A Rostrocaudal Muscular Dystrophy Caused by a Defect in Choline Kinase Beta, the First Enzyme in Phosphatidylcholine Biosynthesis*

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Muscular dystrophies include a diverse group of genetically heterogeneous disorders that together affect 1 in 2000 births worldwide. The diseases are characterized by progressive muscle weakness and wasting that lead to severe disability and often premature death. Rostrocaudal muscular dystrophy (rmd) is a new recessive mouse mutation that causes a rapidly progressive muscular dystrophy and a neonatal forelimb bone deformity. The rmd mutation is a 1.6-kb intragenic deletion within the choline kinase beta (Chkb) gene, resulting in a complete loss of CHKB protein and enzymatic activity. CHKB is one of two mammalian choline kinase (CHK) enzymes (α and β) that catalyze the phosphorylation of choline to phosphocholine in the biosynthesis of the major membrane phospholipid phosphatidylcholine. While mutant rmd mice show a dramatic decrease of CHK activity in all tissues, the dystrophy is only evident in skeletal muscle tissues in an unusual rostral-to-caudal gradient. Minor membrane disruption similar to dysferlinopathies suggest that membrane fusion defects may underlie this dystrophy, because severe membrane disruptions are not evident as determined by creatine kinase levels, Evans Blue infiltration, and unaltered levels of proteins in the dystrophin-glycoprotein complex. The rmd mutant mouse offers the first demonstration of a defect in a phospholipid biosynthetic enzyme causing muscular dystrophy, representing a unique model for understanding mechanisms of muscle degeneration.

Muscular dystrophies are a variable class of more than 20 human disorders characterized by progressive muscle wasting and weakness resulting from myofiber degeneration and regeneration. Histologically, variation in myofiber size with centrally localized nuclei, fibrosis, and fatty infiltration are common features (1, 2). Despite their common pathologies, the genetic causes, severity, age of onset, and inheritance patterns vary widely among the dystrophies. The Muscular Dystrophy Association currently lists over 40 neuromuscular diseases as targets for its research programs and categorizes them by phenotypic characteristics such as age of onset, affected muscle groups, and inheritance pattern (Muscular Dystrophy Association, www.mdausa.org). In the last 10 years, the genetic mapping and identification of novel skeletal muscle genes, including cytoskeletal, cytosolic, nuclear membrane, sarcosomal and extracellular matrix proteins, has dramatically changed this phenotype-based classification and provided clues as to the molecular basis of these disorders (3). What was once considered a single disease entity such as limb-girdle muscular dystrophy (LGMD) has now been subdivided into seven different molecularly defined autosomal dominant (LGMD1A–1G) and ten autosomal recessive (LGMD2A–2J) diseases. Not surprisingly, many of these genes have converged to define pathways critical for the normal functioning and maintenance of skeletal muscles. The fact that many muscular dystrophy cases exist in which mutations to known dystrophy-causing genes have not been detected suggests that this discovery period in human and model organism genetics will continue to identify novel disease genes and mechanisms. The most common forms of muscular dystrophy result from mutations in genes coding for sarcolemmal and extracellular matrix proteins in the dystrophin-glycoprotein complex (DGC) (4), which acts as a linker between the cytoskeleton of the muscle cell and the extracellular matrix, thus providing mechanical support to the plasma membrane during myofiber contraction (5). The association of a large number of muscular dystrophies with the DGC reflects the need to maintain the structural integrity of the plasma membrane of skeletal muscle. Disruption of DGC components results in a loss of membrane stability and subsequent degeneration of muscle fibers (3). Recently, defects in post-translational glycosylation of membrane proteins have been shown to be causative factors in muscle-eye-brain disease, Fukuyama congenital muscular dystrophy, Walker-Warburg syndrome, congenital muscular dystrophy type 1C, and limb-girdle muscular dystrophy type 2I (6–8). In most mammalian tissues, plasma membrane disruption is a common form of injury due to mechanical stress, and resealing of the damaged membrane is critical for cell survival (9, 10). In skeletal muscle, disruptions of the membrane are more frequent due to the repeated lengthening and shortening of muscle cells during contraction (11). Defects in the dysferlin gene result in limb-girdle muscular dystrophy type 2B (LGMD2B) and its allelic disease Miyoshi myopathy (12) through alter-

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¶ The abbreviations used are: LGMD, limb-girdle muscular dystrophy; B6, C57BL/6J; CHK, choline kinase; Chkb, choline kinase β; CHka, choline kinase α; CK, creatine kinase; COX, cytochrome-c oxidase; CPT, CDP-choline:1,2-diacylglycerol cholinephosphotransferase; Cpt1b, carnitine palmitoyltransferase type 1b; DGC, dystrophin-glycoprotein complex; EBD, Evans Blue dye; H&E, hematoxylin and eosin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; rmd, rostrocaudal muscular dystrophy.
ations in repair-vesicle fusion with the phospholipid membrane, resulting in necrosis of muscle fibers in both humans (13) and mice (14).

We have identified a new spontaneous recessive mouse mutation that leads to a progressive muscular dystrophy with a rostral-to-caudal gradient of severity (rmd, rostrocaudal muscular dystrophy) and a neonatal forelimb bone deformity. By positional cloning we have identified the molecular defect in rmd mutant mice as an intragenic deletion in the choline kinase beta (Chkb) gene, one of two mammalian choline kinase (CHK) enzymes (15). The monomeric CHK proteins combine to form the homo- or hetero-dimeric active forms (15) that catalyze the phosphorylation of choline to phosphocholine in the first committed step in the Kennedy pathway (the cytidine diphosphate (CDP)-choline pathway) for the biosynthesis of phosphatidylcholine (PC) (16). PC is the major phospholipid component of the eukaryotic plasma membrane external leaflet (17), an important precursor for many signaling molecules (18), and is thought to be essential for mammalian survival (19, 20). The immediate biosynthetic product of CHK, phosphocholine, may also be an important secondary messenger in regulating cell growth signaling and proliferation (21). The rmd mouse offers the first demonstration that a defect in a phospholipid biosynthetic enzyme can cause muscular dystrophy, suggesting that membrane phospholipids as well as sarcolemma-associated proteins are critical to the disease process. Mutant rmd mice share several phenotypic features with dysferlin-null mouse models that have a primary defect in sarcolemma repair and are a model for human limb-girdle muscular dystrophy 2B (LGMD2B). Our discovery of a mouse mutation in Chkb will provide important insights into the phospholipid biosynthetic pathways necessary for the development and maintenance of healthy muscle tissue.

**FIGURE 1.** Phenotypic and histological examination of rmd/rmd mice. A, +/+ (left) and rmd/rmd (right) mice, aged 6 days. Note smaller size of mutant, along with deformed forelimb. B, lateral skeletal preparation of +/+ (top) and rmd/rmd (bottom) forelimbs (6 days). No bones appear to be missing, although there is severe deformity of radius and ulna (arrow). C–J, histological hematoxylin and eosin (H&E) stained sections of rmd/rmd muscle aged 66 days (C–F), and 409 days (G–J). Note areas of fatty infiltration (arrow) in hindlimb (D), not present in forelimb (C). Note centralized nuclei (arrows) in both forelimb (E) and hindlimb (F), along with degenerating fibers (arrowheads) in hindlimb along with variations in fiber size. Note severe progression of muscle wasting and extreme fatty infiltration in hindlimb (H) not present in forelimb (G). Centralized nuclei (arrows) in forelimb (I), and extensive fibrosis and degenerated fibers (arrowheads) in hindlimb (J). Scale bars for C, D, G, and H: 200 μm; for E, F, I, and J: 50 μm. Boxes in C, D, G, and H are magnified areas in E, F, I, and J, respectively.
EXPERIMENTAL PROCEDURES

Construction of a High Resolution Genetic Cross and Positional Cloning—The rmd mutation arose in an F2 intercross between N-ethyl-N-nitrosourea-mutagenized C57BL/6J (B6) mice and BALB/cByJ (BALB) mice in a mapping cross for an N-ethyl-N-nitrosourea-induced seizure mutation (Szt1) (22). A high resolution mapping cross was established between the original affected hybrid B6CF1-Chkb<sup>rmd</sup>/Chx mutant and B6. The original affected rmd/rmd mice were unable to breed, and therefore, the rmd line was rescued by transplanting the ovaries of an rmd/rmd female into a severe combined immunodeficient (SCID) C3H/HeJ-Pkdc<sup>rmd</sup> female. The recipient was mated with a B6 male, resulting in +/rmd heterozygous F1 offspring, which were mated for use in positional cloning and recombination analysis of the resulting F2 offspring. A second mapping cross with CAST/Ei was also established in the same manner.

Histological Tissue Analysis—The original ovary donor female B6CF1 rmd/rmd mouse (409d), along with two B6CF2 rmd/rmd mice (aged 66 and 134 days) were perfused with Bouin’s fixative and embedded in paraffin, and tissue sections (8 µm) were examined by hematoxylin and eosin (H&E) staining of hindlimb and forelimb muscle cross-sections, along with sections of diaphragm, intercostal muscles, heart, spinal cord, and sciatic nerve.

Quadriiceps muscle from 6-, 14-, 26-, and 59-day +/+ and rmd/rmd mice (2 each per age) were processed as above with H&E. Centralized and non-centralized nuclei were counted in five random fields (20× or 40×) per mouse per age. Fiber diameter was measured in 20 fibers each in each genotype field using a Nikon E600 microscope equipped with Nikon Plan Fluor 4×/0.13, 20×/0.50, and 40×/0.75 objectives and a SPOT RT color digital camera and imaging software version 3.3.3 (Diagnostic Instruments, Inc.).

Skeletal preparations of B6CF2 +/+ and rmd/rmd mice (2 each, 6 days) were performed according to the methods of O’Brien (23), where bone is stained red and cartilage is stained blue. Briefly, mice were skinned, eviscerated, fixed for 4 days in 100% ETOH, then placed in 100% acetone for 3 days and rinsed with water. Mice were stained 15 days (2 volumes of 0.14% Alcan blue 8Gx, 1 volume of 0.12% alizarin red, 8 volumes of 100% glacial acetic acid, and 50 volumes of 70% ETOH), after which they were transferred to a clearing solution (20% glycerol, 1% KOH) until tissue was completely cleared and then transferred to storage solution (glycerol:ETOH:benzyl alcohol, 2:2:1).

Electron Microscopy—Hindlimb, forelimb, and heart muscle from B6CF2 +/+ and rmd/rmd mice (1 each, aged 30 days) were collected and fixed in 2.5% glutaraldehyde, and 100 nm sections were examined with a transmission electron microscope for mitochondrial abnormalities and accumulation of fusion vesicles on the sub-sarcomembranemembrane. In addition, a second pair of B6CF4 +/+ and rmd/rmd mice (1 each, aged 52 days) were perfused with 3.8% acrolein and fixed in 0.1 M cacodylate buffer, and 90 nm sections were examined as above.

Membrane Integrity—B6.CN9F1 +/+ and rmd/rmd animals (two each, aged 40 days) were injected with Evans Blue Dye (EBD, 10 mg/ml stock in phosphate-buffered saline, pH 7.4, 0.2-µm filter sterilized) intraperitoneally (1 mg/10 g of body weight), along with two (66 days) A/J mice (a dysferlin-deficient model of LGMD2B), and one (39 day) mds animal (known to show infiltration of EBD into muscle fibers) as a positive control. After 12–16 h, animals were euthanized and skinned, and muscles were examined for uptake of EBD. Whole sections of quadriceps muscle were frozen in optimal cutting temperature embedding compound (Sakura Finetechical Co., Torrence, CA) in isopentane chilled in liquid nitrogen, and 8-µm sections were examined by fluorescence microscopy for fluorescent dye uptake into cells.

Immunofluorescence Analysis—Frozen muscle sections (8 µm) from the Evans Blue membrane integrity experiments were thawed, fixed 4 min in 1:1 acetone:methanol (for dysferlin only), rinsed twice for 5 min in TBST, blocked for 1 h in 5% NGS-TBST, incubated overnight at 4°C in 1:20 dilution of anti-dysferlin (Hamlet) or anti-dystrophin (DYS2) mouse monoclonal antibody (Novocastra, Newcastle upon Tyne, UK) in blocking solution, rinsed 3× for 5 min, incubated 1 h at 25°C with 1:100 dilution of Alexa Fluor® 488 anti-mouse secondary (Molecular Probes Inc., Eugene, OR), rinsed 3× for 5 min, and visualized as above.

Creatine Kinase Levels—Blood samples were collected from +/+ and rmd/rmd mice (two each, aged 44 (B6CF4) and 53 days (B6CF2)) and centrifuged, and serum was analyzed for CK levels.

Mitochondrial Function—Skeletal muscle tissue samples from B6CF2 +/+ and rmd/rmd animals (two each, aged 31 days) were frozen in optimal cutting temperature embedding compound in isopentane chilled in liquid nitrogen, sectioned (8 µm), blocked with normal goat serum plus anti-mouse antibody, incubated with anti-mouse COX IV monoclonal antibody (Molecular Probes) for 1 h at 25°C, and followed by 1-h incubation with fluorophore-conjugated Alexa 488 goat anti-mouse secondary antibody (Molecular Probes) at 25°C. Immunofluorescence was observed as previously described.

Northern Blot Analysis and Quantitative Real-time PCR—Northern blots prepared from 1 µg of poly(A+) hindlimb muscle RNA from +/+ and rmd/rmd littermates (one each, 63 days, B6CF2), or from 10 µg of rmd and hindlimb muscle heart, liver, and brown fat total RNA from +/+ and rmd/rmd littermates (one each, 35 days, B6.CN6F1) were probed with a 291-bp probe (Strip-EZ DNATM, Ambion, Inc., Austin, TX) encompassing exons 10 and 11 of the Chkb mRNA: forward, 5′-GGCATTCATCCTTTTCTGGGTT-3′ and reverse, 5′-TAAGAAAGGCTACAGAGGTCGC-3′. To account for differential loading, we probed for housekeeping gene expression using an 847-bp probe to Abt1 (activator of basal transcription): forward, 5′-GAATGCCAGACCTCAGAGGTCGC-3′ and reverse, 5′-TCGCCAGAGGTCGC-3′.

Taqman minor groove binder primers and probes were designed using Primer Express™ (Applied Biosystems Inc., Foster City, CA)
based on the cDNA sequences of Chka (91 bp; F = 5′-ACTAGATCTCCTAGGTATTTGGCTATAATG-3′, R = 5′-GCTTCCGGCTTTCAAGAATTGCAATC-3′, probe = 5′-VIC-TGCTAAAGGTAATATC-3′), Chkb (91 bp; F = 5′-GACGCCGTGGTGCACCTATAG-3′, R = 5′-GTACGCTGGCCCTTCTG-3′, probe = 5′-VIC-CTAGCTGGCCCTTCTG-3′), and Cpt1b (92 bp; F = 5′-GATCTGCTATTTGTATTTTGTCATAATG-3′, R = 5′-AAGAGACCCCCGTAGCATCA-3′, probe = 5′-VIC-TTTGGTCCCGTGGCGG-3′). The internal control was the mouse endogenous TATA-box binding protein (TATA) Taqman probe set (Mm00446973_m1, Applied Biosystems).

Indirect Immunoblotting of Sarcolemmal Membrane Proteins—Forelimb and hindlimb muscle (200 mg each) from B6CF1 rmd/rmd mice (2 each, aged 40 and 57 days) were frozen in liquid nitrogen, ground to a fine powder in a mortar and pestle, suspended in 1.0 ml of SDS solubilization buffer (125 mM Tris-HCl, pH 8.0, 55 mM dithiothreitol, 2% (v/v) SDS, including 1 ml of protease inhibitor mixture (Cat # P-8340, Sigma-Aldrich)) per 10 ml of buffer. Suspensions were vortexed, boiled for 5 min, and centrifuged at 20,800 × g for 2 min. Protein content of the supernatants was determined by the Bradford method (27) with minor modifications. The supernatant fractions were incubated in reaction mixtures of 20 mM Tris-HCl, pH 7.5, 154 mM KCl, and 2 mM 2-mercaptoethanol (including 1 tablet of protease inhibitor mixture (Complete phospholipid content.

Phenotype and Histology of rmd Mutant Mice—A spontaneous recessive mutation, rmd, resulting in a hindlimb weakness and muscle wasting disease, was identified at The Jackson Laboratory. The disease phenotype of rmd/rmd mice is first observable starting at day 6 with mutant mice being visibly smaller than their non-affected littermates and showing an outward rotation of the forelimbs that results from defective bone morphology (Fig. 1A). Skeletal preparations of early forelimbs (P6) showed severe bowing (genu varum) of the ulna and radius (Fig. 1B), whereas hindlimb bone development did not appear to be affected. As the disease progresses, the hindlimb muscles become severely affected, and rmd mice lose significant hindlimb motor control by 2–3 months of age as indicated by dragging of hindlimbs. The forelimb muscles show only minor degeneration despite the forelimb bone abnormality, and therefore the mice are able to access food and water.

Histological examination of the forelimb skeletal muscle reveals very little loss of muscle fibers by 66 days, although some variation in fiber diameter and centralized nuclei, characteristic of degeneration-regeneration of muscle fibers, are observed (Fig. 1, C and E). Hindlimb skeletal muscle, by contrast, reveals signs of severe muscular dystrophy, including centralized nuclei, fatty infiltration, and loss of muscle fibers (Fig. 1, D and F). The disease is extremely mild in the forelimbs, and at 1 year of age, forelimb muscles continue to show only minor defects with little loss of muscle fibers (Fig. 1, G and I). Hindlimb muscles, however, are severely affected at 1 year of age (Fig. 1, H and J). The percentage of fibers with centralized nuclei increased in rmd/rmd hindlimb muscle tissues from <2% at 1 week of age to 18% by just 8 weeks (Fig. 2A). During that same time, +/- hindlimb muscles never showed >2% centralized nuclei. The size of individual fibers was significantly smaller in rmd/rmd than in +/- hindlimb muscles at 2, 4, and 8 weeks, although the variation in fiber size was not different (Fig. 2B). The muscle degeneration does not appear to affect lifespan, because rmd/rmd mice have consistently survived beyond 18 months of age. There is no apparent cardiac or diaphragm involvement in aged (>300 days) rmd/rmd mice, and no morphological abnormalities have been observed in the spinal cord, sciatic nerves, retina, or neuromuscular junctions (data not shown), suggesting that rmd is not a model for neurodegenerative diseases.

In Duchenne and Becker muscular dystrophies, the loss of the dystrophin protein compromises the dystrophin-glycoprotein complex, resulting in damage to the sarcolemmal membrane (31). To determine if similar membrane damage occurs in the rmd dystrophy, we used Evans
blue dye (EBD), a low molecular weight diazo dye that does not cross the sarcolemma into normal skeletal muscle fibers but will diffuse into muscle fibers of a number of models with impaired sarcolemmal integrity, including mdx mice, a dystrophin-deficient animal model for Duchenne muscular dystrophy (32). As expected, skeletal muscle from mdx mice showed pervasive blue coloring grossly, while in contrast, we found that skeletal muscle from both /H11001/H11001/het or rmd/rmd animals showed no significant uptake of EBD. Examination of cryosections by fluorescence microscopy (EBD fluoresces red) revealed no EBD uptake in /H11001/H11001/het myofibers and only scattered single EBD-positive fibers in rmd/rmd or A/J-dysf muscles (Fig. 3A). In contrast, mdx myofibers display widespread uptake in numerous clusters of EBD-positive fibers. These data suggest that the Chkb defect does not lead to extensive damage to the sarcolemmal membrane and that, like dysferlin-deficient A/J mice, repair of normal contraction-induced membrane damage may be impaired.

In a variety of muscular dystrophies, alterations or loss of protein components of the sarcolemmal membrane, or membrane repair vesicles, are observed. We tested the levels of membrane proteins by Western blotting of protein extracts from fore- and hindlimbs of +/+ and rmd/rmd mice with monoclonal antibodies to dystrophin, α-dystrogly-
can, β-dystroglycan, emerin, dysferlin, α-sarcoglycan, and β-sarcoglycan. None of these proteins showed any difference in their level of expression between +/+ and rmd/rmd animals (data not shown). In addition, frozen muscle sections of both rmd/rmd and +/+ animals shows normal immunofluorescent staining of dysferlin (Fig. 3B) and dystrophin (Fig. 3C) at the sarcolemma, suggesting that the rmd dystrophy is unlikely to be caused by secondary reductions of dysferlin or of members of the DGC integral sarcolemmal complex of proteins.

In muscular dystrophies, serum levels of creatine kinase (CK) increase as the enzyme is released from tissues during muscle damage or necrosis. This is most evident in dystrophic mice with compromised sarcolemmal membrane integrity, where levels of plasma CK are significantly increased (20-fold). In contrast, muscle fibers from dysferlin-null mice show only moderately increased CK levels (6-fold higher), even after extensive exercise, which causes CK levels in dystrophic mice to rise dramatically (33). Blood samples from +/+ and mutant 6-week-old rmd mice had CK levels ~3 times higher (average 6507 IU/liter) compared with +/+ littermates (average 2587 IU/liter). Together, CK levels, histology, and EBD membrane integrity results indicate that, although rmd mice develop a progressive muscular dystrophy, impaired sarcolemmal integrity does not appear to be a likely mechanism for the dystrophy.

Ultrastructure studies of muscle fibers in several muscular dystrophies are diagnostic and can reveal abnormalities, including alterations in the z-band structure of the contractile sarcomere (34), however, we found no evidence for sarcomere impairment in rmd/rmd muscles (Fig. 4, A and B). There was no evidence of mitochondrial proliferation, but there were extremely enlarged mitochondria in both hindlimb and forelimb muscles, which can be indicative of mitochondrial myopathy, or may be a secondary pathology to muscle degeneration. The presence of megamitochondria has been reported as a secondary response to pathological processes and metabolic perturbations in many diseases (kidney diseases, Reye's syndrome, heart disease, diabetes, and aging) (35, 36), and megamitochondria were widely observed in skeletal muscles of rmd/rmd animals but not in +/+ littermates (Fig. 4, C and D). To confirm that the appearance of megamitochondria was not a result of a primary mitochondrial defect, we examined cytochrome-c oxidase (COX, complex IV of the respiratory chain) staining of rmd/rmd muscle tissues. Decreased COX staining in muscle fibers has been found to be
characteristic of primary mitochondrial metabolism defects where alterations in any of the COX subunits result in marked reduction of generalized COX staining (37). We found no differences between +/+ and rmd/rmd animals in COX-IV immunostaining (data not shown), which suggests that a primary mitochondrial defect is not responsible for the dystrophy.

rmd, a 1.6-kb Deletion in Chkb—The rmd mutation was mapped to the distal region of chromosome 15 between D15Mit159 and D15Mit171 (human 22q13 orthologous region). We constructed a detailed genetic and physical map through recombination analysis of the rmd locus on chromosome 15 using a total of 550 B6CF2 and 516 CASTCF2 offspring, which narrowed the genetic interval to a 500-kb region containing 19 known or predicted genes (Fig. 5A). Because the gene was mapped to a chromosomal location with no known human or mouse neuromuscular mutations, it suggested that rmd was a novel muscular dystrophy mutation. All 19 genes within the minimal interval were tested for expression differences or sequence variation in rmd homozygotes using quantitative real-time PCR, along with genomic and cDNA sequencing. The only mutation identified in the genetic interval was a 1663-bp intragenic deletion within the Chkb gene that removes the final 26 bp of exon 3 through the first 247 bp of intron 9 (Fig. 5B). We have confirmed through sequencing of reverse transcription-PCR products that the mRNA is transcribed to nucleotide 88 in exon 3 and splices into exon 10 (Fig. 5C). This results in a frameshift that codes for 22 novel amino acid residues after the deletion, truncating the protein within exon 10 and removing the final 50 amino acids. Therefore, if the protein were translated, only the dimerization-binding region would be intact, whereas ATP, Ca2+, and choline binding and the active site domains would be removed. However, Western blot analysis with an N-terminal antibody indicates that no CHKB protein (45 kDa) or mutant-specific truncated protein (17 kDa) is expressed in rmd/rmd tissues (data not shown), nor is any CHKB-specific protein detected by immunoprecipitation and quantitation of CHKB activity (Fig. 6).

Electron microscope analysis of dystrophic muscle with membrane repair defects reveals sites of sarcolemmal disruption associated with underlying accumulations of vesicles (33). Based on the role of Chkb in membrane phospholipid biosynthesis, we hypothesized that alterations in sarcolemmal membranes or subsarcolemmal vesicles may exist in rmd/rmd skeletal muscle. To investigate this, we performed further electron microscopy analyses of sarcolemmal membranes and found significant numbers of subsarcolemmal vesicles in rmd/rmd medial gastrocnemius muscles as compared with the few observed in +/+ littermates (Fig. 7, A and B) similar to those described by Bansal et al. (33). We also found multiple sites of sarcolemma disruption in rmd/rmd mice, which were not observed in +/+ littermates (Fig. 7, C and D).

Choline Kinase Activity and Phosphatidylcholine Levels Are Reduced in rmd/rmd Skeletal Muscles—Total CHK enzymatic activity in 27- to 44-day-old mice was significantly decreased in +/rmd and rmd/rmd mice as compared with +/+ controls (Fig. 8A). Notably, in both the forelimbs and hindlimbs of rmd/rmd mice, CHK activity was undetectable. The results of CHK immunoprecipitation using N-terminal isoform-specific antibodies showed that all residual CHK activity in rmd/rmd mice came from the α/α homodimer. Because CHK catalyzes the first step of the major pathway for PC biosynthesis, we examined whether the decrease in CHK activity might alter PC content or phospholipid ratios in rmd tissues. Although total CHK activity was greatly decreased in rmd/rmd mice, there was no significant change in the total amount of PC or PE in liver, brain, kidney, and heart as measured by high-performance liquid chromatography (Fig. 8, B and C). However, a significant decrease in PC levels in muscle tissue from forelimb (38.2% reduction), and a trend toward lower PC levels in hindlimb (31.2% reduction), was observed in rmd/rmd mice as compared with +/+ controls (Fig. 8B). There were no significant changes in PE levels in either hindlimb or forelimb (Fig. 8C), indicating that generalized phospholipid production is not altered in the rmd mouse, but rather the effect is specific to PC. Decreases in the PC/PE ratio were observed in both forelimb and hindlimb muscles, reflecting the reduction of PC as compared with stable PE levels (Fig. 8D). This may suggest that changes either in the absolute levels of PC or in the lipid composition (represented by PC/PE ratio) are crucial to the progression of dystrophic symptoms in our mouse model.

Because CHK activity was decreased in the liver (in which the CDP-choline pathway has been shown to produce ~70% of PC), we also analyzed cholesterol, cholesterol ester, and triacylglycerol content in the plasma and liver as general measures of plasma lipid packaging and liver function. Significant differences were found between +/rmd and rmd/rmd mice in levels of plasma cholesterol (p = 0.0088) and plasma cholesterol ester (p = 0.0188) but not between +/+ and +/rmd or rmd/rmd mice (Table 1). There were no significant differences between rmd/rmd and +/+ control mice for levels of plasma or liver cholesterol, cholesterol ester, or triacylglycerol (Table 1). This may indicate that the loss of one allele of Chkb in heterozygous animals results in a dysregulation of cholesterol and cholesterol ester synthesis, packaging, or degradation pathways in plasma but that liver functions are not affected. An alternate pathway for PC synthesis in mammalian liver, through the methylation of phosphatidylethanolamine (PE) by phosphatidylethanolamine-N'-methyltransferase, is not active in other tissues (20, 38) and may explain the unaltered status of these lipids in the liver.

Chkb and Carnitine Palmitoyltransferase Type1b (Cpt1b)—Chkb is located ~560 bp upstream from the gene encoding Cpt1b, which is the muscle isoform of the first rate-limiting enzyme involved in catalyzing the transesterification of long-chain fatty acyl-CoAs to long-chain acylcarnitines (39). Because of the close proximity of Chkb and Cpt1b, and the detection of bi-cistronic transcripts containing exons from both genes, several groups have described the potential for interactions between these two genes, including Cpt1b promoter or regulatory elements within Chkb (40–42). We found a greatly increased expression of a truncated Chkb mRNA product in rmd/rmd tissues by Northern blot analyses (data not shown). This was confirmed with real-time PCR anal-
ysis, which showed a 4-fold increase in Chkb mRNA in rmd/rmd hindlimb muscle (ΔCt = −1.96 ± 0.75) as compared with +/+ controls (ΔCt = −0.01 ± 0.16) using the TATA-box-binding protein mRNA as an internal control. Although Northern blots and real-time PCR showed ~50% decreased expression of Cpt1b in rmd/rmd hindlimb muscle (ΔCt = −2.74 ± 0.39) as compared with +/+ controls (ΔCt = −4.03 ± 0.22), we have sequenced the entire open reading frame of the Cpt1b cDNA (2319 bp), along with intron-exon junctions for all 19 exons, and found no sequence variations. In addition, measures of enzymatic activity of CPT (normalized against activity of citrate synthase (nanomoles/min CPT ÷ micromoles/min CS), as described (43)) showed no significant differences in medial gastrocnemius (23.1 ± 5.3, 24.4 ± 2.5, p = 0.40), soleus (27.7 ± 13.8, 35.7 ± 3.8, p = 0.20), triceps (22.7 ± 1.3, 20.1 ± 2.9, p = 0.12), or heart (23.3 ± 5.4, 22.2 ± 2.0, p = 0.38) between +/+ control and rmd/rmd animals, respectively. We have also tested Cpt1b expression in mdx tissues using real-time PCR to determine if the observed decrease in rmd/rmd tissues was a secondary effect due to general muscle degeneration and found no differences in Cpt1b expression in mdx muscles (ΔCt = −3.59 ± 0.13) as compared with +/+ controls (ΔCt = −3.82 ± 0.25). This indicates that down-
involved in the components of the cellular membrane (52–54). Here we present the important interactions between the DGC complex and phospholipid Duchenne muscular dystrophy muscle fibers (51). Evidence points to its smaller isoforms) with the phospholipid regulatory pathways in shown that disruption of dystrophin leads to altered membrane phospholipids could result in defective muscle membranes. It has been hypothesized that up-regulation of membrane phospholipids may be a compensatory reaction to (49) and Refsum disease (50). It has been hypothesized that CHK activity plays a key role in the long term regulation of the Kennedy pathway, and CHK has been found to be first evidence of a genetic defect, Chkb, in a membrane phospholipid biosynthetic pathway resulting in muscular dystrophy. In addition, we show that this dystrophy is not caused by the loss of integral membrane proteins, providing further evidence for a unique role of membrane phospholipids in muscle stability.

alterations in the distribution and content of phospholipids in plasma membranes may also have drastic effects on the proper positioning of membrane-bound proteins and of the stability and shape of asymmetrically localized membrane rafts (55). Overcoming the hydrophobic repulsions between membrane bilayers is the initial step for the fusion process (56), and slight alterations in the distribution of PC species have been found to alter membrane surface properties, and therefore, the ability for vesicles to fuse with the membrane (57). Plasma membrane disruption due to mechanical stress is common in mammalian tissues, and in skeletal muscle, disruptions of the membrane are more frequent due to contraction-induced injury (11). Resealing of damaged membranes by fusion with membrane-repair vesicles is critical for cell survival (9, 10). We have observed disruptions in the sarcolemma of rmd/ rmd muscle sections by electron microscopy, along with an accumulation of large numbers of subsarcolemmal vesicles, similar to those described in dysferlin-null muscular dystrophies. Dysferlin levels in the rmd mouse are not altered, indicating that these pathological features are not due to loss of dysferlin but, rather, may be a function of altered membrane composition affecting vesicle fusion.

The active movement of phospholipids between membrane bilayers is a relatively rapid process, and a loss of asymmetry can lead to disruptions in signal transduction pathways (58) along with alterations in membrane stability (59). Apoptosis is initiated by a redistribution of phospholipids in the membrane, specifically with phosphatidylinerine relocating to the outer leaflet where it can be recognized by macrophages. A comparable process is involved in aging erythrocytes and platelets, which slowly externalize phosphatidylinerine and are subsequently engulfed by macrophages (60, 61). Similarly, inhibition of PC synthesis at the second (CPT: phosphocholine cytidylytransferase) or third (CDP-choline:1,2-diacylglycerol cholinephosphotransferase, CPT) steps of the Kennedy pathway leads to cell cycle arrest at G2 and subsequent apoptosis (18, 45). One way in which cells respond to metabolic stresses is to shift the balance of mitochondrial fission and fusion toward the development of megamitochondria to become more resistant to apoptotic stimuli (62). Alterations in PC/PE ratios have been found in mitochondrial membranes during the development of fusing megamitochondria (63), which may explain the presence of megamitochondria in rmd/rmd hindlimb muscle. We have tested for evidence of apoptosis in rmd/rmd hindlimb muscle using cleaved caspase-3 antibodies on fresh-frozen muscle sections and have found no indication of apoptosis (data not shown), indicating that the muscle degeneration in the rmd mouse is not directly due to the caspase-3 apoptosis pathway.

It has been hypothesized that CHK activity plays a key role in the long term regulation of the Kennedy pathway, and CHK has been found to be

**TABLE 1**

| Tissue | Genotype | Cholesterol | Cholesterol ester | Triacylglycerol |
|--------|----------|-------------|------------------|----------------|
| Plasma | +/+      | 383.1 (11.6)| 497.7 (115.8)    | 289.3 (195.9)  |
|        | rmd/rmd  | 465.5 (76.2)| 613.9 (114.0)    | 491.5 (292.8)  |
| Liver  | +/+      | 13.8 (3.8) | 4.0 (1.3)        | 22.2 (21.3)    |
|        | rmd/rmd  | 15.3 (1.8) | 3.9 (1.4)        | 14.7 (14.0)    |
|        |          | 13.9 (1.8) | 4.8 (1.8)        | 38.2 (24.0)    |

*Means are significantly different from each other (p < 0.05) by analysis of variance.

**TABLE 2**

Real-time PCR expression of Chka mRNA

Chka mRNA levels showed no significant differences in rmd/rmd tissues as compared to littermate +/+ controls (all p > 0.10) by analysis of variance. All reactions were performed in triplicate and normalized to TATA-binding protein endogenous control. n = 2 for all tissues by genotype. Values are means (=S.D.).

| Tissue       | Genotype | ΔCt  |
|--------------|----------|------|
| Forelimb     | +/+      | 5.57 (0.61) |
| Liver        | +/+      | 4.39 (0.24)  |
| Hindlimb     | +/+      | 5.42 (0.22)  |
| Kidney       | +/+      | 4.11 (0.02)  |
| Heart        | +/+      | 1.73 (1.47)  |
| Liver        | rmd/rmd  | 2.56 (2.42)  |
| Kidney       | rmd/rmd  | 2.08 (0.03)  |
| Brown fat    | rmd/rmd  | 2.39 (0.25)  |
| Liver        | rmd/rmd  | 4.10 (0.95)  |
| Liver        | rmd/rmd  | 3.92 (0.66)  |
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inducible in various systems (16). Studies of mutant Chinese hamster ovary cells indicate that CHK is part of the control mechanism for the synthesis and degradation pathways that regulate the levels of PC (64). The loss of CHK activity may result in a compensatory feedback mechanism on expression of the mutant Chkb<sup>rdm</sup> gene, which may explain the up-regulation of the truncated Chkb mRNA. The greatly increased expression of the truncated Chkb gene may interfere with the expression of the neighboring Cpt1b gene at the RNA level even through promoter competition between the two genes, read-through transcription of Chkb into the Cpt1b locus, or loss of a Cpt1b enhancer element in the intragenic Chkb genomic deletion. However, the change in Cpt1b mRNA expression is not reflected in CPT enzymatic activity, and is therefore unlikely to be involved in the etiology of this muscular dystrophy model.

In the mouse liver, the activity ratios of CHK are α/α = 20%, α/β = 60%, β/β = 20%, whereas in the mouse heart they are α/α < 5%, α/β = 25%, β/β = 70% (26). We have found greatly decreased CHK activity in all tissues in our rmd homozygous mice, and yet significant alterations in tissue phospholipid levels, can result in muscular dystrophy. Alterations in tissue phospholipid levels, their roles in the pathogenesis in muscular dystrophy and muscular dystrophy in skeletal muscles, despite the predominance of the β/β dimer in normal heart muscle. The pattern of decreased PC levels and PC/PE change corresponds well with the dystrophic phenotype in Cpt1b<sup>−/−</sup> mice, with no alteration and no apparent phenotype in heart, mild alteration and mild dystrophy in forelimbs, and severe alterations with severe dystrophy in hindlimbs. Based on our results in this mouse model of muscular dystrophy, it appears that there is a differential ability of tissues to compensate for the loss of the β isomorph, either through alternative PC biosynthesis or possibly through decreased PC degradation. Interestingly, we found no significant alteration of Chka mRNA in any tissues (forelimb, hindlimb, heart, liver, kidney, and brown fat) by real-time PCR analysis (Table 2), indicating that the compensatory mechanism is not through an increase in α/α dimer activity. By understanding the tissue-specific pattern of CHKB and CHKA expression and their functions in phospholipid synthesis, their roles in the pathogenesis in muscular dystrophy can be explored. It is clear that choline kinase is an important enzyme for many physiological processes, because alterations or defects have previously been linked to cancer (65) and to obesity and diabetes (66). Our data provide the first evidence that alteration of CHK activity, and alterations in tissue phospholipid levels, can result in muscular dystrophy.

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