FUNCTIONAL ANALYSIS OF A UNIQUE TROPONIN C MUTATION, GLY159ASP, THAT CAUSES FAMILIAL DILATED CARDIOMYOPATHY, STUDIED IN EXPLANTED HEART MUSCLE.

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ABSTRACT

Background. Familial dilated cardiomyopathy (DCM) can be caused by mutations in the proteins of the muscle thin filament. *In vitro* these mutations decrease Ca^{2+}-sensitivity and crossbridge turnover rate but the mutations have not been investigated in human tissue. We have studied the Ca^{2+}-regulatory properties of myocytes and troponin extracted from the explanted heart of a patient with inherited DCM due to the cTnC G159D mutation.

Methods and results. Mass spectroscopy showed that the mutant cTnC was expressed approximately equimolar with wild-type cTnC. Contraction was compared in skinned ventricular myocytes from the cTnC G159D patient and non-failing donor heart. Maximal Ca^{2+}-activated force was similar in cTnC G159D and donor myocytes but the Ca^{2+}-sensitivity of cTnC G159D myocytes was higher (EC_{50} G159D/donor = 0.59). Thin filaments reconstituted with skeletal muscle actin and human cardiac tropomyosin and troponin were studied by *in vitro* motility assay. Thin filaments containing the mutation had a higher Ca^{2+}-sensitivity (EC_{50} G159D/donor = 0.36±0.07) whilst the maximally activated sliding speed was unaltered. In addition, the cTnC G159D mutation blunted the change in Ca^{2+} sensitivity when TnI was dephosphorylated. With wild-type troponin, Ca^{2+}-sensitivity was increased (EC_{50} P/unP = 4.7±1.9) but not with cTnC G159D troponin (EC_{50} P/unP = 1.2±0.2).

Conclusions. We propose that uncoupling of the relationship between phosphorylation and Ca^{2+}-sensitivity could be the cause of the DCM phenotype. The differences between these data and previous *in vitro* results show that native phosphorylation of troponin I and troponin T and other post-translational modifications of sarcomeric proteins strongly influence the functional effects of a mutation.

INTRODUCTION

Dilated cardiomyopathy (DCM) is a common cause of sudden death and heart failure and it is estimated that 20-30% of cases of DCM are caused by mutations in specific proteins[1]. Many cases of inherited 'pure' DCM that are not associated with other symptoms, such as conduction disease, are caused by mutations in contractile proteins including actin, myosin, tropomyosin and all three subunits of troponin [1, 2]. These cardiomyopathy-causing mutations present a unique opportunity to link genotype with phenotype. Since mutations at different sites in several different contractile proteins can produce a single phenotype it has been proposed that mutations causing the same phenotype alter the contractile mechanism in the same way. In support of this hypothesis, a number of DCM mutations have been shown to cause decreased Ca^{2+}-sensitivity linked to reduced troponin C Ca^{2+}-binding affinity and decreased cross-bridge turnover rate *in vitro*[3-9]. However, recent work, studying both recombinant proteins and intact myofibrils, has given results that contradict the simple hypothesis and this raises doubts about the physiological relevance of the early studies [10-13].

It has been noted previously that the functional effect of cardiomyopathic mutations in sarcomeric proteins depends upon a number of extrinsic factors. Predominant amongst these are the level of expression of the mutant protein the species and isoform of the partner proteins used in reconstituted systems [4,14] and the post-translational modifications, particularly phosphorylation, of both the mutated and the partner proteins[11, 15-19]. These factors are often unknown and all of them are difficult to reproduce in the synthetic systems currently studied. Consequently, unravelling the links between genotype and phenotype in familial cardiomyopathies demands study of myopathic human cardiac tissue. The availability of such material from septal myectomy operations on hypertrophic cardiomyopathy (HCM) patients has yielded some information about phenotype-genotype

relationships in *MYH7* and *MYBPC3* mutations [20-24], but no studies have been reported on familial dilated cardiomyopathy mutations.

In this manuscript we report the first functional investigation of cardiac myocytes and isolated troponin from the explanted heart of a patient with familial dilated cardiomyopathy due to the mutation of glycine 159 to aspartic acid in troponin C (*TNNC1* gene) [25]. This is the only DCM-causing mutation that has been found in *TNNC1*[1] and as a result it has been extensively investigated *in vitro*. In experiments using filaments reconstituted from recombinant proteins the mutation has been shown to cause the typical DCM molecular phenotype of reduced Ca^{2+}-sensitivity and slower cross-bridge turnover [8, 9] but, interestingly, there is already evidence from studies using recombinant troponin C exchanged into more organised systems such as skinned skeletal or cardiac muscle fibres that the effects of this mutation on muscle function depend upon partner proteins and upon their phosphorylation levels [11, 19, 26]. We have obtained a tissue sample from a patient with this mutation, and have therefore been able to investigate this disease-causing mutation in a completely native context. This has enabled us to clarify the basic mechanism by which this mutation, and by analogy other DCM-causing mutations, may cause dilated cardiomyopathy in man.

METHODS

Human heart muscle samples

The *TNNC1* G159D mutation associated with DCM was identified in a single family by Mogensen *et al*[1]. A 3-year old child from this family presented with DCM (LVEDD 54mm, LVESD 50mm, EF 20%) and was given a heart transplant. This case has been described in detail by Kaski *et al*. [25]. The explanted heart muscle was cut into 0.5g pieces, was rapidly frozen in liquid nitrogen and stored for further analysis. Local ethical approval was obtained from University College London Hospitals and the Brompton, Harefield & NHLI ethics committees for collection and use of tissue samples. The investigation conformed with the principles outlined in the Declaration of Helsinki. Control heart muscle from 4 non-failing donor hearts was supplied by Prof. C Dos Remedios, University of Sydney, Australia. Ethical approval was obtained from The Brompton, Harefield & NHLI Research Ethics Committee, London and St Vincent's Hospital, Sydney. The donor heart tissue was obtained from hearts where no suitable transplant recipient was found. The donor patients had no history of cardiac disease, a normal cardiac examination, normal ECG and normal ventricular function on echocardiography within 24 hours of heart explantation. Clinical and functional characteristics of the donor heart samples have been previously reported [27].

Skinned myocytes

Skinned myocyte experiments were carried out as described previously [28], with minor modifications noted below. In brief, on the day of use, a section of the human tissue sample was removed from storage in liquid nitrogen and was thawed at 4^{o}C in Ca^{2+}-free relaxing solution containing protease inhibitors (Roche Complete Mini protease inhibitor cocktail tablets, 100µg/L). The tissue was homogenised in a mechanical blender, resulting in a suspension of single myocytes, cell fragments and small groups of cells. Myocytes were

permeabilised by treatment with relaxing solution containing 1% Triton X-100 for 15 min. The myocyte pellet was washed three times in relaxing solution and stored on ice for up to 10 hours.

A single myocyte was attached between a force transducer and a high-speed servomotor with silicone glue [29] and was stretched to a resting sarcomere length of 2.0 μm. Myocytes were gravity-superfused using a 3-barrel pipette attached to a stepper motor [30]. Isometric force was measured as the difference between the steady force during Ca^{2+} activation and the zero force reached during the period when the myocyte was made slack during activation by rapid shortening the myocyte by 20% for 40 ms. All measurements were made at 15°C. To determine the Ca^{2+}-sensitivity of force, isometric force measured in each submaximal Ca^{2+}-activation solution was expressed as a fraction of maximal Ca^{2+}-activated force (determined at 30 μmol/L Ca^{2+}). The force – $[Ca^{2+}]$ relationship data for each myocyte was fitted by a modified Hill equation. Maximal force was normalised to cross-sectional area. Cross-sectional was calculated using the width and depth of the myocyte assuming that the cell had an oval cross-section.

Human cardiac troponin

Human cardiac troponin was prepared from heart myofibrils using an anti-TnI antibody affinity column as described by Messer *et al* [27]. Phosphorylation of troponin I and troponin T was measured in SDS-PAGE using Pro-Q Diamond phosphoprotein specific stain as described previously [27]. Troponin was dephosphorylated by treatment with acid phosphatase [27]. Ca^{2+}-regulation of thin filaments containing human cardiac troponin was studied using the quantitative *in vitro* motility assay. These techniques have been previously described [20, 27].

Mass spectroscopy

Surface-Enhanced Laser Desorption/Ionization (SELDI) mass spectrometry was used to analyse G159D cTnC expression in affinity purified troponin from human heart tissue. Recombinant wild-type and G159D cTnC and troponin extracted from the tissue sample were dissolved in 8mol/L urea, 2mmol/L EDTA and applied to NP20 ProteinChips. The chips were rinsed with water to wash away salt and non-binding impurities and 2 x 1μL of a 50% saturated solution of sinapinic acid in 50% acetonitrile, 0.5% trifluoroacetic acid applied. The chips were read in a PBS IIc SELDI-TOF (Ciphergen, UK) calibrated with bovine insulin, ubiquitin, cytochrome C, myoglobin and serum albumin (Sigma, UK).

Statement

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

RESULTS

Identification and characterisation of troponin with TnC G159D mutation.

The presence of the G159D cardiac troponin C (cTnC) mutation in troponin isolated from the patient sample was confirmed by SELDI mass spectroscopy (Figure 1). SELDI-MS analysis of pure recombinant wild-type cTnC generated a peak at m/z 18,410 whilst the G159D cTnC generated a peak at m/z 18,462 (Figure 1A). These peaks represent singly charged cTnC ions (calculated molecular weights for wt and G159D cTnC being 18402 and 18460 respectively). Spectra of the affinity purified heart troponin showed major peaks at m/z ~18500, 24000 and 34600 consistent with singly charged ions of cTnC, TnI and TnT respectively (Figure 1B). Closer examination of the cTnC peak(s) of the troponin isolated from the patient's heart muscle clearly showed the presence of two cTnC components with different m/z values. These two components are separated by approximately m/z 60 (m/z 18,424 and 18,484) consistent with the 58 Da increase in mass introduced by the glycine to aspartic acid mutation and hence correspond to normal and G159D cTnC (Figure 1A).

We observed that recombinant cTnC and G159D cTnC spectra were additive in mixtures (see supplemental data B) therefore we could estimate the fraction of mutated cTnC in the muscle sample by comparison with synthetic spectra of mixtures at varying ratios. Figure 1C shows that the best fit was obtained with 55% G159D cTnC in the patient heart muscle troponin sample.

The contractile protein composition of myofibrils and isolated troponin from donor and G159D cTnC hearts were indistinguishable, in particular, the troponin C content of myofibrils was the same in all the samples we examined (Figure 2A). Comparison of phosphorylation of MyBP-C, troponin T, troponin I and MLC-2 in myofibrils showed no significant difference between donor and patient troponin (Figure 2A), whilst comparison of phosphorylation of

troponin I and T in SDS-PAGE of isolated troponin likewise showed no differences between donor and G159D cTnC samples (Figure 2B).

Contractility of skinned myocytes

We investigated the myofilament-based contractile function in human skinned ventricular myocytes prepared from 4 control (unused donor) hearts and from the G159D cTnC patient. We found no visible difference in the sarcomeric structure between skinned donor and G159D cTnC myocytes. A range of submaximal Ca^{2+} activating solutions was used to determine myofibrillar Ca^{2+}-sensitivity. There was consistency between the force-pCa curves in several myocytes from each patient and there was no difference in mean EC_{50} values between the 4 donor patients (P = 0.11; one-way ANOVA). The data from all donor patients was therefore combined to give the average force-pCa curve for skinned human donor myocytes (Figure 3A). We found that the Ca^{2+}-sensitivity of G159D cTnC myocytes was significantly greater than for donor myocytes (Figure 3A, Table 1), with mean tension EC_{50} values of 0.49 ± 0.04 μmol/L (n=9 myocytes) and 0.80 ± 0.04 μmol/L (n=22), respectively (P<0.001, unpaired t-test). The steepness of the force-Ca^{2+} relationship was also lower in human G159D cTnC than in donor myocytes, with Hill *n* values of 1.76 ± 0.12 and 2.99 ± 0.18, respectively (P<0.001, unpaired t-test). As shown in Figure 3B, maximum force was found to be similar in G159D cTnC and donor myocytes (P = 0.66, unpaired t-test).

Thin filament regulation measured by in vitro motility assay

Thin filaments containing human cardiac troponin and tropomyosin were studied. G159D cTnC and donor troponin were equally effective at regulating actin-tropomyosin filaments in the *in vitro* motility assay. At low [Ca^{2+}] (1 nmol/L) the thin filament motility for both G159D cTnC and donor troponin was switched off by 40nmol/L troponin, with the fraction motile falling to approximately 0.05. The sliding speed was reduced by 35±5% for G159D

cTnC troponin and 38±7% for donor troponin (means of 7 preparations, p=0.75 in paired t-test). At high [Ca^{2+}] (3.9 µmol/L), 40nmol/L human cardiac troponin increased sliding speeds by 7% over actin-tropomyosin as has been previously observed [31]. In these experiments the increase was not statistically significant and there was also no significant difference between G159D cTnC and donor troponin (results not shown). The mean fraction motile at high Ca^{2+} was not significantly different for the two troponin species (G159D cTnC= 0.83±0.04; donor =0.82±0.05, n=7, P = 0.61). Sliding speed was similarly unaffected by the presence of the mutant cTnC (P = 0.92).

When we measured Ca^{2+}-sensitivity of thin filaments reconstituted with G159D cTnC and donor troponin we found the Ca^{2+}-sensitivity of thin filaments containing G159D cTnC troponin was higher than donor for both the fraction motile and sliding speed parameters when they were compared in the same dual chamber motility cell. As previously found with this assay method, there was considerable variation in the absolute value of EC_{50} between assays with different troponin, myosin and actin preparations, but paired measurements in the same motility cell always produced a consistent difference between G159D cTnC and donor troponin. In the example in Figure 4, showing the results from one troponin preparation, the G159D cTnC curve was shifted to the left and the calculated Ca^{2+}-sensitivity was 4.4-fold higher than donor troponin. The results from three pairs of troponin preparations, shown in supplemental data A1 and summarised in table 1, confirm the higher Ca^{2+}-sensitivity with the mutant troponin with the mean ratio EC_{50} G159D/donor for fraction motile = 0.36±0.07 and sliding speed= 0.12±0.03). The calculated Hill coefficient was very variable and was not significantly different between G159D cTnC and donor troponin (mean n_H=1.4±0.2, n=12, all assays pooled).

The effect of changing troponin phosphorylation levels

Troponin I and troponin T were natively phosphorylated in G159D cTnC and donor troponin (Figure 2) but our previous studies with this mutation used unphosphorylated recombinant troponin [8]. Therefore we determined the effect of complete dephosphorylation of troponin using acid phosphatase. Figure 5 shows two typical measurements. With donor troponin we observed higher Ca^{2+}-sensitivity and decreased sliding speed with dephosphorylated troponin as previously reported[27]. In contrast, native and dephosphorylated G159D cTnC troponins exhibited similar Ca^{2+} sensitivities and there was no significant effect of dephosphorylation on the maximum sliding speed. Thus the dephosphorylated G159D cTnC- containing thin filaments were not responsive to the phosphorylation change shown by the donor troponin. In pooled data from paired measurements with three troponin preparations EC_{50} G159D cTnC/ G159D cTnC dP was 1.24±0.17, p=0.29. When dephosphorylated G159D cTnC and donor troponins were compared directly in paired experiments, the Ca^{2+}-sensitivity of G159D cTnC remained greater than donor (EC_{50} G159 cTnC dP/donor dP = 0.56±0.09, n=2, Table 1 and supplemental data A2).

The effect of changing tropomyosin

Previous studies using unphosphorylated recombinant proteins showed a lower Ca^{2+}-sensitivity with G159D cTnC as well as with other DCM mutations in troponin T and tropomyosin[8]. In contrast, the patient sample of G159D cTnC troponin exhibited a higher Ca^{2+}-sensitivity than donor cTnC, when studied using native human troponin and tropomyosin, and its Ca^{2+}-sensitivity was independent of phosphorylation (Table 1, Figures 3,4,5). To test whether this effect is related to partner proteins, we substituted the native human cardiac tropomyosin used in these studies with a recombinant α-tropomyosin expressed in *E.coli* with an N-terminal Ala-Ser extension to compensate for the absence of the

native N-terminal acetylation (AS-α-tropomyosin) [32]. Recent studies show this tropomyosin species is functionally different from native cardiac tropomyosin [12]. In the presence of AS-α-tropomyosin we observed a 2.6-fold lower Ca^{2+}-sensitivity with G159D cTnC troponin compared with donor troponin, similar to previous experiments using recombinant troponin subunits (Figure 6, Table 1).

DISCUSSION

Mutations that cause familial dilated cardiomyopathy without complications, such as conduction disease, have been found in most of the proteins of the cardiac muscle sarcomere, however only one DCM-associated mutation has so far been identified in *TNNC1* [1]. The cTnC G159D mutation was found in a single family and genotypes were obtained over five generations. This appears to be a particularly malignant mutation, since every individual with the mutation has been affected and in particular the individual studied here required a heart transplant at age 3 [25]. Despite the young age of the patient the tissue sample contained adult isoforms of contractile proteins and contractile protein phosphorylation levels indistinguishable from adult donor heart (Figure 2) [1]. The mechanism by which mutations in thin filament proteins cause DCM has been extensively studied using recombinant proteins and a consistent pattern of decreased Ca^{2+}-sensitivity and slower crossbridge turnover has emerged [8, 33]. This manuscript reports the first study of mutated troponin from human heart and it is evident that the functional effects of the mutation are quite strikingly different from the same mutation studied in synthetic systems.

Mass spectroscopy of the troponin from the heart muscle sample shows clearly that the cTnC G159D mutation predicted from genetic studies is expressed in heart as about half of total cTnC (Figure 1) and SDS-PAGE indicates normal levels of all three troponin subunits in the isolated troponin we have studied (Figure 2), therefore it is likely that the G159D cTnC mutation acts as a poison peptide by changing thin filament regulation. In functional measurements at both the myocyte and myofilament level the mutation causes an increase in Ca^{2+}-sensitivity with no change in crossbridge turnover rate, as assessed by the maximum sliding speed (Figures 3,4, Table 1). These findings are opposite to that previously found with recombinant DCM mutations, where Ca^{2+}-sensitivity and crossbridge turnover rate were

found to be decreased [34]. Increased Ca^{2+}-sensitivity is a characteristic of mutations associated with hypertrophic cardiomyopathy [9], but the patient showed no symptoms of hypertrophy preceding heart failure and this mutation has been consistently linked to the DCM phenotype [1, 25]. Thus this unique study of a DCM mutation obtained from patient muscle is not consistent with the experiments using equivalent mutant recombinant proteins. These paradoxical results seem to suggest that previous *in vitro* experimentation, implicating an association of lower Ca^{2+}-sensitivity and crossbridge turnover rate with the DCM phenotype may be incorrect. It is interesting to note that an investigation of DCM-causing mutations in α-tropomyosin also concluded that decreased Ca^{2+}-sensitivity may not be the primary trigger for the DCM phenotype[12].

Recent evidence suggests that defects in the modulation of Ca^{2+}-sensitivity by troponin I phosphorylation may play a significant role in the mechanism of inherited DCM. In a study of a DCM-causing mutation in cardiac actin (*ACTC* E361G) expressed in a transgenic mouse we have found that the only difference between the transgenic and non-transgenic mouse was that the relationship between Ca^{2+}-sensitivity and troponin phosphorylation was uncoupled in the transgenic mouse [16]. In donor heart troponin, Ca^{2+}-sensitivity was increased when the troponin was dephosphorylated, however we found that Ca^{2+}-sensitivity in the G159D cTnC troponin was almost independent of the level of troponin phosphorylation (Figure 5, Table 1). Therefore, by analogy with the *ACTC* E361G study, we could propose that the uncoupling of the relationship between phosphorylation and Ca^{2+}-sensitivity is the primary cause of this DCM phenotype, at least for this mutation. This provides a novel mechanism for initiation of DCM; if thin filaments do not respond to PKA phosphorylation, the contractile response to β-adrenergic stimulation (increased relaxation rate and power output) will be blunted. This would cause cardiac relaxation rate and contractile reserve to be reduced in the same way as they are in acquired heart failure, where troponin I becomes dephosphorylated, resulting in a

higher than normal Ca^{2+}-sensitivity [27, 35, 36]. The combination of high Ca^{2+}-sensitivity, which has not been seen with other DCM-causing mutations[10, 13, 16], and blunted response to adrenergic stimulation may account for the severity and early onset of DCM with the G159D cTnC mutation [1, 25].

The findings from this human biopsy study of G159D cTnC are compatible with recently published animal studies that used recombinant G159D cTnC in skinned muscle [11, 19]. Biesiadecki *et al.* reported that G159D cTnC specifically blunted the phosphorylation-induced decrease in Ca^{2+}-sensitive tension development, without altering the increase in cross-bridge cycling, when the mutant troponin was exchanged into rat heart trabeculae. Moreover, measurements of Ca^{2+} binding by TnI-TnC complexes *in vitro* have shown that the dependence of Ca^{2+} binding affinity on troponin I phosphorylation was lost in the presence of the G159D cTnC mutation [11, 37].

In contrast to the results from G159D cTnC using native troponin, reported here, this mutation previously produced a decrease in Ca^{2+}-sensitivity or Ca^{2+} affinity when tested with recombinant troponin subunits [8, 9] . We also found a lower Ca^{2+}-sensivity with native troponin containing G159D cTnC when we used recombinant AS-α-tropomyosin in place of native human tropomyosin (Figure 6, Table 1). These findings emphasise the importance of studying the subtle functional consequences of cardiomyopathy-causing mutations in a native context. We have already shown that the effect of a mutation depends on the fraction of mutant protein present and also on the species origin of partner proteins [14]. The current work emphasises that phosphorylation of troponin I and troponin T and post-translational modifications of tropomyosin may strongly influence the functional effect of a mutation[38]. It is therefore vitally important to conduct experiments in as near-native conditions as possible and also, when possible, to check *in vitro* experiments against the results obtained using

native tissue from patients with the corresponding cardiomyopathy.

Could uncoupling of the TnI phosphorylation effect on EC_{50} be a common mechanism of familial DCM? We have shown that this mechanism explains the G159D data and it is also a likely explanation for the results from the ACTC E361G transgenic mouse [6]. In addition the hypothesis is not incompatible with published work, since the relationship between the effect of a mutation and troponin phosphorylation has not yet been tested with human sequence actin, tropomyosin and troponin for any other DCM mutations [8-10,12,13].

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CONFLICT OF INTEREST DISCLOSURES

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TABLE 1 LEGEND

Effect of the *TNNC1* G159D mutation on Ca^{2+}-sensitivity of thin filaments from patient muscle and in reconstituted systems, measured by *in vitro* motility assay.

Footnotes:

Due to variability between preparations of troponin, actin and myosin comparisons can only be made within an experiment (rows) and not between experiments (columns).

Further details are presented in the supplemental data

FIGURE LEGENDS

Figure 1 Detection and quantification of G159D cTnC by SELDI-MS

A) SELDI-MS spectra of troponin C. Normalised peak signal intensity is plotted against mass to charge (m/z) ratio. Overlaid spectra of i) recombinant native cTnC (green), ii) recombinant G159D cTnC (blue) and iii) G159D cTnC human cardiac troponin sample (black).

B) Spectrum of whole human troponin (see figure 2B for SDS-PAGE of this troponin sample). Peaks are seen for troponin C (m/z 18,495), troponin I (m/z 23,956) and troponin T (34,566 m/z). For comparison spectra of TnC mixtures of cTnC and cTnC cG159D are shown in the supplemental data section B.

C) Comparison of the G159D cTnC human cardiac troponin spectrum (thick red line) with synthetic spectra containing 50% G159D cTnC (black), 55% G159D cTnC (blue), 60% G159D cTnC (green) and 70% G159D cTnC (purple).

Figure 2. Comparison of protein content and phosphorylation of native and G159D cTnC mutant myofibrils and purified troponin

A) Myofibrils from donor and G159D cTnC heart muscle stained with Flamingo pink total protein stain and with Pro-Q Diamond phosphoprotein stain. The troponin C content of myofibrils was the same in all the samples we examined (ratio band volume TnC/MLC-2 = 0.353±0.004 for donor heart myofibrils and 0.355±0.006 for G159D TnC myofibrils, n=5). Phosphorylation levels were also the same in donor and G159D cTnC myofibrils: mean Pro-Q Diamond/Flamingo Pink band volumes for 5 samples were as follows: MyBP-C, donor=0.488±0.018, G159D cTnC = 0.489±0.038; Troponin T, donor=0.634±0.08, G159D cTnC = 0.570±0.046; Troponin I, donor 1.19±0.03, G159D cTnC = 1.34±0.04; MLC-2, donor=0.147±0.023, G159D cTnC = 0.132±0.017

B) Purified troponin from donor and G159D cTnC heart muscle stained with Coomassie Blue and Pro-Q Diamond. TnI and TnT are phosphorylated. The level of phosphorylation was measured by comparison with the pure TnI standard. The mean TnI phosphorylation was 2.03±0.01 molPi/mol in donor and 2.23±0.01 in G159D cTnC and mean TnT phosphorylation was 2.83±0.01 molPi/mol in donor and 2.74±0.02 in G159D cTnC (n=7)

Figure 3. Contractile properties of skinned donor and G159D cTnC myocytes.

Ca^{2+}-sensitivity of isometric force. Submaximal forces in each myocyte were expressed relative to the maximum force (at 30 μmol/L Ca^{2+}) in the same myocyte. Data are from 4 donor hearts (21 cells) and the G159D cTnC mutant heart (8 cells). Error bars indicate S.E.M.

Inset: Maximum force in skinned myocytes from human donor (open bar) and G159C cTnC tissue (filled bar).

Figure 4 Comparison of Ca^{2+}- activation of motility of thin filaments reconstituted with native donor or native G159DcTnC troponin.

Donor and G159D cTnC troponin were compared in the same motility cell. Sliding speed (top) and fraction of filaments motile (bottom) derived from the same motility experiment. Points are mean and standard error of 4 measurements made in a single motility cell. ❍ and dotted line, native donor thin filaments. Hill equation fits to sliding speed: EC_{50}=0.074±0.134 μmol/L, n_H = 5.9±2.0, fraction motile: EC_{50}=0.044±0.002 μmol/L, n_H= 1.38±0.06. ● and solid line, G159D cTnc thin filaments. Hill equation fits to sliding speed: EC_{50}=0.019±0.093 μmol/L, n_H= 9.2±20, fraction motile: EC_{50}=0.010±0.010 μmol/L, n_H= 2.9±6.2.

Figure 5 Effect of dephosphorylation on Ca^{2+}-sensitivity measured by IVMA with donor or G159D cTnC troponin

Donor and G159D cTnC were assayed in separate experiments. Points shown are mean and standard error of 4 measurements made in a single motility cell.

A Donor heart troponin. ❍ native troponin, ❑ dephosphorylated troponin.

B G159D cTnC troponin. ● native troponin, ■ dephosphorylated troponin.

C Table showing calculated fits of the data to the Hill equation.

D SDS-PAGE of native and dephosphorylated troponin stained with Pro-Q Diamond showing that TnT and TnC are fully dephosphorylated by acid phosphatase treatment.

Figure 6 Ca^{2+}-sensitivity of thin filaments reconstituted with AS α-tropomyosin and donor or G159D cTnC troponin

The figure shows the sliding speed (top) and fraction of filaments motile (bottom) derived from the same motility experiment. Points are mean and standard error of 4 measurements made in a single motility cell. ❍ and dotted line, thin filaments containing AS-α-tropomyosin and donor troponin. Hill equation fits to fraction motile: EC_{50}=0.149±0.031 μmol/L, n_H= 1.2±0.2, sliding speed: EC_{50}=0.120±0.081 μmol/L, n_H= 5.5±4.6. ● and solid line, thin filaments containing AS-α tropomyosin and G159D cTnC troponin. Hill equation fits to fraction motile: EC_{50}=0.40±0.07 μmol/L, n_H= 4.4±3.9, sliding speed: EC_{50}=0.36±0.15 μmol/L, n_H= 1.4±0.7.

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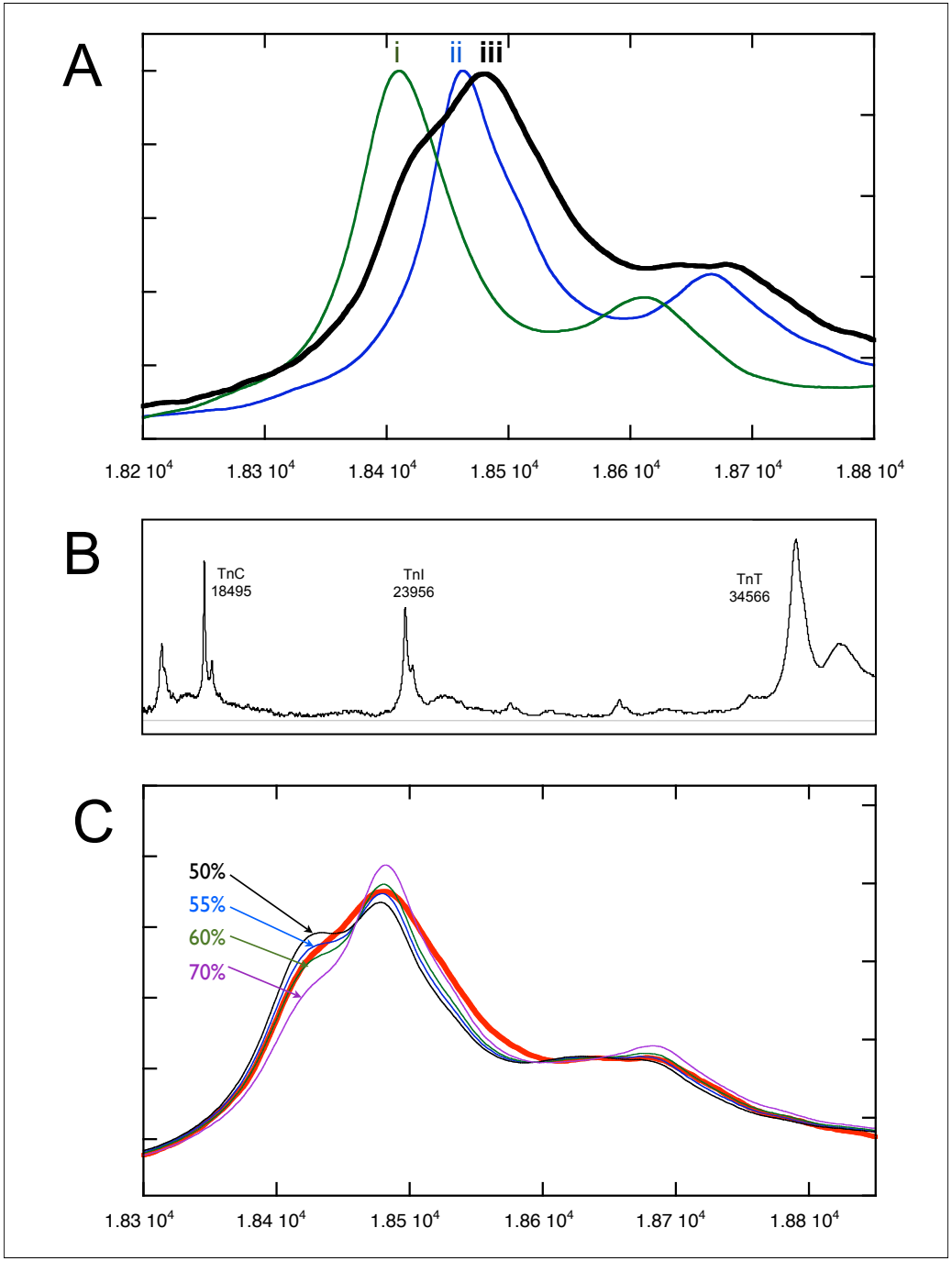
system	method		Control	G159D	n (paired experiments)	Ratio EC_{50} G159D/control
Human heart muscle	Skinned myocytes	Force $EC_{50,}$ µmol/L	0.80±0.01 (n=22)	0.48±0.01 (n=8)	-	0.60
Human heart Troponin and tropomyosin	Motility of thin filaments	Sliding speed EC_{50} µmol/L	0.12±.07	0.014±0.03	3	0.12±0.03
		Fraction motile EC_{50}, µmol/L	0.14±0.05	0.05±0.03	3	0.36±0.07
Dephosphorylated human heart Troponin and tropomyosin	Motility of thin filaments	Sliding speed EC_{50} µmol/L	nd	nd		
		Fraction motile EC_{50}, µmol/L	0.12±.01	0.07±0.01	2	0.56±0.09
Human heart Troponin, recombinant tropomyosin	Motility of thin filaments	Sliding speed EC_{50} µmol/L	0.21±0.05	0.38±0.12	3	1.9±0.5
		Fraction motile EC_{50}µmol/L	0.15±0.03	0.31±0.13	5	2.0±0.8
Recombinant Troponin (50%), Human heart Tropomyosin (Mirza et al.)	Motility of thin filaments	Sliding speed EC_{50} µmol/L	0.32±0.19	0.50±0.1	3	1.5±0.2
		Fraction motile EC_{50} µmol/L	0.25±0.03	0.40±0.03	3	1.5±0.1


A
i
ii
iii
1.82 10^4
1.83 10^4
1.84 10^4
1.85 10^4
1.86 10^4
1.87 10^4
1.88 10^4
B
TnC
18495
TnI
23956
TnT
34566
C
50%
55%
60%
70%
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1.84 10^4
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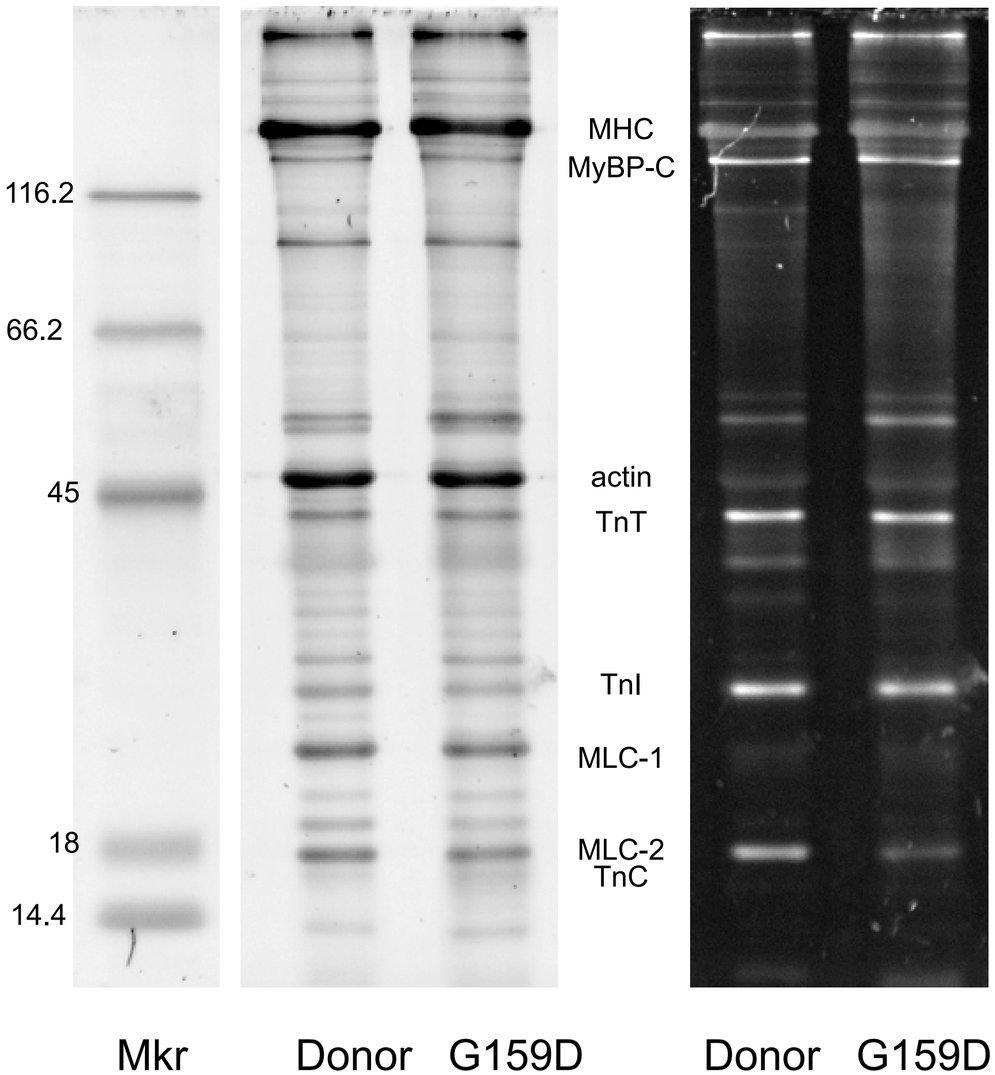
1.87 10^4
1.88 10^4

A


Flamingo
Pro-Q Diamond
116.2
66.2
45
18
14.4
MHC
MyBP-C
actin
TnT
TnI
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MLC-2
TnC
Mkr
Donor
G159D
Donor
G159D

B

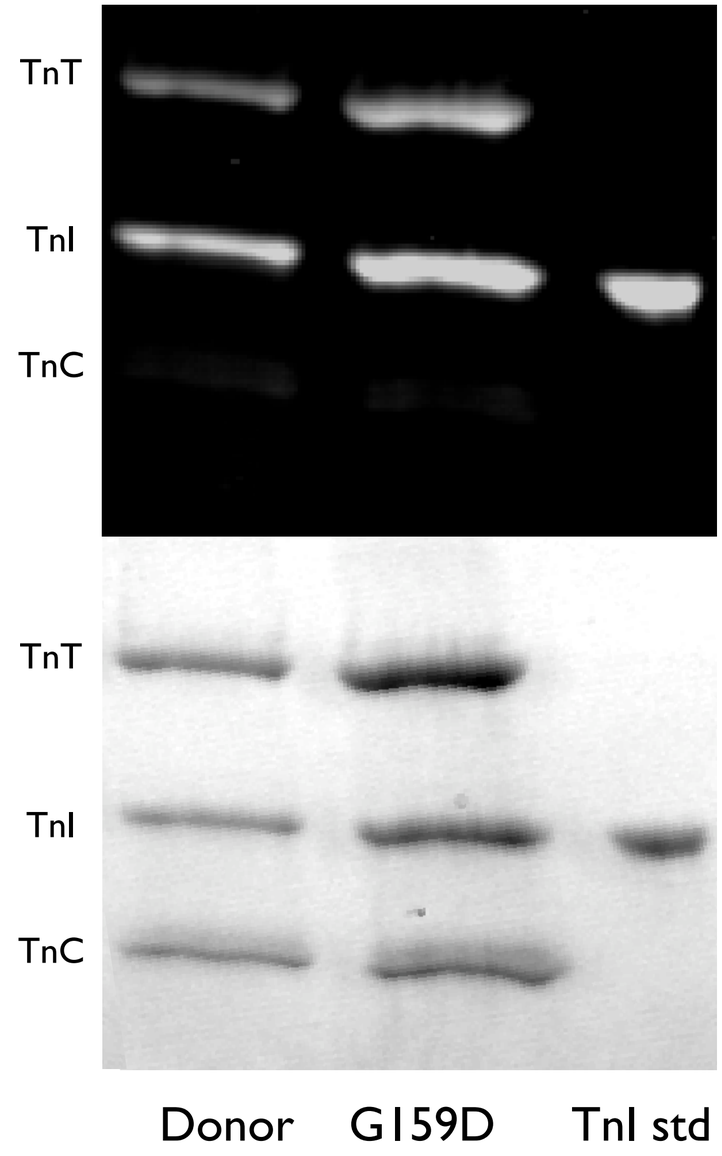

TnT
TnI
TnC
Pro-Q Diamond
TnT
TnI
TnC
Coomassie Blue
Donor
G159D
TnI std

Figure 2

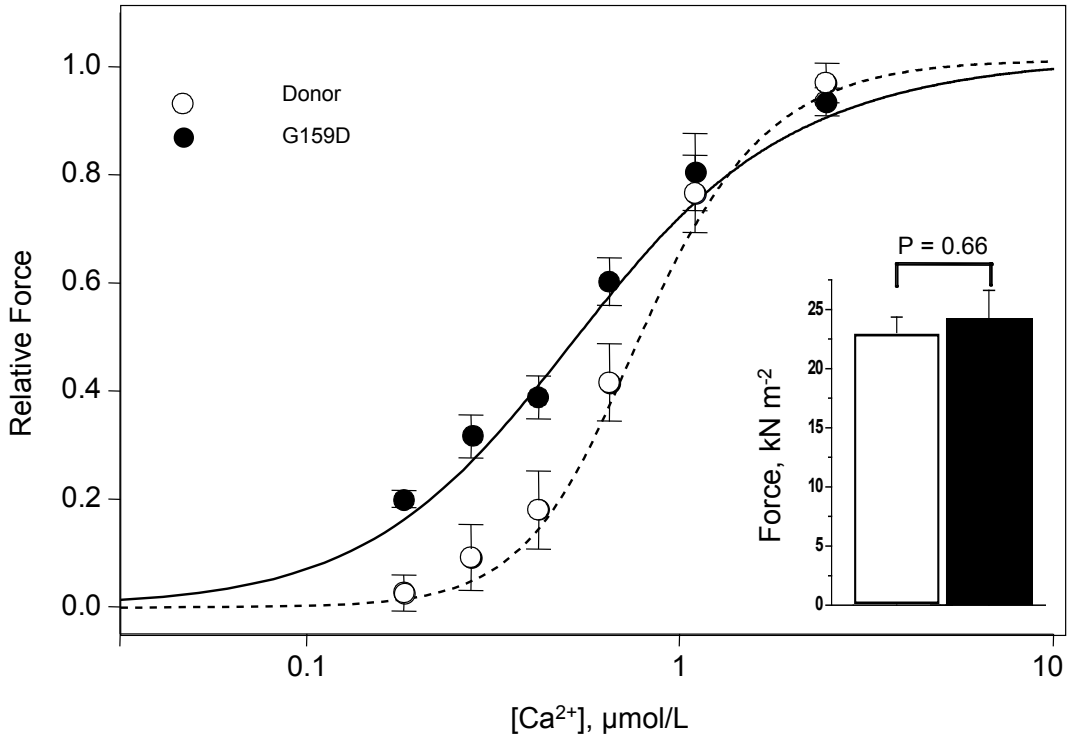
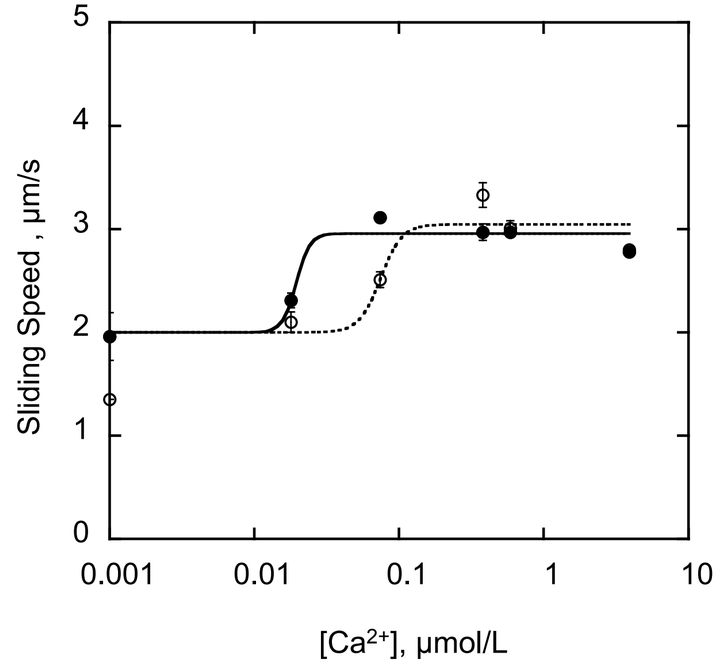

Donor
G159D
Relative Force
1.0
0.8
0.6
0.4
0.2
0.0
0.1
1
10
[Ca2+], µmol/L
P = 0.66
Force, kN m-2
25
20
15
10
5
0

Figure 3


Sliding Speed , µm/s
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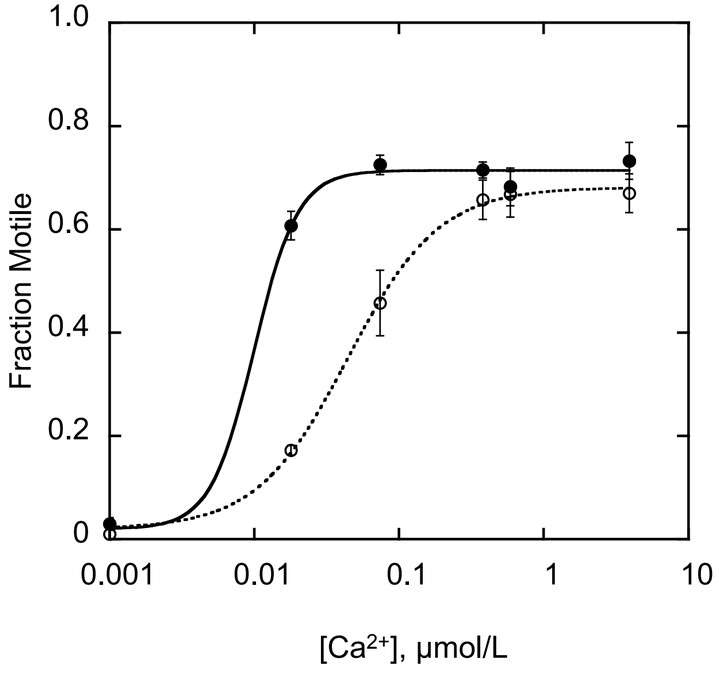
4
3
2
1
0
0.001
0.01
0.1
1
10
[Ca2+], µmol/L


Fraction Motile
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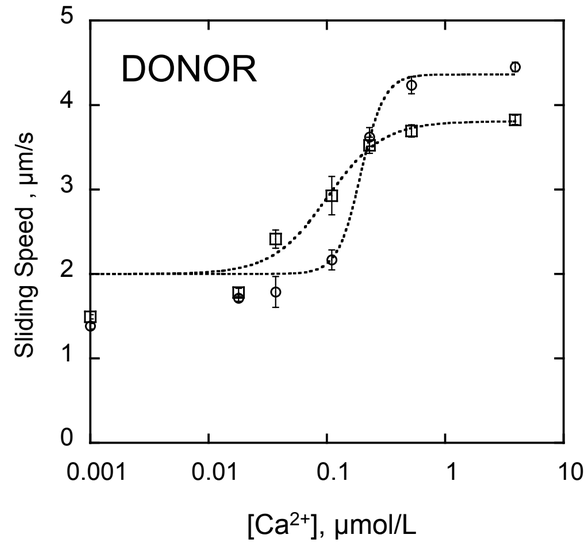
0.2
0
0.001
0.01
0.1
1
10
[Ca2+], µmol/L

Figure 4

A



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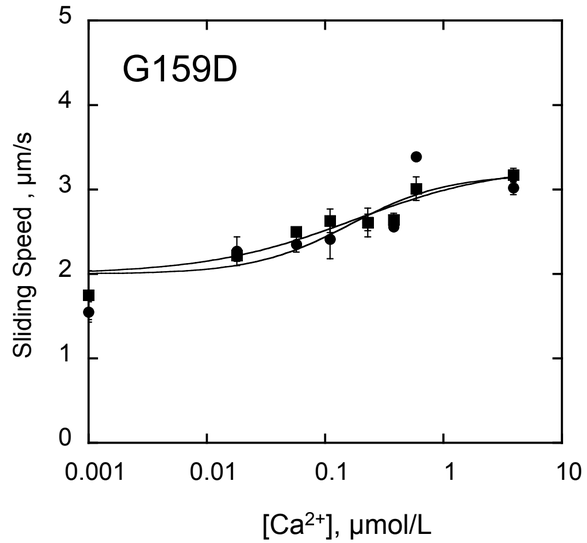
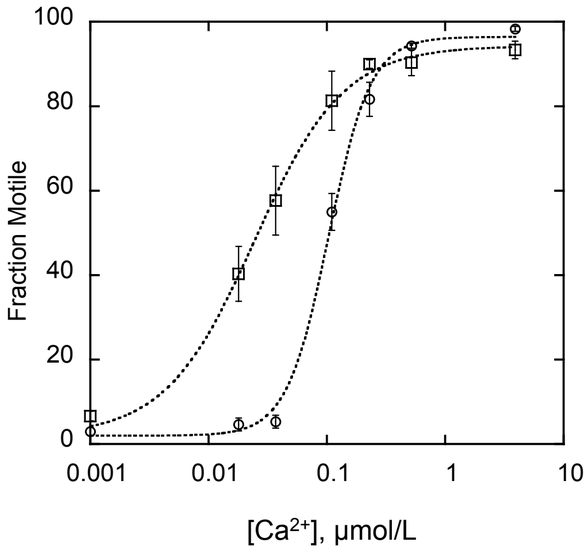
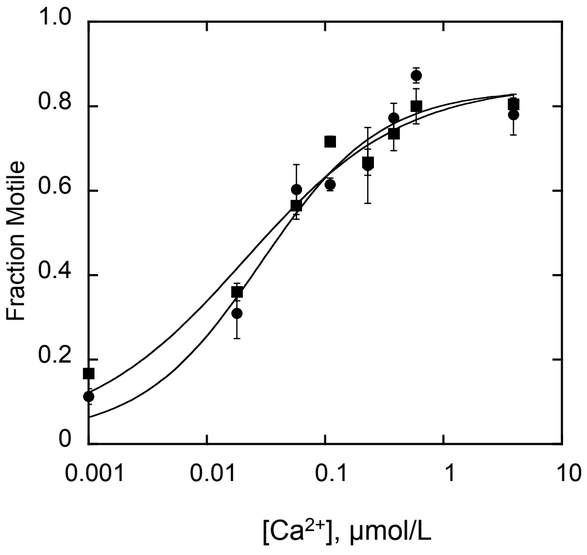


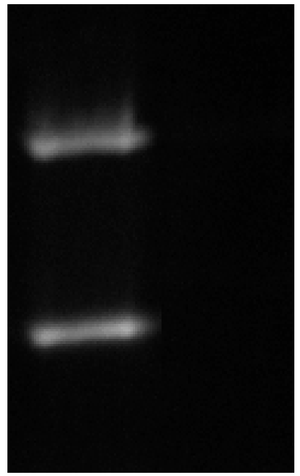




C

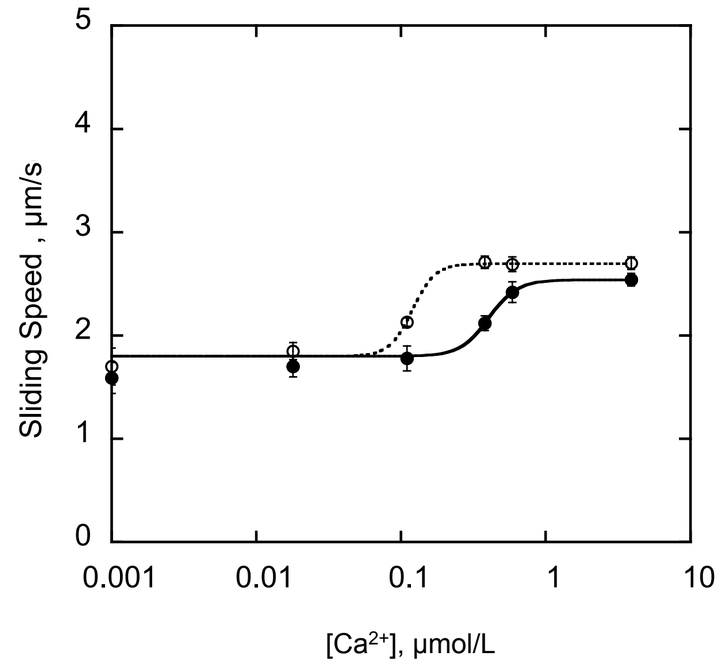
	Donor troponin			G159D cTnC troponin		
	Native	Dephos-phorylated	Ratio n/dP	Native	Dephos-phorylated	Ratio n/dP
Sliding speed at 3.9µMol/L Ca^{2+}, µm/sec	4.4±0.2	3.8±0.2	**1.15**	2.0±0.4	2.2±0.9	**0.91**
Sliding speed EC_{50}, µMol/L	0.171±0.017	0.063±0.012	**2.7**	0.178±0.185	0.164±0.165	**1.08**
Fraction motile EC_{50}, µMMol/L	0.103±0.007	0.026±0.002	**3.9**	0.035±0.014	0.033±0.009	**1.06**

D



Native Dephos-phorylated

FIGURE 5


Sliding Speed , µm/s
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4
3
2
1
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0.01
0.1
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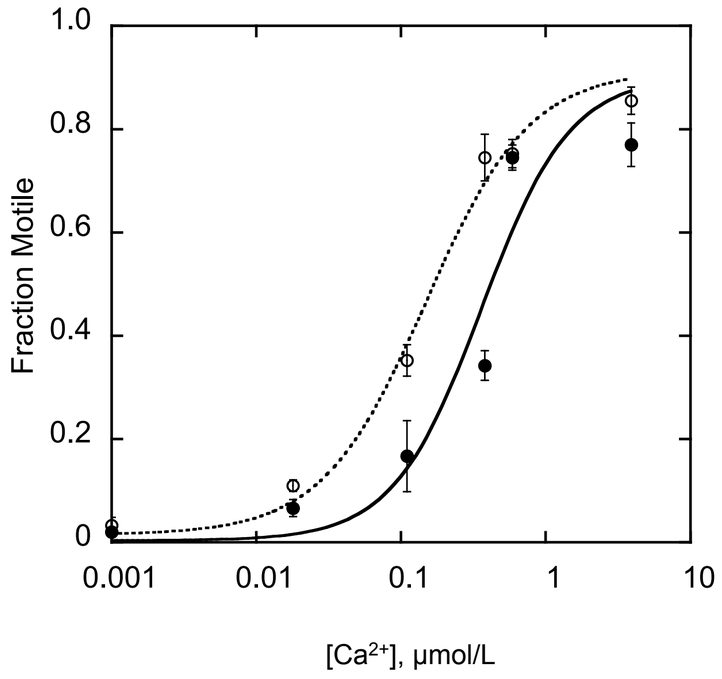
10
[Ca2+], µmol/L


Fraction Motile
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0.4
0.2
0
0.001
0.01
0.1
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10
[Ca2+], µmol/L

Figure 6