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Characterization of the Biologically Important Interaction Between Troponin C and the N-Terminal Region of Troponin I

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Abstract The N-terminal regulatory region of Troponin I, residues 1–40 (TnI 1–40, regulatory peptide) has been shown to have a biologically important function in the interactions of troponin I and troponin C. Truncated analogs corresponding to shorter versions of the N-terminal region (1–30, 1–28, 1–26) were synthesized by solid-phase methodology. Our results indicate that residues 1–30 of TnI comprises the minimum sequence to retain full biological activity as measured in the acto-S1-TM ATPase assay. Binding of the TnI N-terminal regulatory peptides (TnI 1–30 and the N-terminal regulatory peptide (residues 1–40) labeled with the photoprobe benzoylbenzoyl group, BBRp) were studied by gel electrophoresis and photochemical cross-linking experiments under various conditions. Fluorescence titrations of TnI 1–30 were carried out with TnC mutants that carry a single tryptophan fluorescence probe in either the N- or C-domain (F105W, F105W/C domain (88–162), F29W and F29W/N domain (1–90)) (Fig. 1). Low Kd values ($Kd < 10^{-7}$ M) were obtained for the interaction of F105W and F105W/C domain (88–162) with TnI 1–30. However, there was no observable change in fluorescence when the fluorescence probe was located at the N-domain of the TnC mutant (F29W and F29W/N domain (1–90)). These results show that the regulatory peptide binds strongly to the C-terminal domain of TnC. J. Cell. Biochem. 83: 99–110, 2001.

Key words: Troponin; TnI-TnC interaction; peptide

The contraction of striated skeletal muscle results from the sliding motion of myosin along the actin filaments whereby chemical energy in the form of ATP is used [Huxley and Hanson, 1954; Huxley and Niedergerke, 1954]. This mechanism is regulated by Ca^{2+} and requires the regulatory protein troponin and tropomyosin (TM) [Ebashi and Endo, 1968; Endo and Obinata, 1981]. Troponin is a complex of three proteins: troponin C (TnC), which binds Ca^{2+}; troponin T (TnT), which interacts with tropomyosin and anchors troponin to the thin filament, and troponin I (TnI), the inhibitory component capable of binding to actin filament and inhibiting the actomyosin ATPase activity [Leavis and Gergely, 1984; Zot and Potter, 1987; Da Silva et al., 1993; Grabarek et al., 1992; Tobacman, 1996; Miki et al., 1998].

The regions of TnI involved in interactions with TnC were first identified by Syska et al. [1976] as three cyanogen bromide fragments, CN4 (residues 96–116), CN5 (residues 1–21), and CF2 (residues 1–47) that bound to a TnC-Sepharose affinity column. However, only the CN4 fragment containing residues 96–116, named the inhibitory peptide, was able to bind to actin-TM and inhibit the acto-S1-TM ATPase activity [Syska et al., 1976]. Although, it is very well documented that the TnI inhibitory region (residues 104–115) interacts with TnC [Talbot

Abbreviations used: TnC, troponin C; TnI, troponin I; Ip, TnI inhibitory peptide Ac-TnI (104–115) amide; BBRp, N α-benzoylbenzoyl TnI (1–40 residues) amide; TM, tropomyosin; S1, myosin subfragment 1; acto-S1, actin and myosin subfragment 1; TFA, trifluoroacetic; DTT, dithiothreitol; Ac, acetylated N-terminus; Amide, amidated C-terminus.

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and Hodges, 1979, 1981a,b; Katayama and Nozaki, 1982; Cachia et al., 1983, 1986; Van Eyk and Hodges, 1987, 1988], the region on TnC that interacts with the inhibitory region of TnI is not clearly defined. Several physical experiments and chemical crosslinking studies have indicated that the TnI inhibitory peptide interacts with the C-domain of TnC [Weeks and Perry, 1978; Chong and Hodges, 1981, 1982b; Cachia et al., 1983, 1986; 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; Li et al., 2000; Mercier et al., 2000]. However, other crosslinking results demonstrated that residues from the regulatory Ca^{2+}-binding site II in the N-terminus of TnC (residues 46–78) form crosslinks with TnI inhibitory segment residues 92–167 [Leszyk et al., 1990]. Kobayashi et al. [1991] have shown that a mutant TnC containing a single Cys at residue 57 and modified with 4-maleimidobenzophenone can be crosslinked to the inhibitory region of TnI. Recently, similar cross-linking experiments have demonstrated that a single mutant TnC containing either a single Cys residue or a single 4-maleimidobenzophenone at various position in the N-terminal lobe of TnC itself is capable of forming cross-bridging with the inhibitory region of TnI [Leszyk et al., 1998; Luo et al., 1998]. To determine the binding site on TnC for the TnI inhibitory peptide (Ip), we have previously constructed a photoactivatable radioactive TnI peptide (BB-Ip) by solid-phase methodology which crosslinked to TnC in the presence and absence of calcium. From this work we had proposed a computer generated three-dimensional structure of the TnC C-domain-Ip complex [Ngai et al., 1994]. The Ca^{2+}-dependent switch controlling muscle contraction and relaxation is thought to involve an alternating interaction of the TnI inhibitory region (residues 104–115) between sites on actin-TM and TnC [Van Eyk and Hodges, 1988].

Although, this inhibitory site of TnI 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]. In our previous studies on the biological activity of the N-terminal region of TnI, we found that the TnI N-terminal peptides interact with TnC in Ca^{2+}-dependent manner and they are not capable of interacting with the actin filament. However, when bound to TnC, the TnI N-terminal peptides could prevent the ability of TnC to neutralize the inhibition of the acto-S1-TM ATPase activity caused by either TnI or the TnI inhibitory peptide (Ip), residues 104–115 [Ngai and Hodges, 1992]. In the present study, the minimum sequence in the TnI N-terminal regulatory region that can retain biological function is identified as residues 1–30. Investigations on the binding of the TnI N-terminal peptide to TnC were performed using gel electrophoresis, photolysis, and fluorescence studies.

MATERIALS AND METHODS

Preparation of Muscle Proteins

Rabbit skeletal TnC was prepared by the procedure of Chong and Hodges [1982a]; Chong and Hodges [1982b]. 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^{-1} mg^{-1} of S1. Construction, Isolation, and nomenclature of TnC mutants (F105W, F105W/C domain (88–162), F29W and F29W/N domain (1–90)) (Fig. 1) have been described by Li et al. [1994]. The purity of all proteins was checked by SDS–urea-polyacrylamide gel electrophoresis [Chong et al., 1983] and reversed-phase chromatography. The concentrations 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

The peptide sequences used in this study (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

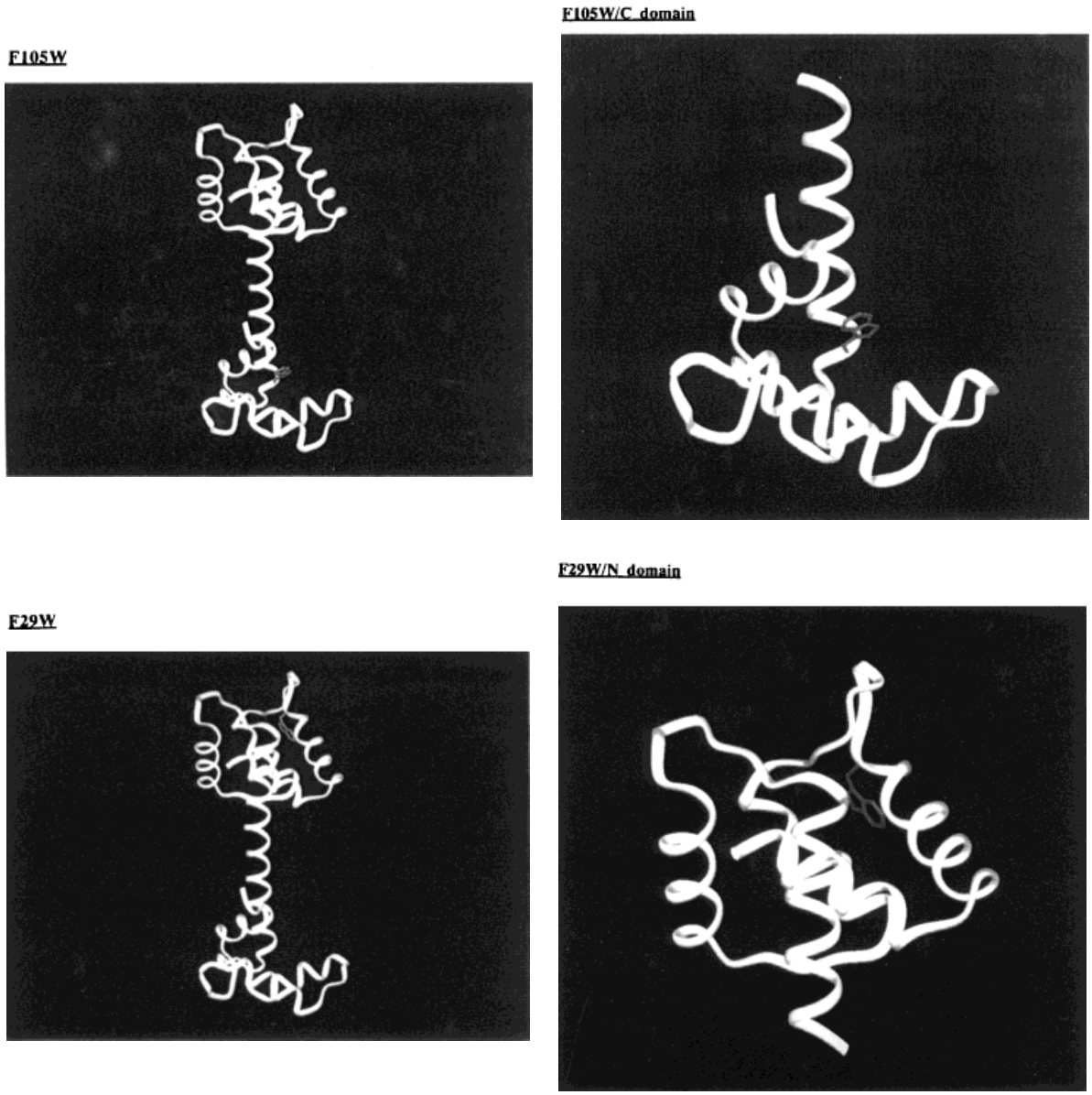

Fig. 1. TnC mutants (F105W, F105W/C domain (88–162), F29W, and F29W/N domain (1–90)). TnC mutant, polypeptide backbone in white ribbon and the corresponding trytophan probe is indicated in grey.

position with the t-butyloxycarbonyl (Boc-) group (Bachem, Philadelphia, PA). The following side-chain protecting groups were used: Arg(Tosyl), Asp(OBzl), Glu(OBzl), 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/g 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 s, 2) 50% TFA in DCM for 18 min, 3)

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
                                  (TnI 1–40)
          1        10        20        30
Ac-GDEEKRNRAITARRQHLKSVMLQIAATELE-amide
                                  (TnI 1–30)
          1        10        20
Ac-GDEEKRNRAITARRQHLKSVMLQIAATE-amide
                                  (TnI 1–28)
          1        10        20
Ac-GDEEKRNRAITARRQHLKSVMLQIAA-amide
                                  (TnI 1–26)
          1        10        20        30        40
BB-GDEEKRNRAITARRQHLKSVMLQIAATELEKEEGRREAEK-amide[b]
                                                BBRp (TnI 1–40)
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[a]These sequences are from primary sequence of rabbit skeletal troponin I [Wilkinson and Grand, 1975, 1978].
[b]The TnI regulatory peptide (TnI 1–40, Rp) was synthesized with Gly-1 labeled [α-^{14}C] and covalently linked to a benzoylbenzoyl moiety (BB) to yield the BBRp peptide.

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 s, 10) one DMF wash, 11) three DCM washes, 12) second coupling period 30 min, 13) one DMF wash, 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-6 V 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 $\times$ 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 per 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). For the BBRp synthesis, a radioactive label was incorporated at position 1 with Boc-Gly [α-^{14}C] and covalently linked to a benzoylbenzoyl (BB) moiety to yield the BBRp peptide with a specific radioactivity of 750 cpm/nmole.

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 continu-

ously 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 at 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.

Photolysis Experiment

TnC (30 μM) and BBRp (60 μM) at a molar ratio of 1:2 were pre-incubated under three conditions : 1) 20 mM Tris–HCl, 3 mM EDTA (metal ion free), 2) 20 mM Tris–HCl, 0.1 mM EGTA and 5 mM $MgCl_2$ (Mg^{2+}-buffer), and 3) 20 mM Tris–HCl, and 3 mM $CaCl_2$ (Ca-buffer) at pH 7.0 under nitrogen atmosphere. The mixtures were allowed to equilibrate (while stirring) at 4°C for 1 h. The equilibrated mixtures were subjected to photolysis by exposing the mixture to ultraviolet irradiation for 1 h at 4°C in an RPR 208 preparative reactor (Rayonet, The Southern New England Ultraviolet Co., Middletown, CN) equipped with RPR 350 nm lamps. Completeness of the photochemical crosslinking reaction was monitored by gel electrophoresis.

Pre-Incubation Conditions for Protein and/or Peptide Interaction Studies on Polyacrylamide Gel Electrophoresis

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, and 5 mM $MgCl_2$ or 3 mM $CaCl_2$ at pH 7.0 under a nitrogen atmosphere, and allowing to equilibrate (while stirring) at 4°C for at least 1 h before running the assay.

Polyacrylamide Gel Electrophoresis

Alkaline polyacrylamide slab gel electrophoresis was performed by the modified method of Head and Perry [1974] using 10% polyacrylamide, containing either 3 mM $CaCl_2$ ($+Ca^{2+}$) or 5 mM $MgCl_2$ with 0.1 mM EGTA ($-Ca^{2+}$) in 20 mM Tris/124 mM glycine buffer at pH 8.6.

Fluorescence Determination

The intrinsic Trp fluorescence was determined by using a Perkin-Elmer MPF-44B fluorescence spectrophotometer equipped with the DCSU-2 corrected spectra accessory, which allows for automatic subtraction of fluorescence due to solvent. The instrument was operated in the ratio mode, and thermostated cells were maintained at 20°C. Detection of fluorescence was effected at 90 degree to the excitation beam. The emission and excitation wavelengths were 301 and 282 nm, respectively [Lakowicz, 1983]. Aliquots of TnI 1–30 peptide were added to the initial 1,000 μl solution of TnC mutant. The concentrations of TnC mutant are in the range of 4–5 μM. Emission spectra were determined from 280 to 400 nm. Solutions of proteins were obtained by initially denaturing TnC mutants in 6M guanidine hydrochloride in the presence of excess EDTA and DTT [Golosinska et al., 1991; Pearlstone et al., 1992], and subsequent dialysis of the protein against a buffer consisting of 50 mM Tris–HCl, 50 mM KCl, and 2 mM $CaCl_2$ at pH 7.1 under nitrogen atmosphere (1 mM DTT was added to the final dialysis buffer). The purified TnI 1–30 peptide was dissolved in the same buffer. Prior to spectral analysis, TnC and TnI 1-30 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 using a Beckman 6300 acid analyzer.

RESULTS

Effect of TnI N-Terminal Peptides on the Ability of TnC ($+Ca^{2+}$) to Release Ip (104–115) Inhibition of Acto-S1-TM ATPase

In agreement with our previous investigation [Ngai and Hodges, 1992], all TnI N-terminal peptides by themselves are not capable of interacting with the actin-TM filament and have no inhibitory effect on the acto-S1-TM ATPase activity. In Figure 2 (panel A), the acto-S1-TM ATPase activity was initially inhibited with TnI inhibitory peptide (Ip), followed by the release of inhibition by TnC in the presence of Ca^{2+} and in the absence or presence of TnI N-terminal peptides [TnI 1–40, TnI 1–30, TnI 1–28, and TnI 1–26]. In the presence of Ca^{2+}, TnC fully released the inhibition induced by Ip (from 26 to 100% ATPase activity). The interac-

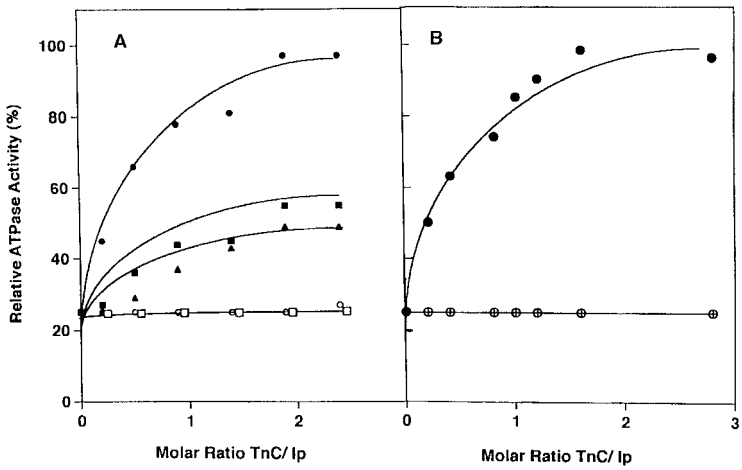


Fig. 2. Effect of the N-terminal TnI peptide on the ability of TnC (Ca^{2+}) to release the acto-S1-TM ATPase inhibition by TnI inhibitory peptide (Ip). The S1, actin and TM concentrations were 3, 1.5, and 0.22 μM, respectively, giving a molar ratio of 14:7:1. Ip at 0.5 μM is present to result the 20% ATPase activity at the beginning of the titration. Panel A: (●) TnC; (■) TnC+TnI 1–26 [1:1.5]; (▲) TnC+TnI 1–28 [1:1.5] (○) TnC+TnI 1–30 [1:1.5]; (□) TnC+TnI 1–40 [1:1.5]; Panel B: (○) TnC+TnI 1–30 [1:1.5]; (+) TnC+BBRp [1:1.5]; (●) TnC.

tion of the N-terminal TnI peptides with TnC (pre-incubation of TnC with the TnI N-terminal peptides) prevented TnC from fully releasing the acto-S1-TM ATPase inhibition induced by TnI inhibitory peptide (Ip). Our present data demonstrated that TnI 1–30 possesses the maximum ability in preventing TnC from releasing the Ip induced ATPase inhibition (identical biological activity with that of TnI 1–40). Therefore, we conclude that TnI residues 1–30 comprise the minimum sequence for the biological activity of the TnI N-terminal region.

Effect of BBRp on the Ability of TnC ($+Ca^{2+}$) to Release Ip (104–115) Inhibition off the Acto-S1-TM ATPase

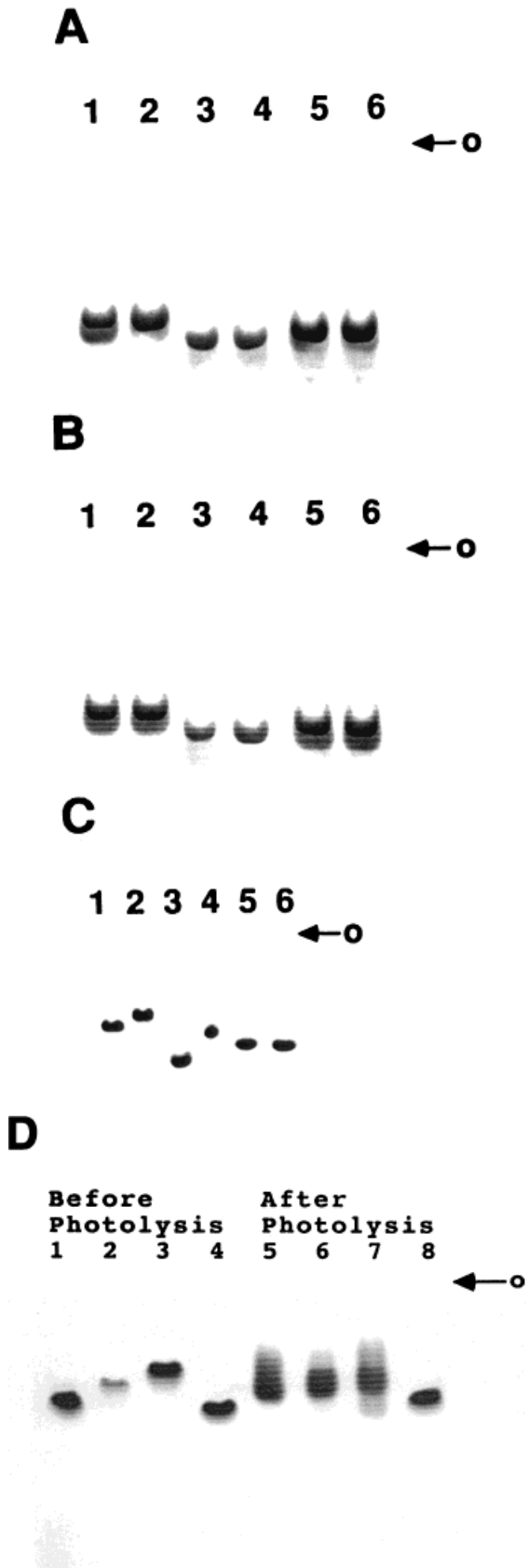
TnI N-terminal regulatory peptide (residues 1–40) was synthesized with α-^{14}C-labeled Gly at position 1 and covalently linked benzoylbenzoyl (BB) moiety at the N-terminus to yield a photoactivatable radioactive peptide (BBRp). The biological activity of BBRp on the acto-S1-TM ATPase assay was studied (Fig. 2, panel B). BBRp retains its ability in preventing TnC from neutralizing the Ip induced inhibition of the ATPase activity (identical activity with that of TnI 1-30).

Interaction of TnC Mutants With TnI 1–30

Analysis of the interaction between TnI 1–30 peptide and TnC mutants (TnC (1–162), TnC C-domain (88–162), and TnC N-domain (1–90)) by native gel electrophoresis is shown in Figure 3 (gel A–C). Figure 3, gel A and B demonstrate that TnI 1–30 is not capable of maintaining a stable complex with TnC mutants (intact TnC, TnC C-domain, and TnC N-domain) in either metal free or Mg^{2+} buffer. Figure 3, gel C, however, indicates that TnI 1–30 can result a stable complex with either intact TnC or TnC C-domain in the presence of Ca^{2+} and no complex formation is observed with TnC N-domain in the presence of Ca^{2+}. We concluded that the major binding site on TnC for the N-terminal regulatory region of TnI is located at the C-domain of TnC. Our results are consistent with a previous observation that TnI residues 1–116 (TnI_{1-116}) and residues 1–98 (TnI_{1-98}) are interacting predominantly with TnC C-domain [Farah et al., 1994].

Complex Formation Between TnC and TnI N-Terminal Peptide Before and After Photolysis

Figure 3, gel D, lanes 2 and 3 show that both TnI 1–30 and BBRp are capable of forming a stable non-covalent complex with TnC in the presence of Ca^{2+}. However, photolysis of a mixture of the native rabbit skeletal TnC and BBRp (1:2 molar ratio) in either metal free, Mg^{2+} or Ca^{2+} buffers yielded a covalent TnC/BBRp complex (Fig. 3, gel D; lanes 5, 6, and 7, respectively). Analysis of the complex by native gel electrophoresis indicated that photochemical crosslinking between TnC and the BBRp peptide was complete and that the complex isolated by reversed-phase HPLC consisted of TnC and BBRp in a 1:1 molar ratio when measured by amino acid analysis and radio-



activity. The finding that TnC/BBRp covalent complex could be formed with all states of TnC (apo, Mg^{2+} and Ca^{2+}) indicates the regulatory region of TnI (residues 1–40) is always capable of interacting with TnC under the above buffering conditions (strongest interaction in the presence of Ca^{2+}) and the N-terminus of BBRp is in close enough proximity to allow for crosslinking to occur during photolysis.

Impact of TnI 1–30 on the Fluorescence of TnC Mutants [F105W, F105W/C Domain (88–162), F29W, and F29W/N Domain (1–90)]

There is no significant fluorescence change observed upon binding of TnI 1–30 to the two TnC F29W mutants [F29W and F29W/N domain (1–90)] which carry a single fluorescence probe at residue-29 of the N-terminal domain of TnC. Emission fluorescence spectra of F105W and F105W/C domain (88–162) are shown in Figure 4. Both spectra of the TnC mutants have a common λ_{max} at 335 nm. However, upon

Fig. 3. Polyacrylamide gel electrophoresis. The native 10% polyacrylamide gel was polymerized in a Tris/glycine buffer, pH 8.6 and DTT (1 mM) at i) 1 mM EGTA (metal free buffer), ii) 1 mM EGTA and 5 mM $MgCl_2$ (Mg^{2+} buffer), or iii) 5 mM $CaCl_2$ Ca^{2+}*. **A:** TnC/TnI 1–30, TnC C-domain/TnI 1–30 and TnC N-domain/TnI 1–30 interaction in metal free buffer: **Lane 1**, TnC (1 nmole); **Lane 2**, TnI 1–30 (1.5 nmole), and TnC (1 nmole) equilibrated for 1 h; **Lane 3**, TnC C-domain (2 nmole); **Lane 4**, TnI 1–30 (3 nmole) and TnC C-domain (2 nmole) equilibrated for 1 h; **Lane 5**, TnC N-domain (2 nmole); **Lane 6**, TnI 1–30 (3 nmole), and TnC N-domain (2 nmole) equilibrated for 1 h. **B:** TnC/TnI 1–30, TnC C-domain/TnI 1–30, and TnC N-domain/TnI 1–30 Interaction in Mg^{2+} buffer: **Lane 1**, TnC (1 nmole); **Lane 2**, TnI 1–30 (1.5 nmole) and TnC (1 nmole) equilibrated for 1 h; **Lane 3**, TnC C-domain (2 nmole); **Lane 4**, TnI 1–30 (3 nmole) and TnC C-domain (2 nmole) equilibrated for 1 h; **Lane 5**, TnC N-domain (2 nmole); **Lane 6**, TnI 1–30 (3 nmole), and TnC N-domain (2 nmole) equilibrated for 1 h. **C:** TnC/TnI 1–30, TnC C-domain/TnI 1–30 and TnC N-domain/TnI 1–30 Interaction in Ca^{2+} buffer: **Lane 1**, TnC (1 nmole); **Lane 2**, TnI 1–30 (1.5 nmole) and TnC (1 nmole) equilibrated for 1 h; **Lane 3**, TnC C-domain (2 nmole); **Lane 4**, TnI 1–30 (3 nmole) and TnC C-domain (2 nmole) equilibrated for 1 h; **Lane 5**, TnC N-domain (2 nmole); **Lane 6**, TnI 1–30 (3 nmole) and TnC N-domain (2 nmole) equilibrated for 1 h. **D:** Photolysis experiment : **Lane 2** and **Lane 3** correspond to the study of complex formation before photolysis in the presence of Ca^{2+}. **Lanes 5–7** correspond to the study of covalently crosslinked TnC/BBRp complex after photolysis. **Lanes 1**, **4**, and **8** are TnC controls (1 nmole). Lane 2 shows the non-covalent TnC/TnI 1–30 complex formed by mixing TnI 1–30 (0.75 nmole) and TnC (0.5 nmole) after equilibration for 1 h. Lane 3 shows the non-covalent TnC/BBRp complex formed by mixing BBRp (1.5 nmole) and TnC (1 nmole) after equilibration for 1 h. Lane 5, 6, and 7 show TnC/BBRp covalently linked complex (1 nmole) formed in metal free buffer, in Mg^{2+} buffer, and in Ca^{2+} buffer, respectively. TnI peptides do not enter gel.

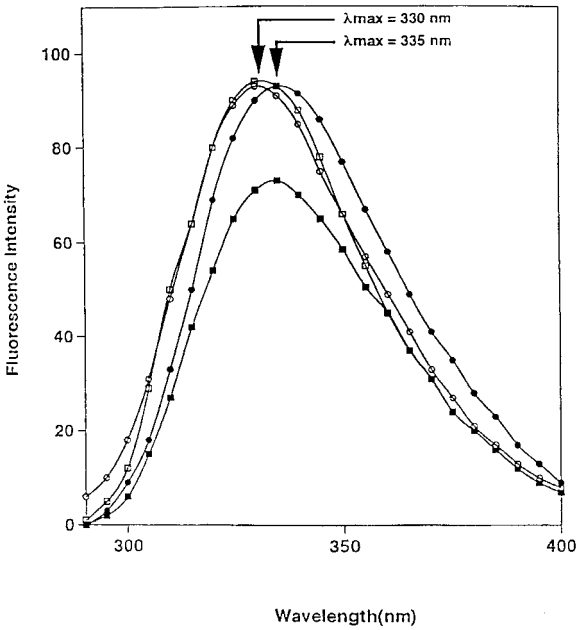


Fig. 4. Fluorescence spectra. Emission fluorescence spectra: (●) F105W; (○) F105W+TnI 1–30; (■) F105W/C domain (88–162) and (□) F105W/C domain (88–162)+TnI 1–30. λ_{max} for the spectra of both F105W and F105W/C domain (88–162) is at 335 nm. λ_{max} for the spectra of both F105W+TnI 1–30 and F105W/C domain (88–162)+TnI 1–30 is at 330 nm as indicated in the figure. Concentration for F105W is 4.6 μM and that of F105W/C domain is 4.25 μM. Buffer conditions are described in Materials and Methods section. No observable fluorescence changes upon binding of TnI 1–30 to the two TnC F29W mutants [F29W and F29W/N domain (1–90)], which carry a single fluorescence probe at residue-29 of the N-terminal domain of TnC (result not shown).

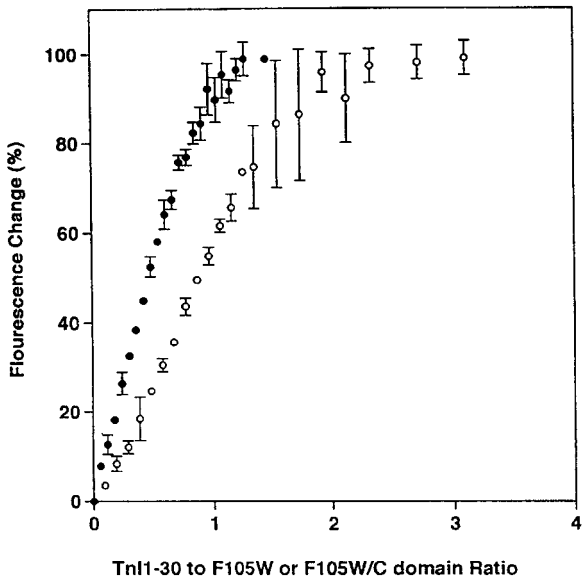


Fig. 5. Fluorescence titration. Experiments monitoring Trp-105 emission at 330 nm with stepwise addition of TnI(1–30). The average data points are shown (●) F105W and (○) F105W/C domain (88–162), with the range of values indicated by bars.

binding of TnI 1–30 onto the corresponding TnC mutant, there is a blue shift in the spectra of the complex formed and a common λ_{max} at 330 nm for both F105W and F105W/C domain (88–162) (Fig. 4). This indicates that the local environment of Trp-105 in either F105W or F105W/C domain (88–162) is altered upon the binding of TnI 1–30.

Fluorescence Titration Experiments of the TnC Mutants [F105W and F105W/C domain (88–162)] With TnI 1–30

The addition of TnI 1–30 to the TnC mutants resulted in a series of different fluorescence emission spectra which allow us to determine the binding constant of TnI 1–30 to the corresponding TnC mutant. The binding curves for both TnC mutants are shown in Figure 5, which demonstrated that TnI 1–30 binds more tightly to F105W than F105W/C domain (88–162). The titration was complete at about a 1 to 1 molar ratio of TnI 1–30 to F105W. The evaluation of the binding constant is based on the application of the following modified equation [Berger and Levit, 1973] that is based on the mass action law. Linearization of the equation assists in obtaining the parameters Co and Kd graphically.

$$\mathrm{Rp}\left(\frac{\mathrm{F_{max}}}{\mathrm{F}}-1\right)=\mathrm{Co}\left(1-\frac{\mathrm{F}}{\mathrm{F_{max}}}\right)+\mathrm{Kd}$$

Where Rp is the concentration of TnI 1–30 added (μM, 10^{-6}M), F is the corresponding observed fluorescence, F_{max} is the maximum fluorescence change observed, Co (slope term in Fig. 6) is the protein concentration (μM, 10^{-6}M), Kd (the y-intercept in Fig. 6) is the dissociation constant (μM, 10^{-6}M). Kd values for both of the F105W and F105W/C domain (88–162) towards the interaction of TnI 1–30 are found to be less than 10^{-7} M^{-1}.

DISCUSSION

Studies on the binding behavior of the TnI 1–30 with TnC in either the presence or absence of Ca^{2+} allow us to understand the Ca^{2+}-dependent TnI/TnC interaction within the troponin complex. By knowing this, we can understand more about the influence of divalent cations on the TnI/TnC interaction. For this purpose, the troponin I N-terminal regulatory region (1–40)

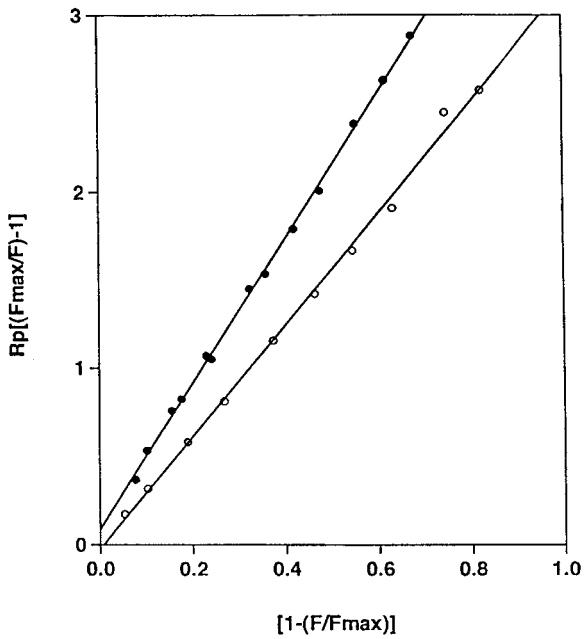


Fig. 6. Determination of the binding constant for F105W and F105W/C domain (88–162). (●) F105W; (○) F105W/C domain (88–162). Ka for both intact F105W and F105W/C domain (88–162) are greater than 10^7 M^{-1}.

was synthesized with Gly-1 labeled [α-14C] and a covalently linked benzoylbenzoyl (BB-) moiety at the N-terminus to yield the photoactivatable radioactive peptide (BBRp) to determine the feasibility of crosslinking it to rabbit skeletal TnC. Results of the photochemical crosslinking experiment demonstrated that BBRp was able to crosslink to TnC molecule in its apo, Ca^{2+} or Mg^{2+} states. In other words, the TnI N-terminal regulatory sequence (residues 1–40) is always capable of interacting with TnC regardless of the metal ion state of TnC. This is consistent with our previous ATPase results which show that the TnI N-terminal regulatory peptides can prevent the release of Ip inhibition by TnC in both Ca^{2+} and Mg^{2+} ATPase buffer [Ngai and Hodges, 1992].

Farah et al. [1994] demonstrated that a TnI mutant ($TnI_{103-182}$) which has in its primary sequence the inhibitory region of TnI (residues 104–115) extended to the C-terminus (residues 116–182) is capable of regulating the acto-S1-TM ATPase activity in a calcium dependent manner. Another two TnI mutants; TnI_{1-98}, which carries the N-terminal domain of TnI (residues 1–98), and TnI_{1-116} (residues 1–116) are both capable of interacting with TnC in either the presence or absence of Ca^{2+}. However, stable TnC/TnI mutant complex formation with either whole TnC or TnC C-domain can only be detected with TnI mutants that carry the N-terminal region (residues 1–98). Our present result indicates a similar observation that TnI 1–30 can form a stable complex with both TnC or C-domain in the presence of Ca^{2+} and no complex formation was observed with TnC N-domain.

Sheng et al. [1992] expressed a TnI recombinant deletion mutant (TnId57), which lacks residues 1–57, in a bacterial expression system. This TnI mutant inhibited acto-S1-ATPase activity and such inhibition could be fully neutralized by TnC in the presence of Ca^{2+} (100% ATPase activity) and partially neutralized by TnC in the absence of Ca^{2+} (50% ATPase activity). Affinity chromatographic studies of this TnI mutant TnId57 indicates that TnId57 appears to lack the region of TnI required for Ca^{2+} -Mg^{2+} site-dependent interaction and has a weakened metal independent interaction.

The above studies have demonstrated that the N-terminal region of TnI interacts mainly with the C-domain of TnC (the Ca^{2+}/Mg^{2+} sites III and IV of TnC) and they both concluded that the N-terminal region of TnI plays a structural rather than a functional role in the Ca^{2+} sensitive control of the actomyosin ATPase. However, they both fail to explain the ability of the N-terminal TnI peptides (TnI 1–30 and TnI 1–40) which when bound to TnC prevents the ability of TnC to release the inhibition of the acto-S1-TM ATPase activity caused by the inhibitory region of TnI. Our native PAGE gel studies indicate once again TnI N-terminal regulatory region (residues 1–40 or residues 1–30) is capable of forming a very tight complex with TnC in the presence of Ca^{2+}. The displacement of Ip from the hydrophobic groove of TnC C-domain upon addition of TnI 1–30 or TnI 1–40 agrees with the observed low Kd values (less than 10^{-7} M^{-1}) for TnI 1–30 towards the interaction of both of the F105W and F105W/C domain (88–162). This reveals that the TnI N-terminal region may be a negative effector of the regulatory process. Wang et al. [1990] demonstrated that binding of cations to the high affinity sites (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). Grabarek et al. [1986] and Rosenfeld and Taylor [1985] showed that the binding of Ca^{2+} to the low affinity sites altered the

environment around Cys 98 in the C-terminal domain. These findings indicated that the N- and C-terminal domains of TnC are communicating with each other upon the binding of Ca^{2+} or Mg^{2+}. Therefore, there must be a modulating event that governs the release of the TnI inhibitory region (Ip) from TnC by the N-terminal region of TnI. Although the N-domain of TnC contains the Ca^{2+}- regulatory sites that control muscle contraction, the two important regions of TnI (Ip, residues 104–115 and TnI 1–30, residues 1–30) bind to the C-domain. This raises the question of how Ca^{2+}-binding to the N-domain of TnC transmits this information to the C-domain of TnC.

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