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Structural and Functional Studies on Troponin I and Troponin C Interactions

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Abstract Troponin I (TnI) peptides (TnI inhibitory peptide residues 104–115, Ip; TnI regulatory peptide resides 1–30, TnI1–30), recombinant Troponin C (TnC) and Troponin I mutants were used to study the structural and functional relationship between TnI and TnC. Our results reveal that an intact central D/E helix in TnC is required to maintain the ability of TnC to release the TnI inhibition of the acto-S1-TM ATPase activity. Ca^{2+}-titration of the TnC-TnI1–30 complex was monitored by circular dichroism. The results show that binding of TnI1–30 to TnC caused a three-folded increase in Ca^{2+} affinity in the high affinity sites (III and IV) of TnC. Gel electrophoresis and high performance liquid chromatography (HPLC) studies demonstrate that the sequences of the N- and C-terminal regions of TnI interact in an anti-parellel fashion with the corresponding N- and C-domain of TnC. Our results also indicate that the N- and C-terminal domains of TnI which flank the TnI inhibitory region (residues 104 to 115) play a vital role in modulating the Ca^{2+}-sensitive release of the TnI inhibitory region by TnC within the muscle filament. A modified schematic diagram of the TnC/TnI interaction is proposed. J. Cell. Biochem. 83: 33–46, 2001.

Key words: Troponin; Tnl-TnC interaction; peptide

The calcium sensitive control of striated muscle contraction is in part regulated by the interaction of troponin and tropomysin in the muscle thin filament [Ebashi and Endo, 1968; Endo and Obinata, 1981]. Troponin is composed of three proteins which interact strongly with one another: troponin I (TnI), the inhibitory subunit which inhibits the Mg^{2+}-activated ATPase of actomyosin, troponin C (TnC), the calcium binding subunit which neutralizes the inhibition of TnI, and troponin T (TnT), which anchor the troponin complex to tropomyosin. It is the Ca^{2+}- dependent conformational changes that occur between the TnC and TnI subunits of the troponin complex that play the most important role in the event of skeletal muscle contraction and relaxation [Leavis and Gergely, 1984; Grabarek et al., 1992 [reviews]; Tobacman, 1996; Miki et al., 1998].

TnI interacts with both tropomyosin and actin [Potter and Gergely, 1974; Hitchcock, 1975] and is responsible for the inhibition of the Mg^{2+}-ATPase activity of actomyosin [Hartshorn and Mueller, 1968; Schaub and Perry, 1969; Greaser and Gergely, 1971; Perry et al., 1972]. Inhibition of the actomyosin ATPase activity is neutralized when calcium-saturated TnC forms a complex with TnI [Perry et al., 1972; Weeks and Perry, 1978; Chong et al., 1983]. Syska et al. [1976] first demonstrated that three TnI fragments, CN4 (residues 96–117), CN5 (residues 1–21), and CF2 (residues 1–47), were all capable of binding to a TnC-Sepharose affinity column but only the CN4 fragment (residues 96–117) was capable of interacting with acto-tropomysin and inhibit the acto-S1-TM ATPase activity [Syska et al., 1976]. TnI CN4 fragment (resides 96–117) has gained attention for extensive investigation and

Abbreviations used: TnC, troponin C; TnI, troponin I; Ip, TnI inhibitory peptide Ac-TnI (104–115) amide; TnI1–30, TnI regulatory peptide, 1–30; TM, tropomyosin; S1, myosin subfragment 1; acto-S1, actin and myosin subfragment 1; HPLC, high-performance liquid chromatography; RPC, reversed-phase chromatography; TFA, trifluoroacetic; DTT, dithiothreitol; Ac, acetylated N-terminus; Amide, amidated C-terminus.

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it demonstrated that TnI residues 104–115 (Ip) comprise the minimum sequence necessary for the inhibition of actomyosin ATPase activity [Talbot and Hodges, 1979, 1981a, b]. It has been concluded that the Ca^{2+}-dependent switch between muscle relaxation and contraction involves a switching event of the TnI inhibitory region (resides 104–115) between actin- TM and TnC, respectively [Van Eyk and Hodges, 1988]. A computer generated three-dimensional model demonstrating the interaction between TnC C-domain and the TnI inhibitory region (peptide) has been proposed by Ngai et al. [1994]. Although the inhibitory site of TnI (residues 104–115) is of major importance, it is not the only Ca^{2+}- sensitive TnI/TnC binding site [Syska et al., 1976; Weeks and Perry, 1978; Grabarek et al., 1981; Ngai and Hodges, 1992; Tripet et al., 1997; Abbott et al., 2000]. We have demonstrated the biological important interaction found between the N-terminal region of TnI (residues 1–40) and TnC and indicated that the N-terminal domain of TnI is not just playing a structural role for anchoring troponin complex in the thin filament via TnT [Ngai and Hodges, 1992; Tripet et al., 1997; Abbott et al., 2000; Mercier et al., 2000]. Recent investigation in our laboratory on the TnI N-terminal regulatory region has further delineated the TnI N-terminal biologically active sequence to residues 1 to 30.

In the present study, TnI peptides and recombinant mutants of TnC and TnI were used to further investigate the Ca^{2+}-sensitive TnI/TnC interaction that governs the event of muscle contraction and relaxation.

MATERIALS AND METHODS

Preparation of Muscle Proteins

Rabbit skeletal TnC was prepared by the procedure of Chong and Hodges [1982a,b]. Rabbit cardiac α-TM was purified as described by Pato et al. [1981]. G-actin was extracted and purified from rabbit skeletal muscle acetone powder as described previously [Spudich and Watt, 1971]. Myosin subfragment (S1) was prepared by the method of Weeds and Taylor [1975], as modified by Talbot and Hodges [1981a]. The S1 purification by DEAE-cellulose chromatography provided two fractions: S1(A1) and S1(A2). These fractions were pooled for use in the acto-S1-TM ATPase assay. The K^+-EDTA ATPase activity of the S1(A1, A2) preparations varied between 500 and 600 nmol of Pi/min/mg of S1. Construction, isolation, and nomenclature of recombinant TnC [TnC (1–162), TnC C domain (88–162), TnC N domain (1–90)], and TnI [wt-TnI (residues 1–182, TnI_{1-116} (residues 1–116) and $TnI_{103-182}$ (residues 103–182)] has been described by Li et al. [1994] and Farah et al. [1994], respectively. The purity of all proteins was checked by reversed-phase chromatography and sodium dodecyl sulfate (SDS)–urea–polyacrylamide gel electrophoresis (PAGE) [Chong et al., 1983]. The concentration of all proteins and synthetic peptides were determined by amino acid analysis, except S1 which was determined by absorbance [Yagi et al., 1967].

Peptide Synthesis and Purification

All peptides (Table I) were prepared using the standard procedures for solid-phase peptide synthesis [Erickson and Merrifield, 1976] on an Applied Biosystems 430A solid phase peptide synthesizer (Foster City, CA). Peptides were synthesized following the general procedure for solid-phase synthesis described by Hodges et al. [1988]. All amino acids used were protected at the α-amino position with the *t*-butyloxycarbonyl (Boc-) group (Bachem, Philadelphia, PA). The following side-chain protecting groups were used: Arg(Tosyl), Asp(OBzl), Glu(OBzl),

TABLE I. Amino Acid Sequences of TnI Peptides[a]

TnI Inhibitory Peptide 104–115 (Ip)

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      104       115
Ac-GKFKRPPLRRVR-amide
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TnI N-terminal peptides

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       1         10        20        30        40
Ac-GDEEKRNRAITARRQHLKSVMLQIAATELEKEEGRREAEK-amide
                          (TnI1–40)

       1         10        20        30
Ac-GDEEKRNRAITARRQHLKSVMLQIAATELE-amide
                          (TnI1–30)
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[a]These sequences are from primary sequence of rabbit skeletal troponin I [Wilkinson and Grand, 1975, 1978].

His(DNP), Lys(2-ClZ), and Thr(Bzl). All amino acids were double coupled using dicyclohexylcarbodiimide generated symmetric anhydrides in dimethylformamide (DMF) for the first coupling and dichloromethane (DCM) in the second coupling to co-poly (styrene, 1% divinylbenzene) benzhydrylamine-hydrochloride resin at a substitution of 0.9 mmol of NH_2/gm of resin (Bachem, Philadelphia, PA). Any incomplete couplings (99.2% yield or less as determined by a quantitative ninhydrin test) were coupled a third time manually using Boc-amino acids: [2-(1H-benzotriazol-1-y1)-1,1,3,3-tetramethyluronium hexafluorophosphate]: 1-hydroxybenzotriazole: 4-methylmorpholine: active sites on resin 2:2:2:3:1 in N-methylpyrrolidone. The following steps were performed in the reaction vessel for each double coupling: (1) deprotection of the Boc-group with 33% trifluoroacetic acid (TFA) in DCM for 80 sec, (2) 50% TFA in DCM for 18 min, (3) three DCM washes, (4) 10% diisopropylethylamine (DIEA) in DMF 1 min (5) 10% DIEA in DMF 1 min, (6) five DMF washes, (7) first coupling 30 min, (8) three DMF washes, (9) 10% DIEA in DMF for 45 sec, (10) one DMF wash, (11) three DCM washes, (12) second coupling period 30 min, (13) one DMF wash, and (14) five DCM washes. If required, final acetylation was performed on the instrument using acetic anhydride: DIEA: mmol of peptide resin 50:20:1 for 10 min, then 100:20:1 for 5 min in DCM. The completed peptides were cleaved from the resins with anhydrous hydrogen fluoride (20 ml/g of peptide resin) in the presence of 10% anisole and 1% ethanedithiol for 1 h at -4°C using type 1B HF-Reaction Apparatus (Peninsula Laboratories Inc., Belmont, CA). The peptide-resin was then washed three times with diethylether (25 ml each). Then, the cleaved peptide was extracted from the resin with neat acetic acid (three times 25 ml each) and then lyophilized. The peptides were dissolved in 25% acetonitrile 75% water (80 mg/5 ml), and sonicated for 10 min. Neat acetic acid was added dropwise while sonicating until the sample cleared (5–10%). The samples were then spun down at 14,000 rpm for 2 min using an Eppendorf centrifuge 5414C (Fisher Scientific). The supernatant was then syringe filtered using a Millex-6V 0.22 μM filter unit (Millipore, Bedford, MA). This solution was then purified using reversed-phase chromatography using an Applied Biosystems 400 solvent delivery system and a 783A programmable absorbance detector connected to a Synchropak RP-4 (250×21.2 mm I.D.) reversed-phase column (Synchrom Inc., Lafayette, IN) operated at a flow rate of 2 ml/min with a linear AB gradient where solvent A was 0.05% aqueous TFA and solvent B was 0.05% TFA in acetonitrile. The gradient rates varied between 0.1–0.5% B/min depending on the sample load used [Burke et al., 1991; Hodges et al., 1991]. The sample loads varied between 20 and 50 mg/run. The fractions were then analyzed using a HP1090 Liquid Chromatography (Hewlett Packard, Avondale, PA) using the above solvent system at 2% B/min starting in 100% solvent A on a Zorbax R_x-C8 2.1 mm $\times$ 15 cm (Rockland Technologies, Giberstville, PA). The homogeneity of the purified peptide was determined by reversed-phase chromatography, amino acid analysis using a Beckman 6300 High Performance Analyzer (Allendale, NJ) and mass spectrometry using a BioIon 20 Plasma Desorption Time of Flight Mass Spectrometer (Uppsala, Sweden).

ATPase Assay

ATPase assays were performed using an automatic pH-stat apparatus consisting of a Brinkman Metrohm 614 Impulsomat, 655 Dosimat, 635 Dosigraph, and 635 pH meter with a 1 ml syringe. Assay samples 2 ml in volume, were placed in glass vials and stirred continuously at 25°C. The acto-S1-TM ATPase activities were measured in a buffer consisting of 5 mM Tris, 30 mM KCl, 0.1 mM EGTA, 5 mM $MgCl_2$,and 2.5 mM disodium ATP, pH 7.8. For experiments requiring the presence of calcium the same buffer was used except the 0.1 mM EGTA was replaced with 3 mM $CaCl_2$. The titrant was 5–10 mM standardized KOH. A single assay vial of acto-S1-TM was titrated with the protein(s) or peptide(s) in the same buffer, and the effect of accumulated protein or peptide on the ATPase activity was determined after each consecutive addition of the protein or peptide.

Size Exclusion and Reversed-Phase Chromatography

Mixtures of skeletal TnC (or recombinant TnC mutants) and TnI1–30 were dissolved in a buffer consisting of 20 mM Tris-HCl, 50 mM KCl, 3 mM $CaCl_2$, and 0.1 mM DDT at pH 6.8 pre-incubated at 4°C under nitrogen atmo-

sphere for 1 h. The TnC/TnI1–30 (or TnC domain/TnI1–30) mixtures were loaded onto a high performance size-exclusion column, Altex TSK G2000 SW (7.5 mm I.D. × 30 cm) (Beckman Inc., Berkeley, CA) in a buffer consisting of 20 mM Tris, 100 mM KCl, in the presence of 3 mM $CaCl_2$ and 0.1 mM DTT, pH 6.8, at a flow rate of 0.4 ml/min at room temperature. Peaks of interest were collected and analyzed by microbore reversed-phase chromatography on a microbore column (Aquapore RP-300 (C_8), 100 × 1.0 mm I.D. 300 Å pore size and 7 μm particle size) (Brownlee Labs., CA). The peptides and proteins were eluted from the column by employing a linear A-B gradient (2% B/min) where eluent A is 0.05% aqueous TFA and eluent B is 0.05% TFA in acetonitrile (pH 2.0), flow-rate, 0.2 ml/min at room temperature. To calculate the peptide/protein ratio in the complex, the peak areas of peptide and TnC (or TnC mutant) obtained on separation of the complex by the microbore reversed-phase chromatography were compared to the peak areas of standard solutions of peptide and TnC (or TnC mutant). The quantity of peptide or protein in the standard solutions was determined by amino acid analysis and used to calculate an instrument and column dependent extinction coefficient (mAu/nmole) for both TnC and peptide.

Circular Dichroism Spectra Determination

Rabbit skeletal TnC was prepared by the method of Chong and Hodges [1982a]. The purity of the proteins was checked by both reversed-phase HPLC and SDS–urea–PAGE [Chong et al., 1983]. It is important that the TnC be in its apo state to avoid erroneously high ellipticity values for the Ca^{2+}-free state that which would affect interpretation of the Ca^{2+}-dependent induced helical structure. In our present study, TnC was first treated in 6 M guanidine hydrochloride in the presence of excess EDTA and DTT to remove Ca^{2+} or any divalent [Golosinska et al., 1991; Pearlstone et al., 1992] and followed by subsequent dialysis of the protein against a buffer consisting of 20 mM MOPS, 1 mM EGTA, and 50 mM KCl at pH 7.12 under nitrogen atmosphere. During the final dialysis, 1 mM DTT was added to the dialysis buffer and the purified TnI1–30 peptide was dissolved in the same buffer. Prior to spectral analysis, TnC and TnI1–30 samples were centrifuged in pre-rinsed Spin-X tubes (Costar) equipped with 0.22-μm nylon filter. The protein and peptide concentrations were determined by amino acid analysis. The circular dichroism (CD) measurements were conducted on a JASCO J-720 spectropolarimeter (Jasco Inc., Easton, MD) interfaced to an Epson Equity 386/25 and conducted by Jasco software. The thermostated cell holder was maintained at 25°C with a Lauda RMS circulating water bath (Lauda, Westbury NY). The instrument was routinely calibrated with ammonium d-(+)-10 camphor sufonate at 290.5 nm, and with d-(−)-pantoyllactone at 219 nm. Each sample was scanned ten times and noise reduction applied to remove the high frequency before calculating molar ellipicities. The voltage to photomutiplier kept below 250 nm were 0.02 and 0.05 (calibrated for pathlength). The concentrations of the skeletal TnC varied between 22 and 30 M in the absence and presence of 1 M equivalent of TnI1–30 peptide. The experimental data were analyzed by using a computer software program designed to determine biphasic binding curves (program kindly provided by Dr. B.D. Sykes, University of Alberta). The fitting program analyzed data in the form of the following equation:

$$Z = f_1 \frac{[Ca^{2+}]^{n_1}}{[Ca^{2+}]^{n_1} + Kd_1^{n_1}} + f_2 \frac{[Ca^{2+}]^{n_2}}{[Ca^{2+}]^{n_2} + Kd_2^{n_2}}$$

where, Z is the percent change in spectral feature; f_1 and f_2, the fraction of change attributed to the high and low affinity sites respectively; n_1 and n_2, the Hill coefficients; Kd_1 and Kd_2 are the apparent dissociation constants of the low and high affinity sites respectively.

PAGE

Unless otherwise stated, all pre-incubated mixtures of proteins and peptides were obtained by dissolving the corresponding components in a buffer consisting of 20 mM Tris-HCl, 50 mM KCl, 0.1 mM EGTA, 5 mM $MgCl_2$, or 3 mM $CaCl_2$ at pH 7.0 under nitrogen atmosphere, and were allowed to equilibrate (while stirring) at 4°C for at least 1 h before running the assay. Alkaline PAGE was performed by the modified method of Head and Perry [1974] on gel slabs made from 8% polyacrylamide gel, containing either 3 mM $CaCl_2$ (in the presence of calcium) or 5 mM $MgCl_2$ with 0.1 mM EGTA (in the absence of calcium) in 20 mM Tris/124 mM glycine buffer at pH 8.6.

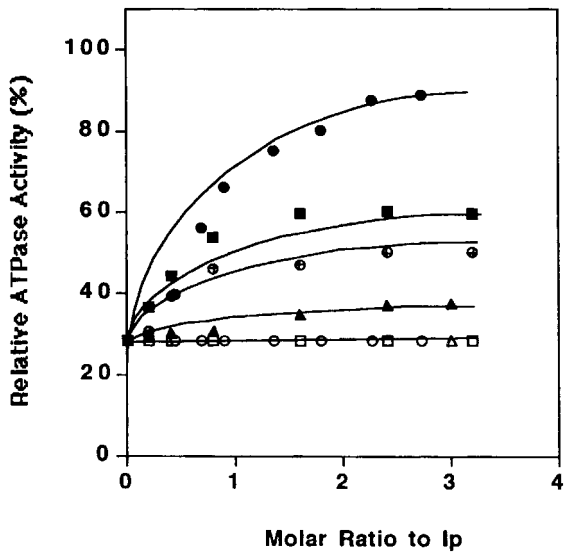


Fig. 1. Effect of N-terminal TnI peptide on TnC mutant (Ca^{2+}) in release of acto-S1-TM ATPase inhibition by TnI inhibitory peptide (Ip). The S1, actin, and TM concentrations were 3, 1.5, and 0.22 μM, respectively, giving a molar ratio of 14:7:1. (●) TnC; (■) TnC C-domain; (▲) TnC N-domain; (⊕) TnC C-domain and TnC N-domain at a 1:1 molar ratio; (○) TnC + TnI1–30; (□) TnC C-domain + TnI1–30; (Δ) TnC N-domain + TnI1–30.

RESULTS

Effect of TnI1–30 on Recombinant TnC Mutants in Release of the Acto-S1-TM ATPase Inhibition by TnI Inhibitory Peptide (Ip)

The acto-S1-TM ATPase activity was inhibited with TnI inhibitory peptide, residues 104–115 (Ip), followed by the release of inhibition by TnC mutant in the presence of Ca^{2+} and in the absence or presence of TnI N-terminal peptide, TnI1–30 (Fig. 1). In the presence of Ca^{2+}, the intact wild type chicken TnC fully released the inhibition induced by Ip (from 28 to 90% ATPase activity) in a manner similar to that of rabbit skeletal TnC (not shown). The recombinant chicken TnC C-domain only partially releases the Ip inhibition (from 28 to about 50% ATPase activity) whereas, the recombinant TnC N-domain was poorly effective in releasing the Ip inhibition (from 28 to 38% ATPase activity). The binding of TnI1–30 to TnC and TnC C-domain prevented neutralization of the Ip inhibition. Interestingly, there was only a partial release of the Ip induced inhibition (from 28 to 43% ATPase activity) by a molar equivalent mixture of C- and N-domains (pre-incubated at 1 to 1 M ratio of C- and N-domains for 1 h before the titration); the result is similar to that of the C-domain (Fig. 1). These results indicate intact D/E helix which links the two domains of TnC is essential in providing the optimum interacting interface for Ip for full biological function of the TnC molecule. In addition, only intact chicken TnC is capable of partially neutralizing the Ip induced inhibition in the absence of Ca^{2+} (results not shown).

Study of the Interaction Between TnI1–30 and TnC Mutants by Size-Exclusion (SEC) and Reversed-Phase Liquid Chromatography (RPC)

The interactions between TnI1–30 and recombinant TnC mutants in the presence of Ca^{2+} were further studied using HPLC methodology. Figure 2, panel A (left) shows the SEC run of the pre-formed TnC/TnI1–30 complex. The complex peak was collected from the size-exclusion run followed by reversed-phase chromatography. TnC and TnI1–30 were easily separated on RPC (panel A, right). The complex (TnC/TnI1–30) was shown by RPC to consist of a 1:1 ratio of TnC and TnI1–30 (integration of peak areas). These results indicate that the shorter version of TnI N-terminal peptide, TnI1–30 (residues 1 to 30) was still capable of maintaining a stable complex with TnC ($+Ca^{2+}$) during SEC and this is in agreement with our previous investigation using larger TnI N-terminal regulatory peptide, residues 1–40 [Ngai and Hodges, 1992]. Panel B (left) shows the SEC run of the pre-formed TnC C-domain-TnI1–30 complex. The complex peak was collected from the size-exclusion run followed by reversed-phase chromatography (panel B, right). The TnC C-domain/TnI1–30 complex was shown by RPC to consist of a 1:1.5 ratio of TnC and TnI1–30 respectively (integration of peak areas). Although these results demonstrated the ability of TnI1–30 in maintaining a stable complex with TnC C-domain ($+Ca^{2+}$) during SEC, the presence of a stable complex of TnI1–30 with TnC N-domain could not be demonstrated (see panel C, left and right). In addition, there is no stable complex formation by SEC for both intact TnC and TnC C-domain in the absence of Ca^{2+}. These observations indicate a significant reduced binding affinity between TnI1–30 and TnC or TnC C-domain in the absence of Ca^{2+}. This demonstrates the Ca^{2+}-sensitive nature of complex formation between TnC and TnI N-terminal peptides.

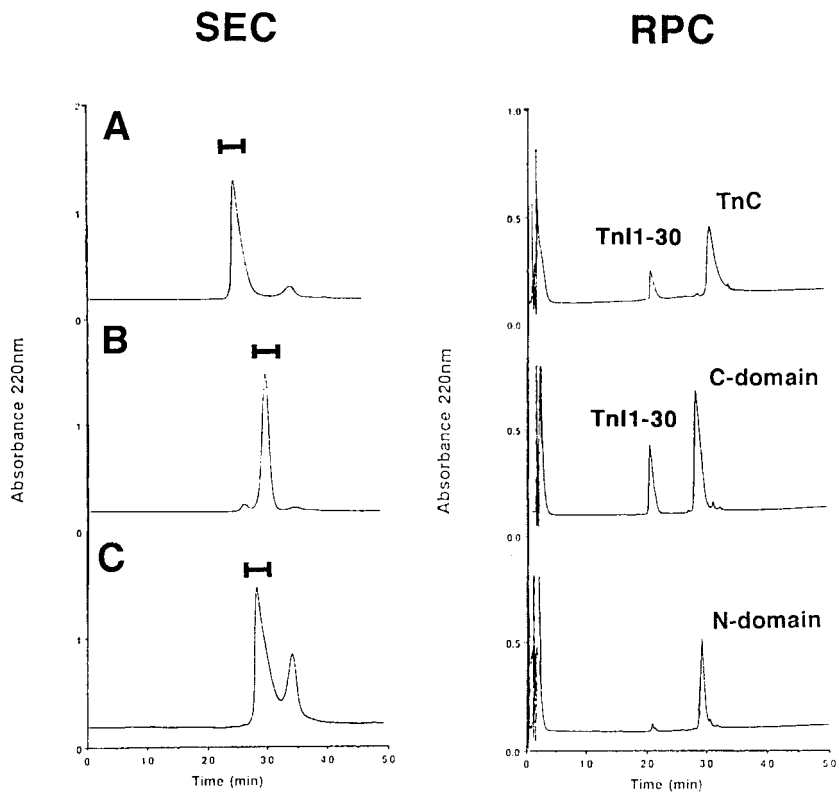


Fig. 2. Study of the interaction between TnI1–30 and TnC mutant by HPLC. **Panel A** (left), TnC (8 nmoles in 100 µl) and TnI1–30 (16 nmoles in 80 µl) were pre-incubated together (1:2 TnC/TnI1–30 molar ratio) for 1 h to form a TnC/TnI1–30 complex in the presence of Ca^{2+}. The pre-incubated mixture (150 µl) was loaded onto the SEC column and the complex (indicated by the horizontal bar on the left panel) was collected and loaded onto the microbore reversed-phase column (panel A, right). **Panel B** (left), TnC C-domain (16 nmoles in 100 µl) and TnI1–30 (32 nmoles in 160 µl) were pre-incubated together (1:2 C-domain/TnI1–30 molar ratio) for 1 h to form a TnC C-domain/TnI1–30 complex in the presence of Ca^{2+}. The pre-incubated mixture (220 µl) was loaded onto the SEC column and the complex (indicated by the horizontal bar on the left panel) was collected and loaded onto the microbore reversed-phase column (panel B, right). **Panel C** (left), TnC N-domain (32 nmoles in 200 µl) and TnI1–30 (64 nmoles in 320 µl) were pre-incubated together (1:2 N-domain/TnI1–30 molar ratio) for 1 h in the presence of Ca^{2+}. The pre-incubated mixture (500 µl) was loaded onto the SEC column and the peak of interest (indicated by the horizontal bar on the left panel) was collected and loaded onto the microbore reversed-phase column (panel A, right). See Materials and Methods section for conditions of incubation and running buffers for the SEC and RPC runs.

Ca^{2+} Titration Profile of Rabbit Skeletal TnC and the TnC/TnI1–30 Complex as Monitored by far-UV Circular Dichroism

The far UV CD calcium titration curves of rabbit skeletal TnC and the TnC/TnI1–30 complex are shown in Figure 3. The apparent binding constants for Ca^{2+} of the low and high affinity sites of TnC are shown in Table II. The pKd_1 values for the low affinity sites (sites I and II) of TnC and TnC/TnI1–30 complex are 5.408 and 5.519 respectively. However, for that portion of the titration curve attributable to the high affinity sites, the structural transition of the TnC/TnI1–30 complex was shifted to higher pCa value with a pKd_2 value of 7.617 as compared with a value of 7.154 for the rabbit skeletal muscle TnC protein. This shift in $-\log K_2$ values demonstrates an increase in Ca^{2+} affinity (three-fold) of sites III and IV of the TnC/TnI1–30 complex (Ka values of 1.4×10^7/M and 4.2×10^7/M for TnC and TnC/TnI1–30 complex, respectively).

TABLE II. Effect of TnI Regulatory Peptide (TnI1–30) on the pKd Values for the Low- and High-Affinity Ca^{2+} Binding Sites of the Skeletal TnC

	Low-affinity site	High-affinity site
	pKd_1	pKd_2
TnC	5.408	7.154
TnC/TnI1–30	5.519	7.617

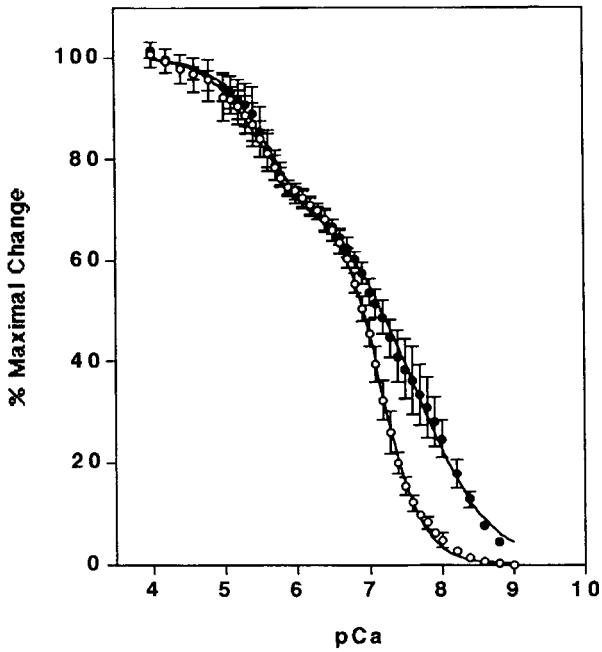


Fig. 3. Effect of TnI regulatory peptide, TnI1–30 on Ca^{2+} binding to skeletal TnC monitored by circular dichroism. The percent maximal change in ellipticity of TnC in the presence (•) and absence (○) of TnI1–30 is plotted vs. pCa value. A 1:1 molar equivalent of TnI1–30 and TnC was used and the curves were calculated by a computer program that best fits the experimental data to a curve composed of two binding constants. See method section for experimental conditions.

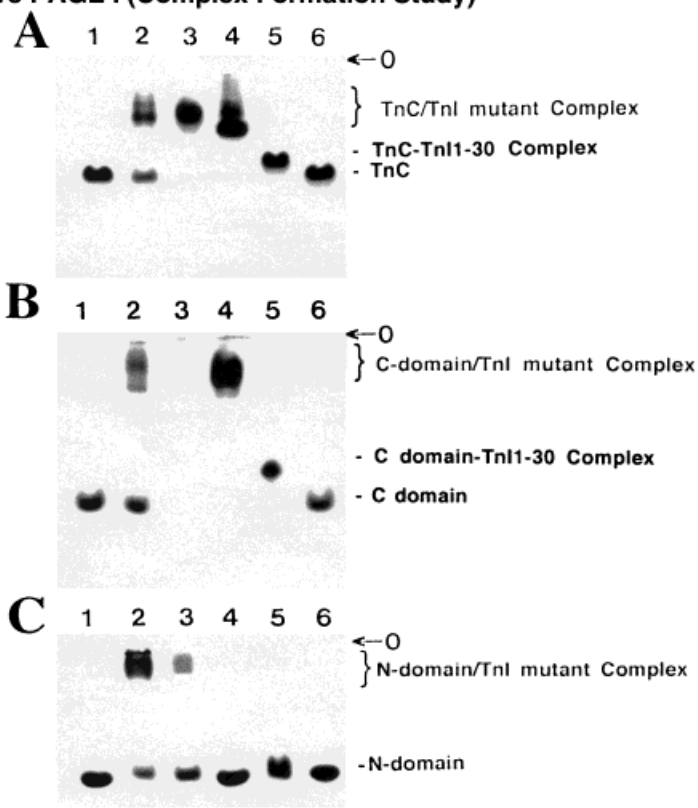
Effect of TnI1–30 on the Interaction Between Recombinant TnC and TnI Mutants

Complex formation studies. Farah et al. [1994] demonstrated that the N-terminal region of TnI interacts with C-domain of TnC and C-terminal region of TnI interacts with N-domain of TnC. Results from Figure 4a is in agreement with their results and we have incorporated the TnI N-terminal regulatory peptide (TnI1–30) into the binding studies.

Figure 4a (panel A) indicates that all TnI truncated analogs (TnI, $TnI_{103-182}$, TnI_{1-116}, and TnI1–30) are capable of forming a stable complex with TnC on the native gel ($+Ca^{2+}$). However, TnI does not bind as tightly as the other TnI truncated analogs that lack either the C or N-terminal region (TnI_{1-116} and $TnI_{103-182}$) or carries only the N-terminal regulatory sequence, residues 1–30 (TnI1–30). All TnI analogs (TnI, $TnI_{103-182}$, TnI_{1-116}, and TnI1–30) are capable of interacting with the C-domain in the presence of Ca^{2+} (Fig. 4a, panel B). Interaction between $TnI_{103-182}$ and C-domain does not result in a discrete band on the native gel. Importantly, only TnI and $TnI_{103-182}$ can form stable complexes with the N-domain of TnC ($+Ca^{2+}$) (Fig. 4a, panel C).

Competition studies. As shown in Figure 4b, panel A, left and right, TnI1–30 can displace TnI from either the TnC/TnI complex or TnC C-domain/TnI complex (lane 4 on both gels). Since native TnI molecule carries the same sequence of TnI1–30 in its primary structure, it suggests that the presence of TnI region other than residues 1–30 in the native TnI molecule reduces the strength of the TnC/TnI interaction. Figure 4b, panel B, left, shows that TnC/$TnI_{103-182}$ interaction is tighter than TnC/TnI1–30 interaction for the fact that TnI1–30 is no longer able to displace $TnI_{103-182}$ from the TnC/$TnI_{103-182}$ complex (lane 4). The absence of the TnI region spanning residues 1 to 102 results in a tight interaction between $TnI_{103-182}$ and TnC, in other words, the presence of residues 1–102 in TnI reduces the strength of TnI/TnC interaction. However, consider that there is no overlapping sequence found between $TnI_{103-182}$ and TnI1–30; $TnI_{103-182}$ interacts predominantly with the TnC N-domain (Fig. 4a, panel C) [Farah et al., 1994], whereas TnI1–30 interacts predominantly with the C-domain of TnC. It may suggest the formation of a ternary complex consists of TnC, TnI1–30, and $TnI_{103-182}$. However, it is also possible that $TnI_{103-182}$ when bound to TnC N-domain results in weakening the interaction between TnI1–30 and the C-domain of TnC. C-domain-TnI1–30 complex is not affected with the introduction of $TnI_{103-182}$ (Fig. 4b, lanes 4, panel B, right). In fact, C-domain/$TnI_{103-182}$ interaction is not as strong as C-domain-TnI1–30 interaction (Fig. 4b, lane 2 and 3, panel B, right). These verify the fact that TnI N- and C-domains flanking the inhibitory region interact in an antiparallel fashion with the corresponding N- and C-domain in TnC. Figure 4b, panel C (right) shows that TnC/TnI_{1-116} interaction is again stronger than the TnC/TnI1–30 interaction. In agreement with the above observation that deletion of the C-terminal region of TnI actually enhances the TnI/TnC interaction. In addition, TnI1–30 (residue 1–30) and TnI_{1-116} (residue 1–116) are competing with the same binding site on TnC (both TnI_{1-116} and TnI1–30 bind predominantly to C-domain [Ngai and Hodges, 1992; Farah et al., 1994].

(a) Native PAGE I (Complex Formation Study)



(b) Native PAGE II (Competition Study)

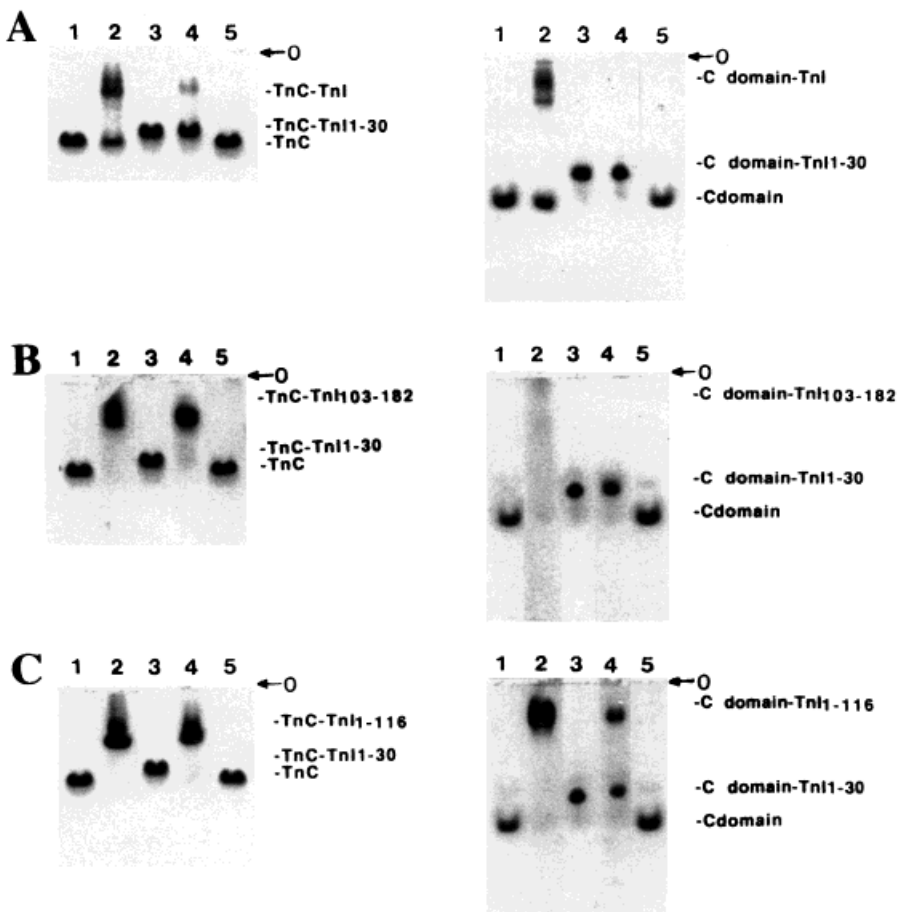


Figure 4b, panel C (right), indicates that both C-domain-TnI_{1-116} and C-domain-TnI1–30 complexes can co-exist (lane 4). However, based on the intensity of the two bands corresponding to the two complexes (TnC C-domain/TnI_{1-116} and TnC C-domain/TnI1–30 complexes) in lane 4, we can see that the C-domain-TnI1–30 complex prevails and this could be explained by the fact that the presence of the TnI sequence other than TnI1–30 together with the inhibitory region of TnI actually weaken the TnC C-domain/TnI_{1-116} interaction. Nevertheless, their presence strengthens the TnC/TnI_{1-116} interaction.

DISCUSSION

Previous investigations on TnI/TnC interactions demonstrated that two regions of TnI (residues 1–40 and 104–115) interact with TnC where three regions of TnC (residues 49–61, 89–100, and 127–138) can interact with TnI [Syska et al., 1976; Weeks and Perry, 1978; Talbot and Hodges, 1979, 1981a, b; Evans and Levine, 1980; Grabarek et al., 1981; Katayama and Nozaki, 1982; Leavis and Gergely, 1984, review; Van Eyk and Hodges, 1988]. Wang et al. [1990] demonstrated that binding of cations (Ca^{2+} or Mg^{2+}) to the high affinity sites (III and IV) of a mutant TnC altered the environment around the amino acid at position 57 in the N-terminal domain (Sites I and II). Rosenfeld and Taylor [1985] and Grabarek et al. [1986] showed that the binding of Ca^{2+} to the low affinity sites (I and II) altered the environment around Cys 98 in the C-domain of TnC. TnC in the presence of TnI adopts a more compact conformation in solution [Wang et al., 1987] than in the crystal structure of TnC [Herzberg and James, 1985, 1988; Sundaralingam et al., 1985]. Studies have indicated that the TnI inhibitory region (residues 104–115 (Ip) or a longer version of Ip (residues 96–116)) can interact with both the C-terminal domain of TnC [Weeks and Perry, 1978; Chong and Hodges, 1982a, b; Leavis and Gergely, 1984; Drabikowski et al., 1985; Tao et al., 1986; Leszyk et al., 1987, 1988; Lan et al., 1989; Van Eyk et al., 1991; Swenson and Fredricksen, 1992] and N-terminal domain of TnC [Leszyk et al., 1990; Kobayashi et al., 1991]. The above observations suggested that the TnI inhibitory region may form a single binding site between the N- and C-domain of TnC. This is supported by our results in which intact D/E helix of TnC is important in maintaining the full biological function of TnC in interacting with the TnI inhibitory peptide, since neither TnC C-domain nor TnC N-domain were able to fully release the Ip inhibition of

Fig. 4. PAGE. The native polyacrylamide gel (10% cross-linked) was polymerized in a Tris/glycine buffer, pH 8.6 with Ca^{2+} (5 mM) and DTT (1 mM). **a:** Native PAGE I (Complex Formation Study)- (Panel A) TnC/TnI analogs* Interaction: **Lane 1**, TnC (1 nmole); **Lane 2**, TnI (1.5 nmole) and TnC (1 nmole) equilibrated for 1 h; **Lane 3**, $TnI_{103-182}$ (1.5 nmole) and TnC (1 nmole) equilibrated for 1 h; **Lane 4**, TnI_{1-116} (1.5 nmole) and TnC (1 nmole) equilibrated for 1 h; **Lane 5**, TnI1–30 (1.5 nmole) and TnC (1 nmole) equilibrated for 1 h; **Lane 6**, TnC (1 nmole). (Panel B) C-domain/TnI analogs Interaction: Lane 1, C-domain (1 nmole); Lane 2, TnI (1.5 nmole) and C-domain (1 nmole) equilibrated for 1 h; Lane 3, $TnI_{103-182}$ (1.5 nmole) and C-domain (1 nmole) equilibrated for 1 h; Lane 4, TnI_{1-116} (1.5 nmole) and C-domain (1 nmole) equilibrated for 1 h; Lane 5, TnI1–30 (1.5 nmole) and C-domain (1 nmole) equilibrated for 1 h; Lane 6, C-domain (1 nmole). (Panel C) N-domain/TnI analogs Interaction: Lane 1, N-domain (1 nmole); Lane 2, TnI (1.5 nmole) and N-domain (1 nmole) equilibrated for 1 h; Lane 3, $TnI_{103-182}$ (1.5 nmole) and N-domain (1 nmole) equilibrated for 1 h; Lane 4, TnI_{1-116} (1.5 nmole) and N-domain (1 nmole) equilibrated for 1 h; Lane 5, TnI1–30 (1.5 nmole) and N-domain (1 nmole) equilibrated for 1 h; Lane 6, N-domain (1 nmole). **b:** Native PAGE II (Competition Study) - (Panel A, left) TnI/TnC/TnI1–30 Interaction: Lane 1, TnC (1 nmole); Lane 2, TnI (1.5 nmole) and TnC (1 nmole) equilibrated for 1 h; Lane 3, TnI1–30 (2 nmole) and TnC (1 nmole) equilibrated for 1 h; Lane 4, TnI (1.5 nmole), TnC (1 nmole) and TnI1–30 (2 nmole) equilibrated for 1 h; Lane 5, TnC (1 nmole). (Panel A, right) TnI/C-domain/TnI1–30 Interaction: Lane 1, C-domain (1 nmole); Lane 2, TnI (1.5 nmole) and C-domain (1 nmole) equilibrated for 1 h; Lane 3, TnI1–30 (2 nmole) and C-domain (1 nmole) equilibrated for 1 h; Lane 4, TnI (1.5 nmole), C-domain (1 nmole) and TnI1–30 (2 nmole) equilibrated for 1 h; Lane 5, C-domain (1 nmole). (Panel B, left) $TnI_{103-182}$/TnC/TnI1–30 Interaction: Lane 1, TnC (1 nmole); Lane 2, $TnI_{103-182}$ (1.5 nmole) and TnC (1 nmole) equilibrated for 1 h; Lane 3, TnI1–30 (2 nmole) and TnC (1 nmole) equilibrated for 1 h; Lane 4, $TnI_{103-182}$ (1.5 nmole), TnC (1 nmole) and TnI1–30 (2 nmole) equilibrated for 1 h; Lane 5, TnC (1 nmole). (Panel B, right) $TnI_{103-182}$/C-domain/TnI1–30 Interaction: Lane 1, C-domain (1 nmole); Lane 2, $TnI_{103-182}$ (1.5 nmole) and C-domain (1 nmole) equilibrated for 1 h; Lane 3, TnI1–30 (2 nmole) and C-domain (1 nmole) equilibrated for 1 h; Lane 4, $TnI_{103-182}$ (1.5 nmole), C-domain (1 nmole) and TnI1–30 (2 nmole) equilibrated for 1 h; Lane 5, C-domain (1 nmole). (Panel C, left) TnI_{1-116}/TnC/TnI1–30 Interaction: Lane 1, TnC (1 nmole); Lane 2, TnI_{1-116} (1.5 nmole) and TnC (1 nmole) equilibrated for 1 h; Lane 3, TnI1–30 (2 nmole) and TnC (1 nmole) equilibrated for 1 h; Lane 4, TnI_{1-116} (1.5 nmole), TnC (1 nmole) and TnI1–30 (2 nmole) equilibrated for 1 h; Lane 5, TnC (1 nmole). (Panel C, right) TnI_{1-116}/C-domain/TnI1–30 Interaction: Lane 1, C-domain (1 nmole); Lane 2, TnI_{1-116} (1.5 nmole) and C-domain (1 nmole) equilibrated for 1 h; Lane 3, TnI1–30 (2 nmole) and C-domain (1 nmole) equilibrated for 1 h; Lane 4, TnI_{1-116} (1.5 nmole), C-domain (1 nmole) and TnI1–30 (2 nmole) equilibrated for 1 h; Lane 5, C-domain (1 nmole). *TnI analogs do not enter gel.

the acto-S1-TM ATPase activity (Fig. 1). Our results is comparable with earlier work using proteolytic fragments of TnC on the studies of TnC/TnI interaction [Grabarek et al., 1981]. Nevertheless, our studies together with the above investigations have suggested that the major TnC binding sites for the inhibitory region of TnI is located on the C-domain of TnC and we have proposed a computer generated three-dimensional structure of the TnC C-domain/Ip complex [Ngai et al., 1994].

More attention has been given to the biologically important interaction between TnC and the N-terminal region of TnI [Ngai and Hodges, 1992; Sheng et al., 1992; Farah et al., 1994; Krudy et al., 1994; Dong et al., 1997; Saijo et al., 1997; Tripet et al., 1997; Van Eyk et al., 1997; Filatov et al., 1998; Vassylyev et al., 1998a,b; Abbott et al., 2000; Mercier et al., 2000] since it was first identified by Syska et al. [1976]. In our previous investigation, we have shown that synthetic peptides corresponding to the N-terminus of TnI were able to interact with TnC and prevent TnC from neutralizing TnI or TnI inhibitory peptide (Ip) induced inhibition of acto-S1-TM ATPase activity. This raises the question of how the N-terminal region of TnI governs the release of the inhibitory region of TnI within the TnI/TnC complex and be fitted into the Ca^{2+}-sensitive control of muscle contraction and relaxation event.

Perry et al. [1975] had proposed two mechanisms to describe how TnC may interact with TnI to neutralize the inhibitory activity of TnI. First it was postulated that the two sites (referred to as TnI1–30 (TnI residues 1–30) and Ip (TnI residues 104–115) in this study) are located on the troponin I molecule so that the binding of one site with TnC takes place without physical obstruction of the other. In this case, the interaction of TnC at the N-terminal region of TnI in the presence leads to a conformational change that causes modification in the molecule in the region of Ip so that the Ip site is no longer available for interaction with actin. Though this scheme did not propose the strong interaction of TnC with the inhibitory peptide Ip in the presence of Ca^{2+}[Van Eyk and Hodges, 1988], this interaction is compatible with Perry's proposal. However, the large conformational changes in TnI were ruled out by studies showing that Ca^{2+} induced changes in the TnC/TnI complex were only slightly greater than the sum of those in the separate subunits as measured by circular dichroism [McCubbin and Kay, 1973].

The second scheme requires that regions of troponin I (Rp and Ip) that interact with TnC and actin are located close together on the surface of the TnI molecule so that Ca^{2+}-induced interaction with TnC effectively prevents actin from interacting with the inhibitory region (Ip). Compatible with this proposal is that TnI Ip region is able to bind to actin and inhibit the actomyosin ATPase activity and bind to TnC causing the release of the ATPase inhibition [Talbot and Hodges, 1979, 1981a,b; Katayama and Nozaki, 1982; Cachia et al., 1983, 1986; Van Eyk and Hodges, 1987, 1988]. It has been proposed that one of the chemical switches in muscle regulation involves the binding of Ip to actin preventing the S1-actin interaction (muscle relaxation). In the presence of Ca^{2+}, conformational changes in TnC result in the release of inhibition via TnC binding to the Ip region, thus allowing the interaction of S1 and actin (muscle contraction).

Binding of TnI1–30 to TnC was shown to prevent the ability of TnC to interact with the inhibitory region, Ip in neutralizing the inhibition of the acto-S1-TM ATPase activity caused by Ip [Ngai and Hodges, 1992]. Adding to our previous findings are: (1) TnI N-terminal region interacts predominantly with the C-domain of TnC, (2). It can interact with TnC in its apo, Mg^{2+}, or Ca^{2+} states [Ngai and Hodges, 2001] and upon the binding of TnI1–30 to TnC, there is an increase in Ca^{2+}- affinity at the high affinity sites (sites III and IV) of the TnC protein, and (3) The increasing orders of the strength of interaction between TnC and TnI analogs are Ip/TnC < TnI/TnC < TnI1–30/TnC < ($TnI_{103-182}$/TnC, TnI_{1-116}/TnC) respectively. Our studies on the TnI/TnC interaction are summarized in Figure 5. This model agrees with results from two other investigations on the biological function of the TnI N-terminus [Sheng et al., 1992; Farah et al., 1994] as well as our previous studies of the biological function of the TnI N-terminal regulatory peptide (residues 1–40). In additions, Vassylyev et al. [1998b] recently demonstrated that TnC in complexing with TnI1–47 has a compact globular shape, in contrast to the extended dumb-bell shaped molecule of intact TnC. In the crystal structure of TnC-TnI1–47 complex, the C-terminal end of the TnI1–47 is tightly bound in the hydrophobic pocket of the TnC C-lobe. We can incorporate

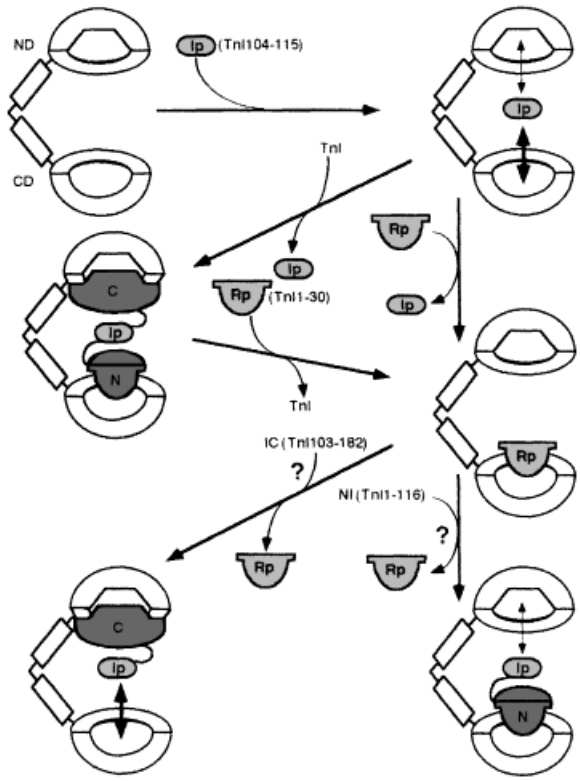


Fig. 5. Summary for TnI/TnC Interaction. TnC molecule is represented by a cross-section of the curved dump bell figure and ND represents N-domain and CD represents C-domain of TnC; N (shaded), TnI residues 1 to 98; Ip, TnI residues 104 to 115; C (shaded), TnI residues 120 to 182; TnI, TnI residues 1 to 182; Rp, TnI residues 1 to 30; TnI_{1-116}, TnI residues 1 to 116; $TnI_{103-182}$, TnI residues 103 to 182. Thick double-headed arrow represents strong interaction and thin double-headed arrow represents weaker interaction [All of the described TnI-TnC interactions are Ca^{2+} dependent (the strength of interaction increases with an increase in Ca^{2+} concentration), see text for detail].

our modified model for the TnC/TnI interaction into the regulation of muscle contraction and relaxation event. We propose that the TnI inhibitory sequence, residues 104–115 (Ip) is the Ca^{2+}-dependent switch and it is exposed on the surface of troponin I where it is available for interaction with either TnC or acto-TM. At low Ca^{2+} concentration where only the high affinity sites (sites III and IV) of the C-domain of TnC are filled with Ca^{2+} or Mg^{2+}, only the N-terminal region of TnI interacts with the C-domain of TnC anchors the whole troponin complex on to the acto-TM complex through its interaction with TnT [Sheng et al., 1992]. The interaction between the TnI N-terminal region and the C-domain of TnC excludes the Ip region together with C-terminal region of TnI from binding to the N-domain of TnC ($-Ca^{2+}$). The inhibitory region (Ip) is then available for interaction with the thin filament, blocking the acto-S1 interaction and resulted inhibition of the acto-S1-TM ATPase activity (muscle relaxation). At high Ca^{2+} concentration, the N-domain of TnC that carries the low affinity sites (I and II) are now filled with Ca^{2+} and interaction between the TnC N-domain and the TnI C-terminal region together with the TnI inhibitory sequence (residues 104–115) is very much strengthened in such a way that the Ip region is no longer available for interaction with the thin filament and the acto-S1 interaction is released and resulted in enhancement of interaction of the acto-S1-TM ATPase activity (muscle contraction). Our findings also indicate that interaction of the C-terminal region together with the Ip of TnI with the TnC N-domain also results a displacement of the TnI N-terminal regulatory peptide from interacting with the C-domain of TnC. These are very interesting findings because the TnI N-terminal region also involves in the interaction with TnT [Hitchcock et al., 1981, Chong and Hodges, 1982b]. It is possible that during the event of muscle contraction and relaxation, the attachment of the troponin complex to the acto-tropomyosin filament through N-terminal could results in amplifying or spreading the Ca^{2+}-induced signaling transmission along the thin filament during muscle contraction and relaxation event.

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