The Calcium Induced Switch in the Troponin Complex Probed by Fluorescent Mutants of Troponin I

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Protein Structure

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ABBREVIATIONS

DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis (beta-aminoethyl ether)-N,N,N',N',-tetraacetic acid; IPTG, isopropylthiogalactoside; Kd, dissociation constant; MOPS, 4-morpholinepropanesulfonic acid; *n*, the Hill constant; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; Tn, troponin complex; TnC, skeletal troponin C; TnI, skeletal troponin I; TnT, skeletal troponin T; Tris, tris (hydroxymethyl) aminomethane.

RUNNING TITLE

The Calcium Induced Switch in the Troponin Complex.

SUMMARY

The Ca^{2+}-induced transition in the troponin complex (Tn) regulates vertebrate striated muscle contraction. Tn was reconstituted with recombinant forms of troponin I (TnI) containing a single intrinsic 5-hydroxytryptophan (5HW). Fluorescence analysis of these mutants of TnI demonstrate that the regions in TnI that respond to Ca^{2+} binding to the regulatory N-domain of TnC are the inhibitory region (residues 96 to 116) and a neighboring region that includes position 121. Our data confirms the role of TnI as a modulator of the Ca^{2+} affinity of TnC; we show that point mutations and incorporation of 5HW in TnI can affect both the affinity and the cooperativity of Ca^{2+} binding to TnC. We also discuss the possibility that the regulatory sites in the N-terminal domain of TnC might be the high affinity Ca^{2+}-binding sites in the troponin complex.

KEYWORDS

5-hydroxytryptophan; Ca^{2+} binding protein; fluorescence; troponin; skeletal muscle

INTRODUCTION

The regulation of striated muscle contraction in vertebrates is accomplished by troponin (Tn), a protein associated with actin in the thin filament. Tn is a complex composed of three polypeptide subunits: troponin C (TnC) has the Ca^{2+}-binding sites, troponin I (TnI) has the inhibitory function, and troponin T (TnT) is the actin-tropomyosin binding component. Tn works as a sensor of intracellular calcium concentration. Stimulation of the muscle leads to Ca^{2+} increase and calcium binding to TnC removes the inhibition of the muscle contraction promoted by TnI. The conformational transition undergone by Tn enables the regulation of muscle contraction [1 - 3].

TnC has two globular domains connected by an α-helix and each domain has two Ca^{2+}-binding sites (EF-hand motifs) [4]. The Ca^{2+}-binding properties of isolated TnC are well known. Sites III and IV in the C-domain (carboxy terminal) bind Ca^{2+} with higher affinity, sites I and II in the N-domain (amino terminal) bind Ca^{2+} with lower affinity [5, 6]. The association between TnC and TnI was shown to be anti-parallel [7]. The C-domain of TnC interacts structurally with the N-terminal region of TnI [8, 9]. The Ca^{2+}-loaded N-domain has a higher affinity for TnI and triggers a chain of conformational rearrangements that moves the inhibitory region of TnI, residues 96 to 116, away from actin [10]. The full regulatory properties are only achieved in the presence of TnT [8].

This article describes the use of fluorescent mutants of TnI to investigate the Ca^{2+}-induced switch in Tn. Each mutant contains a single intrinsic 5-hydroxytryptophan (5HW), a tryptophan (Trp) analog. The unique 5HW can be selectively monitored in the presence of several Trp and works as a site-specific probe for conformational rearrangements [11, 12]. Our

results demonstrate that the inhibitory region and the adjacent region including residue 121 of TnI undergo conformational transitions triggered by Ca^{2+}. Further, the data enables us to better understand the influence of TnI on the calcium binding properties of TnC. We also report for the troponin complex a surprisingly high Ca^{2+}-affinity assigned to the regulatory sites in the N-domain of TnC.

EXPERIMENTAL PROCEDURES

Construction of TnI mutants

The oligonucleotide-mediated mutagenesis technique [13, 14] was used to replace the single Trp codon at position 160 in the chicken fast skeletal muscle cDNA cloned into the phage M13 [15]. It generated the phage M13-TnIW160F (TnITrp-less) which was used as template to construct two other mutants. M13-TnIF106W and M13-TnIF177W respectively had Phe-106 and Phe-177 mutated to Trp (Fig. 1a). The mutagenic primers used were: W160F 5'-TGGGTGACTTCAGGAAGAACA-3', F106W 5'-GGGCAAGTGGAAGAGGCCA-3', F177W 5'-GAAGAAGATGTGGGAGGCCGG-3'. The mutant TnI cDNA inserts were released by digestion with the restriction enzymes Nde I and Bam HI, and subcloned in the expression vector pET3a [16]. The mutants TnIY79W, TnIF100W and TnIM121W were engineered by PCR (for details see Higuchi [17]) using the vector pET-TnIW160F (TnITrp-less) as template. Trp replaced respectively Tyr-79, Phe-100, and Met-121 (Fig. 1a). The oligonucleotides used were: Y79W 5'-GGATGAGGAAAGGTGGGACACAGAG-3', Y79W(rev) 5'-TCACCTCTGTGTCCCACCTTTCCTC-3', F100W 5'-GAGCCAGAAGCTGTGGGACCTGAG-3', F100W(rev) 5'-GCCCCTCAGGTCCCACAGCTTCTG-3', M121W 5'-GTCTGCTGATGCCTGGCTGCGTG-3', M121W(rev) 5'-CAGGGCACGCAGCCAGGCATCAG-3', T7 promoter, 5'-TACGACTCACTATAGGGAGACCAC-3', T7 terminator, 5'-TAGTTATTGCTCAGCGGTGGCAGC-3'. The digestion of the amplification products with Nde I / Bam HI released the complete cDNA of TnI allowing subcloning in pET3a [16]. All mutations were confirmed by DNA sequencing [18].

Protein preparation

The 5HW was incorporated into recombinant proteins using the *Escherichia coli* lineage CY (DE3) pLys S [12]. This is a lineage auxotrophic for Trp [19], which was modified for use with the pET system [16]. The proteins were expressed with the following protocol: A transformed colony with the desired vector was grown in 50 mL minimal media (M9) plus 50 mg / L L-tryptophan, 200 mg / L carbenicillin, and 200 mg / L chloramphenicol succinate, at 37 ^{o}C. This culture was used to inoculate 4 L of the same media. When the OD_{600} of the culture reached 0.8 - 1.0, the bacteria were recovered by centrifugation (3,000 xg, 4 ^{o}C, 15 min). The bacteria were then resuspended in the same media with 0.4 mM IPTG and without L-tryptophan. After 15 min 100 mg / L L-5-hydroxytryptophan was added. The culture of bacteria was incubated for 3 h and collected by centrifugation. The purification proceeded as described for recombinant TnI [15]. All mutants of TnI behaved as TnI in purification steps (data not shown) and had the same electrophoresis polyacrylamide gel mobility (Fig. 2). The amount of purified TnI with 5HW incorporated was between 5 to 10 mg / L of culture. The 5HW incorporation ratio for this method was estimated to be higher than 90 % [12]. Recombinant TnT was obtained as described [8]. Recombinant TnC [15] and the mutants of TnC, TnCF29W, [20] or TnCD30A, TnCD66A, TnCD106A, and TnCD142A [7] are described elsewhere. All forms of TnC were prepared as in Fujimori et al. [21].

The ability of TnC to form a stable complex with each mutant TnI was visualized through urea / PAGE [7, 22]. The concentration of protein was determined with the technique described by Hartree [23]. The SDS / PAGE was done as described in Laemmli [24].

Troponin complex reconstitution

The binary and ternary (Fig. 2c) complexes were reconstituted as previously described [7] with some modifications. Equimolar amounts of protein were mixed and sequentially dialyzed against the following buffers: a) 50 mM Tris-HCl pH 8.0, 4.6 M urea, 1 M KCl, 50 μM $CaCl_2$, 0.01 % NaN_3, 10 mM 2-mercaptoethanol; b) 50 mM Tris-HCl pH 8.0, 2 M urea, 1 M KCl, 50 μM $CaCl_2$, 0.01 % NaN_3, 10 mM 2-mercaptoethanol; c) 50 mM Mops pH 7.0, 1 M KCl, 5 μM $CaCl_2$, 0.01 % NaN_3, 10 mM 2-mercaptoethanol; and three times against the fluorescence buffer: d) 50 mM Mops pH 7.0, 100 mM KCl, 1 mM EGTA, 0.01 % NaN_3, 10 mM 2-mercaptoethanol. The aggregated proteins were removed by centrifugation (10,000 xg, 15 min, 4 ^{o}C).

Fluorescence experiments

Fluorescence spectra were determined with a Hitachi F-4500 spectrofluorimeter. For the excitation spectra, the emission was collected at 340 nm. For the emission spectra, the excitation was at 315 nm. The band slits were always 5 nm for both emission and excitation. The samples were diluted in fluorescence buffer to a concentration of 2 μM, in a final volume of 1.5 mL. We allowed the protein to equilibrate for 20 min at 25 ^{o}C before initiating the experiment. Fluorescence buffer plus 5 mM $CaCl_2$ or 50 mM $CaCl_2$ was used in the titration experiments. The free Ca^{2+} concentration was calculated using the software "Sliders" [25]. A single scan was performed for each Ca^{2+} addition and the total area of the emission spectra between 325 and 345 nm was used to plot the titration curves.

RESULTS

We produced six different recombinant TnIs with a single 5HW in positions we aimed to investigate: TnIY79**H**W, TnIF100**H**W, TnIF106**H**W, TnIM121**H**W, TnI160**H**W, and TnIF177**H**W (Fig. 1a, and Materials and Methods). Binary and ternary troponin complexes were reconstituted for fluorescence analysis from their recombinant subunits (Fig. 2c). The advantage of this strategy is that the 5HW can be selectively excited between 310 - 320 nm in the presence of several Trp (Fig. 3a). Therefore, the fluorescence of the single 5HW in TnI can be monitored in the presence of three Trp from TnT [26]; TnC does not contain Trp [27].

The urea / PAGE experiment permits visualization of the TnC-TnI interaction (Fig. 2). Due to its negative charge TnC enters in the gel, the positively charged TnI does not. The interaction between TnC and TnI is so strong when calcium is present (0.5 mM $CaCl_2$) that TnC is able to carry TnI into the gel [7, 22]. In the absence of calcium (0.5 mM EDTA or 10 mM $MgCl_2$ / 1 mM EGTA, data not shown) TnC enters alone. All TnI mutants exhibit the same behavior as TnI. This demonstrates that the mutations and the incorporation of 5HW in TnI do not strongly affect the Ca^{2+}-dependent interaction with TnC.

Regions of TnI sensitive to calcium binding to TnC

To determine which regions of TnI are sensitive to Ca^{2+} binding to TnC, we compared the fluorescence emission spectra of the reconstituted complexes in the absence and presence of calcium. Because changes in the environment around a fluourophore affect its fluorescent properties, the 5HW is a site-specific probe for allosteric modifications within Tn. The highest variation obtained is a 70 % increase in the fluorescence of the ternary complex Tn-TnIM121**H**W in the calcium-saturated state (pCa 4) as compared to the Apo state (Fig. 3c). The

presence of Ca^{2+} also promotes a consistent 12 % increase in the emission spectra of Tn-TnIF100**H**W (Fig. 3b). Two binary complexes TnC-TnIF106**H**W and TnC-TnIM121**H**W (data not shown) present significant variation in fluorescence emission. The complexes with TnIY79**H**W, TnI160**H**W, and TnIF177**H**W, however, are not sensitive to the addition of calcium (i.e. the fluorescence intensity changes are lower than 3 %). In summary, the data from TnI fluorescent mutants shows that the portion of TnI that responds to Ca^{2+} binding to TnC is the inhibitory region plus a neighboring region that includes position 121 (Fig. 1a).

Following the identification of the complexes that display a fluorescence signal, Ca^{2+}-titration experiments were performed. Two important parameters are acquired, the affinity for Ca^{2+}, dissociation constant (Kd), and the cooperativity (*n*) of Ca^{2+} binding (Table 1). The TnC-TnIF106**H**W shows a curve characterized by an initial decrease in the fluorescence intensity (-6 %, $Kd_1 = 4.5 \times 10^{-8}$ M) followed by an increase (3 %, $Kd_2 = 2.8 \times 10^{-6}$ M, Fig. 4b). Therefore, TnIF106**H**W may be a probe for calcium binding to both domains of TnC. The parameters for Tn-TnIF100**H**W are in agreement with the first part of the curve of TnC-TnIF106**H**W for both Kd and *n* (Fig. 4a). Positions 100 and 106 are part of the inhibitory region and respond to the same event, Ca^{2+} filling a high affinity class of sites. The probe at position 121 of TnI shows a Kd consistent with the occupancy of a lower affinity Ca^{2+}-binding sites with a very high cooperativity, $n \sim 2$. This value indicates that two sites are occupied by Ca^{2+} at nearly the same time. Although we analyzed TnC-TnIM121**H**W as a one step curve, this binary complex shows a decrease at low pCa in the titration curve (fig 4b). This decrease may also be an indication of Ca^{2+} binding to a different class of sites.

The TnC mutant TnCF29**H**W (Phe-29 was mutated to Trp and 5HW incorporated) is a probe for Ca^{2+} filling the sites in the N-domain [20, 28]. The presence of TnI increases the Ca^{2+}-

affinity of the regulatory sites of TnC by one order of magnitude, and TnT has no further effect (Fig. 4c and Table 1). Although the Kd values acquired are only slightly different in comparison with the respective TnIM121**H**W binary and ternary complexes, TnCF29**H**W does not display Ca^{2+}-cooperative binding. It appears that there are three different sets of data: one for probes in the inhibitory region of TnI, another for the probe at position 121 of TnI, and a third for the probe in the N-domain of TnC.

Identification of the TnC domain perceived by the TnI mutants

To determine whether the observed variation in Kd and *n* is due to mutations or different phenomena, Tn was reconstituted with a set of four TnC mutants combined with TnIF100**H**W or TnIM121**H**W. There is an Asp involved in metal ion coordination in the first position of all EF-hands of TnC. This allowed each one of the Ca^{2+}-binding sites to be disrupted by an Asp to Ala replacement: TnCD30A (site I), TnCD66A (site II), TnCD106A (site III), and TnCD142A (site IV) [7, 29].

Neither the calcium affinity nor the cooperativity displayed by TnC are affected by mutations in sites III and IV. The Tn with a disrupted site IV (TnCD142A) shows the same calcium titration curve as the respective complex with TnC. Similarly, TnCD106A, that prevent Ca^{2+} binding to site III, has no effect in the curve of TnIF100**H**W and only slightly lower the intensity change of TnIM121**H**W. This small decrease in the intensity change is likely due to inter-domain communication. It demonstrates that the probes at positions 100 and 121 of TnI are not sensitive to calcium binding to structural sites III and IV in the C-domain of TnC (Fig. 5).

The complexes reconstituted with TnCD30A are characterized by a lower amplitude of fluorescence variation, the affinity constants, however, are not affected. All complexes with

TnIM121**H**W where TnC has two functional sites in the N-domain show strong cooperativity (nearly 2, Table 1, Fig. 4, and Fig. 5b). However, TnCD30A has only one functional site in the regulatory domain and cooperativity would be impossible; in fact TnCD30A drops the *n* value to 1 (Fig. 5b). This implies that the presence of a 5HW in position 121 of TnI promotes cooperativity among the regulatory sites of TnC. Figures 5a and 5b clearly show the strong disturbance of the calcium titration curve shapes upon replacement of Asp-66 by an Ala. Recent data have confirmed that this mutation severely decreases the Ca^{2+} affinity of the regulatory domain of TnC, affecting not only site II but also site I [30]. These results indicate that the inhibitory region and position 121 of TnI are sensitive to the calcium-triggering signal from the N-domain of TnC.

Fluorescence analysis was undertaken for this group of Asp to Ala TnC mutants, and TnIF106**H**W or TnIF106W (the same TnI mutant with Trp instead of 5HW, data not shown). As TnC does not contain Trp [27], the fluorescence of the single Trp of TnIF106W can be selectively excited at 295 nm. TnIF106W follows the same pattern as TnIF106**H**W. The variation in the fluorescence signal is, however, slightly larger, characterized by a 10 % decrease in the first part of the curve and a 4 % increase in the second part (data not shown). Disruption of sites I and in particular site II modifies the first part of the signal. This indicates that the high affinity Ca^{2+}-signal is related to the N-domain. Further, the disruption of the sites in the C-domain affects the lower affinity part of the signal. The difference in the second part of the curve, however, is too small to permit any firm conclusion.

All three data sets, the results for 5HW in the N-domain of TnC, in the inhibitory region of TnI, and at position 121 of TnI, followed the Ca^{2+} binding to the N-domain of TnC. The variation in Kd and *n* are likely due to the mutations rather than Ca^{2+} binding to different sites.

Previous studies have shown site directed point mutations in TnC that altered the Ca^{2+}-binding properties of TnC [20, 21, 31]. Here we present evidence that point mutations in the TnI alter the dissociation constant and the cooperativity of Ca^{2+} binding to TnC. This study further elucidates the TnI modulatory role in the TnC Ca^{2+}-affinity.

DISCUSSION

Several studies have reported the use of naturally occurring fluorescent amino acids, Tyr or Trp, or the use of proteins labeled with extrinsic attached probes to analyze ligand binding, protein-protein interaction and folding pathways [6, 20, 32 - 38]. However, the use of Tyr and Trp is limited because the interpretation of the data becomes difficult if more than one is present. The use of attached extrinsic fluorescent probes may lead to protein structural alterations due to their relative large size and potential for forming or disrupting interactions. The incorporation of 5HW and other non-naturally occurring amino acid analogs into a protein seems to be a good alternative. They can be used as site-specific probes, with an expected lower conformational damage [11, 12, 28]. We demonstrate here that it is possible to construct fluorescent recombinant mutants of TnI that have their emission spectra affected by Ca^{2+} binding to TnC, a different polypeptide chain. We were able to follow the fluorescent signal to investigate the information of Ca^{2+}-binding to the regulatory sites in TnC transmitted to TnI and to analyze the modulatory effect of TnI on Ca^{2+}-binding properties of TnC.

The calcium-induced switch

The regulatory TnC domain loaded with calcium exposes a hydrophobic surface [38, 39]. Recently, many works have pointed out that the part of TnI that interacts with this hydrophobic pocket is a region adjacent to the C-terminal end of the inhibitory sequence [28, 36, 40 - 43]. Furthermore, Met-121 of TnI has been considered a fundamental residue in this interaction [9, 42, 43]. The fluorescence changes of 5HW at position 121 promoted by Ca^{2+} support this idea. Consequently, the inhibitory region, positions 96 to 116 [10], may bind elsewhere, instead of the hydrophobic pocket [7, 34 – 36, 44]. Our findings show that TnC-TnIF106**H**W and Tn-

TnIF100**H**W are sensitive to Ca^{2+} binding to the regulatory domain of TnC. It demonstrates that even if the positions 100 and 106 of TnI do not interact directly with the N-domain, calcium promotes conformational rearrangements that are transmitted to the inhibitory region of TnI, the main event in the regulation of muscle contraction. The probes in the N and C-terminal regions of TnI, TnIF79**H**W, TnI160**H**W, and TnIF177**H**W, do not display variation in the fluorescence spectra promoted by Ca^{2+}, this suggests that calcium occupying the TnC sites causes little structural modifications in these regions. The N-terminal region of TnI, positions 1 to 95, seems to have mainly structural function in maintaining the organization of the Tn [7 - 9, 45]. The function of the C-terminal region of TnI is less understood. A mapping of the TnI interactions with the other thin filament proteins obtained by photo-cross-linking is consistent with this scheme [46]

The amplitude of the variation in the emission spectra promoted by Ca^{2+} is different for binary and ternary complexes: TnIF100**H**W shows variation only for the ternary complex, TnIF106**H**W shows variation only forming the binary complex and TnC-TnIM121**H**W presents a 10 % increase while Tn-TnIM121**H**W displays a 70 % increase. These results indicate that TnT causes alterations in the environment around the TnI regions involved in the regulatory process, reflecting the structural flexibility of the middle part of TnI [44].

TnI modulatory effect in TnC Ca^{2+}-affinity

Since the original experiments of Ca^{2+}-binding done by Potter and Gergely [5], it has become clear that TnI modulates the TnC affinity for calcium. At that time, the structure of TnC and the relative independence of the N- and C-domains were unknown [4], and there had been no identification of the low and the high affinity sites. When Leavis et al. [6] used proteolytic

fragments of TnC to identify the high affinity sites in the C-domain and the low affinity sites in the N-domain, it was assumed to be the case for TnC-TnI and Tn as well. It has been considered that TnI increases the Ca^{2+}-affinity of both domains by one order of magnitude. Several studies have supported the conclusions for TnC alone [20, 21, 35, 37, 47, 48].

The 5OH mutants allowed us to investigate the Ca^{2+}-affinity of TnC when forming the troponin complex using full-length proteins. However, the results are puzzling. The Tn-TnCF29**H**W and TnCF29**H**W-TnI show one order of magnitude increase in the regulatory sites affinity for calcium in comparison with TnCF29**H**W alone (Table 1 and Fig. 4c, [28]). This is in agreement with the scenario described above. It is important to note that Phe-29 is part of the hydrophobic surface exposed in the open (Ca^{2+}-loaded) N-domain [38, 39]. There are evidences that this position influences the Ca^{2+}-affinity of the N-domain [30], and the replacement of Phe by Trp impairs the regulatory properties of TnC [49]. It is difficult to explain how the presence of 5OH at position 121 can promote cooperativity among sites I and II. Regardless, the work of other researches showed that position 121 can be photo-cross-linked with residues in the hydrophobic pocket [42], that alterations in Met-121 or in the region nearby reduce the Ca^{2+}-dependent interaction with TnC [43], and also indicated the importance of the TnI residues 117 to 129 to modulate the Ca^{2+}-affinity of the N-domain [28]. Accordingly, it is not surprising that the 5OH at position 121 has an effect on the Ca^{2+}-binding properties.

The experiments with the D to A TnC mutants clearly determined that the probes in the inhibitory region follow Ca^{2+} binding to the N-domain of TnC (Fig. 5). To make these results compatible with the traditional view, the substitution of both Phe-100 and Phe-106 for 5HW would have to promote an extra increase in the Ca^{2+}-affinity of sites I and II. As discussed before, the inhibitory region may not interact directly with the N-domain. Consequently, one

alternative explanation is that alterations in those positions would not affect the N-domain Ca^{2+}-binding properties. Never such high Ca^{2+}-affinity values, ~ 3 x 10^{-8} M, had been related to sites I and II of TnC. Also, the N-domain was linked to the first part of the bimodal Ca^{2+}-titration curves of the binary complexes. Together, these could be evidence that the high affinity sites are in the N-domain when TnC is bound to TnI. The literature has little information about the Ca^{2+}-affinity of each domain of TnC when bound to TnI, perhaps because it has not been previously considered. Data from extrinsic attached probes, usually on Cys-98 of TnC, are sensitive to Ca^{2+} binding to the two classes of sites, and the authors interpreted the high affinity sites being in the C-domain and the low affinity in the N-domain of TnC. Nevertheless an absolute assignment could not be made [32, 33 and references there in]. Other workers have reported that the Ca^{2+}-affinity of the structural C-domain increases when in the presence of a molar excess of the inhibitory peptide [34 - 36], however this may be a non-physiological interaction [9, 44, 48].

It was tempting to propose a hypothesis that the regulatory sites I and II of TnC are the higher Ca^{2+}-affinity sites in troponin complex. Nevertheless, we are convinced that carefully planed experiments using whole troponin and direct assignment of each Ca^{2+}-binding site are required to solve the question. Our data showed that small modifications, like a point mutation and a quite non-invasive probe in TnI, can affect both affinity and cooperativity of the TnC Ca^{2+}-binding sites. Further more, we should be aware that as the properties of free TnC are not equal to the TnC in troponin complex, in the same way, conclusions reached for Tn alone might not represent the thin filament conditions, where Tn is likely to be strongly affected by the interaction with actin-tropomyosin.

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TABLES

Table 1. Fluorescence emission titration curves parameters.

The data from Ca^{2+}-titration of fluorescence emission was adjusted to the equation: $\Delta F = (\Delta F_{max} \times [Ca^{2+}]^n) / (Kd^n + [Ca^{2+}]^n)$, ΔF is the fluorescence variation, ΔF_{max} is the maximum fluorescence variation, Kd is the apparent Ca^{2+} dissociation constant and n is the Hill coefficient. *Only for TnC-TnIF106**H**W we used an equation that describes a biphasic curve: $\Delta F = (\Delta F_{max1} \times [Ca^{2+}]^{n1}) / (Kd_1^{n1} + [Ca^{2+}]^{n1}) + (\Delta F_{max2} \times [Ca^{2+}]^{n2}) / (Kd_2^{n2} + [Ca^{2+}]^{n2})$, ΔF is the fluorescence variation, ΔF_{max1} is the maximum fluorescence variation, Kd_1 is the apparent Ca^{2+} dissociation constant and n1 is the Hill coefficient for the first part of the curve, ΔF_{max2} is the maximum fluorescence variation, Kd_2 is the apparent Ca^{2+} dissociation constant and n2 is the Hill coefficient for the second part of the curve, shown between parenthesis. The values presented are the average and standard deviation of three independent titrations.

Complex	ΔF_{max}	Kd (M)	n
TnC-TnIF106**H**W *	-6 % (+3 %)	4.5 ± 0.3 e -8 (2.8 ± 0.5 e -6)	1.2 ± 0.2 (1.0 ± 0.3)
Tn-TnIF100**H**W	+ 12 %	3.1 ± 0.7 e -8	1.0 ± 0.1
TnC-TnIM121**H**W	+ 10 %	4.7 ± 1.1 e -7	2.0 ± 0.4
Tn-TnIM121**H**W	+ 70 %	3.3 ± 0.1 e -7	1.9 ± 0.1
TnCF29**H**W	+ 500 %	7.6 ± 1.6 e -6	1.0 ± 0.1
TnCF29**H**W-TnI	+ 500 %	6.4 ± 0.4 e -7	1.1 ± 0.1
Tn-TnCF29**H**W	+ 450 %	5.8 ± 0.1 e -7	1.0 ± 0.1

FIGURE LEGENDS

Fig. 1. Schematic model of TnC and TnI. (A) The structural Ca^{2+}-binding sites III and IV of TnC are represented as empty balls. The regulatory Ca^{2+}-binding sites I and II of TnC are represented as gray balls. The inhibitory region of TnI is highlighted in dark gray, the proposed modulatory region of TnI is highlighted in light gray. The original amino acid residues of each mutated positions in TnI are indicated. In each mutant only one position was mutated to Trp represented as empty bars. The natural Trp replaced by Phe in all double mutants is represented by a filled bar. The anti-parallel interaction of TnI and TnC is illustrated. (B) Comparison of the structure of Trp and 5HW. Our recombinant protein expression system incorporates 5HW in Trp codon positions.

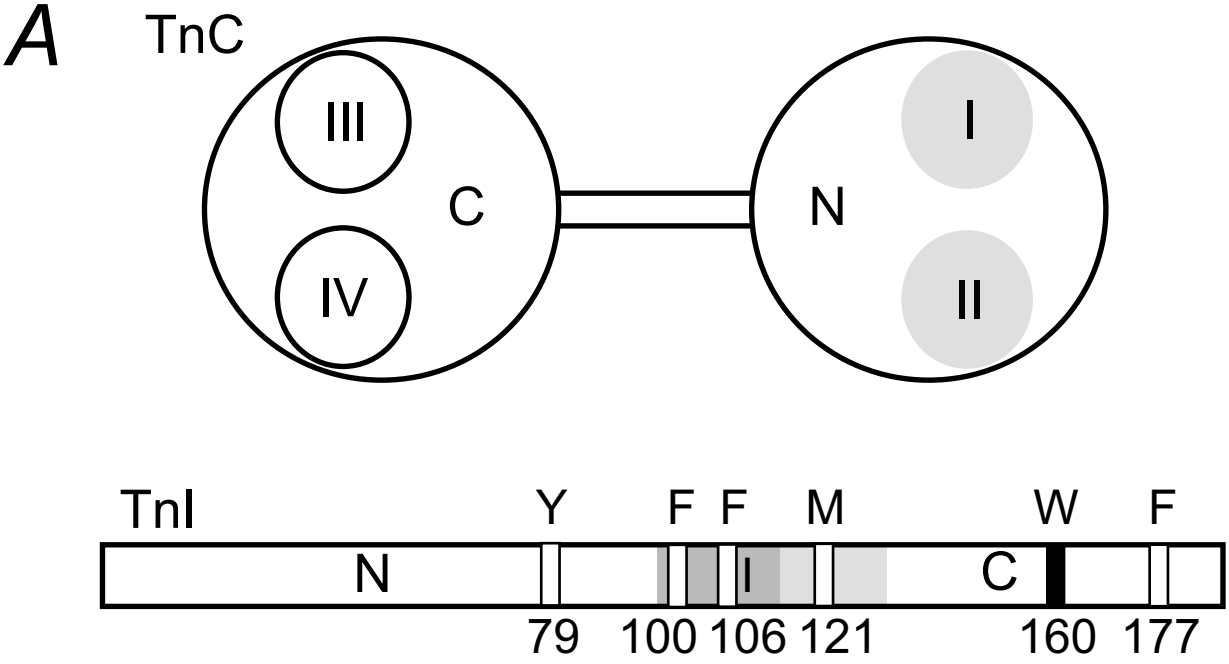
Fig. 2. Urea / PAGE analysis and reconstitution of troponin complexes. The ability of each mutant TnI to bind TnC was assessed by urea / PAGE in the presence of (A) 0.5 mM EDTA and (B) 0.5 mM Ca^{2+}. In the absence of Ca^{2+} only the band of free TnC is visible in the gel. When Ca^{2+} is present there is a second band corresponding to the binary complex, TnC-TnI. Lane 1, TnC; lane 2, TnC-TnI; lane 3, TnC-TnITrp-less; lane 4, TnC-TnIY79**H**W; lane 5, TnC-TnIF100**H**W; lane 6, TnC-TnIF106**H**W; lane 7, TnC-TnIM121**H**W; lane 8, TnC-TnI160**H**W; lane 9, TnC-TnIF177**H**W. (C) SDS / PAGE of the reconstituted ternary complexes with all TnI mutants, TnC and TnT. Lane 1, Tn; lane 2, Tn-TnITrp-less; lane 3, Tn-TnIY79**H**W; lane 4, Tn-TnIF100**H**W; lane 5, Tn-TnIF106**H**W; lane 6, Tn-TnIM121**H**W; lane 7, Tn-TnI160**H**W; lane 8, Tn-TnIF177**H**W.

Fig. 3. The 5HW fluorescent mutants of TnI. (A) Comparison between the fluorescence excitation spectra of Tn (.....) and Tn-TnI160**H**W (——). The thin doted line shows that the single 5HW of TnI160**H**W can be selectively excited at 315 nm in the presence of three Trp from TnT. As TnI160**H**W has the wild-type sequence, these two complexes are different only with respect to the hydroxyl group present in 5HW. Two ternary troponin complexes reconstituted with fluorescent mutants of TnI were sensitive to Ca^{2+}-binding: (B) Tn-TnIF100**H**W and (C) Tn-TnIM121**H**W showed significant increase in the fluorescence emission spectra in the Ca^{2+}-saturated state, pCa 4, (——) compared to the Apo state (.....).

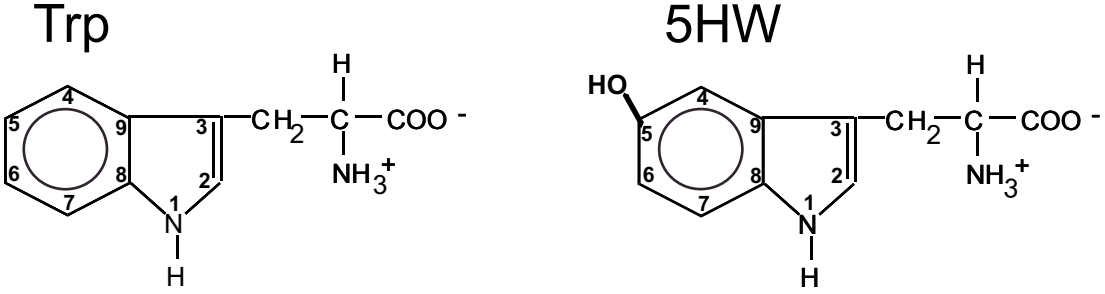
Fig. 4. Calcium titration of the fluorescent troponin complexes. (A) Ternary complexes Tn-TnIF100**H**W and Tn-TnIM121**H**W; (B) Binary complexes TnC-TnIF106**H**W and TnC-TnIM121**H**W; (C) TnCF29**H**W, TnCF29**H**W-TnI and Tn-TnCF29**H**W. The data is an average of three independent experiments, the error bars show the respective standard deviation. Lines are the best fit for the equations presented in Table 1.

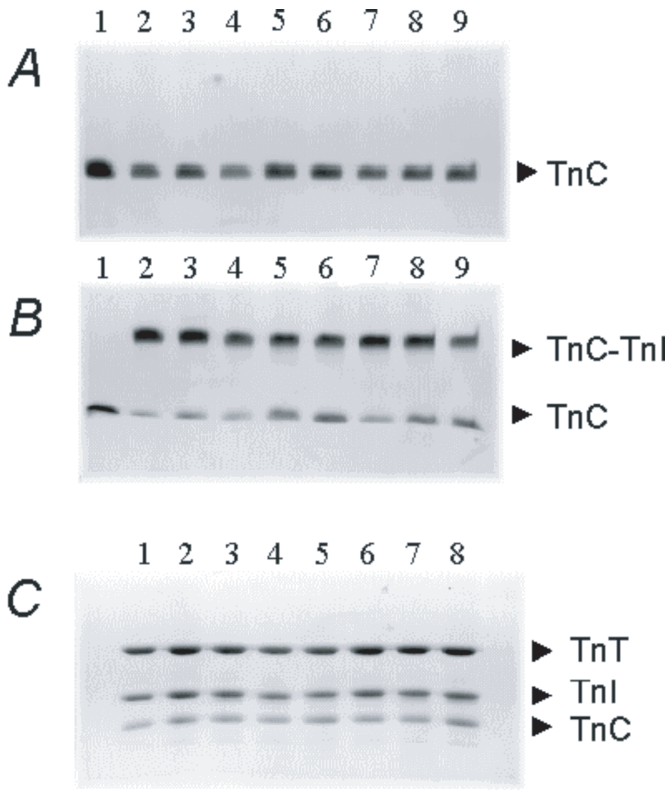
Fig. 5. Calcium titration of ternary troponin complexes with the fluorescent TnI and TnC, TnCD30A, TnCD66A, TnCD106A, TnCD142A. (A) Ternary troponin complexes with TnIF100**H**W. (B) Ternary troponin complexes with TnIM121**H**W. The data is an average of three independent experiments; the error bars show the respective standard deviation.

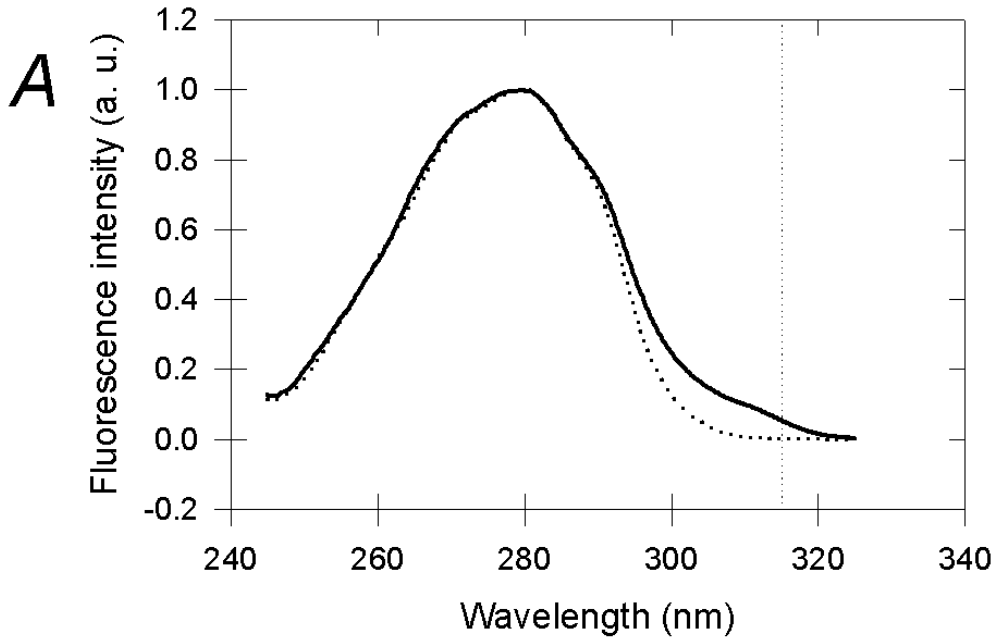
A

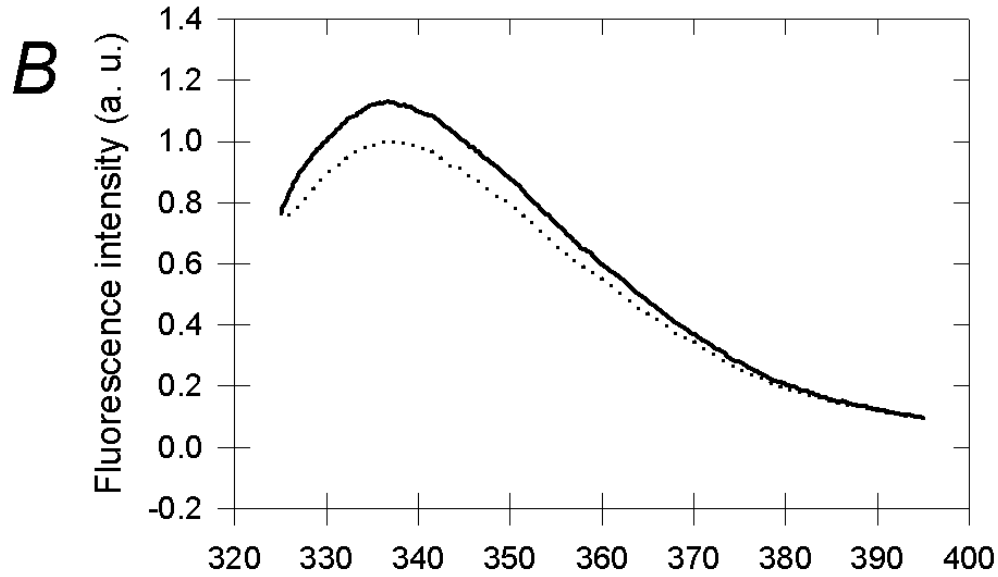

TnC
III
IV
C
N
I
II
TnI
Y
F
F
M
W
F
N
I
C
79
100
106
121
160
177

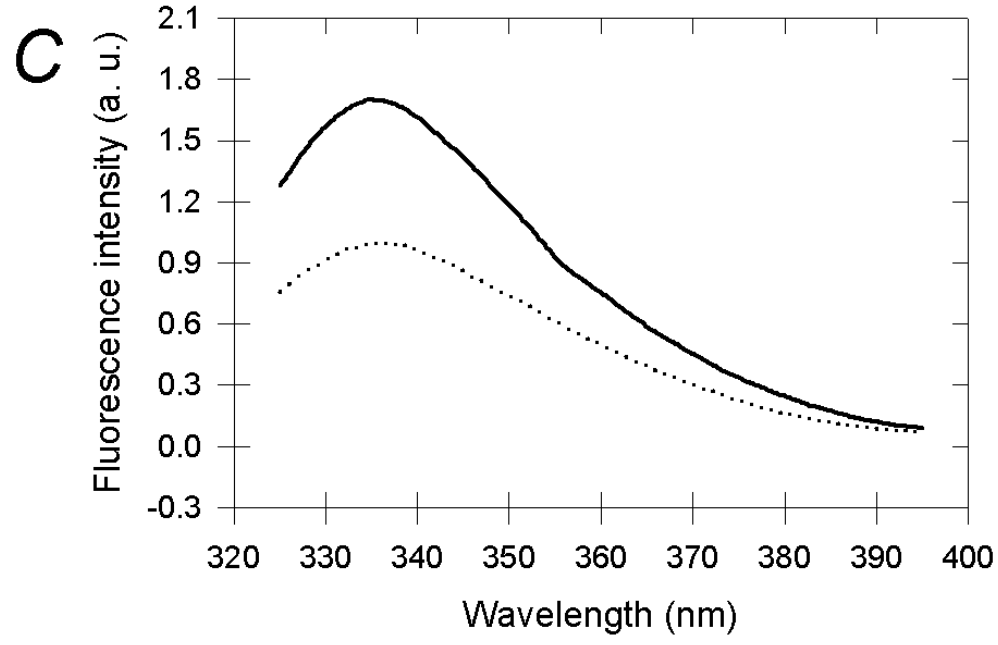
B

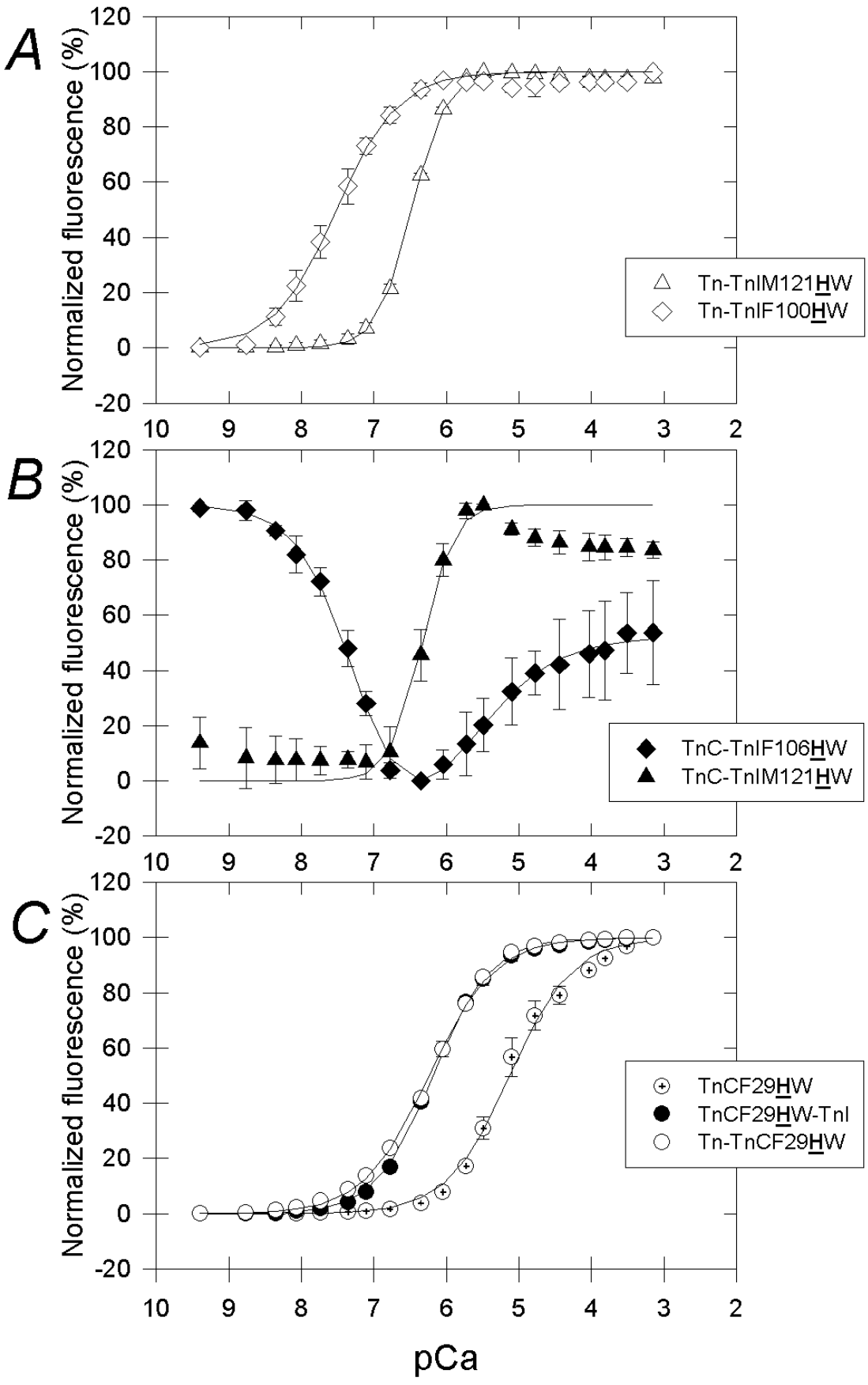

Trp
5HW
HO
H
COO -
NH3+
CH2
N
H

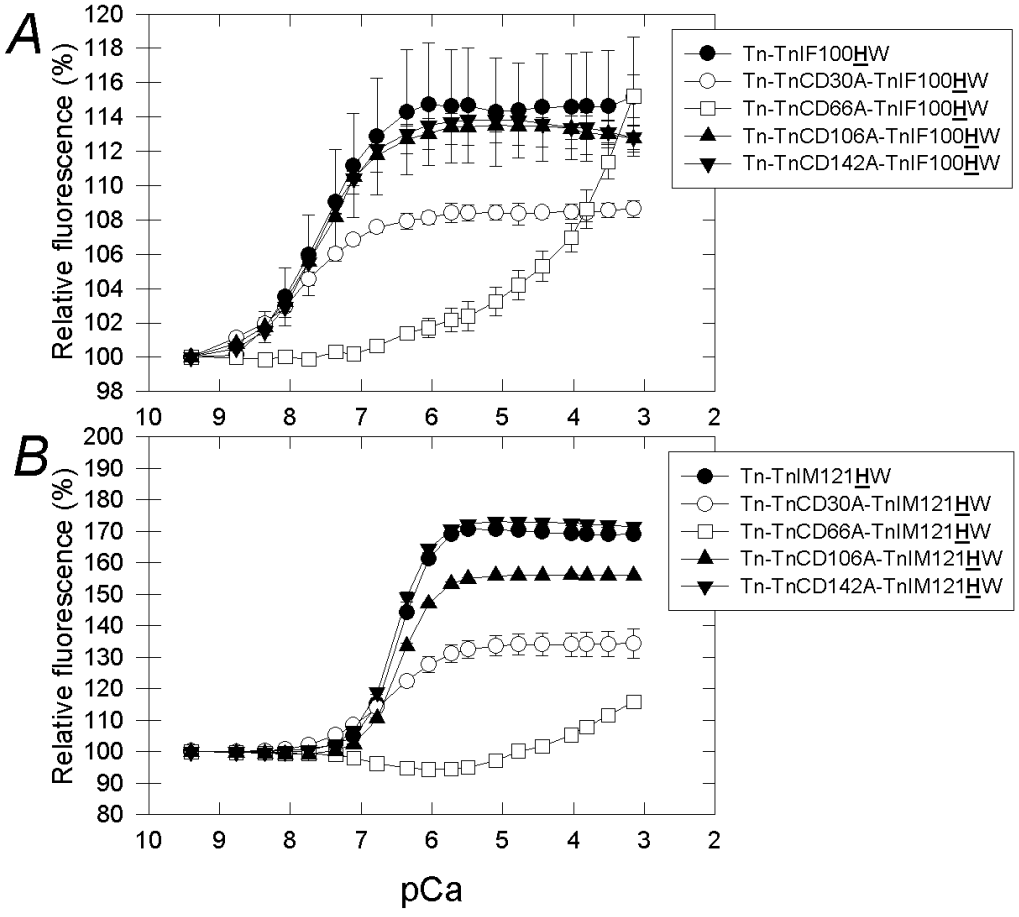

1 2 3 4 5 6 7 8 9
A
TnC
1 2 3 4 5 6 7 8 9
B
TnC-TnI
TnC
1 2 3 4 5 6 7 8
C
TnT
TnI
TnC


A
Fluorescence intensity (a. u.)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
240
260
280
300
320
340
Wavelength (nm)


B
Fluorescence intensity (a. u.)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
320
330
340
350
360
370
380
390
400


C
Fluorescence intensity (a. u.)
2.1
1.8
1.5
1.2
0.9
0.6
0.3
0.0
-0.3
320
330
340
350
360
370
380
390
400
Wavelength (nm)


A
B
C
Normalized fluorescence (%)
120
100
80
60
40
20
0
-20
10
9
8
7
6
5
4
3
2
pCa
Tn-TnIM121HW
Tn-TnIF100HW
TnC-TnIF106HW
TnC-TnIM121HW
TnCF29HW
TnCF29HW-TnI
Tn-TnCF29HW


A
B
Relative fluorescence (%)
pCa
Tn-TnIF100HW
Tn-TnCD30A-TnIF100HW
Tn-TnCD66A-TnIF100HW
Tn-TnCD106A-TnIF100HW
Tn-TnCD142A-TnIF100HW
Tn-TnIM121HW
Tn-TnCD30A-TnIM121HW
Tn-TnCD66A-TnIM121HW
Tn-TnCD106A-TnIM121HW
Tn-TnCD142A-TnIM121HW