ANt derivative, the maximal force was similar to the ANtAKGK derivative was to the right of that for CaM.

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**Phosphodiesterase (PDE) Activation with TnC Derivatives**—As an additional probe for CaM mimicry by the TnC derivatives, we assayed for the competencies of the mutants in activating the PDE enzyme for catalyzing the hydrolysis of cAMP into AMP in *vitro*. The data points shown in Fig. 3B were all determined at 5 μM protein concentration, indicating maximal activity (% Vmax). As with tension regulation in smooth muscle (Fig. 3A), only the double mutant ΔNtΔKGK was found to be effective in PDE assay. The maximal PDE activity with this derivative was 75 ± 2% Vmax (mean of four experiments).

Fig. 4 shows the complete dose-response curves for PDE activation. Each PDE activation curve represents the mean of two observations in the same experiment. The curve for the ΔNtΔKGK derivative was to the right of that for CaM. The derived Kd values from the mean of four such independently conducted experiments were 0.94 and 209 nM for CaM and the mutant, respectively. These values are listed in the top two rows of Table III.

**Modifications of the Central Helix: Further Mutagenesis**—Although the crystal structure of CaM is dumbbell-shaped, the presence of a 26-residue synthetic target peptide (putative M13 peptide) duplicating the CaM-binding domain of skeletal muscle myosin light chain kinase (residues 577–602 in MLCK) forces a globular conformation in solution. This global transformation of CaM is accompanied by disruption of the central helix linker resides 75–83 (KMKDSTDSEE in CaM). The residues identified as particularly labile were "DTDS" (the numerals corresponding to CaM sequence) occurring sequentially. The corresponding linker in TnC has the sequence, 85MKEDAKGK89 (see Fig. 2). A notable difference is 85EDAKGK92 in TnC in the location of DTDS in calmodulin. The effect of EDA→DTD conversion in combination with the KGK deletion is investigated presently. Three constructs from the previous set were used as precursors: 1) ΔNt, 2) ΔKGK, and 3) ΔNtΔKGK, with superimposed 85EDA89→DTD conversion. These hybrids are listed in Table I under the subheading "Supplemental central helix mutants." The assay used was the PDE activity. Wild-type holo-STnC4 and bovine calmodulin were also probed under the same conditions. Fig. 5 shows the results with 5 μM protein in each case.

Compared with the corresponding precursors, all three hybrids (two double variants and one triple) with the 85EDA89→DTD conversion showed strikingly higher effect on PDE activity. For instance, even wild-type TnC hybrid with 85EDA89→DTD conversion showed a significant activation of PDE activity (Fig. 5, compare with third and second bars). Additional deletion of the N-helix had no effect in this case (compare fourth and third bars).

The most pronounced effect in this batch of mutants was manifest with derivatives that combined the KGK-deletion; thus, the 85EDA89→DTDΔKGK double mutant indicated phosphodiesterase activity close to that with CaM (fifth bar...
that while a TnC variant including concomitant deletions of TnC conversion. These conclusions are based on the findings that the diminished length of the central helix could be a partial determinant of the CaM. The extra =KGKW triplex in the linker of TnC was the source of elongated central helix by approximately 4.5 Å. Our results indicate that these residues are also central in the mechanisms inhibiting the TnC mimicry of CaM, but that the diminished length per se was not a significant factor. Possibly their contribution to the net charge imbalance in the central helix could be a partial determinant of the CaM-TnC conversion. These conclusions are based on the findings that while a TnC variant including concomitant deletions of the N-helix and KGKW trplex mimicked CaM in two separate assays (smooth muscle contractility and the phosphodiesterase activity), but when the N-helix and the SEE3 triplex, instead of KGKW, were deleted, the conversion was substantially ineffective.

The presence of EDAA7 contiguous with KGKW within the TnC linker, in place of DTD in calmodulin, was identified as an additional possible determinant in the functional singularity of TnC. Indeed, the EDAA7→DTD mutation was most effective for CaM mimicry when the KGKW triplex was deleted as well (creating the sequence DTDS). Several lines of evidence in the literature (see below) suggest that the CaM linker is easily bent to accommodate a target peptide and, moreover, that the DTDS arrangement was involved in this compliancy. By showing directly that TnC can be converted into quasi-CaM as a net result of DTD implantation, the present study provides firm evidence for the dominant role of these central helix residues.

A flexible central helix in CaM is indicated in several studies. Cross-linking with bismaleimidohexane confined the separation between the N- and C-domains of CaM to within 19 Å (compared with the crystalline separation of 37 Å), and the cross-linked CaM activated MLCK as effectively as non-linked CaM (Persechini and Kretsinger, 1988). Furthermore, as mentioned earlier, bending of the central helix by unfolding of the linker is also observed in multidimensional NMR and x-ray structures of CaM complexed with target peptides derived from the sequences of CaM-binding domains of the skeletal muscle or the smooth muscle MLCK (26-residue peptide mimicking the skeletal MLCK for NMR structure in Ikura et al. (1992); 20-residue peptide from smooth muscle MLCK for crystal structure in Meador et al. (1992)). It would appear that all four EF-hands in CaM are able to act in a concerted manner after binding Ca2++. Interestingly, recent molecular dynamics simulation studies of CaM also suggest a highly flexible central helix (Mehler et al., 1991). Our present findings suggest that the TnC central helix is less pliant than CaM, yielding thereby a conformation suitable exclusively for interaction with Tnl in muscle. Evidently, in TnC, the N-terminal EF-hands must act separately from their C-terminal counterparts.

Although direct structural information from NMR and/or crystals of target peptide-TnC complex, analogous to CaM, will be essential in elucidating the TnC central helix pliancy in the physiological milieu, the findings of Trewella and coworkers (Trewella et al., 1990; Blechner et al., 1992) of small angle x-ray scattering are insightful. Their observations on two target peptides (PhK 5 and PhK 13) based on disparate regulatory domains of the catalytic subunit of phosphorylase kinase, indicated that CaM was compacted only with one (PhK 5) and not with the other (PhK 13), nor even when both peptides were present concurrently (Trewella et al., 1990). This supports the view that central helix flexibility will guide the final conformation of the regulator-target complex, which would suggest that the conformation adapted by the TnC-Tnl complex is also highly specific.

CONCLUSIONS

In conclusion, we provide compelling evidence that CaM and TnC differ in their central helix properties. The present findings also indicated that in the special case of mutants with EDAKGKW→DTD conversions, the capacity to regulate phosphodiesterase activity was the same whether or not N-helix was deleted (Fig. 5). This was different from the effects observed on deleting the KGKW triplex, in which case the manipulation of N-helix was important. Presumably,
maximal compliance of the central helix was already attained with DTD residues and N-helix could not further modify this parameter. Alternatively, it is possible that the effects of N-helix deletion were different in the two sets of mutants but that these were unresolved in the selected measurements of the two assays used here.

In view of the present discussion on the key role of central helix in the functions of CaM and TnC, significant variety in the tension regulations by cardiac and skeletal TnC isoforms will be of continuing interest (such as in Sr²⁺ activations, and in the possible function of TnC in the Frank-Starling mechanism; e.g. Babu et al. (1987, 1988b)). Whether the central helix double mutation (rabbit muscle 86EDA87-*bovine cardiac DDS) reinforces the dominating influence of the highly differentiated structure of Ca²⁺-deficient cardiac site 1 (Gulati et al., 1992), in governing the heart function, would be worth investigating.

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