Two mutations in troponin I that cause hypertrophic cardiomyopathy have contrasting effects on cardiac muscle contractility

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We investigated the effects of two mutations in human cardiac troponin I, $Arg^{145} \rightarrow Gly$ and $Gly^{203} \rightarrow Ser$, that are reported to cause familial hypertrophic cardiomyopathy. Mutant and wild-type troponin I, overexpressed in *Escherichia coli*, were used to reconstitute troponin complexes in vanadate-treated guinea pig cardiac trabeculae skinned fibres, and thin filaments were reconstituted with human cardiac troponin and tropomyosin along with rabbit skeletal muscle actin for *in vitro* motility and actomyosin ATPase assays. Troponin containing the $Arg^{145} \rightarrow Gly$ mutation inhibited force in skinned trabeculae less than did the wild-type, and had almost no inhibitory function in the *in vitro* motility assay. There was an enhanced inhibitory function with mixtures of 10–30 % [Gly^{145}]troponin I with the wild-type protein. Skinned trabeculae reconstituted with troponin I containing the $Gly^{203} \rightarrow Ser$ mutation and troponin C produced less Ca^{2+}-activated force (64 ± 8 % of wild-type) and demonstrated lower Ca^{2+} sensitivity [ΔpCa_{50} (log of the Ca^{2+} concentration that gave 50 % of maximal activation) 0.25 unit ($P < 0.05$)] compared with wild-type troponin I, but thin filaments containing [Ser^{203}]-troponin I were indistinguishable from those containing the wild-type protein in *in vitro* motility and ATPase assays. Thus these two mutations each result in hypertrophic cardiomyopathy, but have opposite effects on the overall contractility of the muscle in the systems we investigated, indicating either that we have not yet identified the relevant alteration in contractility for the $Gly^{203} \rightarrow Ser$ mutation, or that the disease does not result directly from any particular alteration in contractility.

Key words: calcium, motility, regulation.

INTRODUCTION

Hypertrophic cardiomyopathy (HCM) is the most frequently occurring inherited cardiac disorder, affecting up to 1 in 500 of the population [1]. The hallmark abnormality is unexplained cardiac hypertrophy, particularly of the interventricular septum, which is associated with significant sarcomeric disarray. However, the disease is variable in the severity and timing of the appearance of symptoms.

If HCM is a consequence of the expression of an abnormal protein (a 'poison peptide'), then abnormal functioning of the mutated protein should be detectable for the purified protein when examined in a suitable contractility test system. This has proven to be the case for mutations in myosin heavy chain [2], tropomyosin [3] and troponin T [4,5]; however, mutations in troponin I have only been studied using the ATPase assay [6]. We therefore investigated the effects of two HCM-associated mutations in troponin I on the functional properties of cardiac muscle thin filaments in two assays that measure the contractile properties of actomyosin: chemically skinned muscle fibres and the *in vitro* motility assay.

Troponin I is central to thin filament regulation, since it is an actin-binding inhibitory protein which on its own inactivates thin filaments. Ca^{2+} sensitivity is conferred on troponin by the troponin C subunit, which is an EF-hand Ca^{2+}-binding protein. At micromolar Ca^{2+} concentrations troponin C binds strongly to troponin I and thus prevents it from binding and inhibiting actin. Troponin T forms the scaffolding of this regulatory switch by binding to troponin C, to troponin I and very strongly to tropomyosin. Regulatory conformational changes in actin, troponin I and troponin C are transmitted efficiently to tropomyosin by troponin T. Since each tropomyosin molecule is in contact with seven actin monomers, inhibitory and activating signals are transmitted co-operatively along the thin filament. The integrated nature of the troponin–tropomyosin switch explains how mutations in any component of it can lead to a common outcome.

Kimura et al. [7] identified six HCM-associated mutations in the cardiac troponin I gene. Five of these mutations ($Arg^{145} \rightarrow Gly$, $Arg^{145} \rightarrow Gln$, $Arg^{162} \rightarrow Trp$, $Gly^{203} \rightarrow Ser$ and $Lys^{206} \rightarrow Gln$) are missense mutations, and one mutation causes the deletion of one codon (Lys^{183}). Subsequently an additional missense mutation ($Ser^{199} \rightarrow Asn$) has been described [8], along with a mutation that causes the replacement of the C-terminal eight amino acids with 19 nonsense residues (Glu^{202} truncation) [9].

Here we examine the effects of two of these HCM-associated mutations in human cardiac troponin I. The first, in which a positively charged arginine residue at position 145 is replaced with a neutral glycine, is in the highly basic inhibitory region. This mutation has been shown to reduce the inhibition of the actin-activated myosin MgATPase and increase the residual Ca^{2+} sensitivity in a reconstituted actomyosin system and in myofibrils [6,10]. However, this mutation does not seem to

Abbreviations used: actin(φ), rhodamine–phalloidin-labelled actin; DTT, dithiothreitol; HCM, hypertrophic cardiomyopathy; HMM, heavy meromyosin; pCa_{50}, log of the Ca^{2+} concentration that gave 50 % of maximal activation; S-1, subfragment-1.

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increase the affinity of troponin I for troponin C [6]. The second mutation is in the C-terminal region of human cardiac troponin I at position 203, where glycine is replaced by a serine residue. To date no studies have investigated this mutation, which is not located in a currently defined interaction site, but which is in the region that is truncated in stunned myocardium. A comparison of these two mutations, which are in different functional regions of troponin I and yet apparently produce the same phenotype, should provide insight into the mechanism by which mutations lead to HCM.

We have studied the effects of introducing recombinant human cardiac troponin I containing these mutations on troponin I function using two techniques that measure contractility directly. First, we have prepared chemically skinned guinea pig cardiac trabeculae fibres in which the native troponin I and troponin C subunits have been extracted by vanadate treatment and replaced with native and mutant troponin I and troponin C [11]. This technique enables us to measure effects on isometric force generation in an intact myofibrillar matrix. We have also used the *in vitro* motility assay to study how the unloaded movement of thin filaments over myosin is controlled by native and mutant troponin. The advantages of this technique are that, as thin filaments are reconstituted entirely from pure proteins, we can ensure that all regulatory proteins have human cardiac sequences and we can control the ratio of wild-type to mutant troponin I. This has proven to be a significant factor in assessing the nature of the functional perturbation caused by the mutant protein [3,4]. We have found that the $Arg^{145} \rightarrow Gly$ mutation seriously impairs the inhibitory ability of troponin I in all three systems. In contrast, the $Gly^{203} \rightarrow Ser$ mutation has no detectable effect in the unloaded *in vitro* motility assay or on actomyosin ATPase, but significantly decreases the maximal force and Ca^{2+} sensitivity of isometric tension in chemically skinned muscle fibres. These results indicate the value of using a variety of techniques that measure different aspects of contractility when assessing HCM-associated mutations.

EXPERIMENTAL

Human cardiac troponin subunits

The previously described pMW172 bacterial expression construct containing human cardiac troponin I cDNA [6,12] was altered using a two-step PCR-based oligonucleotide-directed mutagenesis protocol to introduce the HCM mutations $Arg^{145} \rightarrow Gly$ and $Gly^{203} \rightarrow Ser$. The wild-type and mutant troponin I proteins were overexpressed in *Escherichia coli* strain BL21(DE3)pLysS [13], and purified using cation-exchange and hydroxyapatite chromatography [6].

Troponin complexes were made by mixing recombinant troponin subunits (dissolved in 6 M urea) in a molar ratio of 1:1:3 (troponin T/troponin I/troponin C). After 3 h of dialysis into 6 M urea, 1 M KCl, 0.1 mM $CaCl_2$, 10 mM Mes, pH 6.0, and 5 mM dithiothreitol (DTT) at 4 °C, the mixtures were then dialysed overnight against 1 M KCl, 0.1 mM $CaCl_2$, 10 mM Mes, pH 6.0, and 5 mM DTT at 4 °C. Finally, the proteins were dialysed without stirring for 4 h into 50 mM KCl, 4 mM $MgCl_2$, 25 mM imidazole, pH 7.4, and 5 mM DTT. Each buffer contained a mixture of leupeptin, chymostatin and pepstatin (2 μg/ml each). To separate aggregated protein, the mixtures were centrifuged at 300000 ***g*** at 4 °C for 20 min.

Human cardiac tropomyosin was prepared from human myocardium as described by Purcell et al. [14]. Appropriate ethical approval was obtained for the use of this tissue.

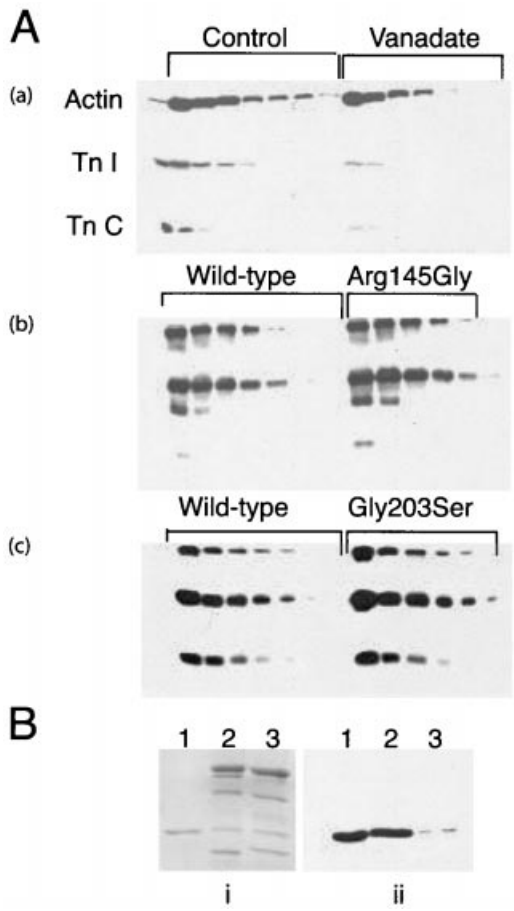

Figure 1 Western blot analysis of protein content of muscle fibres

(**A**) Western blots were performed on muscle fibres at the end of the reconstitution experiment. Each blot shows two fibres that were each loaded several times in a series of 2-fold dilutions. In panel (**a**) the first lane contains standards for actin, troponin I (Tn I) and troponin C (Tn T). This panel shows the effect of vanadate treatment on a bundle of fibres compared with a control untreated fibre that was adjacent to the vanadate-treated fibre in the same heart. Panel (**b**) shows the reconstitution of troponin I and troponin C into a vanadate-treated fibre, comparing wild-type troponin I and troponin I containing the $Arg^{145} \rightarrow Gly$ mutation. Panel (**c**) shows a comparison between fibres reconstituted with wild-type troponin I and troponin I containing the $Gly \rightarrow Ser^{203}$ mutation. (**B**) Lane 1, troponin I standard; lane 2, human cardiac myofibrils; lane 3, guinea pig cardiac myofibrils. Shown are an SDS/15%-PAGE gel (i) and a Western blot treated with the anti-(human cardiac troponin I) antibody (ii).

Actin–tropomyosin-activated myosin subfragment-1 (S-1) ATPase assay

Actin–tropomyosin-activated myosin S-1 ATPase rates were measured using 0.5 μM rabbit skeletal myosin S-1, 7 μM rabbit skeletal actin and 1 μM rabbit α-tropomyosin in ATPase buffer (5 mM Pipes, pH 7.0, 3.87 mM $MgCl_2$ and 1 mM DTT) containing 3 mM ATP at 37 °C in the presence of 0–4 μM troponin I or 0–2 μM troponin complex, as described previously [6]. Each assay was carried out in triplicate and on at least three separate preparations of troponin I.

Animals and tissue preparation

Guinea pigs were humanely killed by CO_2 asphyxiation according to institutional guidelines, and the heart was removed quickly and placed in iced modified Tyrode's solution [15].

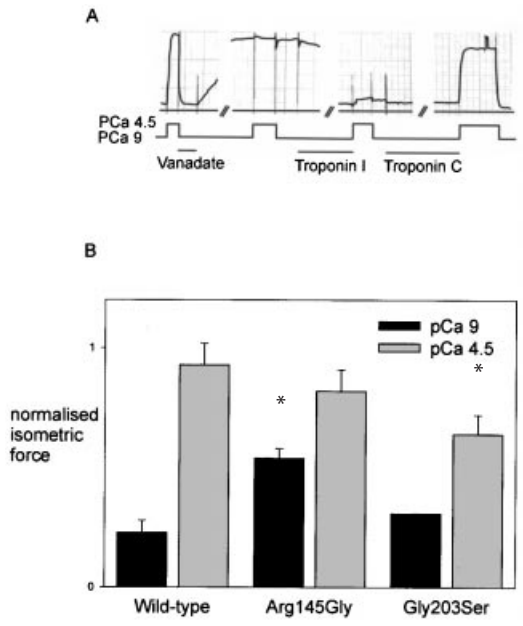

Figure 2 Reconstitution of guinea pig cardiac trabeculae with troponin I and troponin C

(**A**) Tension tracing of a guinea pig trabecula showing the effects of treatment with vanadate followed by troponin I and troponin C. (**B**) Inhibition and Ca^{2+} sensitivity of reconstituted guinea pig cardiac trabeculae with wild-type and mutant troponin. Force is expressed relative to the force at pCa 9 after vanadate treatment. Mean force for a number of fibres is shown, and the error bars indicate S.E.M. ($n = 6$–9). The data at pCa 9 were obtained from fibres after treatment with 75 μM wild-type or mutant recombinant human cardiac troponin I for 60 min. Relative force at pCa 4.5 was measured following treatment of the fibres with recombinant troponin C for 60 min. *Significant difference between wild-type and mutant troponin I.

Small bundles of trabeculae were dissected from the left ventricles of the hearts at 8 °C and placed in glycerinating solution [50 % (v/v) glycerol in relaxing solution (described below)] with 1 % Triton X-100 for 24 h at 4 °C. Then they were transferred to fresh glycerinating solution and stored at −20 °C for up to 2 weeks. An aluminium T-clip was attached to each end of the fibre, and the fibre was mounted on two hooks for tension measurements as described previously [15]. The temperature was maintained at 12 °C for all experiments.

Solutions

Three standard solutions were used for all fibre experiments. The exact concentrations of the individual constituents were calculated using a computer program based on that of Fabiato and Fabiato [16]. Relaxing solution contained 60 mM Bes, 10 mM EGTA, 1 mM free Mg^{2+} and 5.5 mM MgATP. The ionic strength was adjusted to 150 mM with potassium propionate and the pH was adjusted to 7.2 with KOH. Phosphocreatine (10 mM) and creatine kinase (15 units/ml) were added to maintain the free MgATP concentration. EDTA-free Complete™ protease inhibitors (Roche Molecular Biochemicals) were added to all solutions. Activating solution also contained 10 mM $CaCl_2$. A relaxing solution without the Ca^{2+} buffer (EGTA) was used as a pre-activating solution. All solutions for the Ca^{2+} sensitivity experiments were made up in the same way.

Troponin I was used in a low-ATP relaxing solution (0.5 mM NaATP), since the ATP concentration in the normal relaxing solution caused problems with regard to the solubility of troponin I.

Fibre treatments

Following a test contraction in Ca^{2+}, troponin I and troponin C were extracted as described by Strauss et al. [17]. Briefly, the fibres were treated with 10 mM vanadate in relaxing solution for 5–10 min. After the fibres were returned to relaxing solution they slowly developed tension independently of Ca^{2+} to >75 % of that obtained with the initial Ca^{2+} activation, due to the selective removal of troponin I and troponin C (see Figures 1 and 2). Fibres were then treated with 75 μM recombinant human cardiac troponin I for up to 90 min until tension had relaxed to a steady state. They were then treated with 50 μM recombinant human cardiac troponin C for 60–90 min to restore Ca^{2+} sensitivity to the fibres, and then the Ca^{2+} sensitivity was tested with a range of Ca^{2+} concentrations. The Ca^{2+} concentration curve was constructed twice for each fibre for the full range of Ca^{2+} concentrations, with stepwise increasing concentrations of Ca^{2+} from pCa 6.0 to 5.9 to 5.8 to 5.7 etc. pCa was then changed from 4.5 directly to 9, maintained at 9 for 5–10 min and then increased again to 4.5 in a stepwise manner. The mean of the two values for tension at each Ca^{2+} concentration was calculated as a single data point and plotted as mean tension relative to the mean tension at pCa 5.

Gel electrophoresis and Western blotting

At the end of each experiment, the fibres were removed from the hooks, cut away from the T-clips and stored in sample buffer containing 2 % SDS, 10 % glycerol, 5 % 2-mercaptoethanol, 62.5 mM Tris/HCl, pH 6.8, and 0.05 % Bromophenol Blue, and stored at −20 °C until analysis. Prepared samples were boiled for 5 min and then diluted in sample buffer to provide a range of loadings for each sample. The samples were run on 12 % (w/v) acrylamide Tris/HCl 'Ready Gels' (Bio-Rad), using the Mini-gel system. The running buffer consisted of 3 g/litre Tris base, pH 8.3, 14.4 g/litre glycine and 1 g/litre SDS. The proteins were then transferred to nitrocellulose for Western blotting using a wet transfer apparatus (Bio-Rad) with 5.82 g/litre Tris base, pH 9.2, and 2.93 g/litre glycine in 20 % (v/v) methanol for 16 h at 30 V. A mixture of primary antibodies was used: anti-(α-sarcomeric actin) (Sigma) diluted 1:2000, anti-(human cardiac troponin I) diluted 1:1000 and anti-(human cardiac troponin C) diluted 1:2000 (clone 197 and clone 7G4 respectively; Santa Cruz Biotechnology). These were detected on X-ray film using sheep anti-(mouse IgG) antibody linked to horseradish peroxidase (Amersham Life Science) and Renaissance Chemiluminescence Reagent (NEN).

In vitro motility assay

The movement of thin filaments over a bed of immobilized heavy meromyosin (HMM) was investigated using the *in vitro* motility assay, as described previously [4,18], using skeletal muscle HMM on coverglasses coated with silicone by soaking in 0.2 % dichloromethylsilane in chloroform. F-actin was labelled with rhodamine–phalloidin [designated as actin(φ)], as described by Kron et al. [19]. Complexes of F-actin(φ) with tropomyosin and with tropomyosin–troponin were formed at 10 × assay concentration: 100 nM actin(φ), 400 nM tropomyosin and 0–1000 nM troponin were mixed and used in the motility assay within 30 min.

The HMM was treated each day to remove rigor deadheads by co-sedimentation with actin and ATP [19], and was then used within 3 h. Two 50 μl aliquots of HMM at 100 μg/ml were

infused in buffer A (50 mM KCl, 25 mM imidazole/HCl, pH 7.4, 4 mM $MgCl_2$, 1 mM EDTA and 5 mM DTT) to provide a coating of immobilized HMM on the coverslip. This was followed by 2×50 μl of buffer B (buffer A containing 0.5 mg/ml BSA) and then 2×50 μl of 10 nM actin(φ)-associated tropomyosin–troponin in buffer A. Volumes of 50 μl of buffer C (buffer B containing 0.1 mg/ml glucose oxidase, 0.02 mg/ml catalase, 3 mg/ml glucose and 0.5 % methylcellulose in the presence or absence of troponin at assay concentration) and 50 μl of buffer D (buffer C containing 1 mM ATP) were then infused. The Ca^{2+} concentration was varied by incorporating Ca^{2+}/EGTA buffers in the final assay buffers C and D. The movement of actin(φ)–tropomyosin filaments over the immobilized skeletal muscle HMM was observed under a Zeiss epifluorescence microscope ($63 \times /1.4$ objective) with a DAGE-SIT-68 camera and recorded on video tape. Four 15 s videos were recorded in each cell before any significant photobleaching occurred. Videos were digitized and the movement was analysed to determine the fraction of filaments moving and the velocity of motile filaments using the automatic tracking program described by Marston et al. [20].

Statistical analysis

Unless indicated otherwise, pooled data are expressed as means ± S.E.M. Student's *t*-test was used for statistical analysis of the data. A P value of < 0.05 was considered to indicate significant differences between groups.

RESULTS

Fibre studies

Reconstitution of contractility with wild-type and mutant troponin I

The troponin I and troponin C in guinea pig trabeculae was replaced with recombinant human subunits using the vanadate technique. At the end of each experiment we examined the protein content of the muscle fibres. Figure 1(A) shows three Western blots where each membrane has been treated with mixed antibodies for human cardiac troponin I and troponin C and α-sarcomeric actin. The anti-actin antibody was used to standardize for loading differences between lanes. The actin content of the fibres remained almost unchanged following vanadate treatment; any decrease would result in a significant reduction in force, which we did not observe. Following vanadate treatment, 84 ± 6 % of the native troponin I had been removed. Panels (b) and (c) of Figure 1(A) show that, following treatment with troponin I and troponin C, the fibres were reconstituted with the same amount of both wild-type and mutant troponin I. These fibres underwent a number of changes of solution in order to construct two pCa curves following treatment with human cardiac troponin I, which should have washed out any protein that was non-specifically bound within the fibre. The higher density of the troponin I and troponin C bands in fibres that had been reconstituted with human recombinant proteins is due to the high sensitivity of the antibody for the human sequence. This was demonstrated by a comparison of myofibrils extracted from human heart and guinea pig trabeculae (Figure 1B). Coomassie Blue staining demonstrated that the troponin I content of the two samples was identical; however, in the Western blot the guinea pig myofibrils were barely labelled by the anti-(human troponin I) antibody. From this we could deduce that the quantity of recombinant human cardiac troponin I in the reconstituted fibres was almost identical with the quantity of native troponin I in the fibres before vanadate treatment.

Figure 2 shows the contractile properties of fibres that were reconstituted with human cardiac troponin I. Following vanadate

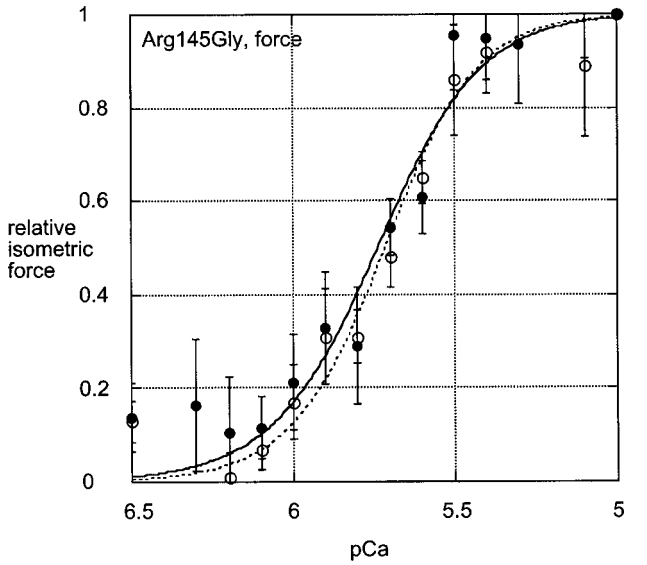

Figure 3 Force–pCa relationship for fibres reconstituted with $Arg^{145} \rightarrow Gly$ human cardiac troponin I

Fibres were reconstituted with either wild-type (○) or $Arg^{145} \rightarrow Gly$ (●) human cardiac troponin I for up to 90 min, and then with cardiac troponin C for 60 min. Force is expressed relative to force at pCa 5. Values are means ± S.E.M. of six experiments. The lines are the best fits of the points to the Hill equation: wild-type troponin I, $pCa_{50} = 5.72 \pm 0.03$ and $h = 2.97 \pm 0.47$; [Gly^{145}]troponin I, $pCa_{50} = 5.74 \pm 0.04$ and $h = 2.92 \pm 0.38$.

treatment, guinea pig trabeculae fibres contracted in the presence of ATP, independently of the Ca^{2+} concentration, producing at least 75 % of the force obtained before treatment. Figure 2(A) shows a tension trace demonstrating the functional effects of extraction of troponin I and troponin C from the fibres. Following treatment with exogenous wild-type human cardiac troponin I and troponin C, full Ca^{2+}-sensitive regulation was restored to the fibres.

In Figure 2(B), all values for force are shown relative to the force at pCa 9 following treatment with vanadate for a number of fibres (Ca^{2+}-independent tension). When vanadate-treated fibres were incubated with 75 μM recombinant human cardiac troponin I at pCa 9, the Ca^{2+}-independent tension declined slowly, taking about 45 min to reach a stable tension, which was 23 ± 5 % ($n = 8$) of the Ca^{2+}-independent tension. For comparison, untreated fibres relaxed to 91 ± 4 % of maximum tension at pCa 9. The fibres were then treated with 50 μM recombinant cardiac troponin C in relaxing solution for up to 90 min. Following treatment with troponin C, the fibres contracted repeatedly in response to Ca^{2+}.

Wild-type human cardiac troponin I inhibited Ca^{2+}-independent tension by 77 ± 5 % ($n = 8$). Troponin I containing the $Arg^{145} \rightarrow Gly$ mutation inhibited Ca^{2+}-independent tension by 46 ± 4 % ($n = 9$). Therefore the mutant [Gly^{145}]troponin I inhibited Ca^{2+}-independent tension significantly less well than did the wild-type protein ($P < 0.05$). Human cardiac troponin I containing the $Gly^{203} \rightarrow Ser$ mutation inhibited Ca^{2+}-independent tension by 69 ± 0.1 % ($n = 7$), which was not significantly different from the effect of wild-type troponin I ($P = 0.25$).

Also shown in Figure 2 is the restitution of force following treatment with troponin C for fibres treated with wild-type and mutant troponin I. For wild-type troponin I and troponin C, the Ca^{2+}-activated force was 93 ± 9 % ($n = 8$) of the Ca^{2}-independent tension. For [Gly^{145}]troponin I the Ca^{2+}-activated force was

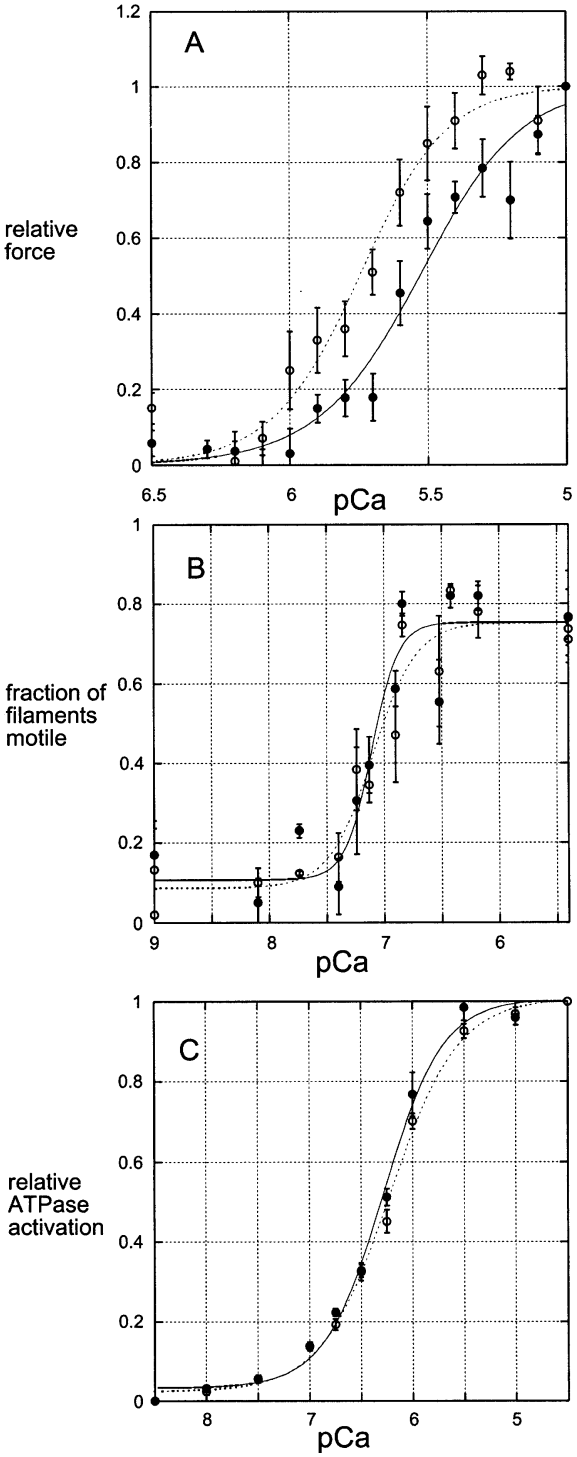

Figure 4 Comparison of the regulation by Ca^{2+} of thin filaments containing wild-type or mutant Gly^{203} → Ser troponin I in three different assays

(**A**) Force–pCa relationship in skinned fibres. Fibres were reconstituted with either wild-type (○) or Gly^{203} → Ser (●) troponin I, and isometric force was measured as described in the legend to Figure 3. Values are means ± S.E.M. of five experiments. The wild-type data are five data sets taken from the data in Figure 3. The lines are the best fits of the points to the Hill equation: wild-type troponin I, $pCa_{50} = 5.75 \pm 0.03$ and $h = 2.73 \pm 0.41$; [Ser^{203}]troponin I, $pCa_{50} = 5.53 \pm 0.04$ and $h = 2.36 \pm 0.35$. (**B**) Ca^{2+}-dependent regulation of thin filament sliding in the *in vitro* motility assay. The motility of thin filaments reconstituted with wild-type (○) or Gly^{203} → Ser (●) troponin I was compared in dual-chamber motility cells over a range of Ca^{2+} concentrations; 10 nM actin, 40 nM human cardiac tropomyosin and 75 nM human cardiac troponin were present in the motility cell. Pooled results from two experiments are shown. Values (means ± S.E.M.) of the fraction of motile filaments determined from four places in a motility cell are plotted. The data were fitted to the Hill equation: wild-type troponin I, $pCa_{50} = 7.10 \pm 0.07$ and $h = 2.28 \pm 0.75$; [Ser^{203}]troponin I, $pCa_{50} = 7.11 \pm 0.06$ and $h = 3.71 \pm 1.64$. (**C**) Ca^{2+}-dependent regulation of actin–tropomyosin-activated myosin ATPase. Thin filaments reconstituted with wild-type (○) and Gly^{203} → Ser (●) troponin I were compared. Actin–tropomyosin-activated myosin ATPase was measured in the presence of 2 μM troponin. For each experiment ATPase was normalized thus: pCa 4.5 values = 1 and pCa 8.5 values = 0. Values are means ± S.E.M. of normalized data from five separate experiments. The actin–tropomyosin-activated myosin ATPase rate at pCa 4.5 was 12.4 s^{-1}, and that at pCa 8.5 was 1.2 s^{-1}. Wild-type troponin I, $pCa_{50} = 6.26 \pm 0.03$ and $h = 1.25 \pm 0.13$; [Ser^{203}]troponin I, $pCa_{50} = 6.31 \pm 0.03$ and $h = 1.39 \pm 0.21$.

$82 \pm 9\%$ ($n = 7$) and for [Ser^{203}]troponin I it was $64 \pm 8\%$ ($n = 7$) of the Ca^{2+}-independent tension. Thus both mutations in human cardiac troponin I appear to decrease the level of activation when compared with the wild-type protein, but this difference was only statistically significant for the Gly^{203} → Ser mutation ($P < 0.05$).

Ca^{2+} sensitivity of the reversal of inhibition

Figure 3 shows the Ca^{2+} activation curve for wild-type troponin I and for the Arg^{145} → Gly mutant. Relative fibre tension was normalized to the fibre tension at pCa 5 for both the wild-type and mutant troponin I. For wild-type troponin I the mean of six measurements for the log of the Ca^{2+} concentration that gave 50% of maximal activation (pCa_{50}) was 5.72 ± 0.03, and the Hill coefficient, h, which is a measure of the co-operativity of the system, was 2.97 ± 0.47. For [Gly^{145}]troponin I the pCa_{50} was 5.74 ± 0.04, with $h = 2.92 \pm 0.38$. Therefore the Arg^{145} → Gly mutation had no effect on Ca^{2+} sensitivity compared with the wild-type protein when reconstituted into guinea pig trabeculae fibres. The difference in Hill coefficient between wild-type and [Gly^{145}]troponin I was not significant ($P = 0.68$).

Figure 4(A) shows the Ca^{2+} activation curve for troponin I containing the Gly^{203} → Ser mutation compared with that for the wild-type protein. For wild-type troponin I the mean of the five measurements of the pCa_{50} was 5.75 ± 0.03, with $h = 2.73 \pm 0.41$. However, for [Ser^{203}]troponin I the mean pCa_{50} was 5.53 ± 0.04, with $h = 2.36 \pm 0.35$. This mutation has thus made the reversal of inhibition less sensitive to Ca^{2+}, with a decrease in the pCa_{50} of 0.22 unit compared with wild-type troponin I. This difference in pCa_{50} between wild-type human cardiac troponin I and the protein containing the Gly^{203} → Ser mutation was significant ($P < 0.05$), but the h values were not significantly different ($P = 0.52$).

In vitro motility studies

The movement of actin filaments over a bed of rabbit skeletal HMM was regulated by Ca^{2+}, troponin and tropomyosin, as expected. The addition of native human cardiac tropomyosin increased the filament velocity by $20 \pm 3\%$ ($n = 3$), reaching a plateau at 20 nM tropomyosin; we therefore routinely used 40 nM tropomyosin in our assays. At pCa 5 and 28 °C, 80–90% of actin–tropomyosin filaments were moving at 3–4 μm/s. The addition of human cardiac troponin reconstituted from recombinant I, T and C subunits produced a further increase in velocity of up to 40%, which reached a plateau at 15–30 nM troponin, in accordance with our previous measurements [4]. Incorporation of the Arg^{145} → Gly or the Gly^{203} → Ser mutation into troponin I had no significant effect on this enhancement of velocity (results not shown).

We added increasing concentrations of reconstituted troponin to actin–tropomyosin at pCa 9. As observed previously, the

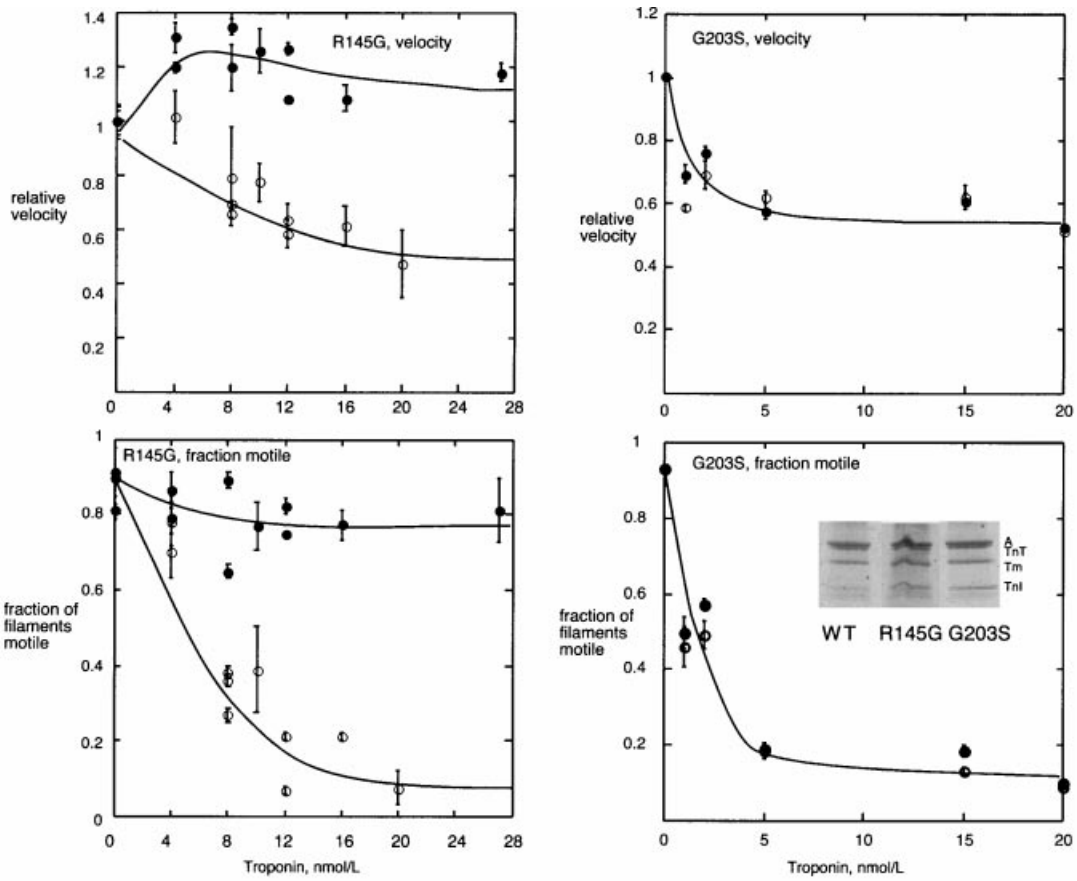

Figure 5 ***In vitro*** **motility study of cardiac muscle thin filaments reconstituted with wild-type, Arg^{145} → Gly or Gly^{203} → Ser human cardiac troponin I at pCa 9**

Troponin was reconstituted with wild-type (○) or Arg^{145} → Gly (●) human cardiac troponin I (left panels), or with wild-type (○) or Gly^{203} → Ser (●) human cardiac troponin I (right panels). Increasing concentrations of reconstituted troponin were mixed with 10 nM actin and 40 nM human cardiac tropomyosin. Pooled data from three separate experiments are shown. In each experiment, four measurements of velocity (upper panels; expressed relative to velocity of actin–tropomyosin filaments) and the fraction of filaments that were motile (lower panels) were made at different locations within the same motility cell. The lines are freehand Bezier curves through the points. The inset in the lower right panel shows binding of troponin I (TnI), troponin T (TnT) and tropomyosin (Tm) to actin (A) under the conditions of the motility assay. Thin filaments were reconstituted with 20% biotin–phalloidin-labelled actin/80% actin(φ). Thin filaments were selected from the motility assay mixture (100 nM actin) with streptavidin-coated Dynabeads, which were recovered by a magnetic separator. PAGE of the thin filaments is shown. Troponin I is clearly incorporated into the thin filament at this concentration. WT, wild-type.

fraction of filaments that were motile fell to less than 0.1, and the velocity of those thin filaments that were motile was reduced by 40–50 %. Maximum inhibition of motility, observed at 15–30 nM troponin, was approx. 95 %, as found previously in this system [21]. In some experiments we increased the tropomyosin concentration at a saturating troponin concentration, but there was no increase in the degree of inhibition. When human cardiac troponin I containing the Arg^{145} → Gly mutation was present in reconstituted troponin, inhibitory activity was virtually absent (Figure 5). Addition of [Gly^{145}]troponin had no effect on the fraction of filaments motile, and the speed of filament movement increased by up to 30 %. Control experiments confirmed that the binding of wild-type and mutant troponin subunits to actin–tropomyosin was identical under these conditions, and thus [Gly^{145}]troponin I binds to actin but does not inhibit motility. This behaviour is very similar to that observed with a truncated troponin T lacking the C-terminal 28 amino acids. In contrast, the mutation Gly^{203} → Ser in troponin I did not affect the inhibitory response significantly (Figure 5); both troponin subunit binding to actin and inhibition of motility at pCa 9 were indistinguishable from those with the wild-type protein.

We determined Ca^{2+} activation curves for thin filament motility with wild-type troponin and the [Ser^{203}]troponin I mutant. Thin filaments containing mutant troponin were compared with those containing normal troponin in dual-chamber motility cells over a range of Ca^{2+} concentrations in random order. The results were fitted to the Hill equation. In four separate experiments we could detect no significant difference between wild-type and [Ser^{203}]-troponin, in contrast with the results obtained in desensitized muscle fibres (Figure 4B). The mutant [Gly^{145}]troponin I could not be studied in this assay, since the fraction of filaments motile was the same at pCa 5 and pCa 9 (Figure 5).

In vivo, it is likely that the wild-type and mutant troponin I are both expressed and incorporated into troponin, and so there is the potential for interaction between the two species. To examine this, we studied the regulatory effects of troponin containing different ratios of wild-type and mutated troponin I at a constant total troponin concentration. Mixtures of wild-type and Arg^{145} → Gly mutant troponin I gave a markedly biphasic response when the total troponin concentration was partially saturating. We performed this experiment on three thin filament preparations; Figures 6(A) and 6(B) show a single experiment. When the

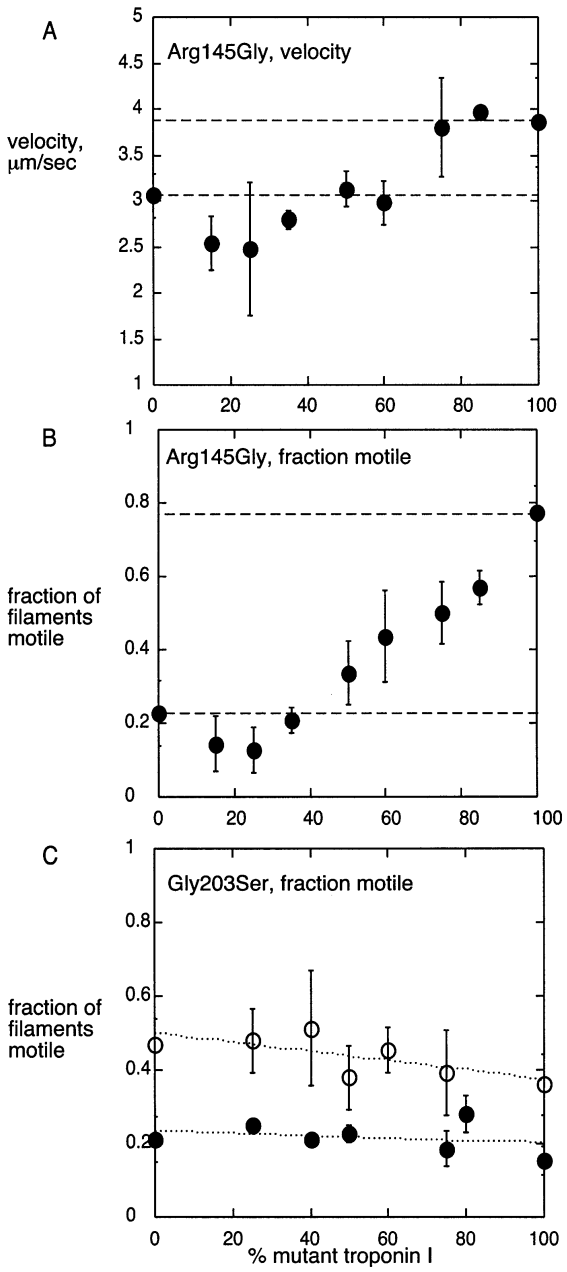

Figure 6 ***In vitro*** **motility regulatory properties of troponin containing different ratios of wild-type and mutant troponin I at pCa 9**

Filaments contained 10 nM actin and 30 nM tropomyosin. (**A**, **B**) Effects of various mixtures of wild-type and [Gly[145]]troponin I. Filament velocity (**A**) and fraction of filaments motile (**B**) were measured at pCa 9 in the presence of a mixture of troponin containing wild-type and [Gly[145]]troponin I (total concentration 40 nM). Data are means ± S.E.M. of four measurements from a single experiment. Concentrations of [Gly[145]]troponin I of 10–30% reduced the fraction of filaments motile and also decreased filament velocity, whereas 60% or more [Gly[145]]troponin I resulted in a progressive switch on of the filaments (high fraction motile and high velocity). The broken lines indicate the motility parameters with 100% wild-type and 100% mutant troponin I. (**C**) Troponin containing mixtures of wild-type and Gly[203] → Ser human cardiac troponin I. The fraction of filaments motile was measured at pCa 9 in the presence of a mixture of troponin containing wild-type and [Ser[203]]troponin I. Results are means ± S.E.M. of four measurements from two experiments (○, 8 nM troponin; ●, 25 nM troponin), starting at different levels of inhibition. The lines are linear regression fits to the data.

mutant accounted for between 0 and 20 % of total troponin I, the velocity and fraction of filaments motile declined in all three experiments, indicating better inhibition than with wild-type troponin. The decrease in motility and velocity was significant ($P = 0.037$). The reversal of inhibition due to the Arg[145] → Gly mutation only became apparent when more than 50 % of the mixture was mutant protein. The non-linear response parallels that obtained in the actomyosin ATPase assay [6]. In contrast, mixtures of wild-type and mutant [Ser[203]]troponin gave a linear response with both fully and partially saturating troponin, with no indication of interactions between the two types of troponin I on the same filament (Figure 6C).

ATPase studies

We determined Ca^{2+} activation curves for actin–tropomyosin activation of ATPase with troponin containing wild-type and [Ser[203]]troponin I. The pCa_{50} values for wild-type troponin (6.26 ± 0.03) and for troponin containing the Gly[203] → Ser mutation (6.31 ± 0.03) were virtually identical (Figure 4C).

DISCUSSION

We have investigated the functional effects of two mutations in human cardiac troponin I which have been described in association with HCM, but which result in slightly different phenotypes. Patients with the Arg[145] → Gly mutation develop the characteristic ventricular hypertrophy of HCM, while three affected individuals shown to carry the Gly[203] → Ser mutation manifested hypertrophy only at the cardiac apex [7]. The two mutations are in different parts of the troponin I sequence with different known functions, and therefore it is likely that the functional effects of the two mutations are different. We have used three techniques in parallel, since, by looking at the effects of mutations on isometric force in muscle fibres, on filament movement using *in vitro* motility assays and on the actomyosin MgATPase, we could highlight different aspects of the regulatory mechanism of the thin filament.

All three experimental techniques were designed to allow direct comparisons between wild-type and mutant protein under identical conditions, where the only variable is the sequence of the troponin I component. We therefore conclude that the differences that we have observed between wild-type and mutant protein are caused by the mutations affecting the functional properties of the troponin I.

For troponin I containing the Arg[145] → Gly mutation, the three techniques gave very similar results: impaired inhibition of isometric tension in muscle fibres and greatly reduced switch-off of filament motility and ATPase at pCa 9. In contrast, for troponin I containing the Gly[203] → Ser mutation, the ATPase and motility assays, which measure properties of unloaded thin filaments, gave a different result from isometric force measurements. This mutation induced a small decrease in maximum force at pCa 5 and a significant decrease in the Ca^{2+} sensitivity of isometric force in muscle fibres, yet no differences between the wild-type and mutant proteins were detected in *in vitro* motility and actomyosin ATPase assays.

Arg[145] → Gly mutation

We replaced native troponin I and troponin C in chemically skinned guinea pig trabeculae fibres with mutant and wild-type human cardiac troponin I and wild-type human cardiac troponin C. This technique maintains the myofibrillar structure of cardiac muscle and is the closest approach possible to the *in vivo* situation, while also permitting the protein components to be manipulated directly. When the mutant [Gly[145]]troponin I was incorporated into muscle fibres, we clearly observed a substantial decrease in Ca^{2+} regulation due to the failure of the muscle to

relax properly at pCa 9, while the residual Ca^{2+}-dependent change in force was slightly more Ca^{2+}-sensitive than with wild-type troponin I. When studied using the *in vitro* motility assay, thin filaments incorporating the $Arg^{145} \rightarrow Gly$ mutant troponin I were completely unregulated. Binding measurements showed that this was not due to the absence of mutant troponin I from the thin filaments assayed. The abolition of the inhibitory function of troponin I was also observed in acto-S-1 ATPase assays [6], but ATPase assays on myofibrils in which native troponin I had been replaced by mutant troponin I showed only a partial loss of inhibitory function [10].

A likely reason for the less marked effect of the $Arg^{145} \rightarrow Gly$ mutation on the control of force in muscle fibres and myofibrils is that, following treatment of guinea pig trabeculae with vanadate, not all native cardiac troponin I is extracted. Therefore in the skinned trabeculae fibre system we are looking at a mixture of native guinea pig troponin I and mutant recombinant human troponin I. As a similar situation occurs in the heart muscle of affected patients, we examined the properties of troponin mixtures containing both wild-type and $Arg^{145} \rightarrow Gly$ mutant troponin I in the *in vitro* motility assay at pCa 9 (Figures 6A and 6B). We found that no loss of inhibition was observed until over 60 % of the troponin I was mutant protein. This could explain the partial loss of inhibition seen in guinea pig trabeculae if up to 30 % wild-type troponin I was present.

These observations may account for the results of a recently published study on transgenic mice overexpressing wild-type or [Gly^{145}]troponin I [22]. The viability of the transgenic mice depended upon the level of mutant transgene expression. The lines showing highest expression died before birth, whereas mice with the lowest level of expression had a normal lifespan unless subjected to stress. These results parallel those obtained with transgenic mice expressing the mutation resulting in truncated troponin T [23]. Both mutations lead to a complete loss of relaxation when studied in an *in vitro* motility assay [4], indicating that this is a potentially lethal mutation and that patients with the mutation probably only express the mutated protein at low levels. Unfortunately, no investigation of the level of expression of these HCM-associated mutations has yet been possible.

The $Arg^{145} \rightarrow Gly$ mutation is located within a region of troponin I that has been shown to be essential for Ca^{2+}-controlled inhibition. Indeed, the sequence R^{136}GKFKRPTL**R**RVR^{148} itself can substitute for whole troponin in vanadate-treated cardiac muscle [24]. The replacement of Arg^{112} with Gly in the equivalent peptide of the rabbit skeletal muscle troponin I sequence (residues 104–115) has been shown to reduce the inhibitory properties of the troponin I peptide by 90 % [25]. Thus the effects of the $Arg^{145} \rightarrow Gly$ mutation on the function of thin filaments that we have observed are in agreement with previous structure–function studies on troponin I.

$Gly^{203} \rightarrow Ser$ mutation

The effects of the $Gly^{203} \rightarrow Ser$ mutation in troponin I on troponin function were much less marked in our assay systems than those of the $Arg^{145} \rightarrow Gly$ mutation. The *in vitro* motility assay and actomyosin ATPase assay could not detect any differences between mutant and wild-type troponin I. However, incorporation of [Ser^{203}]troponin I and troponin C into vanadate-desensitized fibres resulted in a large decrease in Ca^{2+} sensitivity and a decrease in maximum force compared with wild-type protein.

Gly^{203} is located seven amino acids from the C-terminus of troponin I. The sequence S^{199}GME**G**RKK^{206} is highly conserved between isoforms in humans and also between species, suggesting that it has an important functional role [26,27]. There have been a number of reports of C-terminal deletions in cardiac troponin I which affect troponin function. Deletion of the C-terminal 17 amino acids, which is believed to occur in the heart during myocardial stunning [28], results in less force and a decreased Ca^{2+} sensitivity in the trabeculae of transgenic mice which contain 9–17 % truncated troponin I [29]. This change is quite similar to that observed with troponin I containing a point mutation at Gly^{203}. However, another series of experiments in which ATPase was measured in vanadate-treated myofibrils substituted with troponin I mutants found that a 12-amino-acid C-terminal deletion had no effect, whereas a 24-amino-acid deletion actually increased Ca^{2+} sensitivity in ATPase assays [30]. Ca^{2+}-regulated interactions have recently been identified between troponin C and the extreme C-terminal 20 amino acids of troponin I [31,32]. Thus it appears that this extreme C-terminus of troponin I may be involved in an interaction with troponin C.

The reason why an effect of the $Gly^{203} \rightarrow Ser$ mutation is not seen in *in vitro* assays is probably related to the experimental system. We could produce no evidence that mixtures of mutated and wild-type troponin I behave differently from either of the components, or that [Ser^{203}]troponin I bound anomalously (either too much or too little) in skinned trabeculae. One difference between the skinned trabeculae system and the *in vitro* motility assay is that, in the trabeculae, actin, myosin and troponin are organized into a sarcomeric array and are present at high concentrations (0.2–0.5 mM). The sarcomere is an optimal system for co-operative interactions between contractile and regulatory proteins, whereas at the extremely low concentrations and low myosin/actin ratios present *in vitro* such co-operativity is largely absent. Another difference between the techniques is that isometric force is measured in guinea pig trabeculae, whereas in the *in vitro* motility and ATPase assays filaments are unloaded. Thus different aspects of contractility are measured by the different techniques. The discrepancy between force measurements with troponin I-(1–193) in trabeculae and ATPase measurements with troponin I-(1–199) in myofibrils may be an example of this [29,30]. Such results illustrate the importance of applying a variety of techniques to studying the same mutant protein in order to understand functional changes caused by HCM-associated mutations.

Relationship of functional change to phenotype

It is noteworthy that the two mutations that we studied, which have different functional effects *in vitro*, appear to result in differing phenotypes *in vivo* [7]. We have not yet investigated the behaviour of the mutant troponins after phosphorylation by cAMP-activated protein kinase, and a recent publication indicates that there can be interaction between mutation sites and phosphorylation sites [33]. On the other hand, it is possible that the clinical characterization of HCM is not precise enough, and that hypercontractile ($Arg^{145} \rightarrow Gly$) and hypocontractile ($Gly^{203} \rightarrow Ser$) mutations actually result in different phenotypes. The recent discovery that mutations in actin, myosin, tropomyosin and troponin T can lead to either HCM or dilated cardiomyopathy phenotypes indicates that this is indeed possible [34–36]. It is an exciting prospect that we may in the future be able to link specific alterations in troponin function to distinctive pathologies.

The paradoxical observations on contractility may be explained by the suggestion that the primary lesion that triggers hypertrophy is a mismatch between ATP supply and demand, due to increased tension cost and lower efficiency, as has been

demonstrated for troponin T mutants [37,38]. Until recently, all the known genes implicated in HCM encoded proteins that are components of the cardiac muscle sarcomere. However, mutations that result in HCM have now been discovered in the γ_2 subunit of AMP-activated protein kinase. It appears that these mutations directly affect the ability of the heart to maintain an adequate availability of ATP under conditions of increased demand [39]. These findings are consistent with the proposal that HCM is caused by a failure to maintain normal ATP homoeostasis within the myocyte.

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