The kyphoscoliosis (ky) mouse is deficient in hypertrophic responses and is caused by a mutation in a novel muscle-specific protein

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Received 4 October 2000; Revised and Accepted 6 November 2000 DDBJ/EMBL/GenBank accession no. AJ293727

The ky mouse mutant exhibits a primary degenerative myopathy preceding chronic thoraco-lumbar kyphoscoliosis. The histopathology of the ky mutant suggests that Ky protein activity is crucial for normal muscle growth and function as well as the maturation and stabilization of the neuromuscular junction. Muscle hypertrophy in response to increasing demand is deficient in the ky mutant, whereas adaptive fibre type shifts take place. The ky locus has previously been localized to a small region of mouse chromosome 9 and we have now identified the gene and the mutation underlying the kyphoscoliotic mouse. The ky transcript encodes a novel protein that is detected only in skeletal muscle and heart. The identification of the ky gene will allow detailed analysis of the impact of primary myopathy on idiopathic scoliosis in mice and man.

INTRODUCTION

As many as 1–2% of the population at 16 years of age show abnormal spinal curvature, with ~0.6% of these requiring orthotic or surgical correction (1). Concordance for adolescent idiopathic scoliosis (AIS) in monozygotic twins has been found to be significant but not complete, supporting a genetic aetiology for a multifactorial disorder (2,3). The inheritance of severe forms of this disease (degrees II–IV) can be described by a model that assumes a dominant major gene effect (4), although support for any specific mode of inheritance is limited. Fundamental understanding of the primary causes of AIS is hampered because patients usually present in early puberty with already well developed curves. Moreover, without the identification of specific gene defects from linkage studies, the early pathological processes in man are impossible to investigate. One approach to dissecting the genetic basis of AIS is to identify mouse mutants showing scoliosis and appraise them as candidates for models of AIS (5,6).

Currently no mouse mutant fully satisfies all requirements for acceptance as a model for AIS; however, the autosomal recessive mouse mutant kyphoscoliosis (ky) is a useful system for examining the neuromuscular and skeletal mechanisms by which spinal curvature develops (7). Chronic thoraco-lumbar kyphoscoliosis in homozygous ky/ky (Fig. 1a) is preceded by a single postnatal phase of muscle fibre degeneration and regeneration within postural slow contracting muscles (e.g. soleus) (8). The serum enzyme levels of pyruvate kinase and total creatine kinase have been measured in ky/ky mice and found to be within normal limits (8). All ky/ky muscles are smaller, slower contracting and weaker than controls (9). Biochemical analysis has shown that in muscles of ky/ky mice there are dramatic shifts to the expression of contractile protein isoforms typical of slow muscle, including myosin heavy chains (MHCs) and myosin light chains (MLCs) (10).

In order to gain insights into the mechanisms leading to scoliosis, the pathological changes provoked by the ky mutation have
BDL  
ky/ky

BDL  
ky/ky

BDL Soleus  
ky/ky Soleus

40 days

100 days

f

Mean muscle fibre diameter (μm)

BDL  k/ky  BDL  k/ky  BDL  k/ky  BDL  k/ky

Type 1  Type 1A  Type 2A  Type 2

(*) Significant change (P=0.0001)
been further characterized. Also, in the present study, the ky gene has been identified by positional cloning and the causative mutation unveiled.

RESULTS

MHC isoform expression shifts in ky/ky mice

The expression shifts in the MHC isoforms in the mutant background have been further characterized by immunolocalization of MHC1 in both wild-type BDL and ky/ky muscle fibres (Fig. 1b–d). For example, ky/ky soleus muscle shows a progressive and eventually complete shift to type 1 fibres expressing only MHC1 that is not due to selective fast muscle fibre death. The distribution of two other MHCs (MHC2A and 2B) showed complementary changes with complete loss of fast myosin expression (data not shown).

Response to compensatory overload

Whereas the body weight of ky/ky mice is ~80% of that of age-matched BDL controls, both soleus and extensor digitorum longus (EDL) muscle weights are as low as 25% (10). Thus, we predicted that ky/ky soleus muscles might be exposed to an endogenously generated overload arising from the disproportionate body mass. If normal muscles are overloaded in vivo they can respond by adapting the type of MHC and MLC expressed and also by undergoing hypertrophy to generate increased force. We hypothesized that the small size of ky/ky muscles might reflect a defect in their ability to adapt to load and that the extreme shifts to a slow contractile phenotype were generated in order to increase their resistance to fatigue. We therefore examined the effect of surgical compensatory overload imposed on the EDL muscle in vivo in order to induce muscle fibre hypertrophy. The EDL was chosen as it does not undergo necrosis and regeneration in ky/ky and thus any changes could be separated from this event. There is a complete absence of a hypertrophic response to 5 weeks of compensatory overload in the ky EDL, whereas the mean fibre diameters of type 2A and 2B fast muscle fibres increase up to 17.8% in wild-type BDL mice (Fig. 1f).

Neuromuscular junction remodelling

ky/ky muscles such as soleus, whose muscle fibres necrose and regenerate, show motoneuron sprouting in the absence of motoneuron loss (8). We have now examined the distribution of acetylcholine receptors in neuromuscular junctions (NMJs) of ky/ky soleus and show that they are abnormally arranged in numerous small patches rather than the well ordered structures typical of wild-type NMJs (Fig. 2a). The EDL muscle is normal in this respect. Moreover, in vivo imaging shows that NMJs within sternomastoid, a muscle that in ky/ky mice does not undergo necrosis and regeneration, are persistently unstable showing both synapse loss and formation within individual adult muscles (Fig. 2b). Thus, NMJ disorganization in ky/ky muscles is not a simple consequence of muscle fibre remodelling. Although this may reflect a secondary adaptive change to a primary muscle abnormality, it does not rule out a role for Ky in NMJ stabilization.

Positional cloning and identification of the ky mutation

We have undertaken a positional cloning effort to identify the mouse ky mutation. High resolution genetic and physical mapping of the ky locus has led to the identification of a putative muscle-specific transcription unit from the ky non-recombinant interval (11). Using a combination of rapid amplification of cDNA ends and muscle cDNA library screening, a 5.3 kb composite of the candidate gene transcript was assembled. A perfect Kozak’s consensus sequence (12) surrounds the putative ATG, indicating the beginning of an open reading frame (ORF) encoding a 73 kDa protein. Although 3′-untranslated region (3′-UTR) probes showed additional bands on northern blots, possibly identifying alternatively spliced products, probes from the predicted coding region identified only a single band in skeletal and heart muscles (Fig. 3a). Semi-quantitative RT–PCR analysis revealed that this transcript is present at significantly lower levels in ky/ky mice compared with wild-type (Fig. 3b). Northern blot analysis also demonstrated a very low level of expression of this muscle-specific gene in ky/ky mice (Fig. 3b). Sequencing of the candidate gene in ky/ky mice identified a GC deletion affecting codon 24 downstream of the putative first ATG (Fig. 4). Other mouse strains, including the parental wild-type strain BDL, C57BL/6, Mus spretus, 129, C3H and BALB/c showed the expected wild-type sequence across this region. The GC deletion alters the reading frame leading to a premature stop codon at position 105.

The Ky protein belongs to a new family of transglutaminase-like proteins

The human ky orthologue, identified through expressed sequence tag matches and mapped using the Genebridge4 radiation hybrid panel, falls into a conserved region of synteny on 3q21, between markers D3S3086 and D3S3108 (data not shown). Two rounds of PSI-BLAST (13) database searches using an E-value inclusion threshold of 10−3 revealed significant similarity to five predicted proteins: Drosophila melanogaster CG13435, Caenorhabditis elegans K02C4.4, Saccharomyces cerevisiae Ydl117wp, Schizosaccharomyces pombe C9G1.06C and Synechocystis sp. sll1681. Our own studies (Fig. 4) and those of others (14) demonstrate that these five proteins each contain a domain homologous to human transglutaminase.
With the exception of *C. elegans* K02C4.4, which contains substitutions of key active site residues, these transglutaminase homologues are likely to possess protease functions (14). The *Saccharomyces cerevisiae* Ky homologue Ydl117wp has recently been renamed CYK3 because of its role in cytokinesis. This novel cytokinetic factor possibly contributes to secretory targeting or septal deposition during yeast cell division. Furthermore, genetic interactions also argue for CYK3 having a central role in cell division (15).

**DISCUSSION**

The mutation identified in this paper has unveiled a novel muscle-specific protein that plays a key role in skeletal muscle’s normal response to mechanical load. Our results show that the signalling mechanisms controlling changes in contractile protein biochemistry are intact in *ky/ky*. The complete shift to type I fibres expressing only MHC1 that occurs in *ky/ky* mice probably reflects an adaptive change of postural muscles to the endogenous overload caused by the low muscle-to-body weight ratio (10). Adaptive change is also observed in AIS where muscles from the convex and concave sides of the curvature show different fibre type proportions (16–18), probably reflecting response to changing demand (19).

Fibre conversions and hypertrophy are part of the normal synchronized response of skeletal muscle to functional overload or chronic increase in demand. Interestingly, there is a complete absence of a hypertrophic response to compensatory overload in the *ky* EDL. To our knowledge *ky* is the only published mouse mutant to exhibit the absence of a hypertrophic response to overload *in vivo*. As such, our results show that the Ky protein is an essential component of the muscle hypertrophy pathway. Given that fast-to-slow fibre transformations are normal in *ky*, the *ky* mouse provides genetic evidence for the independent regulation of these two cellular processes.

We have also shown that the NMJs of *ky/ky* mice are not normally differentiated. Muscles undergoing fibre remodelling (e.g. soleus) show a striking pattern of acetylcholine receptor distribution with no typical endplate localization, although clustering still occurs in many isolated patches. The uninterrupted loss and formation of adult sternomastoid NMJs shown by *in vivo* imaging demonstrates that NMJ instability is not a consequence of muscle fibre remodelling, as sternomastoid is spared the necrosis and regeneration processes affecting other muscle groups in *ky/ky* mice. These data are most consistent with the changes seen at *ky/ky* NMJs being an adaptive response to intense muscle activity; similar changes have been observed in intensely exercised rats (20). Nevertheless, our results do not rule out the possibility that Ky plays a role in the maintenance of stability in maturing NMJs.

Although proximal muscle weakness is often found in human scoliotic patients (21, 22) there are no detailed functional studies to date that demonstrate a similar muscle dysfunction in humans to the one provoked by the *ky* mutation in the mouse. Nevertheless, in the light of the results shown in this paper, we hypothesize that mutations in the human homologue of *ky* will lead to weakness of postural muscles and therefore it is a candidate for a group of familial AIS. Moreover, further elucidation of the function of this protein may shed light on the molecular pathogenesis leading to scoliosis in humans.

**MATERIALS AND METHODS**

**Production of *ky* mutant stock**

The *ky* recessive mutation arose in the BDL strain of laboratory mice. Homozygote *ky/ky* mice were produced from heterozygous parents and were readily identified because muscle weakness and subsequent chronic scoliosis prevents *ky/ky*.
mutant mice from executing a normal placing reflex. In addition, each ky/ky mouse was autopsied to confirm its muscle phenotype. Muscle fibres from the whole of the posterior compartment of the lower hind limb were examined. The ky/ky phenotype was characterized by the presence of active muscle fibre necrosis, regeneration as indicated by the presence of small diameter fibres that are highly basophilic and the retention of central nucleation (8).

Expression analysis
Multiple tissue northern blots (Clontech) were probed with ky cDNA 3'-UTR and ORF probes (Fig. 3a) following the manufacturer’s recommendations. The 3'-UTR probe was generated with primers forward 5'-AAGCAGATTAGCCCAAGCAG-3' and reverse 5'-GGCTGTGTCTAGCGAGTGG-3'. The ORF probe was generated with primers forward 5'-TGAGGACCCAGAAGCCAAC-3' and reverse 5'-AGCTTTATCCCGTTCTTCTCTC-3'. For expression analysis, samples were obtained from 40- to 50-day-old ky/ky and wild-type BDL mice. Northern blots were made using 500 µg of total RNA isolated from the whole of the posterior compartment of the lower hind limb using the Trizol method (Sigma). Semi-quantitative RT-PCR analysis was carried out on first strand cDNA generated with Superscript II (Gibco BRL) from 500 ng of poly(A)+ RNA purified from total RNA using mini-columns (Qiagen), according to the manufacturer’s recommendations. RT–PCR reactions for ky (forward 5'-GGGATGAAGCTGGAGGTGTA-3', reverse 5'-ATTAGGGGTGTCTGGGCTCT-3'), desmin (forward 5'-GACGCTGTGAACCAGGAGTT-3', reverse 5'-ACGAGCTAGAGTGCTGCTGAT-3') and myogenin (forward 5'-CCTGCCCTGAGTTGAGAGAG-3', reverse 5'-CGGCAGCTTTTACAAAACAACA-3') were performed at increasing numbers of cycles (31, 33, 35, 37, 39 and 41 cycles) and the optimal number of cycles reflecting the concentration of each transcript in the first strand cDNA template was empirically determined on agarose gels.

Histology
Tissues were fixed by immersion in 10% neutral buffered formalin for 24 h, dehydrated in ethanol, cleared in xylene and embedded in paraffin wax. Sections of 4 mm thickness were

Figure 3. Identification and analysis of the ky transcript. (a) (Top) A diagram of the ky composite cDNA drawn to scale. Black solid bars depict the position and length of probes used in northern blots below. (b) (Left) Northern blot using total RNA from BDL and homozygous ky/ky mice probed with ky and Desmin as an internal loading control; (right) combined semi-quantitative RT–PCR for ky and two controls (desmin and myogenin). RT–PCR reactions were independently calibrated for each gene (see Materials and Methods). To facilitate comparison, the PCR products for ky, desmin and myogenin have been combined and loaded together.
dewaxed, brought to water via an ethanol series and stained with haematoxylin and eosin.

**Immunohistochemistry**

Tissues were frozen in isopentane cooled in liquid nitrogen prior to cryosectioning. Standard indirect immunohistochemistry was employed using primary mouse monoclonal antibodies specific to MHC isoforms: BAD5 for MHC1, SC-71 for MHC2A and BF-F3 for MHC2B (23). Primary antibodies were localized using an FITC-conjugated polyclonal anti-mouse IgG (Sigma) and visualized using an Olympus PROVIS microscope.

**Whole mount muscle histochemistry**

Whole mounts of muscles for confocal microscopic imaging were prepared, following dissection, by pinning out under slight tension onto layers of Sylgard polymer. Muscles were stained in 20 μg/ml α-bungaroxin–tetramethylrhodamine isothiocyanate (α-BTX–TRITC) (Molecular Probes) dissolved in phosphate-buffered saline (PBS) (pH 7.4, 15–30 min room temperature). Muscles were washed three times in PBS (15 min) with agitation. Muscles were fixed in 1% freshly prepared phosphate-buffered paraformaldehyde (pH 7.4, 15 min) prior to three further PBS washes (15 min each). They were then teased into small bundles containing ∼100–150 fibres. Bundles were immediately mounted in Vectashield UV-free aqueous mountant under 1 μm thick coverslips laid onto Vectabond-coated slides.

**Confocal microscopy**

Confocal imaging was performed using two different systems. Initially, images were captured using an MRC500 confocal
visualization system (BioRad). Subsequently images were captured using a DVC-250 Viewscan confocal microscope (BioRad). The configuration, means of alignment and means of regulating the laser input of the MRC 500 confocal microscope have been described in detail elsewhere (24). Samples were illuminated with either the 488 nm or the 515 nm line of the argon ion laser, selected with an appropriate band pass filter of a 10 nm bandwidth. The images were collected with a 565 nm long pass filter placed before the detector. For the MRC500 images were collected for an average of 160 scans of the laser and the number of scans used by the Viewscan was empirically determined for each sample. Images from several planes of focus 0.6–1 µm apart were collected and presented in a single image where the vertical pixel that exhibited the maximum brightness in the data set was displayed.

**In vivo microscopy**

Sternomastoid muscles were isolated from mice and visualized as described previously (25). Essentially, 10- to 15-week-old **ky/ky** and **BDL (+/+)** mice were anaesthetized with a single intraperitoneal injection of chloral hydrate (0.5–0.6 mg/kg body wt) or a mixture (5.0 ml/kg body wt) containing 0.17 mg of ketamine (Ketaset; Aveco) and 1.7 mg of xylazine (Anased; Lloyd Laboratories) per millilitre of 0.9% sodium chloride solution. The anaesthetized mouse was placed on its back on the stage of a modified microscope so that the upper part swung away. The mouse was then intubated and mechanically ventilated for the duration of the experiment. A midline incision was made from the sternum to the apex of the mandible, and the left sternomastoid muscle was exposed by lateral deflection of the skin and salivary glands. The muscle was gently lifted on a small platform (23). Nerve terminals and muscle fibres were stained for 3 min with a 4–10 µM solution of 4-Di-2-Asp-TRITC. After staining, the wound was washed with lactated Ringer’s solution and a coverslip was lowered on the surface of the muscle. With the microscope swung back with lactated Ringer’s solution of non-blocking concentrations (2–4 µl/mI) of α-BTX–TRITC. After staining, the wound was washed and the NMJs were visualized with a small platform (23). Nerve terminals and muscle fibres were visualized with lactated Ringer’s solution and a coverslip was lowered on the surface of the muscle. With the microscope swung back into its normal position, the NMJs were visualized with conventional epifluorescence and video microscopy. Using a low-light-level SIT camera (Dage/MTI) with a Trapix digital image where the vertical pixel that exhibited the maximum brightness in the data set was displayed.

**Morphological and statistical analysis**

The least diameter of 200 muscle fibres randomly sampled from sections stained with antisera to **MHC1**, **MHC2A** and **MHC2B** was measured from digitized images using NIH Image software. These data were analysed statistically using Student’s t-test to determine significant differences between loaded and unloaded muscles.

**Accession numbers**

GenBank accession numbers are as follows: **D.melanogaster** CG13435 (7302360), **C.elegans** K02C4.4 (3878111), **Saccharomyces cerevisiae** Ydl117wp (6320086), **Schizosaccharomyces pombe** CG1l.06C (3183389) and **Synechocystis** sp. sll1681 (1651866); **M.musculus** ky (A293727).

**ACKNOWLEDGEMENTS**

We wish to thank Dr Leslie Bridges (University of Leeds) for the image of the normal neuromuscular junction and Dr Alan Entwistle (Ludwig Institute, London) for assistance with confocal imaging. This research was supported by grants from the MRC, SERC, BBSRC and the Charing Cross and Westminster Hospital Trustees.

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