Published in final edited form as:

Neuromuscul Disord. 2006 November ; 16(11): 782–791.

Muscle cell and motor protein function in patients with a IIa myosin missense mutation (Glu-706 to Lys)

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Abstract

The pathogenic events leading to the progressive muscle weakness in patients with a E706K mutation in the head of the myosin heavy chain (MyHC) IIa were analysed at the muscle cell and motor protein levels. Contractile properties were measured in single muscle fiber segments using the skinned fiber preparation and a single muscle fiber *in vitro* motility assay. A dramatic impairment in the function of the IIa MyHC isoform was observed at the motor protein level. At the single muscle fiber level, on the other hand, a general decrease was observed in the number of preparations where the specific criteria for acceptance were fulfilled irrespective of MyHC isoform expression. Our results provide evidence that the pathogenesis of the MyHC IIa E706K myopathy involves defective function of the mutated myosin as well as alterations in the structural integrity of all muscle cells irrespective of MyHC isoform expression.

Introduction

Myosin is a major structural component of skeletal muscle and is considered to be the molecular motor that converts free energy derived from the hydrolysis of ATP into mechanical work. It is a hexamer comprised of two myosin heavy chain (MyHC) subunits of approximately 220 kD molecular weight and four myosin light chain (MyLC) subunits, two regulatory and two essential MyLC, of approximately 20 kD each. The MyHCs found in mammalian skeletal muscle myofibrils are all isoforms of the class II myosin family. At least ten MyHCs have been identified in striated muscles from mammals, i.e., β /slow (I), α -cardiac, slow-tonic, embryonic, fetal, IIa, IIx (IIc), IIb, a super-fast extraocular and masticatory myosin isoform (1,2). Three of these are expressed in normal adult extrafusal human limb and trunk muscles, i.e., β /slow or type I, and the two fast MyHCs, IIa and IIx (1).

The coding sequence for the adult MyHC isoforms is among the most heavily amplified in mammalian species and the resulting polypeptide generally accounts for 15-25% of the total body protein content. In spite of the abundance of myosin and its critical role in motor function, there are only a few reports in the literature describing neuromuscular disorders where the motor protein myosin is the primary cause of the disease. However there is an increasing awareness of both acquired and hereditary myosin myopathies, i.e., “myosinopathies” (3). The

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acute quadriplegic myopathy (AQM), or critical illness myopathy, is an example of an acquired myosinopathy. It is associated with generalized muscle paralysis and partial or complete loss of myosin and myosin-associated proteins caused by a dramatic down-regulation of myosin synthesis at the transcriptional level and enhanced protein degradation in response to pharmacological treatment in modern anesthesia and intensive care (4). The recently described selective myosin loss in experimental studies of cancer cachexia may represent another acquired myosinopathy caused by altered cytokine activation of myosin synthesis at the gene level and/or proteolysis via the ubiquitin-dependent proteasome pathway (5). The very small number of myopathies identified in humans that have been associated with myosin mutations need not be due to the absence of myosinopathies. Indeed, the large size of the 17p13.1 locus within the MyHC gene would predict a prominent role for MyHC mutations in human myosinopathies (3). There is accordingly reason to believe that this group of myopathies may have been misdiagnosed and lumped together with other neuromuscular disorders. Experimental animal studies have shown distinct patterns of muscle dysfunction in IIX and IIB MyHC null mice, while heterozygous mice are clinically normal (6). Missense mutation in the β /slow MyHC gene causes a familial hypertrophic cardiomyopathy-like disease in heterozygous mice, indicating that missense mutations are more deleterious than nulls (7). In humans, numerous mutations have been reported in the β /slow MyHC gene in patients with familial hypertrophic cardiomyopathy and the vast majority of these mutations are located in the motor domain of the myosin molecule (8). In these patients, a central core disease has also been observed in skeletal muscle, i.e., in the type I fibers expressing the β /slow MyHC (9). However, pure skeletal muscle myosinopathies have not been reported until recently (10,11). These include different mutations in the distal region of the β /slow MyHC affecting primarily skeletal muscle and a mutation of the IIA MyHC (10,12-16).

An “autosomal dominant myopathy with congenital contractures, ophthalmoplegia and rimmed vacuoles” linked to the MYH2 gene on chromosome 17p13.1 has been reported by Darin and co-workers (15). This missense mutation is in a highly conserved region of the motor domain of the type IIA MyHC isoform and gives rise to an amino acid shift from a negatively charged (Glu-706) to a positively charged (Lys) residue (14,17) and the progression and severity of the disease correlates with type IIA MyHC expression (18).

It is hypothesized that this missense mutation in the motor domain causes a malfunction of the actin-myosin interaction in fibers expressing type IIA MyHC, which might be manifested by reduced cross-bridge force-generating capacity and/or slowing of actin propagation over myosin. To test this hypothesis, the function of the adult MyHC isoforms expressed in human skeletal muscle will be explored at the cellular and molecular levels in patients with the IIA MyHC mutation and compared with healthy controls.

Materials & Methods

Patients, control subjects and muscle fiber preparations

Muscle fibers from four patients carrying the MYH2 E706K mutation were investigated and compared with results from 26 healthy male and female controls ranging in age between 18 and 80 years (46 ± 26 yrs) (18,19). Patient 1 was a 63-year-old man with joint contractures at birth. He had slowly progressive proximal muscle weakness since childhood. At investigation he had moderate muscle weakness and external ophthalmoplegia, and was able to rise from sitting position with hand support. The muscle biopsy showed marked variation in the diameter of the fibers, which frequently showed numerous internalized nuclei. There was a moderate increase in interstitial connective tissue and occasional regenerating muscle fibers. Patient 2 was a 38-year-old woman with multiple joint contractures at birth. Since childhood mild, proximal muscle weakness had persisted without obvious progression. She also had external ophthalmoplegia and hand weakness. She could rise from a sitting position without support.

The muscle biopsy showed moderate variation in muscle fiber size. Fibers frequently showed numerous internalized nuclei. There was slight increase in interstitial connective tissue, but no regenerating fibers. Patient 3 was a 36-year-old woman with multiple joint contractures at birth. Since childhood she had mild proximal muscle weakness with slow progression. There was a predominant back insufficiency and she also had external ophthalmoplegia. She was able to rise from a sitting position without support. The muscle biopsy showed moderate variability of fiber size and slight increase of interstitial connective tissue. Muscle fibers with internalized nuclei were frequent, but there were no regenerating fibers. Patient 4 was a 47-year-old man with slight muscle weakness with onset in adulthood. His major complaint was back insufficiency and he also had hand weakness and external ophthalmoplegia. He was able to rise from a sitting position without support. The muscle biopsy showed slight but pathologic variation in fiber size and many fibers showed internalized nuclei. There were no regenerating fibers and no increase of interstitial connective tissue.

Muscle samples were taken from the lateral portion of the quadriceps (vastus lateralis) muscle in both patients and controls. Muscle samples were obtained by open biopsy in the patients and by the percutaneous conchotome method in the control subjects with the understanding and consent of the patients/subjects. The muscle sample was immediately placed in an ice-cold relax solution (in mmol/l: 100 KCl, 20 Imidazole, 7 MgCl₂, 2 EGTA, 4 ATP, pH 7.0; 4°C). Small bundles of ~25-50 fibers were dissected free from the muscle and tied to a glass microcapillary tube at ~110% resting length. The bundles were then placed in a skinning solution (relax solution containing glycerol; 50:50 v/v) at 4°C for 24 h and subsequently stored at -20°C for use within 3 weeks, or treated with cryoprotectant (sucrose) solution for long-term storage at -80°C as described earlier (20). At the beginning of a set of experiments, a sucrose treated frozen bundle was desucrosed by stepwise lowering the sucrose concentration of the relax solution until no sucrose remained. The bundle was thereafter stored in relax solution with glycerol at -20°C for a maximum of two weeks. Muscle samples were treated in an identical way in patients and controls. Short muscle fiber segments were used in all biochemical and contractile analyses and identical results are obtained irrespective of if samples are taken by the open or percutaneous muscle biopsy technique. The Ethical Committee at Gothenburg University and the Pennsylvania State University's Institutional Review Board approved all aspects of this study.

Single fiber contractile measurements

On the day of an experiment, the fibers were placed for 30 min in a relaxing solution containing 0.5% Brij-58 (polyoxyethylene 20 cetyl ether; Sigma Chemical Co.). The fibers were mounted in an experimental apparatus similar to one described previously (21,22), leaving an average fiber segment length of 2.02 ± 0.51 mm (mean $\pm$ SD, range 1.05-3.60 mm) exposed to the solution between connectors to a force transducer (Model 403, Cambridge Technology, Inc., U.S.A.) and a DC torque motor (Model 300H, Cambridge Technology, Inc.). The apparatus was mounted on the stage of an inverted microscope (Zeiss Axiovert-35, Carl Zeiss, Oberkochen, Germany). While the fiber was in the relaxing solution, the sarcomere length (SL) was set to 2.76 ± 0.04 μ m (range 2.66-2.85 μ m) by adjusting the overall segment length. The segments were observed at a magnification of 320 $\times$. Prints of the fiber segments were taken with a videoprinter (P71E, Mitsubishi Electric Corp., Japan). The sarcomere length, the segment width and the length of segment between the connectors were measured directly from the microscope via a TV-overlay with the aid of a digitizer connected to a microcomputer (Videoplan, Kontron Bildanalys GmbH, Munich, Germany). The final magnification with the image analysis system on the TV screen was 1,480 $\times$. Fiber SL was measured routinely in the fibers during maximal activation. Fiber depth was measured by recording the vertical displacement of the microscope nosepiece while focusing on the top and bottom surfaces of the fiber. Cross-sectional fiber area (CSA) was calculated from the width and depth, assuming

an elliptical circumference, and was corrected for the 20% swelling that is known to occur during skinning (22). Specific tension was calculated as maximum tension (P_0) normalised to CSA.

Relaxing and activating solutions contained (in mM): 4 MgATP, 1 free Mg^{2+} , 20 imidazole, 7 EGTA, 14.5 creatine phosphate and sufficient KCl to adjust the ionic strength to 180. The pH was adjusted to 7.0. The free Ca^{2+} concentrations were 10^{-9} M (relaxing solution) and $10^{-4.5}$ M (maximum activation solution) and are expressed as pCa ($-\log[Ca^{2+}]$). Apparent stability constants for Ca^{2+} -EGTA were corrected for temperature and ionic strength (23). The computer program of Fabiato(23) was used to calculate the concentrations of each metal, ligand and metal-ligand complex. Immediately preceding each activation, the fiber was immersed for 10-20 s in a solution with a reduced Ca^{2+} -EGTA buffering capacity (24). This solution was identical to the relaxing solution except that EGTA was reduced to 0.5 mM, which resulted in more rapid attainment of steady tension during subsequent activation and helped to preserve the regularity of cross-striations during activation.

V_0 was measured by the slack-test procedure (25). Fibers were activated at pCa 4.5 and once steady tension was reached, various amplitudes of slack (ΔL) were rapidly introduced (within 1-2 ms) at one end of the fiber. The time (Δt) required to take up the imposed slack was measured from the onset of the length step to the beginning of tension redevelopment. For each amplitude of ΔL , the fiber was re-extended while relaxed in order to minimize non-uniformity of sarcomere length. A straight line was fitted to a plot of ΔL versus Δt , using a least-squares regression, and the slope of the line was recorded as V_0 for that fiber. Maximum active tension (P_0) was calculated as the difference between the total tension in the activating solution (pCa 4.5) and the resting tension measured in the same segment while in the relaxing solution. All contractile measurements were carried out at 15°C. The contractile recordings were accepted in subsequent analyses if a V_0 value was based on linear regressions including four or more data points, and data were discarded if r for the fitted line was less than 0.97, if P_0 changed more than 10% from first to final activation, or if SL during isometric tension development changed by more than 0.10 μm compared with SL while the fiber was relaxed (22).

In vitro motility assay

The single fiber *in vitro* motility system has been described in detail elsewhere (26,27). In brief, a short muscle fiber segment was placed on a glass slide between two stripes of grease and a nitrocellulose-coated coverslip was placed on top, creating a flow-cell of approximately 2 μl volume. Myosin was extracted from the fiber segment through addition of a high-salt buffer (0.5 M KCl, 25 mM Hepes pH 7.6, 4 mM $MgCl_2$, 4 mM EGTA, 2 mM ATP, 1% β -mercaptoethanol). After a 30 minute incubation on ice, a low-salt buffer (25 mM KCl, 25 mM Hepes pH 7.6, 4 mM $MgCl_2$, 1 mM EGTA, 1% β -mercaptoethanol) was applied, followed by BSA (1 mg/ml). Non-functional myosin molecules were blocked with fragmented F-actin and rhodamine-phalloidin (Rh-Ph; Molecular Probes Inc., OR) labeled actin filaments were subsequently infused into the flow-cell followed by motility buffer (2 mM ATP, 0.1 mg/ml glucose oxidase, 23 $\mu g/ml$ catalase, 2.5 mg/ml glucose, 0.4% methyl cellulose in low-salt buffer) to initiate movement. The pH of the buffers was adjusted with KOH and the final ionic strength of the motility buffer was 71 mM. The flow-cell was placed on the stage of an inverted epifluorescence microscope (Olympus IX 70, Olympus America Inc. NY) and thermostatically controlled at 25°C. Actin movements were filmed with an image intensified SIT camera (SIT 66, DAGE-MIT Inc., USA) and recorded on tape with a VCR.

Motility data analysis

From each single fiber preparation, 20 actin filaments moving with constant speed in an oriented motion were selected for speed analysis. Recordings and analysis were only performed

from preparations in which >90% of the filaments moved bi-directionally. The center of a filament was tracked and the speed was calculated from 20 frames at an acquisition rate of 5 or 1 frame(s)/s depending on the fiber type, using an image analysis package (OPTIMAS 6.0, Optimas Corp., USA). The average speed and standard deviation of the 20 filaments were calculated. Since the standard deviation in this group of filaments was small (between 10-15% of the mean), the average velocity was taken as representative for the muscle fiber.

Electrophoretic separation of myosin heavy and light chain isoforms

The MyHC composition of a muscle fiber or muscle biopsy sample was determined by 6% SDS-PAGE. The total acrylamide and Bis concentrations were 4% (w/v) in the stacking and 6% in the running gel, respectively, and the gel matrix included 30% glycerol. Electrophoresis was performed at 120 V for 24 h with a Tris-glycine electrode buffer (pH 8.3) at 15°C (SE 600 vertical slab gel unit, Hoefer Scientific Instruments, USA).

The gels were silver-stained and subsequently scanned in a soft laser densitometer (Molecular Dynamics, Sunnyvale, CA) with a high spatial resolution (50 μ m pixel spacing) and 4096 optical density levels (21,28,29). The volume integration function (ImageQuant software v3.3, Molecular Dynamics) was used to quantify the amount of protein on the 6% gels.

Statistics

Means, standard deviations of means (SD) and correlation coefficients (r) of linear regressions were calculated from individual values by standard procedures. A two-tailed t-test was used for comparisons of two groups and one-way analysis of variance (ANOVA) was used for comparisons among more than two groups. Differences were considered significant at $p < 0.05$.

Results

Muscle fiber size

In previous studies on patients with the IIa MyHC missense mutation, morphological muscle biopsy analyses at the light and electron microscopic level have shown an irregular intermyofibrillar network and rimmed vacuoles in type 2A fibers, increased variability in size of muscle fibers, increased interstitial fat and connective tissue and internalized myonuclei (14,15,18). A short summary of the clinical picture and histopathological findings are listed in Materials & Methods. In this context it is interesting to observe that the structural abnormalities were predominantly in 2A fibers, but central nuclei also observed in other fiber types as well (Fig. 1). In individual membrane permeabilized muscle fiber segments, the size of individual muscle fibers can be determined and related to MyHC isoform expression. The advantage with this approach is that muscle fiber size is measured at a fixed sarcomere length. The β /slow (type I) and the two fast (type IIa and IIx) MyHC isoforms were expressed in all patients (Fig. 2). In both male and female patients, increased muscle fiber size variability was observed independent of MyHC isoform expression and there was no sign of muscle atrophy. On the contrary, the average size of fibers expressing the type I MyHC isoform was approximately twice as large as the corresponding cell type in age- and gender-matched controls. Muscle cells expressing different fast MyHC isoforms in patients were of the same size or slightly larger than in the controls (Table 1). One muscle cell segment in a male patient co-expressed all adult human limb muscle MyHC isoforms (IIIa-IIx). This MyHC isoform expression was not observed in any of the muscle fiber segments from the controls and it is anticipated that this rare MyHC combination reflects a MyHC isoform transformation (2).

Single fiber contractility

A total of 97 membrane permeabilized single muscle fiber segments were attached to the force transducer and servomotor in the experimental set-up. However, only four of the 97 fibers (4%)

success rate) fulfilled the criteria for acceptance (see Materials and Methods). An unacceptable decrease in force during repeated maximum activations at high calcium concentration (pCa 4.5) and fibers breaking during activation were the dominating causes for not accepting fibers, i.e., 65% of the fibers broke or lost more than 10% of P_0 during repeated activations (Table 2).

MyHC isoform expression was determined in 73 of the 97 fibers and in all four fibers that fulfilled the criteria for acceptance. Among the 73 fibers, 59% expressed type I, 27% type IIa and 3% type IIx MyHC. The other fibers expressed two or three MyHC isoforms, i.e., type I and IIa (7%), IIa and IIx (3%) and I, IIa and IIx (1%). All four fibers with acceptable contractile recordings expressed the β /slow (type I) MyHC isoform. Three of the four fibers had V_0 's (0.94, 1.10, 1.11 ML/s), which were within the range of the control fibers expressing the type I MyHC isoform (0.21-1.47 ML/s; $n=351$), but they were all faster than the average V_0 in the control fibers (0.55 ± 0.20 ML/s). The V_0 (1.79 ML/s) in the fourth fiber was slightly faster than contractile speed of control fibers, resulting in a significantly ($p < 0.001$) faster average V_0 in the patient fibers (1.24 ± 0.38 ML/s). The specific tensions in these four fibers were within the normal range, i.e., 26 ± 7 N/cm² (18-33 N/cm²; Fig. 3). In spite of the fact that only 4% of all fibers fulfilled the criteria for acceptance, many muscle fibers did develop specific tensions which were within the normal range irrespective of MyHC isoform expression, i.e., prior to unacceptable loss of force during repeated activations, non-uniform sarcomere lengths or weak correlations when calculating V_0 (Fig. 3). However, the recordings from the patients failing to reach the level of acceptance have not been used in any statistical comparisons due to the risk of including false measurements.

In vitro motility

Single fiber *in vitro* motility assays were prepared in a total of 349 fiber segments from patients carrying one mutated allele of the type IIa MyHC gene. The MyHC isoform expression was determined in 344 of these fibers and the relative distribution of fibers expressing different isoforms or combinations of isoforms is shown in Table 2. In accordance with single fiber measurements, approximately half of the fibers expressed the type I MyHC isoform and one third of the fibers expressed the IIa MyHC isoform alone. The remaining fibers co-expressed two or three isoforms and 1% of the fibers expressed the IIx isoform (Table 2). It should be noted that the success rate of the current single fiber *in vitro* motility assay is modest due to stringent criteria for acceptance, i.e., bi-directional motility of the vast majority of moving filaments, and the success rate typically varies between 30 and 50% in normal control preparations independently of the MyHC isoform expressed in the fiber. In muscle fibers expressing the type I, IIa or IIx MyHC isoforms, the number of muscle fibers was sufficiently large to allow an estimate of success rate. In the muscle cells expressing the type I MyHC isoform, the success rate was similar to that seen in control fibers. A significantly lower success rate (3-4%) was, on the other hand, observed in muscle cells expressing the IIa MyHC or a combination of type IIa and IIx MyHCs (Table 2). In preparations expressing the IIa MyHC isoform and classified as unacceptable, 38% showed no motility (cross-bridges in rigor confirmation, "dead heads"), 43% demonstrated numerous "dead heads" together with randomly moving actin filaments or filaments showing "stop and go" motility, and 19% showed numerous moving filaments but in only a small fraction of these preparations (4%) were filaments moving bidirectionally without any "stop and go" motility. In preparations co-expressing the IIa and IIx MyHC isoforms and classified as unacceptable, 4% showed no motility, 46% demonstrated numerous "dead heads" together with filaments moving randomly or showing "stop and go" motility, and 59% showed an acceptable number of moving filaments, but they were moving randomly and/or numerous filaments showed "stop and go" motility.

Actin filament speeds propelled by the type I MyHC were typically within the normal limits (Fig. 4) and there was no significant difference in the average motility speed between patients

($0.73 \pm 0.13 \mu\text{m/s}$) and controls ($0.78 \pm 0.22 \mu\text{m/s}$). In acceptable IIA MyHC preparations, motility speeds ($1.16 \pm 0.12 \mu\text{m/s}$) were slower ($p < 0.001$) in all the patient fibers than in any preparation in the controls ($2.61 \pm 0.28 \mu\text{m/s}$) independent of the age and gender of the patient (Fig. 4). Motility speed ($4.05 \mu\text{m/s}$) in the single acceptable preparation expressing the type IIX MyHC isoform from patients was within the normal range (Fig. 4). The motility speed and percent IIA MyHC in the few fibers co-expressing type I and IIA MyHCs did not differ between patients ($0.80 \pm 0.17 \mu\text{m/s}$; 31%) and controls ($1.01 \pm 0.50 \mu\text{m/s}$, 49%). In the hybrid fibers co-expressing the IIA and IIX MyHCs, motility speed and percent IIX MyHC were similar in patients ($3.24 \pm 0.53 \mu\text{m/s}$; 68%) and controls ($3.40 \pm 0.65 \mu\text{m/s}$, 55%). The motility speed in the fiber co-expressing type I (58%), IIA (4%) and IIX (37%) was $1.44 \mu\text{m/s}$, i.e., a motility speed within the range of fibers co-expressing type I and IIA MyHC isoforms Fig. 4).

Discussion

The study presents data on myosin function at the protein and cellular level in patients carrying a missense mutation in the gene coding for the type IIA myosin. Analyses at the motor protein level demonstrate a significant negative effect on the function of the IIA MyHC isoform while other myosin isoforms had normal function. Analyses at the cellular level, on the other hand, indicate that the IIA MyHC mutation is associated with impairments in muscle cell function, which goes beyond structure-function changes in the IIA MyHC isoform. These findings suggest complex mechanisms underlying the symptoms of “autosomal dominant myopathy with congenital contractures, ophtalmoplegia and rimmed vacuoles”.

Patients with the autosomal dominant myopathy, first described by Darin and co-workers (15), are characterized by multiple joint contractures at birth, mild myopathy at childhood and adolescence, and a progressive course at middle age with proximal muscle weakness, ophtalmoplegia and elevated creatine kinase serum levels. The histopathological abnormalities in muscle tissue increase during the progression of the disease and include central nuclei, fiber size variability and focal disorganization of myofilaments (inclusion bodies) in type 2A fibers (14,15,18). By screening the MyHC-IIA gene, a mutation was detected in affected family members, i.e., a negatively charged glutamate replaced by a positively charged lysine residue in the SH1 helix of the myosin molecule (14). This mutation is in a region of the myosin molecule which is 100% conserved in both conventional and unconventional myosins (14). This region is close to the catalytic domain that undergoes a conformational change during ATP hydrolysis. The altered charge in this region, induced by the mutation, may have a significant effect on motor protein function. The present observations demonstrating a significant decline in motility speed as well as an increased number of dysfunctional IIA myosin molecules in the *in vitro* motility assay support this hypothesis. The slowing of motility speed together with a relatively well maintained specific tension at the cellular level indicate that the impaired muscle function in these patients is not primarily caused by an impaired thick filament assembly or myosin-actin binding. An altered conformational change of the IIA myosin molecule during ATP hydrolysis appears to be a more likely cause underlying the impaired IIA myosin function.

By introducing probes to the *Dictyostelium discoideum* myosin class II motor domain, myosin function has been studied in detail and interpreted in relation to motor domain crystal structures. Zeng and co-workers (30) recently constructed the equivalent mutation to the E706K mutation in human IIA MyHC in *Dictyostelium discoideum* myosin converter region, E683K. It was predicted that the change in amino acid charge would result in charge repulsion instead of attraction. This would in turn lead to a kinked and/or more unstable helix region and a reduced range of motion of the converter domain. Studies on nucleotide binding and hydrolysis indicated a 3-fold reduction of the hydrolysis step and slowing of the acto-myosin dissociation leading to a higher duty ratio in the cycle and a reduced contractile speed (30). Thus, the slowing

observed in the *in vitro* motility speed in the mutated IIa myosin from the patients is consistent with the “braking” action suggested by the *Dictyostelium* E683K mutation, but the reduction in motility speed was less than the 3-fold reduction anticipated from the *Dictyostelium* experiments. However, the smaller reduction in motility speed is most probably related to the fact that in the heterozygous subjects the motility speed is determined by the combined action of both the native and mutated IIa MyHCs.

Contractile recordings at the muscle cell and motor protein levels were included only when specific criteria for acceptance were fulfilled (see Materials and Methods). At the single muscle fiber level, the criteria originally introduced by Moss (22) were used for acceptance. According to these criteria, high reproducibility, e.g., when comparing two segments of the same muscle fiber from both rabbit (21) and human skeletal muscle (31), has been reported in contractile recordings from single membrane permeabilized muscle fibers. In the single muscle fiber *in vitro* motility assay, originally developed within our group (27, 32,33), motility speed was measured from actin filaments which were only moving parallel or anti-parallel to the flow in the experimental chamber. These filaments move slightly faster, but more importantly, they show significantly less variable motility speed than randomly moving filaments (27). When restricting analyses to actin filaments moving parallel or anti-parallel, reproducibility equal to that seen at the single muscle fiber level (21,31) has been reported, e.g., when comparing motility speed between two halves of the same muscle fiber (32,34). Due to the necessary strict acceptance criteria, numerous preparations were discarded from both patient and control subjects. The risk associated with eliminating preparations representing a subpopulation of normal muscle cells or motor proteins by excessively stringent criteria is negligible compared with the advantage of removing false measurements. However, an increased number of preparations failing to reach the level of acceptance may reflect pathological changes at the muscle cell or motor protein levels, thus we have compared the success rate in preparations from patients and controls in an attempt to monitor this possibility.

The mutated and native alleles of the IIa MyHC isoform are equally expressed at the transcript level in patients with the E706K mutation (18). In patients carrying the E706K mutation, the IIa MyHC mRNA levels consistently exceeded the IIa MyHC protein levels, while IIa myosin mRNA and protein levels were roughly the same in healthy controls (18). It was therefore suggested that the disproportionately high IIa MyHC transcript levels reflected an increased turnover of the mutated IIa myosin (18). The amount of mutated versus native IIa myosin may accordingly vary between different 2A muscle fibers. The relative amounts of mutated versus native myosin in the different muscle cells investigated in this study are not known. A considerable effort was made to separate the mutated vs. the native myosin using 2D gel electrophoresis and mass spectrometry, but we were not able to obtain reproducible results using either approach. The slowing of actin filament speed propelled by the motor protein and the high rate of unacceptable recordings in preparations expressing IIa or co-expressing IIa and IIx myosin indicate a significant amount of mutated IIa MyHC in all these preparations.

Proteolytic degradation products of myofibrillar proteins have been suggested to have a toxic effect on muscle cell structure leading to impaired function and eventually apoptotic effects (35). The increased turnover of the mutated IIa myosin (18) may accordingly result in an increased amount of toxic byproducts that may have a general effect on muscle cell structure and function leading to the increased number of preparations failing to reach the level of acceptance.

At the motor protein level, the disproportionately high number of unsuccessful recordings was restricted to preparations expressing the IIa MyHC isoform. At the cellular level the number of preparations that fulfilled the criteria for acceptance was also very low (4%). However, in contrast to recordings at the motor protein level, the poor success rate at the cellular level

affected all fiber types irrespective of the MyHC isoform expression. This 4% success rate should be compared with the 75% success rate typically obtained when performing contractile recordings from normal human muscle fiber segments by the experienced scientist conducting all contractile recordings in this study and having more than eight years experience with this experimental setup. The extremely low success rate in all fiber types suggests that the mechanisms underlying the phenotype of the inclusion body myopathy with the E706K mutation is not restricted to structural and functional changes in the converter region of the IIA myosin molecule. The increased frequency of structural abnormalities during activation with high calcium concentrations, such as uneven cross-striation pattern, partial or complete disruption of the muscle fiber segments, suggest alterations in cytoskeletal elements. The involvement of other proteins is supported by the progressive nature of the disease in adulthood and the mild phenotype in children. While the missense mutation of the *MYH2* gene is causing the myopathy, other genes and proteins may play important roles as modifiers of the symptoms. The polygenic nature of phenotypes such as muscle mass, varying according to the age (36, 37), may contribute to certain deviations from the mendelian type of inheritance of this myosinopathy in some patients, such as the conversion of recessive to a dominant effect over the life span. It can be hypothesized that allelic variations of other genes influenced the ability of muscle tissue to adapt to the primary mutation. Adoption of the polygenic concept might help to identify pathways that could be targeted to delay and/or alleviate symptoms of the disease.

The involvement of muscle fiber types other than 2A, expressing the IIA MyHC isoform, is supported by pathological changes in all muscle fiber types in severely affected muscles from the oldest patients carrying the E706K mutation, although rimmed vacuoles were restricted to muscle fibers expressing the IIA MyHC isoform (14). In young patients with a milder phenotype, on the other hand, morphological changes were restricted to 2A fibers. The muscle samples in this study were taken from affected muscles in adult patients, albeit not with a severe phenotype. The present observations suggest that changes in muscle cell function precede changes in muscle fiber morphology, representing a later stage in the pathological process.

The four muscle cells that fulfilled the criteria for acceptance expressed the β /slow (type I) MyHC isoform had average shortening velocities that were significantly faster than in controls. We have observed a similar increase in shortening velocity in hypertrophic muscle cells expressing the β /slow MyHC isoform in overused slow-twitch motor units from patients with a prior polio lesion (38,39). It is suggested that the faster V_0 and larger size of fibers expressing the type I MyHC isoform in patients carrying the IIA myosin missense mutation than in the control subjects are secondary to a functional overload (38,39). *In vitro* motility observations indicate that the faster shortening velocities at the muscle cell level were not secondary to changes in the function of the β /slow MyHC isoform.

In conclusion, the *MYH2* E706K mutation has a significant negative effect on the function of the IIA myosin isoform consistent with the “braking” action suggested by the *Dictyostelium* E683K mutation. However, single fiber contractile measurements indicate that the phenotype of this autosomal dominant myopathy is caused by changes that go beyond the IIA MyHC and probably affects structural elements in all muscle fiber types.

Acknowledgements

This study was supported by grants from NIH (AR45627, AR47318) and Swedish Research Council (8651) to LL and AFM and Swedish Research Council (7122) to AO. Authors are grateful to Dr. D.A. Blizard for the English editing of the manuscript.

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Figure 1.

Serial cross sections of muscle biopsy from patient 1 showing increased variability of fiber size and slight increase of interstitial connective tissue. Major structural changes are confined to type 2A muscle fibers (two of them are indicated as 2A) but structural alterations with internalized myonuclei are also present in some type 1 fibers (arrows). a) Haematoxylin-eosin b) ATPase pH 4.6 c) NADH-tetrazolium reductase

Figure 2. Electrophoretic separation of MyHC isoforms from 10 μ m cross-sections from a healthy control subject (C) and the four patients with the IIa MyHC mutation (1-4). The two fast (type IIa and IIx) and the β /slow (type I) MyHC isoforms are indicated on the silver stained 6% SDS-PAGE. Only the section corresponding to the MyHC region of the gel is included.

Figure 3. Specific tension in muscle cells expressing different MyHC isoforms from control subjects (open symbols) and from patients carrying the type IIa myosin missense mutation (filled symbols). All fibers from the control subjects fulfilled the criteria for acceptance (see Materials & Methods), but only 4 of the 96 fibers from the patients were acceptable and all these fibers expressed the type I MyHC.

Figure 4. Actin filament motility speed propelled by different myosin heavy chain isoforms extracted from single muscle fiber segments from control subjects (open symbols) and from patients carrying the type IIa myosin missense mutation (filled symbols).

Cross-sectional area calculated from depth and width measurements and assuming an elliptical cross-section of individual muscle fibers. All measurements were performed at a sarcomere length between 2.65 and 2.85 μm .

Table 1

Group	n	Type I (μm^2)	Type I-IIa (μm^2)	Type IIa (μm^2)	Type IIax (μm^2)	Type IIx (μm^2)	Type I-IIax (μm^2)
Male patients	2	7500 $\pm$ 2080 (n=22)	9330 (n=1)	4910 $\pm$ 1440 (n=9)	7100, 8570 (n=2)	6240, 7130 (n=2)	1260 (n=1)
Female patients	2	5420 $\pm$ 2700 (n=20)	3430 $\pm$ 1360 (n=4)	3550 $\pm$ 1000 (n=10)			
Male controls	9	3640 $\pm$ 1080 (n=54)	3210 $\pm$ 1220 (n=5)	4640 $\pm$ 540 (n=11)	4140 $\pm$ 1300 (n=16)	4070 $\pm$ 870 (n=6)	
Female controls	8	2960 $\pm$ 960 (n=57)	2840 $\pm$ 560 (n=12)	2580 $\pm$ 530 (n=19)	2020 (n=1)	2080, 2350 (n=2)	

Values are means $\pm$ S.D. and the total number of measured fibers is given within parentheses.

Total number of single muscle fibers preparations (Single fiber) and *in vitro* motility assays (Motor protein) in the four patients with the IIa myosin missense mutation. Fibers are separated according to expression of MyHC isoform. Fibers which could not be reliably identified on the gels are represented as unidentified (UI). The total number of fibers used in the single fiber and motor protein preparations are given together with the absolute (#) and relative (%) number of recordings fulfilling the criteria for acceptance (+) are given within each fiber type.

		I	I +	I/IIa	I/IIa +	IIa	IIa +	IIax	IIax +	IIx	IIx +	I-IIax	I-IIax +	UI	UI +
Single fiber	#	43	4	5	0	20	0	2	0	2	0	1	0	18	0
	%	47	9	5	0	22	0	2	0	2	0	1	0	20	0
Motor protein	#	191	70	10	4	104	4	32	6	4	1	3	1	5	0
	%	55	37	3	40	30	4	9	19	1	25	1	30	1	0