THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 36, Issue of September 4, pp. 23448–23453, 1998
Printed in U.S.A.

Identification of Troponin C Antagonists from a Phage-displayed Random Peptide Library*

(Received for publication, May 15, 1998)

Heather Hanson Pierce‡, Fred Schachat§¶, Philip W. Brandt‖, Christian R. Lombardo, and Brian K. Kay‡ ‡‡§§**

*From the ‡Curriculum in Genetics and Molecular Biology, the **Macromolecular Interactions Facility, and the ‡‡Department of Biology, University of North Carolina, Chapel Hill, North Carolina 27599, the ¶Department of Cell Biology, Duke University Medical School, Durham, North Carolina 27710, and the ‖Department of Anatomy and Cell Biology, Columbia University Medical School, New York, New York 10032*

Affinity purification of a phage-displayed library, expressing random peptide 12-mers at the N terminus of protein III, has identified 10 distinct novel sequences which bind troponin C specifically. The troponin C-selected peptides yield a consensus binding sequence of (V/L)(D/E)*XLKXXLXX*LA. Sequence comparison revealed as much as a 62.5% similarity between *ϕ*T5, the peptide sequence of the phage clone with the highest level of binding to troponin C, and the N-terminal region of troponin I isoforms. Biotinylated peptides corresponding to library-derived sequences and similar sequences from various isoforms of troponin I were synthesized shown to bind troponin C specifically. Alkaline phosphatase fusion proteins of two of the phage clone sequences bound troponin C specifically, and were specifically competed by both library-derived and native troponin I peptides. Measurement of equilibrium dissociation constants of the peptides by surface plasmon resonance yielded dissociation constants for troponin C as low as 0.43 μM for pT5; in contrast, dissociation constants for calmodulin were greater than 6 μM for all peptides studied. Nondenaturing polyacrylamide gel electrophoresis demonstrated that pT5 formed a stable complex with troponin C in the presence of calcium. We also found that the pT5 peptide inhibited the maximal calcium-activated tension of rabbit psoas muscle fibers.

Members of the EF-hand family of calcium-binding proteins regulate the interaction of proteins in a wide variety of cellular processes, from cell cycle progression to muscle contraction (1, 2). In particular, troponin C (TnC),[1] the major regulatory protein in striated muscle contraction, is the calcium-binding component of the ternary troponin complex. It regulates the actomyosin activity in a calcium-dependent manner, through its association with tropomyosin and actin. Since TnC does not interact directly with either of these proteins, it must transmit the calcium signal to the thin filament by its interaction with the other two components of the troponin complex, troponin I (TnI) and troponin T (TnT). TnI, the inhibitory subunit, inhibits the actomyosin ATPase and binds actin, while TnT binds tropomyosin. Because TnC is dependent on TnI and TnT for transmission of the calcium signal to the actin filament, studying the protein-protein interactions of TnC is critical to understanding the function of this molecule.

We investigated the characteristics of peptides that interact with TnC using a phage-displayed combinatorial peptide library. Affinity selection of phage-displayed peptide libraries, a technique based on screening a library of foreign peptides displayed on the surface of M13 bacteriophage (3–6), has become a useful tool in recent years for characterizing protein-protein interactions. Due to the physical linkage of the expressed peptide and its genetic sequence, libraries with diversities of 10^8 to 10^{10} peptides (7) have been rapidly screened for a wide variety of applications. To date, they have been used to map antibody epitopes and mimotopes (5, 6, 8–10), and to discover peptide ligands for membrane receptors (11) and cytosolic proteins (12–14).

In this publication, we describe the affinity selection of a phage-displayed library of random 12-mers with immobilized rabbit fast skeletal TnC, and the isolation of novel sequences which bind TnC. The sequences have a consensus motif of (V/L)(D/E)*XLKXXLXX*LA, and are similar to a sequence within the region of TnI, which is believed to play a role in the calcium-dependent interaction with TnC (15–17). We have shown that TnC-selected peptides from a phage-displayed library and similar sequences from TnI bind TnC in a specific and biologically relevant manner.

EXPERIMENTAL PROCEDURES

Random 12-mer Library Construction—The 12-mer random peptide library was constructed as described (18) to yield 10^9 different recombinant M13 phage, which were plate-amplified to generate the library.

Preparation of TnC—TnC was purified either from rabbit muscle as described (20) or following bacterial expression by Phenyl-Sepharose chromatography as described by George *et al.* (21).

Isolation of TnC-binding Phage—Affinity selection of the 12-mer library was used to isolate peptides that bound TnC as described (19). Briefly, rabbit fast skeletal TnC was immobilized on polystyrene microtiter plates, and nonspecific binding was blocked by adding bovine serum albumin (BSA). After three rounds of affinity purification, TnC binding phage were eluted with 50 μl of 50 mM glycine·HCl (pH 2.0) and neutralized with 50 μl of 200 mM sodium phosphate (pH 7.4). Recovered phage were plated, and individual plaques isolated from bacterial lawns. The DNA of isolates was prepared from overnight liquid cultures with QIAprep Spin Miniprep Kits (Chatsworth, CA), and the culture supernatants were stored at 4 °C for confirmation of TnC binding activity (22). DNA was sequenced with an oligonucleotide primer upstream of the gene III cloning site of the M13 vector m663 (23).

* This work was supported in part by National Institutes of Health Grant R01 EY11377. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed: Dept. of Cell Biology, Duke University Medical School, Durham, NC 27710.

§§ Present address: Dept. of Pharmacology, University of Wisconsin, 1300 University Ave., Madison, WI 53706-1532.

[1] The abbreviations used are: TnC, troponin C; TnI, troponin I; CaM, calmodulin; TnT, troponin T; BSA, bovine serum albumin; AP, alkaline phosphatase; PBST, 0.1% Tween 20 in phosphate-buffered saline; RU, resonance units; hcTnI, human cardiac TnI-(44–59); msTnI, mouse fast skeletal TnI-(13–28); dTnIA, normal *Drosophila* TnI; dTnIV, mutant *Drosophila* TnI; ELISA, enzyme-linked immunosorbent assay; Ip, troponin I inhibitory peptide-(104–115); Rp, troponin I regulatory peptide-(1–40); MOPS, 4-morpholinepropanesulfonic acid.

Synthetic Peptides—Peptides, corresponding to sequences from native TnI isoforms and sequences displayed by TnC-selected phage, were synthesized by the Peptide Synthesis Facility at the University of North Carolina, Chapel Hill, NC. The C termini of the peptides were amidated, and a four-residue linker (GSGS) was added at their N termini prior to biotinylation. The sequences included: pT3, biotin-GSGSSRVDELKLKLSTLVSR-NH_2; pT5, biotin-GSGSSRLDYLKSSLLHLGSR-NH_2; hcTnI, biotin-GSGSSRKLQLKTLLLQIAKQ-NH_2; msTnI, biotin-GSGSARRQHLKSVMLQIAAT-NH_2; dTnIA, biotin-GSGSERKKKLRLLRKKAAE-NH_2; dTnIV, biotin-GSGSERKKKLRLLLRKKVAE-NH_2; APC, biotin-PKRHSGSYLVTSV-NH_2; Amp1, biotin-GSGSAPTPPKRSSSF-NH_2.

Alkaline Phosphatase Fusion Recombinants and Proteins—Inserts of phage clones T3 and T5 (ϕT3 and ϕT5) were amplified by polymerase chain reaction with primers upstream and downstream of the M13 gene III sequence, double-digested with *Xho*I and *Xba*I, and gel-purified. The bacterial alkaline phosphatase (AP) fusion vector, pMY101 (24), was digested with *Xba*I and *Sal*I, gel-purified, ligated to M13 phage inserts, and the ligation products used to transform electrocompetent DH5αF′ cells. DNA was prepared as above from cultures of individual colonies, and recombinants identified by diagnostic digestion with *Sal*I and confirmed by DNA sequencing. AP fusion proteins were prepared by growing recombinants in liquid culture with ampicillin selection to an optical density (600 nm wavelength) of 0.3, addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM, followed by incubation at 37 °C for 18 h. The conditioned medium was recovered by centrifugation at 4000 × *g* for 10 min.

ELISA Binding Assays—Microtiter wells were coated with 50 ng of rabbit fast skeletal TnC or 50 ng of bovine brain calmodulin (CaM; Calbiochem-Novabiochem Corp., La Jolla, CA) in 100 mM $NaHCO_3$ (pH 8.5) overnight at 4 °C. Nonspecific binding was blocked by incubation with 1% BSA in 100 mM $NaHCO_3$ (pH 8.5) for 1 h at room temperature. The wells were washed with 0.1% Tween 20 in phosphate-buffered saline (PBST). The peptides were added to the blocked wells in a 25-μl volume (for a total reaction volume of 50 μl) and incubated at room temperature for 2 h. For monovalent binding assays, 25 μl of 20 ng/μl streptavidin-linked alkaline phosphatase (Sigma) was added to the microtiter wells following the binding reaction and incubated for an additional 30 min at room temperature, followed by multiple washes with PBST. To detect bound peptides, 50 μl of alkaline phosphatase buffer (1 M diethanolamine (pH 9.8), 1.0 mM $MgCl_2$, 40 μM $ZnSO_4$, 6.5 mM *p*-nitrophenyl phosphate) was added to each well and incubated 10 min at 37 °C to allow for full color development. The optical density of the wells was then measured at 405 nm with a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA).

Protein was adsorbed to microtiter plates as described above. For AP fusion binding assays, 50 μl of conditioned medium was added to 50 μl of 100 μM $CaCl_2$ in PBST in blocked wells and incubated at room temperature for 2 h. For competition assays, 100 μl of conditioned medium and 100 μl of competitor were added to the blocked wells and incubated at room temperature for 2 h. The wells were washed repeatedly with PBST. Bound AP fusion proteins were detected by the addition of 56 μl of alkaline phosphatase buffer, followed by measurement of optical density at 405 nm with a microplate spectrophotometer.

Surface Plasmon Resonance Measurements—Equilibrium binding affinities of TnC for both library-derived and native TnI peptide sequences were determined by measurements of surface plasmon resonance (25) on a BIAcore™ 2000 instrument (Biacore AB). The technique of surface plasmon resonance for characterization of macromolecular interactions has been discussed in several reviews (26–28). In brief, ligands are attached to a carboxymethylated dextran gold surface and analytes are injected over the peptide surface. Binding is measured as an increase in resonance units (RU). Immunopure NeutrAvidin (Pierce)-coated CM5 sensor chips (BIAcore™, Inc.) were prepared to capture the biotinylated peptides onto the surface. NeutrAvidin was dissolved at 1 mg/ml in HBS buffer (20 mM HEPES, 3.4 mM EDTA, 150 mM NaCl, 0.005% P-20, pH 7.4), and diluted 1:20 in 10 mM sodium acetate, pH 6.0. NeutrAvidin was then covalently attached to the carboxyl groups on the surface of the CM5 sensor chip activated with 200 mM 1-ethyl-3-[3-demethylaminopropyl]carbodiimide hydrochloride and 50 mM *N*-hydroxysuccinimide. Following 10 injections of 100 mM HCl (2-min contact time) over the NeutrAvidin-coated chip to achieve a stable base line, biotinylated peptides were immobilized by one or two injections of 100 nM peptide solution. After injection of 10 mM HCl to remove unbound peptide, final surface densities of between 75 and 125 RU resulted.

To measure equilibrium binding affinities, increasing concentrations of TnC or calmodulin were injected over the peptide surface. Running buffer was HBS supplemented with 100 μM $CaCl_2$ in place of EDTA. Injection volumes were 60 μl at a flow rate of 10 μl/min. The steady state binding levels, in RU, were recorded at the end of the injection. The surface was regenerated with 50 mM EDTA, pH 8.0 (2-min contact time). Binding to a control peptide, Amp1,[2] was also measured. These values were used to subtract nonspecific interactions. Background corrected RU values were plotted as a function of added TnC or calmodulin concentration, and nonlinear regression analysis (29) used to determine equilibrium binding constants fit to a single-site binding model (25, 30, 31).

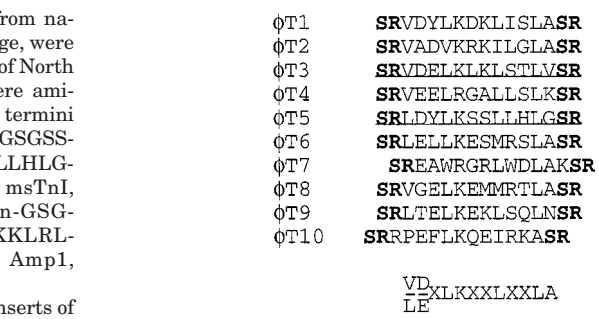

FIG. 1. **Amino acid sequence of peptides displayed by TnC-selected phage from the random 12-mer library.** Residues within the peptides that match the consensus have been aligned. *Bold* residues are fixed in all library clones. Consensus residues (≥50% frequency at a given position) appear at the *bottom* of the figure; sequences included in synthetic peptides are *underlined*.

Nondenaturing Gel Electrophoresis—Analysis of the structural interaction of pT5 with TnC was performed as detailed by Farah *et al.* (17). Purified TnC (6.7 μM) was incubated at room temperature for 30 min with pT5 (6.7, 13.4, and 33.5 μM), human cardiac TnI (6.7 and 13.4 μM), or Amp1 peptide (6.7, 13.4, and 33.5 μM). Samples were then diluted with 1.5 volumes of 41.5 mM Tris, 133 mM glycine (pH 8.6), 2 mM dithoithreitol, 0.02% bromphenol blue, 16.7% glycerol, and 10 mM $CaCl_2$. Samples were analyzed in 8% native gels containing 25 mM Tris, 80 mM glycine (pH 8.6), 0.5 mM $CaCl_2$, and 10% glycerol.

Physiological Analysis—The activation and relaxing solutions and the procedure for isolation of skinned fibers are described by Brandt *et al.* (32). Briefly, the *p*Ca 8 relaxing solution contains (as sodium salts): 9.8 mM EGTA, 0.2 mM CaEGTA, 5 mM MgATP, 5 mM ATP, 7.5 PO_4, 40.1 mM propionate, 17.2 mM SO_4, 10 mM MOPS at pH 7.00. The *p*Ca 4.75 activating solution contains: 10 mM CaEGTA, 5 mM MgATP, 5 mM free ATP, 10 mM MOPS, 7.5 mM PO_4, propionate, and SO_4 to adjust the final solution to a standard ionic strength of 0.200 and monovalent cation concentration of 155 mM. Single fibers were isolated from bundles of rabbit psoas muscle skinned at 0 °C in skinning saline (5 mM K_2H_2EGTA, 2 mM Na_2MgATP, 5 mM Na_2K_2ATP, 130 mM potassium propionate, 5 mM imidazole at pH 7.0). The maximal tension data were collected on an apparatus as described previously (32).

RESULTS

Isolation and Characterization of TnC-binding Phage—In an effort to study the peptide ligand preferences of TnC, we screened a phage display combinatorial peptide library with TnC. The library expressed random peptides 12 amino acids in length at the N terminus of bacteriophage M13 protein III. Immobilized rabbit fast skeletal TnC was used to affinity purify TnC-binding phage from the library. After three rounds of selection, isolated clones were tested for binding to TnC; approximately 90% of the isolates exhibited strong TnC binding activity in an ELISA-based assay. DNA sequences from isolated clones identified 10 unique TnC-binding peptides (Fig. 1). Alignment of these sequences yields a consensus motif of (V/L)(D/E)*X*LK*XX*L*XX*LA, where *X* represents positions that have no apparent conservation. On the basis of these alignment data, we concluded that residues at insert positions 4, 5, and 11 were the most critical for binding to troponin C, since they were the most highly conserved. In *in vitro* phage binding assays,

[2] Amp1, whose sequence is given under "Experimental Procedures," is an SH3 domain-binding sequence from c-Abl.

```
pT5   1 ................SRLDYLKSSLLHLGSR.................. 16
                          ||   ||. || :  .
cTnI 27 RAYATEPHAKKKSKISASRKLQLKTLLLQIAKQELEREAEERRGEKGRAL 76

pT5   1 ............SRLDYLKSSLLHLGSR...................... 16
                      .|  :||| :| : .
sTnI  1 MGDEEKRNRAITARRQHLKSVMLQIAATELEKEEGRREAEKQNYLAEHCP 50
```

FIG. 2. **Native TnI isoforms are similar to peptides displayed by TnC-selected phage.** Alignment of peptide sequence displayed by ϕT5 (pT5) with native TnI isoforms. Human cardiac TnI-(44–59) exhibits 37.5% identity and 56.25% similarity to pT5; mouse fast skeletal TnI-(13–28) exhibits 31.25% identity and 62.5% similarity to pT5.

phage clones T3 and T5 (ϕT3 and ϕT5) exhibited the strongest TnC binding activity. Alignments of these peptide sequences (including the fixed serine and arginine residues from the M13 pIII protein) to known TnC ligands, namely TnI, TnT, and actin, revealed 50–60% similarity to sequences in the N-terminal regions of human cardiac and mouse skeletal TnI (Fig. 2).

Previous studies have shown that in addition to the well characterized interaction of the C-terminal TnI-inhibitory peptide with TnC, there are also important interactions between the N-terminal region of TnI with TnC. Based on the similarity between TnC-selected peptides and sequences in the N-terminal region of TnI, we hypothesized that the corresponding native sequences were sufficient for interaction with TnC. This reasoning was supported by studies which have shown that peptides from this region of TnI can bind TnC and can compete the release of acto-S1-Tm ATPase inhibition by TnC (15, 16). It is also notable that one of the best characterized non-lethal indirect flight mutations in *Drosophila melanogaster*, *heldup*,[2] is due to a single alanine to valine mutation at codon 55 in the homologous N-terminal region of *Drosophila* TnI (33, 34).

In Vitro Peptide Binding Assays—To facilitate characterization of the displayed sequences, biotinylated peptides were synthesized corresponding to the sequences displayed by TnC-selected phage clones (Fig. 1), and are hereafter denoted as pT3 and pT5. Due to their similarity to displayed peptides, sequences from the N-terminal region of native TnI isoforms were also synthesized as biotinylated peptides. The native peptides included human cardiac TnI-(44–59) (hcTnI), mouse fast skeletal TnI-(13–28) (msTnI) and both normal (dTnIA) and mutant (dTnIV) *Drosophila* flight muscle TnI-(42–57) (Fig. 2*B*). To evaluate relative binding strength and specificity, the peptides were assayed for direct binding to immobilized BSA, rabbit fast skeletal TnC, and CaM (Fig. 3). Both of the library-derived peptides bound TnC above background and exhibited very little binding to CaM, a TnC-related protein. All four TnI-derived sequences bound TnC, with hcTnI, dTnIA, and dTnIV exhibiting the most robust binding signals. Binding to CaM was pronounced for hcTnI, dTnIA, and dTnIV, but was very low for msTnI. These data show that TnC-selected peptides from a phage library can bind TnC specifically, and that similar sequences from the N-terminal region of TnI isoforms can also bind TnC.

AP Fusion Binding and Competition Assays—To confirm binding of the library-derived sequences, and to determine whether the biotinylated peptides could compete binding to TnC, insert sequences from ϕT3 and ϕT5 were expressed as bacterial alkaline phosphatase fusions using the vector pMY101 (24). Assays of direct binding to TnC showed that both fusion proteins bound TnC specifically (Fig. 4*A*), confirming that the library-derived sequences were *bona fide* binders. The fusion with highest binding activity, pT5AP, was used to assay the ability of the biotinylated peptides to compete TnC binding activity (Fig. 4*B*). The pT5AP fusion was most easily competed by pT5, with an approximate IC_{50} of 300 nM, followed by hcTnI, pT3, and msTnI. These results agreed with the relative binding strengths determined by peptide ELISA (Fig. 3). The competition data provide evidence that the peptides are binding at, or near, the same site on TnC.

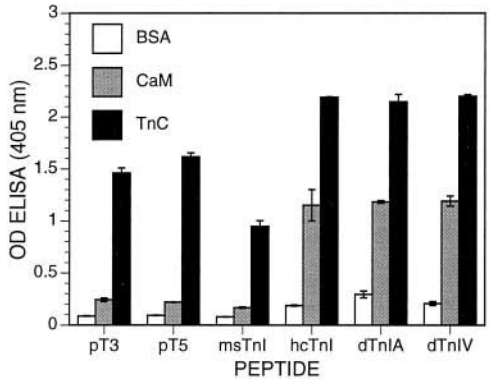

FIG. 3. **Relative binding strength and specificity of synthetic TnC-binding peptides.** Binding to 50 ng of immobilized TnC or CaM, or to BSA, was measured by ELISA. Values represent the average OD at 405 nm for triplicate binding reactions done in the presence of 5 μM peptide and 100 μM calcium.

BIAcore Measurement of Equilibrium Dissociation Constants—Surface plasmon resonance was used to determine equilibrium dissociation constants for native and library-derived peptides. After biotinylated peptides were immobilized on an avidin-coupled sensor chip, increasing concentrations of TnC and CaM were injected over the immobilized peptides in the presence of calcium, and steady state binding was measured in RU. Regeneration of the peptide-coated sensor chips with EDTA resulted in a complete loss of TnC binding, which indicates that the association requires the presence of calcium. Background-corrected RU values were plotted as a function of increasing TnC or CaM (Fig. 5), and equilibrium dissociation constants for each peptide (Table I) were determined from the response data; pT5 had the lowest K_d at 0.43 μM, followed by hcTnI at 0.86 μM, and pT3 at 6.1 μM. The K_d of msTnI could not be measured accurately, presumably because saturating conditions could not be achieved. These dissociation constants correspond roughly to the relative and approximate IC_{50}s measured by AP fusion competition assay (Fig. 4). Only the hcTnI peptide exhibited a detectable response to increasing CaM; however, its K_d was 6 μM. These data confirm that in the presence of calcium the library-derived peptides bind TnC specifically and can exhibit dissociation constants higher than those of the corresponding TnC sequences.

Assay of Interaction by Polyacrylamide Gel Electrophoresis—Head and Perry (35) initiated the use of polyacrylamide gel electrophoresis to demonstrate stable interactions between TnI and TnC, and others have used similar techniques to analyze the interaction of TnI peptides with TnC (15–17). The interaction of pT5 with TnC was conducted using the modification of Farah *et al.* (17). Purified TnC was mixed with pT5, and the products of the binding reactions were resolved by nondenaturing gel electrophoresis (Fig. 6). In the presence of calcium, pT5 associates with TnC in a stable manner, as indicated by the presence of a lower mobility band and a reduced concentration of free TnC. Full-length TnI also associates with TnC in a

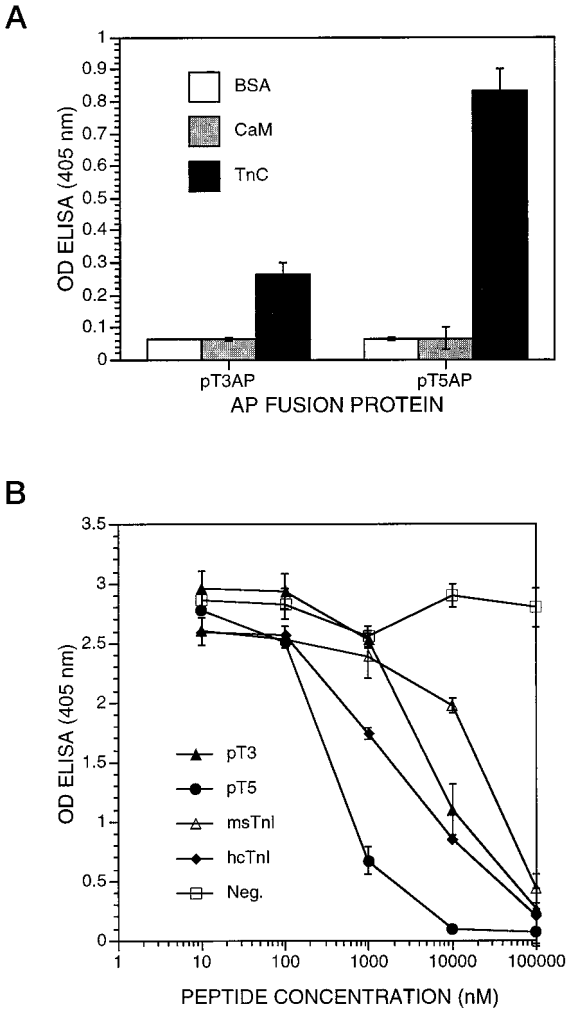

FIG. 4. **Alkaline phosphatase fusion binding and competition assays.** *A*, fusions of insert sequences from ϕT3 and ϕT5 to bacterial alkaline phosphatase (pT3AP, pT5AP) bind TnC specifically by ELISA assay. Fifty nanograms of TnC or CaM were immobilized in microtiter wells. Values represent average OD at 405 nm for duplicate binding reactions done in the presence of 50 μM $CaCl_2$ and 50 μl of conditioned medium. *B*, specific inhibition of AP fusion peptide interaction with TnC by soluble peptide ligands. Fifty nanograms of TnC were immobilized in microtiter wells. Increasing concentrations of competitor peptide were incubated with pT5AP. Values represent average OD at 405 nm for duplicate binding reactions done in the presence of 100 μl of competitor and 100 μl of conditioned medium containing pT5AP. The negative control peptide (*Neg.*) is Amp1, an SH3 domain-binding peptide.

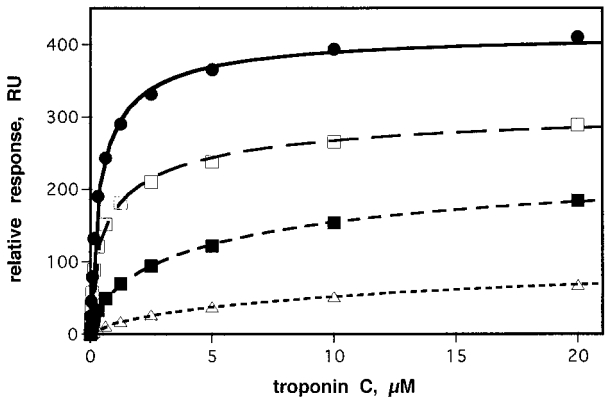

FIG. 5. **Measurement of TnC binding by surface plasmon resonance.** Biotinylated peptides were immobilized on a NeutrAvidin-coated sensor chip, and increasing concentrations of TnC were injected over the immobilized peptides. Steady state binding was measured in RU at the end of each injection. The graph shows background-corrected RU values plotted as a function of increasing TnC concentration: ●, pT5; □, hcTnI; ■, pT3; △, msTnI.

TABLE I

Equilibrium binding constants of troponin C-binding peptides

Equilibrium binding constants for troponin C-binding peptides in the presence of calcium were determined by measurements of surface plasmon resonance. Steady state binding levels (in resonance units) were plotted as a function of added TnC, and data was analyzed by nonlinear regression analysis methods to determine equilibrium binding constants by fitting to a single-site binding model.

Peptide	Sequence	Binding constant
		μM
pT5	SRLDYLKSSLLHLGSR	0.43
hcTnI	SRKLQLKTLLLQIAKQ	0.86
pT3	SRVDELKLKLSTLVSR	6.1
msTnI	ARRQHLKSVMLQIAAT	50[a]

[a] This value is approximate, as the binding constant is probably near or above the limits of the BIAcore™ 2000 (see BIAcore 2000 Instrument Handbook, p. A1, BIAcore AB).

stable manner, as indicated by the presence of bands with reduced mobility and a reduction in the concentration of free TnC. The negative control peptide, Amp1, does not associate with TnC, as indicated by the absence of bands with reduced mobility and the presence of large amounts of free TnC. Similar results were observed in experiments performed with radioactively labeled TnC, generated by transcription-coupled translation in rabbit reticulocyte lysate (results not shown). These results taken together demonstrate that the TnC-selected pT5 forms a stable complex with TnC in the presence of calcium.

Physiological Effects of Peptides—The physiological consequences of naturally occurring TnC-binding peptides have been studied by determining their effects on maximal calcium-induced tension in skinned muscle fibers. The ability of pT5 to disrupt activation of the troponin complex was assessed by measuring its effect on maximal calcium-activated tension in skinned rabbit psoas fibers. Addition of the pT5 peptide to the *p*Ca 4.75 activating solution inhibited maximal calcium tension. As shown in the tension trace in Fig. 7, the maximal tension is reduced by 11, 38, and 66% at pT5 concentrations of 0.2, 0.5, and 1 mM, respectively. Studies on six fibers showed that a 1 mM solution of pT5 reduces maximal tension by 59.5 ± 3.4% (average ± S.E.). Under the same conditions, the negative control peptide APC has no effect on maximal calcium-activated tension. The effect of pT5 is fully reversible, as indicated by the recovery of maximal activated calcium tension following removal of the pT5 solution, washing of the fiber in *p*Ca 8 relaxing solution, and reactivation. These results show that pT5 binds TnC in a biologically relevant manner by demonstrating that it is capable of disrupting the activation function of the troponin complex.

DISCUSSION

In this study, we describe the affinity selection of phage-displayed random peptide library with TnC, and the isolation of 10 distinct sequences that bind TnC with a consensus of (V/L)(D/E)*X*LK*XX*L*XX*LA. Comparison of these sequences with other TnC-binding proteins revealed a striking similarity to a sequence within the N-terminal region of TnI. We have demonstrated that TnC-selected peptides from the phage-displayed library and similar sequences from TnI bind TnC specifically, with binding constants as low as 0.43 μM. In addition, we have shown that a TnC-selected peptide forms a stable complex with TnC in the presence of Ca^{2+}. When further ELISA studies were performed to determine whether the binding of TnC-selected peptides to rabbit fast skeletal TnC was calcium-dependent (data not shown), we found that the binding of library-selected

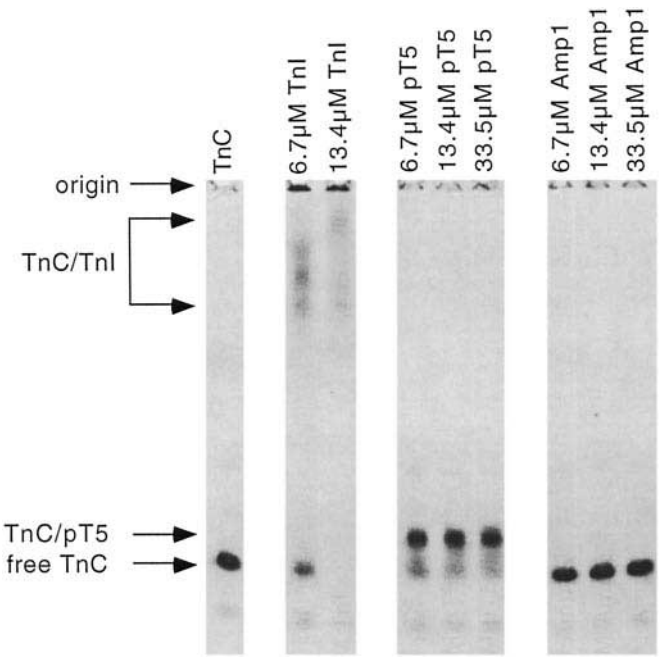

FIG. 6. **Analysis of interaction of pT5 with purified TnC by polyacrylamide gel electrophoresis.** Purified TnC (6.7 μM) was incubated at room temperature for 30 min with pT5 (6.7, 13.4, and 33.5 μM), human cardiac TnI (6.7 and 13.4 μM), or Amp1 peptide (6.7, 13.4, and 33.5 μM). Samples were then diluted with 1.5 volumes of 41.5 mM Tris, 133 mM glycine (pH 8.6), 2 mM dithoithreitol, 0.02% bromphenol blue, 16.7% glycerol, and 10 mM $CaCl_2$. The entire sample volumes were analyzed in an 8% native gels containing 25 mM Tris, 80 mM glycine (pH 8.6), 0.5 mM $CaCl_2$, and 10% glycerol. Bands representing free TnC and TnC complexes are indicated by the *arrows*.

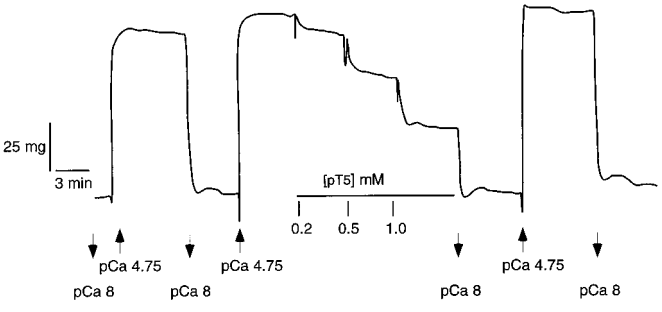

FIG. 7. **The effects of pT5 on maximal calcium-activated tension.** The effect of pT5 on the maximal calcium-activated tension generated by a skinned rabbit psoas fiber is presented in the tracing. A 60-μm diameter fiber was mounted and adjusted to a sarcomere length of 2.5 μm. In activating solution, *p*Ca 4.75, the fiber generates 103 mg of tension, and that tension is continuously reduced on exposure to increasing concentrations of pT5. The effect of pT5 is fully reversible, as indicated by the recovery of maximal tension following removal of the pT5 solution and washing of the fiber in *p*Ca 8 relaxing solution.

peptides and the mouse skeletal TnI peptide (msTnI) appeared to be calcium-dependent, while the binding of peptides from human cardiac TnI (hcTnI) and *Drosophila* TnI (dTnIA, dTnIV) appeared to be calcium-independent.

We also demonstrated that a TnC-selected peptide binds TnC in a biologically relevant manner, as demonstrated by its inhibitory effect on calcium-induced tension generation in skinned muscle fibers. Addition of a 1 mM solution of pT5 resulted in a reduction in maximal tension of approximately 60%. Although the concentration of pT5 necessary to cause the reduction is much higher than its dissociation constant for TnC, it is important to realize that there are many stabilizing interactions among the subunits of the troponin complex. This peptide is homologous to only a portion of the regulatory peptide (Rp) of TnI, and x-ray crystallographic studies on the TnC-regulatory protein complex indicate will that pT5 will only disrupt interactions between TnI and the structural C-terminal domain of TnC (36). This most likely explains why a small peptide, like pT5, requires a high concentration to impair the troponin activation *in situ*. Taken together, these results demonstrate the power of using phage-displayed combinatorial libraries to map native protein-protein interactions (3, 7). The results also implicate that this short N-terminal sequence within TnI plays an important role in the interaction of TnC and TnI *in vivo*, which is supported by the results of previous studies on the interaction of TnC and TnI.

The majority of previously published studies on the interaction of TnC and TnI have concentrated on the TnI inhibitory peptide (Ip), which consists of the C-terminal residues 104–115 of TnI. Several different groups have demonstrated that proteolytic fragments and synthetic peptides of the inhibitory region of TnI mimic the activity of TnI in their ability to inhibit the actomyosin ATPase activity and bind TnC resulting in the release of inhibition (16, 37–43). In recent years, however, a number of studies have also been done on the function of the N-terminal region of TnI. Farah *et al.* (17) and Ngai and Hodges (15) have demonstrated that addition of peptides from the N-terminal region of TnI (TnI-(1–40), TnI-(10–40), and TnI-(20–40)) can prevent TnC from fully releasing the inhibition of actomyosin ATPase induced by TnI or Ip, although the N-terminal peptides do not directly inhibit actomyosin ATPase activity. In addition, both groups have demonstrated that peptides from the N-terminal region of TnI, namely the Rp (residues 1–40) and TnI-(1–98), can form stable complexes with TnC in the presence of calcium.[3] The results of these studies have led to the hypothesis that the N-terminal region of TnI is responsible for the structural interaction with TnC.

Furthermore, Katayama and Nozaki (16) have demonstrated that a short synthetic peptide, consisting of residues 10–25 of the N-terminal region of rabbit fast skeletal TnI, could form a stable complex with TnC in the presence of Ca^{2+}. Taken along with our findings that a synthetic peptide consisting of residues 13–28 of mouse fast skeletal TnI binds TnC, and that a similar TnC-selected peptide can form a stable complex with TnC in the presence of Ca^{2+}, it is reasonable to suspect that the minimal sequence necessary for the interaction of the N-terminal region of TnI and TnC is bounded by these two native peptide sequences. In addition, we believe that we may have identified residues within the N-terminal region of TnI critical for binding to TnC, as they were highly conserved in the library-derived peptides. These results can be extended in the future by mutagenesis of these conserved residues within TnI, as well as performing a systematic scan of peptides spanning this region of TnI, and testing their binding to TnC. The peptides produced in this study will also serve as useful reagents to study the interaction of the N terminus of TnI with truncated forms of TnC to determine where the peptides are binding to TnC.

REFERENCES

1. Means, A. R. (1994) *FEBS Lett.* **347**, 1–4
2. Farah, C. S., and Reinach, F. C. (1995) *FASEB J.* **9,** 755–767
3. Cortese, R., Monaci, P., Nicosia, A., Luzzago, A., Felici, F., Galfre, G., Pessi, A., Tramontano, A., and Sollazzo, M. (1995) *Curr. Opin. Biotechnol.* **6**, 73–80
4. O'Neil, K. T., and Hoess, R. H. (1995) *Curr. Opin. Struct. Biol.* **5**, 443–449
5. Kay, B. K., Adey, N. B., He, Y. S., Manfredi, J. P., Mataragnon, A. H., and Fowlkes, D. M. (1993) *Gene* (*Amst.*) **128,** 59–65
6. Scott, J. K., and Smith, G. P. (1990) *Science* **249,** 386–390
7. Kay, B. K. (1995) *Perspect. Drug Discovery Design* **2,** 251–268

[3] Ngai and Hodges (15) claim that interaction of Rp and TnC is Ca^{2+}-dependent, based on the evidence that they do not form a complex in the absence of Ca^{2+}, but Farah *et al.* (17) have found that TnI-(1–98) and TnC can form a complex in the presence of Ca^{2+} or Mg^{2+}.

8. Cwirla, S. E., Peters, E. A., Barrett, R. W., and Dower, W. J. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 6378–6382
9. Felici, F., Luzzago, A., Folgori, A., and Cortese, R. (1993) *Gene* (*Amst.*) **128,** 21–27
10. Motti, C., Nuzzo, M., Meola, A., Galfre, G., Felici, F., Cortese, R., Nicosia, A., and Monaci, P. (1994) *Gene* (*Amst.*) **146,** 191–198
11. Koivunen, E., Wang, B., and Ruoslahti, E. (1994) *J. Cell Biol.* **124,** 373–380
12. Pierce, H. H., Adey, N., and Kay, B. K. (1995) *Mol. Divers.* **1,** 259–265
13. Dedman, J. R., Kaetzel, M. A., Chan, H. C., Nelson, D. J., and Jamieson, G. A., Jr. (1993) *J. Biol. Chem.* **268,** 23025–23030
14. Sparks, A. B., Quilliam, L. A., Thorn, J. M., Der, C. J., and Kay, B. K. (1994) *J. Biol. Chem.* **269,** 23853–23856
15. Ngai, S., and Hodges, R. S. (1992) *J. Biol. Chem.* **267,** 15715–15720
16. Katayama, E., and Nozaki, S. (1982) *J. Biochem.* (*Toyko*) **91,** 1449–1452
17. Farah, C. S., Miyamoto, C. A., Ramos, C. H. I., da Silva, A. C., Quaggio, R. B., Fujimori, K., Smillie, L. B., and Reinach, F. C. (1994) *J. Biol. Chem.* **269,** 5230–5240
18. Adey, N. B., Sparks, A. B., Beasley, J., and Kay, B. K. (1996) in *Phage Display of Peptides and Protein Domains: A Laboratory Manual* (Kay, B. K., Winter, J., and McCafferty, J., eds) pp. 68–78, Academic Press, San Diego
19. Adey, N. B., Guo, R., Hanson, H. L., Rider, J. E., Sparks, A. B., and Kay, B. K. (1996) *Methods Mol. Cell. Biol.* **6,** 34–45
20. Brandt, P. W., Diamond, M. S., and Schachat, F. H. (1984) *J. Mol. Biol.* **180,** 379–384
21. George, S. E., VanBerkum, M. F., Ono, T., Cook, R., Hanley, R. M., Putkey, J. A., and Means, A. R. (1990) *J. Biol. Chem.* **265,** 9228–9235
22. Sparks, A. B., Adey, N. B., Quilliam, L. A., Thorn, J. M., and Kay, B. K. (1995) *Methods Enzymol.* **255,** 498–509
23. Fowlkes, D. M., Adams, M. D., Fowler, V. A., and Kay, B. K. (1992) *BioTechniques* **13,** 422–428
24. Yamabhai, M., and Kay, B. K. (1997) *Anal. Biochem.* **247,** 143–151
25. Morelock, M. M., Ingraham, R. H., Betageri, R., and Jakes, S. (1995) *J. Med. Chem.* **38,** 1309–1318
26. Myszka, D. (1997) *Curr. Opin. Biotechnol.* **8,** 50–57
27. O'Shannessy, D. (1994) *Curr. Opin. Biotechnol.* **5,** 65–71
28. Fisher, R., and Fivash, M. (1994) *Curr. Opin. Biotechnol.* **5,** 389–395
29. Bevington, P. R., and Robinson, D. K. (1992) *Data Reduction and Error Analysis for the Physical Sciences*, McGraw Hill, New York
30. Myszka, D., Arulananthm, P., Sana, T., Wu, Z., Morton, T., and Ciardelli, T. (1996) *Protein Sci.* **5,** 2468–2478
31. Kalinin, N., Ward, L., and Winzor, D. (1995) *Anal. Biochem.* **228,** 238–244
32. Brandt, P. W., Cox, R. N., and Kawai, M. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77,** 4717–4720
33. Beall, C. J., and Fyrberg, E. (1991) *J. Cell Biol.* **114,** 941–951
34. Prado, A., Canal, I., Barbas, J. A., Molloy, J., and Ferrus, A. (1995) *Mol. Biol. Cell* **6,** 1433–1441
35. Head, J. F., and Perry, S. V. (1974) *Biochem. J.* **137,** 145–154
36. Vassylyev, D., Takeda, S., Wakatsuki, S., Maeda, K., Maeda, Y. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 4847–4852
37. Talbot, J. A., and Hodges, R. S. (1979) *J. Biol. Chem.* **254,** 3720–3723
38. Talbot, J. A., and Hodges, R. S. (1981) *J. Biol. Chem.* **256,** 12374–12378
39. Talbot, J. A., and Hodges, R. S. (1981) *J. Biol. Chem.* **256,** 2798–2802
40. Cachia, P. J., Sykes, B. D., and Hodges, R. S. (1983) *Biochemistry* **22,** 4145–4152
41. Cachia, P. J., Van Eyk, J., Ingraham, R. H., McCubbin, W. D., Kay, C. M., and Hodges, R. S. (1986) *Biochemistry* **25,** 3553–3562
42. Van Eyk, J. E., and Hodges, R. S. (1988) *J. Biol. Chem.* **263,** 1726–1732
43. Van Eyk, J. E., and Hodges, R. S. (1987) *Biochem. Cell Biol.* **65,** 982–988