Systematic mapping of regions of human cardiac troponin I involved in binding to cardiac troponin C: N- and C-terminal low affinity contributing regions

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Abstract The Spot method of multiple peptide synthesis was used to map in a systematic manner regions of the human cardiac troponin I sequence (hcTnI) involved in interactions with its physiological partner, troponin C (cTnC). Ninety-six 20-mer peptides describing the entire hcTnI sequence were chemically assembled; their reactivity with [^{125}I]cTnC, in the presence of 3 mM Ca^{2+}, enabled the assignment of six sites of interaction (residues 19–32, 45–54, 129–138, 145–164, 161–178 and 191–210). For several sites, a good correlation with literature data was obtained, thus validating this methodological approach. Synthetic peptides, each containing in their sequence an interaction site, were prepared. As assessed by BIACORE, all of them exhibited an affinity for cTnC in the range of 10^{-6}–10^{-7} M, except for hcTnI [39–58] which showed a nanomolar affinity. This peptide was also able to block the interaction between hcTnI and cTnC. We therefore postulate that despite the existence of multiple cTnC interaction sites on the hcTnI molecule, only that region of hcTnI allows a stabilization of the complex. Residues 19–32 from the N-terminal cardio-specific extension of hcTnI were also found to be involved in interaction with cTnC; residues 19–32 may correspond to the minimal sequence of the extension which could switch between the N- and C-terminal TnC domains, depending on its phosphorylation state. Finally, two Ca^{2+}-dependent cTnC binding domains within the C-terminal part of hcTnI (residues 164–178 and 191–210) were also mapped. The latter site may be linked with the cardiac dysfunction observed in stunned myocardium.

Key words: Troponin; Interaction; Synthetic peptide; Affinity; Spot method

1. Introduction

Vertebrate skeletal and cardiac muscle contraction are regulated in a Ca^{2+}-dependent manner by the troponin complex through interactions with tropomyosin and the actin filament. The troponin complex consists of three proteins: troponin I (TnI) which inhibits the actomyosin Mg^{2+}-ATPase, troponin C (TnC) which binds Ca^{2+} ions and removes TnI inhibition and troponin T (TnT) which makes primary protein–protein contacts with tropomyosin. Ca^{2+} binding to TnC initiates structural changes and alters protein–protein interactions, leading to muscle contraction. Two isoforms of TnC exist in striated muscle, fast skeletal TnC (sTnC) and slow skeletal, which is identical to cardiac TnC (cTnC). The crystal structure of TnC reveals a dumbbell shaped molecule with its N- and C-terminal domains well separated by a single central α helix [1,2] and four Ca^{2+} binding sites, two in each domain. In cTnC, the Ca^{2+} binding site I, located in the NH_2-regulatory domain, is naturally inactive. The cardiac TnI isoform has a unique N-terminal extension of 32 residues which could be phosphorylated mainly at serine residues 23 and 24 upon β-adrenergic stimulation [3,4]. Compared to the skeletal isoform, much less is known about the regions of cardiac TnI involved in the Ca^{2+}-dependent regulation. An antiparallel arrangement between cTnC and cTnI has been proposed [5]. Consistent with this model, the N-terminal domain of cTnI (residues 33–80) forms a stable binary complex with cTnC-(81–161) [6], and cTnI-(147–163) has been shown to interact with the NH_2-domain of cTnC, stabilizing its open conformation [7]. An inhibitory region (residues 128–148 in human cTnI) has been identified [8] and recent results from Rarick [9] indicate that residues 152–199 of cTnI are essential for full inhibitory activity and Ca^{2+} sensitivity of myofibrillar ATPase activity in the heart.

In order to get a comprehensive view of the regions of hcTnI involved in binding, we report here on the systematic mapping of human cardiac TnI (hcTnI) regions that interact with cTnC. Ninety-six 20-mer peptides describing the entire hcTnI sequence were assembled by the Spot method [10]. Their reactivity with [^{125}I]cTnC was assessed, leading to the localization of six interaction sites. Soluble peptides, containing in their sequence an interaction motif, were synthesized and their affinity for cTnC was measured by surface plasmon resonance analysis.

2. Materials and methods

2.1. Preparation of bovine cTnC

Bovine cTnC whose sequence is identical to the human protein was used. TnC was obtained from deep-frozen bovine cardiac muscle and purified as described by Thulin and Vogel [11]. The semi-purified cTnC obtained was dialyzed against 10 mM Tris pH 7.5, 200 mM NaCl, 3 mM $CaCl_2$, 5 mM DTT buffer and purified by gel filtration

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Abbreviations: hcTnI, human cardiac troponin I; cTnC, cardiac troponin C; Ip, inhibitory peptide; RU, resonance unit; NHS, *N*-hydroxysuccinimide; EDC, *N*-ethyl-*N*′-(dimethylaminopropyl)-carbodiimide

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chromatography on Sephacryl HR100 (Pharmacia), previously equilibrated with the same buffer. TnC was eluted as a single peak at 105 ml and stored until use at −80°C. The purity of the protein was monitored by sodium dodecyl sulfate polyacrylamide (12%) gel electrophoresis. Protein concentration was determined by the Bradford method (Bio-Rad, Munich, Germany).

2.2. Iodination of cTnC

Purified cTnC was radioiodinated by the Bolton and Hunter method [12], with 100 μCi of *N*-succinimidyl 3-(4-hydroxy,5-[^{125}I]iodophenyl)propionate (Amersham) per 100 μg of cTnC. The iodinated mixture was separated by gel filtration chromatography on a PD10 column (Pharmacia), using 10 mM Tris pH 7.5, 200 mM NaCl, 3 mM $CaCl_2$, 5 mM DTT as eluent. The specific activity of the purified labelled cTnC was about 5×10^5 cpm/μg. A control, irrelevant, protein (an anti-carcinoembryonic antigen IgG) was labelled in similar conditions.

2.3. Biotinylation of cTnC

Purified cTnC was biotinylated according to the ECL protein biotinylation module kit protocol (Amersham) with 40 μl of biotinylation reagent per mg of protein. The biotinylated mixture was separated on a PD10 column as described above. Biotinylated cTnC was stored at −20°C.

2.4. Peptide synthesis on cellulose membrane

Ninety-six overlapping 20-mer peptides frameshifted by two residues, representing the complete hcTnI protein sequence [13], were synthesized on a cellulose membrane (Abimed GmbH, Langenfield, Germany) by the Spot technique [10]. The synthesis was performed by using an ASP 412 spotter (Abimed GmbH), as previously described [14].

2.5. Reactivity of cellulose-bound peptides

The set of membrane bound peptides was incubated for 90 min at 37°C with either [^{125}I]TnC (55.5 nM; 5.3×10^5 cpm/ml) or an equivalent amount of [^{125}I]IgG, as a control. The binding of radiolabelled proteins to cellulose-bound peptides was detected by autoradiography of the membrane. The membrane was further treated so as to remove bound protein as described [10] and reused when necessary.

2.6. Synthesis of soluble hcTnI peptides

Peptides were prepared by Fmoc solid-phase synthesis on a AMS 422 robot (Abimed Gmbh) [15] as described [14]. Phosphorylated serine ($S^{(p)}$) was from Novabiochem as well as other Fmoc amino acids. Synthetic peptides corresponding to regions of interaction with cTnC were as follows:hcTnI [15–34] ($_{15}$APAPIRRRSSNYRAYATEPH$_{34}$), hcTnI [15–34 diP] ($_{15}$APAPIRRRS$^{(p)}$S$^{(p)}$NYRAYATEPH$_{34}$), hcTnI [39–58] ($_{39}$SKISASRKLQLKTLLLQIAK$_{58}$), hcTnI [124–143] ($_{124}$EIADLTQKIFDLRGKFKRPT$_{143}$), hcTnI [145–164] ($_{145}$RRVRISADAMMQALLGARAK$_{164}$), hcTnI [159–178] ($_{159}$LGARAKESLDLRAHLKQVKK$_{178}$) and hcTnI [191–210] ($_{191}$WRKNIDALSGMEGRKKKFES$_{210}$). The following control peptides (corresponding to non-binding regions of hcTnI) were also synthesized: hcTnI [73–92] ($_{73}$GRALSTRCQPLELTGLGFAE$_{92}$), hcTnI [89–108] ($_{89}$GFFAELQDLCRQLHARVDKV$_{108}$), and hcTnI [109–128] ($_{109}$EERYDIEAKVTKNITEIADL$_{128}$).

2.7. Real-time interaction analysis by BIACORE

The binding of synthetic hcTnI peptides to immobilized cTnC was measured by using BIACORE 2000 (Biacore AB, Uppsala, Sweden). CTnC (50 μg/ml in 10 mM acetate buffer) was coupled to a flowcell of a CM5 sensor chip (Biacore AB) using the NHS-EDC protocol. The amount of bound cTnC was 569 pg/mm^2. The second flowcell was treated with buffer and activating reagents alone and used as a control. Increasing concentrations of synthetic hcTnI peptides (0.5–80 μg/ml in 10 mM HEPES buffer, 3 mM Ca^{2+} or 10 mM EDTA) were injected onto the two flowcells and the sensorgrams corresponding to the binding of peptides to cTnC and to the control were simultaneously registered: association time 120 s, dissociation time 300 s, flow rate 50 μl/min. After subtraction of the background signal recorded for the control flowcell, the binding level, resonance unit (RU) of each peptide on cTnC was measured 6 s after the end of injection (126 s total time). The K_D constants were obtained from these sensorgrams, using BIAevaluation 3.0 software (Biacore AB) and the global–global or local–global method (simultaneous analysis of the sensorgrams corresponding to respectively all or one peptide concentration). As a control experiment, soybean trypsin inhibitor (a protein unrelated to cTnC, but having a similar molecular weight and isoelectric point) was coupled to a CM5 sensor chip and the binding of the peptide having shown the best binding properties to cTnC was assessed.

2.8. Competitive enzyme-linked immunosorbent assay (ELISA) with hcTnI peptides

Biotinylated cTnC (2.7 nM) was preincubated with synthetic peptides derived from the hcTnI sequence at increasing concentrations (2.7 nM–2.7 μM; molar excess 1–10^3) for 2.5 h at 4°C in TBS (50 mM Tris, 150 mM NaCl, pH 7.5), 0.1% Tween 20, 1% BSA (blocking solution) and 3 mM $CaCl_2$. ELISA plates were coated with 100 μl of 41.6 nM hcTnI (prepared as described [16]) overnight at 4°C in PBS. Plates were washed with PBS, 0.1% Tween and blocked for 90 min at 37°C with blocking solution. The blocking solution was removed and 100 μl of the mixture of cTnC with the synthetic peptides was added to the wells. After incubation for 90 min at 37°C and washing, binding of cTnC was detected by peroxidase-conjugated streptavidin (Amersham) (diluted 1:3000 in blocking solution). After 60 min at 37°C, and washing, color was developed by addition of 100 μl per well of *o*-phenylenediamine (4 mg/ml) containing 0.03% (v/v) hydrogen peroxide in 0.1 M citrate buffer pH 5. The resulting absorbance was measured at 450 nm with an automated microtiter plate reader (Dynatech MR 5000).

3. Results

3.1. Determination of the cardiac TnI regions involved in the interaction with TnC

The capacity of peptides derived from the sequence of hcTnI to bind cTnC was investigated in a systematic manner. The entire 210 amino acid sequence of hcTnI was presented as a set of 96 overlapping 20-mer peptides (2-residues frameshift) synthesized on a cellulose membrane according to the Spot method [10,17]. The reactivity of the hcTnI peptides was probed with [^{125}I]cTnC (55.5 nM; 5.3×10^5 cpm/ml) in the presence of 3 mM $CaCl_2$. Fig. 1A shows the reactivity profile obtained after a 3 h radiographic exposure. As expected from the literature data, cTnC reacted with several hcTnI peptides. These interactions were abolished by an 150-fold molar excess of soluble hcTnI (Fig. 1B) and no binding with any peptide was observed with [^{125}I]IgG, used as an irrelevant protein, tested under the same conditions (Fig. 1D). From Fig. 1A, it appears that six groups of peptides were reactive. The first one included spot peptides 2–11, the second spot peptides 16–23, the third spot peptides 60–68, the fourth spot peptides 69–74, the fifth spot peptides 77–81 and the sixth spot peptides 94–96 (Fig. 1A). Table 1 lists the sequences and the reactivity index of [^{125}I]cTnC reactive peptides. The six interaction sites were tentatively assigned as follows. Site 1 (hcTnI residues 19–32) corresponds to the sequence common to the four reactive peptides from the first group (spot peptides 7–10); site 1 belongs to the N-terminal cardio-specific extension of hcTnI [13]. Site 2 (hcTnI residues 45–54), was the sequence common to spot peptides 18–23. Site 3 (hcTnI residues 129–138), was the sequence common to spot peptides 60–65. Site 4 (hcTnI residues 145–164) corresponds to a single fairly reactive spot peptide (number 73). Addition or elimination of residues from the sequence of this peptide induced an almost complete loss of reactivity, indicating that all amino acids of peptide 73 were critical for the interaction. Site 5 (hcTnI residues 161–178) was established on the basis of the observation that the last two peptides of the fifth set were the most reactive, suggesting that the presence of residues A_{161}, R_{162}, K_{177} and K_{178} was

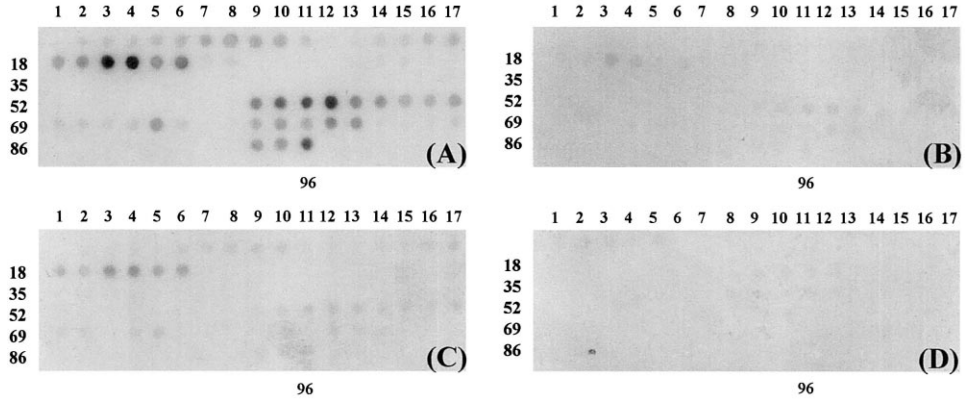

Fig. 1. Reactivity profile of hcTnI peptides assembled by the Spot method with cTnC. The 96 overlapping 20-mer peptides frameshifted by two residues were synthesized by the Spot method. HcTnI peptide reactivity with (A) [^{125}I]cTnC (55.5 nM, 5.3×10^5 cpm/ml) in the presence of 3 mM $CaCl_2$, (B) [^{125}I]cTnC (55.5 nM, 5.3×10^5 cpm/ml) and hcTnI (8.3 μM), (C) [^{125}I]cTnC in the presence of 10 mM EDTA, (D) irrelevant radiolabelled protein ([^{125}I]IgG, 5×10^5 cpm/ml) in the presence of 3 mM $CaCl_2$.

important for binding. Spot peptide 80, in the sequence of which the four residues were present, was more reactive than the spot peptide 79 which did not contain K_{177} and K_{178}. Similarly, spot peptide 82 containing the two K but not the A_{161} and R_{162} showed decreased reactivity in comparison with the spot peptide 81 (Fig. 1A). Site 6 (hcTnI residues 191–210) corresponds to the sequence common to spot peptides 94–96 extended by four residues, because the strongest reactivity of the spot peptide 96 suggested the critical contribution of residues E_{209} and S_{210} for the binding. The Ca^{2+}-dependence of the interaction between TnI and TnC is largely documented in the literature (affinity of 10^6 M^{-1} in the absence of Ca^{2+} and 10^9 M^{-1} in the presence of Ca^{2+} [18,19]). The availability of the set of overlapping peptides from the hcTnI sequence allowed the study of the Ca^{2+}-dependence of cTnC binding to the different hcTnI regions. Removal of Ca^{2+} from the binding buffer resulted in a marked decrease in the binding signal for many peptides, except for spot peptides 20–23 (Fig. 1C). These peptides enclosed site 2, which might correspond to the domain allowing the strong anchorage of TnI to TnC, as reported by Farah et al. [20]. The reactivity of the other five motifs with [^{125} I]cTnC was almost abolished in the presence of EDTA.

3.2. *Affinity determination of the interaction between hcTnI peptides and cTnC by BIACORE analysis*

Based on the sequence of the peptides used in the Spot assay, a series of peptides (as described in Section 2) corresponding to the highly reactive sequences of hcTnI and containing each an interaction site was synthesized by conven-

Table 1
Identification of hcTnI regions involved in cTnC interaction

Spot #	Sequence	Reactivity index[a]	Sequence localisation	Deduced interaction site
7	RPAPAP**IRRRSSNYRAYATE**	++	13-32	IRRRSSNYRAYATE
8	APAP**IRRRSSNYRAYATE**PH	++	15-34	**19-32**
9	AP**IRRRSSNYRAYATE**PHAK	++	17-36	(motif 1)
10	**IRRRSSNYRAYATE**PHAKKK	++	19-38	
18	AKKKSKISAS**RKLQLKTLLL**	++	35-54	RKLQLKTLLL
19	KKSKISAS**RKLQLKTLLL**QI	++	37-56	**45-54**
20	SKISAS**RKLQLKTLLL**QIAK	+++	39-58	(motif 2)
21	ISAS**RKLQLKTLLL**QIAKQE	+++	41-60	
22	AS**RKLQLKTLLL**QIAKQELE	++	43-62	
23	**RKLQLKTLLL**QIAKQELERE	++	45-64	
60	TKNITEIADL**TQKIFDLRGK**	++	119-138	TQKIFDLRGK
61	NITEIADL**TQKIFDLRGK**FK	++	121-140	**129-138**
62	TEIADL**TQKIFDLRGK**FKRP	++	123-142	(motif 3)
63	IADL**TQKIFDLRGK**FKRPTL	+++	125-144	
64	DL**TQKIFDLRGK**FKRPTLRR	++	127-146	
65	**TQKIFDLRGK**FKRPTLRRVR	++	129-148	
73	**RRVRISADAMMQALLGARAK**	++	145-164	RRVRISADAMMQALLGARAK
				145-164
				(motif 4)
77	AMMQALLG**ARAKESLDLRAH**	++	153-172	ARAKESLDLRAHLKQVKK
78	MQALLG**ARAKESLDLRAHLK**	++	155-174	**161-178**
79	ALLG**ARAKESLDLRAHLKQV**	++	157-176	(motif 5)
80	LG**ARAKESLDLRAHLKQVKK**	+++	159-178	
81	**ARAKESLDLRAHLKQVKK**ED	+++	161-180	
94	EVGD**WRKNIDALSGMEGRKK**	++	187-206	WRKNIDALSGMEGRKKKFES
95	GD**WRKNIDALSGMEGRKKKF**	++	189-208	**191-210**
96	**WRKNIDALSGMEGRKKKFES**	+++	191-210	(motif 6)

[a]Peptide reactivity with [^{125}I]-cTnC was determined by visual observation : only fairly (++) and strongly (+++) peptides are listed.

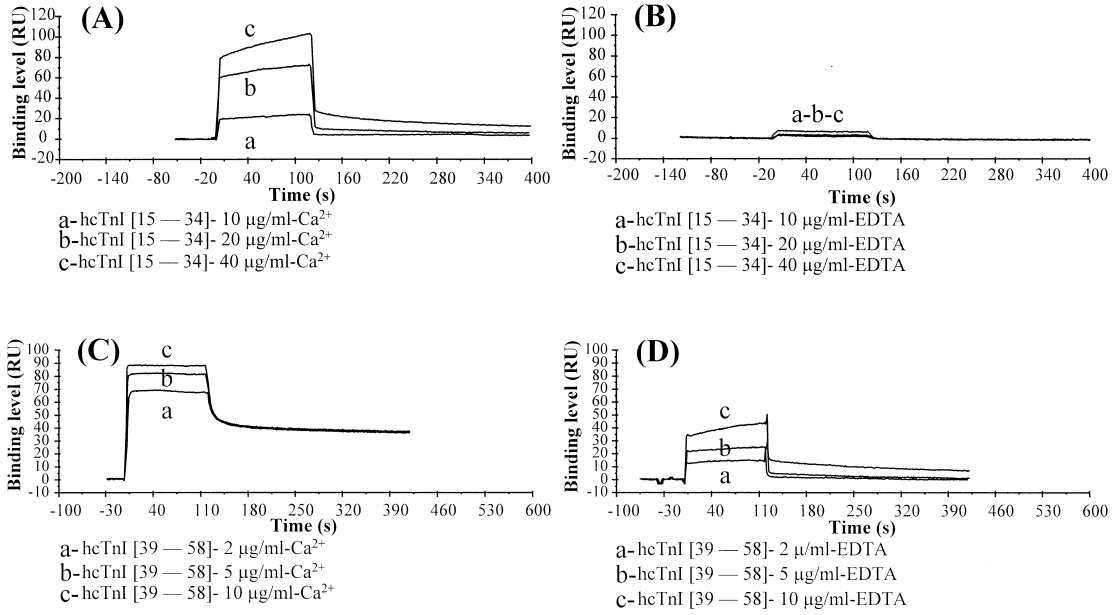

Fig. 2. Sensorgrams representing the surface plasmon resonance analysis of the interaction between immobilized cTnC and hcTnI peptides. A: Binding of hcTnI [15–34] (10, 20, 40 μg/ml) to cTnC, in the presence of 3 mM $CaCl_2$, (B) in the presence of 10 mM EDTA. C: Binding of hcTnI [39–58] (2, 5, 10 μg/ml) to cTnC in the presence of 3 mM $CaCl_2$, (D) in the presence of 10 mM EDTA.

tional solid phase synthesis and analyzed by real-time interaction using BIACORE technology. Increasing concentrations of the peptides were injected onto the two flowcells, as described in Section 2. Typical sensorgrams of the interaction of two soluble hcTnI peptides (hcTnI [15–34] and hcTnI [39–58]) with immobilized cTnC, in the presence of Ca^{2+} and after subtraction of the appropriate controls, are shown in Fig. 2A,C). The binding levels (expressed in RU, measured 126 s after injection) for all the tested peptides are presented in Table 2. Peptides containing an interaction site in their sequence, hcTnI [15–34] (site 1), hcTnI [124–143] (site 3), hcTnI [145–164] (site 4), hcTnI [159–178] (site 5) and hcTnI [191–210] (site 6) bound to immobilized cTnC in a dose-dependent manner. Even at low concentrations, the hcTnI [39–58] peptide, (site 2), bound very strongly to cTnC, suggesting a high affinity. Only very low binding of this peptide to immobilized soybean trypsin inhibitor, an irrelevant protein, was observed (signal < 8 RU at the highest concentration tested). No binding was detectable with peptides derived from sequences of hcTnI corresponding to unreactive spot peptides (hcTnI [73–92], hcTnI [89–108] and hcTnI [109–128]). The deduced equilibrium dissociation constant (K_D) of the interaction with cTnC was in the 10^{-6} M range for all peptides but two: hcTnI [145–164] (1.42×10^{-7} M) and hcTnI [39–58] (3.07×10^{-9} M) (Table 2). The high affinity of peptide hcTnI [39–58] for TnC was confirmed in a separate experiment in which the peptide was coupled to the CM5 sensor chip and the binding of cTnC analyzed ($K_D = 5.72 \pm 1.87\times10^{-9}$ M). The binding of hcTnI [15–34], hcTnI [124–143], hcTnI [145–164], hcTnI [159–178] and hcTnI [191–210] was not even measurable when the BIACORE experiments were performed in the presence of 10 mM EDTA (Table 2 and Fig. 2B). This lack of binding to cTnC

Table 2
Synthetic TnI peptide binding levels and affinities for cTnC measured by surface plasmon resonance

Peptide	Binding level at 126 s (RU)								$K_D \pm$ S.D. (M)	$K_D \pm$ S.D. (M)
	with Ca^{2+}, peptide concentration (μg/ml)				with EDTA, peptide concentration (μg/ml)				with Ca^{2+}	with EDTA
	5	10	20	40	5	10	20	40		
hcTnI [15–34]	NM[a]	4	12	27	NM	0	0	0	$5.17\pm1.79\times10^{-6}$	NM
hcTnI [15–34 diP]	0	0	1	0	0	0	0	ND	NM	NM
hcTnI [39–58]	56	55	56	ND	3	15	39	55	$3.07\pm1.40\times10^{-9}$	$4.95\pm0.07\times10^{-6}$
hcTnI [124–143]	7	17	23	79	0	0	0	ND	$3.02\pm1.61\times10^{-6}$	NM
hcTnI [145–164]	98	118	142	ND[b]	0	8	14	ND	$1.42\pm0.97\times10^{-7}$	NM
hcTnI [159–178]	21	29	43	67	0	1	1	ND	$1.42\pm0.13\times10^{-6}$	NM
hcTnI [191–210]	6	13	25	75	0	1	1	ND	$1.69\pm0.89\times10^{-6}$	NM
hcTnI [73–92]	0	0	0	0	ND	ND	ND	ND	NM	ND
hcTnI [89–108]	0	0	0	0	0	0	0	0	NM	NM
hcTnI [109–128]	0	0	0	0	ND	ND	ND	ND	NM	ND
Control hcTnI [39–58][c]	0	3	5	8	ND	ND	ND	ND	NM	ND

Each K_D value represents the mean ± S.D. of triplicate determinations.
[a]Not measurable.
[b]Not determined.
[c]Binding with soybean trypsin inhibitor.

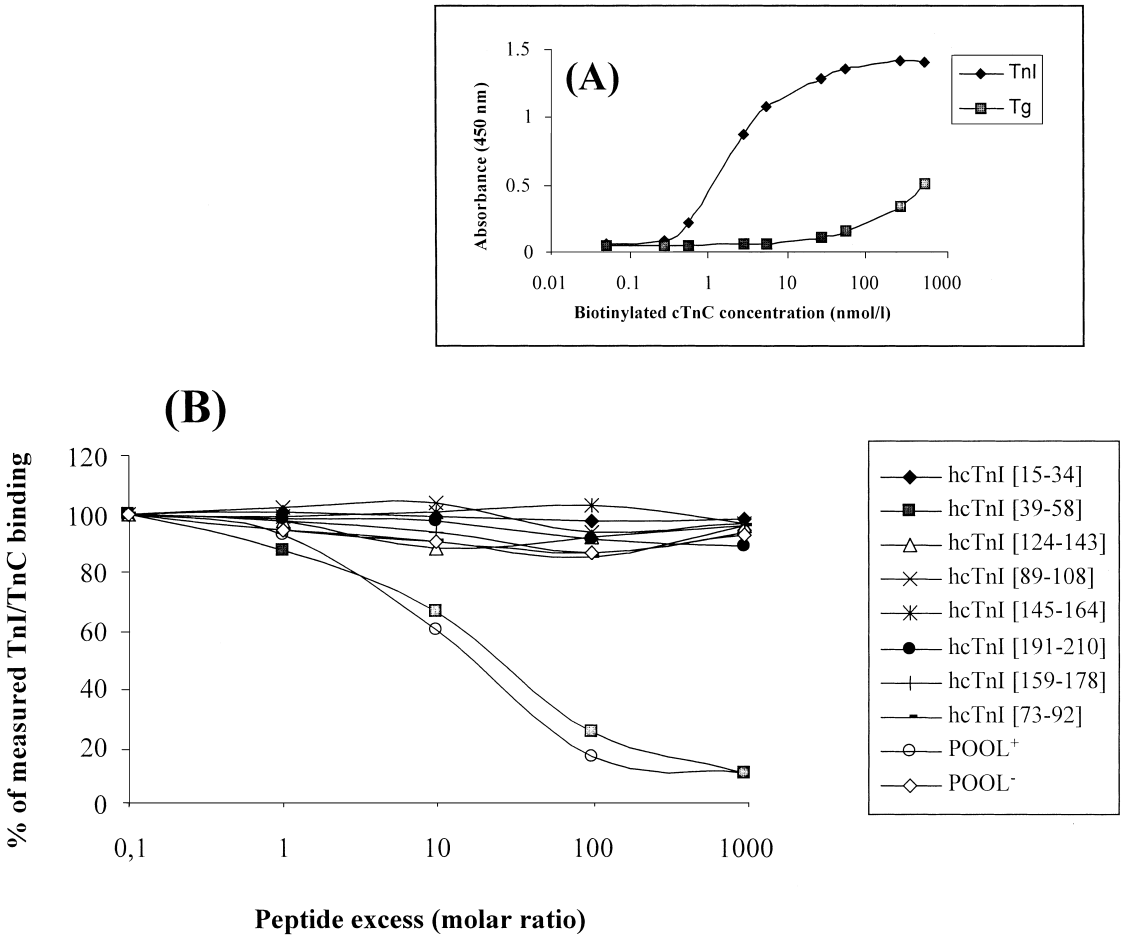

Fig. 3. Inhibition by free synthetic hcTnI peptides of the binding of cTnC to hcTnI in a competitive ELISA. HcTnI was coated onto microtitration plates. A: CTnC binding profile to immobilized hcTnI or to thyroglobulin (Tg), an irrelevant immobilized protein. B: Binding of 2.7 nM biotinylated cTnC was inhibited by increasing concentrations of either a mixture of synthetic peptides designated by $POOL^+$ (mixture of peptides hcTnI [15–34], hcTnI [39–58], hcTnI [124–143], hcTnI [145–164], hcTnI [159–178] and hcTnI [191–210] and by $POOL^-$ (mixture of control peptides, i.e. hcTnI [73–92], hcTnI [89–108], hcTnI [109–128]) or with isolated hcTnI peptides.

confirms the Ca^{2+}-dependence for sites 1, 3, 4, 5 and 6 of hcTnI, as observed in the mapping experiments described above. In the presence of EDTA, only the hcTnI [39–58] peptide was able to interact with cTnC (Fig. 2D). However, its affinity for the protein was greatly decreased (4.95×10^{-6} M) as compared with the value measured in the presence of Ca^{2+} (3.07×10^{-9} M) (Table 2). Therefore cTnC interaction of the site 2 was also Ca^{2+}-dependent, but despite a large drop in the affinity, was still measurable.

3.3. Inhibition of the binding between hcTnI and cTnC by the synthetic hcTnI peptides

The ability of biotinylated cTnC to bind to immobilized hcTnI was assessed by ELISA (Fig. 3A). In the presence of Ca^{2+}, cTnC reacted specifically with hcTnI in a dose-dependent manner. Then, competitive binding assays were performed to examine the ability of the synthetic hcTnI peptides to block the binding of biotinylated cTnC to immobilized hcTnI (Fig. 3B). The mixture of peptides which contained in their sequence an interaction site (mixture of peptides hcTnI [15–34], hcTnI [39–58], hcTnI [124–143], hcTnI [145–164], hcTnI [159–178], and hcTnI [191–210], designated as $POOL^+$) induced a specific inhibition. An almost complete inhibition was obtained at 10^3-fold peptide excess. No inhibition was observed with the $POOL^-$ (a mixture of non-reactive peptides, i.e. hcTnI [73–92], hcTnI [89–108], and hcTnI [109–128]). When single peptides were used instead of the mixture, only the hcTnI [39–58] peptide had an inhibitory effect. The calculated peptide concentration giving 50% inhibition was 65 nM, in accordance with our observation that hcTnI [39–58] had a nanomolar affinity for cTnC.

4. Discussion

It is still not precisely known how the troponin components interact inside the whole troponin complex, but published models indicate that TnI and TnC interact through multiple contacts covering a large part of their surface [20–23]. The aim of our study was to map in a systematic manner all the hcTnI regions involved in cTnC interactions. We chose the Spot format which has previously been shown extremely powerful in mapping protein–protein interaction sites [24]. The study of [^{125}I]cTnC reactivity with 96 overlapping peptides from the hcTnI sequence enabled the assignment of six specific sites of interaction. We assumed that the strongest reactive sequences in the Spot analysis contained an interaction site and we synthesized these sequences as soluble peptides. This allowed us to measure, for the first time in the

same set of experiments, an affinity of cTnC with each region of interaction, as modelled by a 20-mer synthetic peptide. Five peptides had an affinity in the range of 10^{-6}–10^{-7} M, but peptide hcTnI [39–58] showed a much higher (nanomolar) affinity. A mixture of the six binding peptides was able to block the interaction between hcTnI and cTnC. When tested separately, only the hcTnI [39–58] peptide retained this ability. We also found that the interaction of cTnC with hcTnI regions 19–32, 45–54, 129–138, 145–164, 161–178 and 191–210 clearly appeared to be Ca^{2+}-dependent, whatever the methodology used, Spot or BIACORE. The significance of these results will now be discussed in the framework of the current understanding of hcTnI–cTnC interactions.

The first interaction region was represented by the hcTnI [15–34] peptide and belonged to the cardiac-specific N-terminal extension. Serines 23 and 24 of the additional N-terminal segment can be phosphorylated by protein kinase A, producing a decrease in the Ca^{2+} sensitivity of myofibrillar Mg^{2+}-dependent ATPase [25]. Little is known about the molecular mechanism by which diphosphorylated cTnI affects the Ca^{2+} affinity of cTnC. However, an interaction with cTnC could directly influence it [26,27]. The documented antiparallel mode of interaction between the two proteins suggests that the N-terminal extension of cTnI interacts with the C-terminal domain of cTnC. Recently, Gaponenko et al. suggested, from NMR investigations, that the non-phosphorylated cardiac-specific N-terminus of hcTnI interacts with the N-terminal domain of cTnC. This interaction was not observed with the recombinant fragment (Asp^{23}, Asp^{24}) cTnI 1–80, which mimics the phosphorylated form of the protein [28]. In our study, we observed that only one part of the cardiac-specific extension, containing the two adjacent serines, was able to bind cTnC. This region might therefore correspond to the minimal sequence of the extension which could switch between the N- and C- terminal TnC domains depending on its phosphorylation state. The hcTnI [15–34] peptide in its bis-phosphorylated form (hcTnI [15–34 diP]) failed to bind immobilized cTnC either in the BIACORE or the Spot format (Table 2 and data not shown). TnI phosphorylation induced a 2.5–3.5-fold decrease in affinity for cTnC [19,27,29,30]. Furthermore, in agreement with our findings, an NMR study of the binding of TnI peptide (17–30) to cTnC, indicated an affinity constant of about 10^5, lowered by a 100-fold factor by diphosphorylation [31].

The second interaction site represented by the hcTnI [39–58] peptide belongs to the region 33–80 which is known to form a stable binary complex with the C-terminal domain of cTnC [5,6]. In a completely different approach, the sequence 44–59 from hcTnI was identified by the phage-display technology as corresponding to the minimum region of interaction [32]. However, the affinity of the biotinylated 44–59 peptide measured by BIACORE was 8.6×10^{-7} M, versus 3.07×10^{-9} M for the hcTnI [39–58] peptide. This discrepancy could be explained by the different BIACORE formats used (bound peptides in the study of Pierce et al., soluble peptides in our case), and/or by the larger size of our peptide (20 amino acids), which could then better mimic the interaction site. In the antiparallel model of TnI–TnC interaction postulated by Farah et al. [20], region 1–40 of the skeletal TnI isoform (equivalent to sequence 33–80 of hcTnI) is strongly anchored to the C-terminal domain of TnC in the absence and the presence of Ca^{2+}, while the inhibitory and C-terminal region of TnI switch between actin-tropomyosin in the absence of Ca^{2+} and binding sites of TnC in the presence of Ca^{2+}. By analogy with this model, we propose that the region 45–54 identified by the Spot method could correspond to the minimal site of permanent anchorage of hcTnI to cTnC. This would explain the binding persistence in the absence of Ca^{2+}, observed in the two tested formats, BIACORE and Spot. HcTnI [39–58] peptide had the strongest affinity for cTnC (3.07×10^{-9} M); it was also the only peptide able to inhibit the TnI–TnC complex formation with a 65 nM IC_{50}, in keeping with the observation that the skeletal peptide TnI [1–40] was able to displace TnI from the preformed TnI–TnC complex [33]. Therefore, we postulate that, despite the existence of multiple cTnC interaction domains on the hcTnI molecule, only the region of permanent anchorage 45–54 allows stabilization of the complex.

The third site of interaction (129–138) is part of the inhibitory peptide (Ip) (residues 128–148). Our failure to identify exactly the entire Ip sequence was possibly due to the fact that the length of the peptides (20 amino acids) used for the Spot peptide synthesis is shorter than the actual length of Ip (21 amino acids). HcTnI [124–143] peptide binding to the cTnC was Ca^{2+}-dependent, in agreement with literature data showing that the Ip interaction is stronger in presence of Ca^{2+} [23]. Furthermore, hcTnI [124–143] peptide affinity for TnC was 3.02 μM, in the same order of magnitude as that observed by spectral methods ($K_A = 5\times10^5$ M^{-1}, i.e. $K_D = 2$ μM [34]).

The fourth interaction region mimicked by the hcTnI [145–164] peptide contained the end of the Ip sequence and the entire sequence (147–163) described by multinuclear multidimensional NMR spectroscopy as binding to the N-terminal domain of cTnC in the Ca^{2+} saturated state; the cTnI sequence 147–163 interacted with the hydrophobic core of cTnC–Ca^{2+} and stabilized the open conformation of cTnC [7]. This site is analogous to the sequence 115–131 of the skeletal TnI isoform whose cTnC binding in a Ca^{2+}-dependent manner, as determined by affinity chromatography analysis, was clearly demonstrated. This 'second TnC binding site' is critical to allow TnC to fully and rapidly neutralize the actomyosin inhibition caused by various peptides [23]. Our data demonstrate that hcTnI [145–163] binds in a Ca^{2+}-dependent manner to cTnC, probably in its N-terminal domain. Affinity measurement by spectral methods for the peptide 147–163 yielded a macroscopic dissociation constant K_D of 154 ± 10 μM [7]. This affinity is about six times weaker than the affinity of skeletal TnI 115–131 for the skeletal cTnC–Ca^{2+} bound-N-terminal domain [35], and about 1000-fold weaker than the affinity of hcTnI [145–163] peptide binding to entire cTnC. This difference in the measured affinity could result from methodological differences (NMR versus BIACORE) or may be due to the use of one isolated cTnC domain in the study of Li et al. [7] and the entire protein in our case, or, most probably, to the differences in the amino acid sequences of the two peptides under study: the peptide 147–163 of Li et al., was lacking three residues (R_{144}, R_{145} and V_{146}) which are thought to be important for affinity (see discussion in the article by Li et al. [7]).

Our results also show that the reactivity of hcTnI region 161–178 with cTnC is Ca^{2+}-dependent. A recent study by Rarick et al. [9] has shown that the C-terminal region of cTnI is essential for the Ca^{2+}-dependent regulation of cardiac myofilament activation. Their data suggest that the inhibitory

region inhibits about 50% of the ATPase activity, whereas two other regions (residues 152–188 and 189–199) in mouse cTnI contribute 25% each. Their results also indicate the presence of a Ca^{2+}-dependent cTnC binding domain within residues 152–188. Therefore residues 164–178, which we mapped, could correspond more precisely to the second C-terminal cTnC binding domain implicated in cardiac regulation.

Finally, concerning site 6, our data gave hints as to the implication of hcTnI region 191–210 in cTnC binding. In the Olah and Trewelha model [21] and in the cross-linking study published by Kobayashi [36], most of the skeletal TnI molecule is involved in TnC interaction, with the exception of the C-terminal 36 residues. However, it has been found that recombinant mouse cTnI (1–199), complexed with cTnC, totally restored the Ca^{2+} sensitivity of myofibrils, whereas the cTnI (1–188)–cTnC complex only partially restored it and the cTnI (1–152)–cTnC complex did not restore Ca^{2+}-sensitivity at all [9]. These results indicate that residues 152–199 of cTnI are essential for Ca^{2+} sensitivity of myofibrillar ATPase activity in the heart, probably via an interaction with cTnC [9]; our finding of the existence of an interaction site in the 191–210 residues strengthens this hypothesis. Interestingly, transgenic mice expressing TnI (1–193) in their heart were found to develop ventricular dilatation, diminished contractility and reduced myofilament calcium responsiveness, recapitulating the phenotype of stunned myocardium [37]. Furthermore, a product equivalent to rat TnI (1–193) is prominent in the human post-ischemic myocardium. It has been suggested that this modification of the troponin complex may be due to the lack of the C-terminal region and be directly responsible for the myofibrillar dysfunction observed in ischemia [37,38]; our identification of a new interaction site within residues 191–210 is in line with this hypothesis.

In conclusion, our systematic approach of the interaction between hcTnI and cTnC permitted us to map six possible interaction sites with cTnC. These regions are scattered all along the hcTnI sequence. A good correlation between our results and literature data validates the methodological approach used. The existence of a cTnC interaction site localized in the N-terminal cardio-specific extension of hcTnI has been confirmed and more precisely identified. The interaction of this region with cTnC is largely influenced by the phosphorylation state of the protein. We also described residues 39–58 as a high affinity binding site for cTnC. Our data also indicated the occurrence of two Ca^{2+}-dependent cTnC, low affinity, binding domains within the C-terminal part of hcTnI, namely residues 164–178 and 191–210. The latter sequence may be linked with the cardiac dysfunction observed in stunned myocardium.

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