Analysis of myotilin turnover provides mechanistic insight into the role of myotilinopathy-causing mutations

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MFM (myofibrillar myopathies) are caused by mutations in several sarcomeric components, including the Z-disc protein myotilin. The morphological changes typical of MFMs include Z-disc alterations and aggregation of dense filamentous sarcomeric material. The causes and mechanisms of protein aggregation in myotilinopathies and other forms of MFMs remain unknown, although impaired degradation may explain, in part, the abnormal protein accumulation. In the present paper we have studied the mechanisms regulating myotilin turnover, analysed the consequences of defective myotilin degradation and tested whether disease-causing myotilin mutations result in altered protein turnover. The results indicate that myotilin is a substrate for the Ca2+-dependent protease calpain and identify two calpain cleavage sites in myotilin by MS. We further show that myotilin is degraded by the proteasome system in transfected COS7 cells and in myotubes, and that disease-causing myotilinopathy mutations result in reduced degradation. Finally, we show that proteolysis-inhibitor-induced reduction in myotilin turnover results in formation of intracellular myotilin and actin-containing aggregates, which resemble those seen in diseased muscle cells. These findings identify for the first time biological differences between wt (wild-type) and mutant myotilin. The present study provides novel information on the pathways controlling myotilin turnover and on the molecular defects associated with MFMs.

Key words: calpain, degradation, myotilin, muscle, sarcomere, Z-disc.

INTRODUCTION

Muscle contractility is regulated at the levels of calcium homeostasis, cell signalling and through the maintenance of the sarcomere, the smallest contractile unit of the skeletal or cardiac muscle. The sarcomere is a morphologically static structure used to generate and transmit force. However, its individual components are constantly assembled and degraded by carefully regulated molecular mechanisms. Myotilin [1], a sarcomere Z-disc component, forms, together with palladin [2] and myopalladin [3], a family of Ig domain-containing actin-binding proteins. Myotilin consists of two Ig domains flanked by a unique serine-rich N-terminus and a short C-terminal tail, and interacts with Z-disc core structural proteins: actin [4,5], α-actinin [1], filaments [6,7], FATZ (filamin-α, actinin-2, and telothonin-binding protein of Z-disc) proteins [7] and Enigma proteins including ZASP (Z band alternately spliced PDZ-containing protein) [8]. In addition, myotilin interacts with regulators of signalling cascades and MurF ubiquitin ligases [9]. Myotilin cross-links actin filaments into large stable bundles both in vitro and in cells [4,5]. Missense mutations in the MYOT (myotilin) gene, mostly at the N-terminal serine-rich region, cause adult-onset autosomal-dominant myopathy, MFMs (myofibrillar myopathy). The disease typically manifests as distal myopathy, but may also affect proximal muscles and the heart. The typical morphological features include Z-disc alterations, aggregation of dense filamentous myotilin, and desmin-containing material, rimmed vacuoles, and multilayered autophagic vesicles [10–12]. The disease mechanism is still unclear, but may be linked to the structural alterations of the Z-disc caused by dysfunctional proteins, or their abnormal accumulation due to defective degradation caused by mutant ubiquitin (UBB+) and abnormal protein complexes resistant to proteasome degradation [13]. Myotilin-knockout mice are virtually normal, whereas mice with introduced patient mutations develop progressive myofibrillar pathology; mice overexpressing mutant myotilin show more severe muscle degeneration and enhanced myofibrillar aggregation [14–16].

Calpains are a group of calcium-dependent non-lysosomal cysteine proteases expressed ubiquitously in all cells. There are more than a dozen calpain isoforms, some with multiple splice variants. The two main isoforms, calpain 1 and 2, differ primarily in their calcium requirements [17]. Calpain 3 is a more tissue-specific protease expressed in muscle and the brain [18,19]. Disruption of the gene encoding calpain 3 causes muscular dystrophy. Calpains are able to cleave many cytoskeletal proteins and can thus intervene in cytoskeleton regulation, particularly during processes such as adaptive response to exercise or regeneration after muscle wasting. A number of cytoskeletal proteins have been identified as potential calpain substrates in vitro, although not all have been confirmed as in vivo targets. These include myofibrillar Z-disc proteins titin, α-fodrin, α-actinin, desmin, nebulin, filamin C and myosin light chain 1, supporting the idea that calpain participates in sarcomeric remodelling [18,20,21]. Calpains are required to mediate the dissociation of sarcomere proteins from the assembled myofibrillar structure before the UPS (ubiquitin–proteasome system) is able to degrade them [22]. Calpains perform the initial proteolytic cleavage that allows E3 ubiquitin ligases to ubiquitinate the peptides and target them for degradation in the proteasome.

Abbreviations used: GFP, green fluorescent protein; EGFP, enhanced GFP; F-actin, filamentous actin; GST, glutathione transferase; HA, haemagglutinin; mAb, monoclonal antibody; MALDI, matrix-assisted laser-desorption ionization; MFMs, myofibrillar myopathies; MG132, Z-Leu-Leu-Leu-al; MS/MS, tandem MS; TOF, time-of-flight; UPS, ubiquitin–proteasome system; wt, wild-type; Z-LLal, Z-Leu-Leu-H; ZASP, Z band alternately spliced PDZ-containing protein.

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There is evidence that dysregulated protein turnover may play an important role in muscle or heart disease. When the calpain system is inhibited in the heart, as in mice overexpressing the endogenous calpain inhibitor calpastatin, morphological evidence of widespread protein aggregation has been identified along with increased autophagy [23]. The co-ordinated effort by calpain and ubiquitin ligases is also illustrated in models of skeletal muscle atrophy. Ubiquitin ligases, including MuRF1, have proven to be essential in the atrophic process. When calpain inhibitors are introduced into the system sarcomere degradation is inhibited, thereby inhibiting muscular atrophy without reducing the ubiquitin ligase levels [24]. When the degradative capacity of the proteasome is exceeded, harmful proteins can be sequestered in aggresomes or degraded via lysosomal-mediated autophagy [25]. In the present study, we studied the mechanisms that control myotilin turnover. We obtained evidence that myotilin is a calpain substrate and that myotilin is further degraded into small peptides by the proteasomal machinery. We show for the first time that mutated myotilin accumulates in cells in a manner resembling that seen in the myotilinopathy patients, apparently due to inappropriate degradation.

MATERIALS AND METHODS

Plasmids and antibodies

Full-length myotilin-encoding gene (residues 1–498) and its variants were generated by PCR amplification from human myotilin cDNA and subcloned into the pGEX-4T1 GST (glutathione transferase)-fusion vector (Pharmacia) for production of GST-fusion proteins in bacterial cells, and into the pAHP vector [4], pDEST27 (Invitrogen) or pEGFP-C2 [EGFP is enhanced GFP (green fluorescent protein); Clontech] for mammalian expression [5,8]. The mutations Ser55Phe (S55F), Thr57Ile (T57I), and Ser60Cys (S60C) in myotilin were generated with the QuikChange® Site-Directed Mutagenesis Kit (Stratagene) and verified by sequencing. Myotilin was detected with the N-terminal 151 or C-terminal 231 polyclonal antibodies (Figure 1A) [26]. Alternatively, HA (haemagglutinin)-tagged myotilin was detected with an anti-HA mAb (monoclonal antibody; Nordic Biosite) or GST-tagged myotilin with a goat anti-GST antibody (GE Healthcare). Ezrin was detected with the mAb 3C12 (Sigma–Aldrich). F-actin (filamentous actin) was visualized with Alexa Fluor® 568 phallolidin (Invitrogen).

Cell transfections, treatments and quantifications

COS7 cells (A.T.C.C.) were transfected with FuGENE®6 reagent (Roche) and incubated for 48 h before analysis. C2C12-myoblasts (A.T.C.C.) were induced to differentiate into myotubes by shifting to culture medium containing 2% (v/v) horse serum for 7–9 days. After serum-starvation, endogenous calpain activity was induced with the addition of 5 μM ionomycin (Calbiochem) and 10 mM CaCl2 for 1 h. Calpain was inhibited with 10 μM Z-LLal (Z-Leu-Leu-H; PeptaNova) for 2 h. Protein synthesis was inhibited with 100 μg/ml cycloheximide (Sigma), proteolysis with 10 μM MG132 (Z-Leu-Leu-Leu-al; Sigma–Aldrich), proteasomal degradation with 7.5 μM lactacystin (Calbiochem) and lysosomal degradation with 10 μM bafilomycin A (Sigma–Aldrich). Actin filaments were destabilized with 0.5 μM Latrunculin B (Sigma–Aldrich). Cells were analysed as described in [5,8].
The morphology of the phalloidin-stained actin cytoskeleton was analysed from 100 myotilin-transfected cells and the experiment was repeated three times. Three different morphological phenotypes were identified: actin aggregates, actin dots and actin bundles. Actin aggregates were accumulations, whose diameter exceeded 4 μm. Actin dots were accumulations smaller than 4 μm in diameter. Cells considered having actin dots did not contain any aggregates larger than 4 μm. The phenotype was considered as actin bundles when more actin cables than aggregates or dots were visible. During analysis, the investigator was blinded to the sample treatments. The intensity of the Western blots was quantified by TyphoonImager 9400 (GE Healthcare) and analysed by ImageQuantTL2003 software (GE Healthcare). All statistical analyses were performed in Excel with unpaired Student’s t test.

In vitro proteolysis with calpain 1

A 75 mg sample of rat skeletal muscle was pulverized with liquid nitrogen and homogenized by sonication (5W for four cycles of 10 s) in ice-cold reaction buffer (30 mM KCl, 15 mM imidazole, 5 mM NaCl, 1 mM MgCl₂, 1 mM EGTA, 1 mM EDTA, 0.5 mM dithiothreitol and 3 mM CaCl₂, pH 7.5) in the absence of Ca²⁺. The crude homogenates were subsequently incubated as described previously [20]. Proteolysis was initiated by the addition of 1 or 5 units of calpain 1 (Calbiochem) to the reaction mixtures. After incubation for 1, 5 or 30 mins, aliquots were collected and boiled in Laemmli buffer. Mixtures were incubated in the presence of protease inhibitors 10 μM Z-LLal, 75 μM E64D (Sigma), 500 μM calpeptin (Calbiochem), 1 mM PMSF (Merck), 3 μM aprotinin, 5 μg/ml pepstatin A and 2 μg/ml leupeptin (Sigma–Aldrich), or in the absence of Ca²⁺.

GST–myotilin fragments were produced as described previously [5]. Glutathione–Sepharose beads with 4 μg of fusion protein were incubated with 0.4 units of recombinant active calpain 1 (human erythrocytes: Calbiochem) in 30 μl of calpain buffer (20 mM Tris/HCl and 30 μM CaCl₂, pH 7.4) with or without 10 μM calpain inhibitor Z-LLal for 5 min at room temperature (22 °C). The reaction was stopped by adding 25 μl of Laemmli buffer. Proteins were resolved on SDS/PAGE (12% gel), blotted, and detected with anti-myotilin antibodies or with silver staining. Loading of the GST-fusion proteins was controlled by immunoblotting with goat anti-GST antibody. Myotilin fragments were alternatively analysed by MS.

MALDI (matrix-assisted laser-desorption ionization)–TOF (time-of-flight) analyses

The calpain digestion reaction was made directly on the MALDI target plate. A saturated matrix solution CHCA (α-cyano-4-hydroxy cinnamic acid; Sigma) in 33% ACN (acetonitrile)/0.1% TFA (trifluoroacetic acid) was then added. MALDI–TOF analyses were carried out with Autoflex III (BrukerDaltonics) equipped with a SmartBeam™ laser (wavelength of 355 nm), operated in positive and reflective modes. Typically, mass spectra were acquired by accumulating the spectra of 2 000 laser shots and up to 10 000 for MS/MS (tandem MS) spectra. External calibration was performed for molecular assignments using a peptide calibration standard (BrukerDaltonics).

Peptide identifications were performed by searching the peptide monoisotopic masses for peptide mass fingerprints or the amino acid sequence tag for peptide fragments in MS/MS against the GenBank® nr (non-redundant; http://www.ncbi.nlm.nih.gov/) database using Matrix Science’s Mascot (http://www.matrixscience.com/; Matrix Science) or against locally created databases on an intranet server. FlexAnalysis™ and Biotools™ software (Bruker Daltonics) were used to analyse MS data as the search engine interface between raw data transfer and the databases in the Mascot server respectively. The following parameters were set for the searches: 0.1 Da precursor tolerance and 0.5 Da MS/MS fragment tolerance for combined MS/MS searches, oxidized methionine was set as variable modification, the enzyme was set to none.

RESULTS

Calpain-mediated proteolysis of myotilin

To investigate whether myotilin is a substrate for calpain in vivo, COS7 and Ptk-2 (results not shown) cells were transfected with myotilin cDNA. The cells were treated with the calcium ionophore ionomycin and CaCl₂ for 1 h to activate intracellular calpain. Western blot analysis demonstrated that the amount of myotilin decreased after the ionomycin treatment (Figures 1B and 1C). The reduction was partially blocked by the specific calpain inhibitor Z-LLal. No proteolytic fragment from myotilin was detected in cell lysates, indicating that such intermediates are unstable and degraded by cellular factors. There was no significant difference in the calpain degradation of myotilin harbouring myotilinopathy mutations Ser55Phe, Thr57Ile and Ser60Cys.

To confirm that endogenous myotilin is also a calpain substrate, C2C12 myoblasts were differentiated into myotubes for 7–9 days to induce myotilin expression and calpain was activated. Again, calpain activation caused loss of myotilin, which was reversed by the calpain antagonist Z-LLal (Figures 1D and 1E).

To further analyse the calpain-mediated cleavage of myotilin, rat muscle homogenates were incubated with 1 or 5 units of calpain 1 at different time points. Immunoblot analysis with the C-terminal myotilin antibody against amino acids 231–342 showed decay of the 57 kDa full-length protein and appearance of three degradation products (Figures 2A and 2C). Only the upper degradation product of 40 kDa was seen with the N-terminal myotilin antibody against amino acids 1–150 (results not shown). Already after 1 min, 5 units of calpain cleaved myotilin, and after 5 min most of the full-length myotilin was degraded. It was also observed that 1 unit of calpain degraded full-length myotilin in 5 min. To detect the potential endogenous Ca²⁺-dependent proteolytic activity of the rat skeletal muscle homogenates, the tissue homogenates were incubated in the presence of Ca²⁺. Immunoblot analyses revealed that, even in the absence of exogenously added calpain 1, myotilin underwent partial proteolysis after 5 min (calcium buffer, 5 min; Figure 2A). Comparison of the molecular mass distributions of the autoproteolytic fragments with those generated by calpain 1 (calcium buffer, 5 min and 5 units of calpain, 5 min; Figure 2A) revealed matching of the two larger 40 kDa and 32 kDa fragments. The degradation was inhibited by Z-LLal, suggesting that at least a part of the endogenous Ca²⁺-dependent proteolytic activity is mediated by calpains. The degradation was inhibited with the addition of Z-LLal in the lysate before incubation or by incubation in the absence of Ca²⁺.

To test further whether the endogenous Ca²⁺-dependent proteolytic activity is mediated by calpains and/or other proteases, different inhibitors were tested. Rat muscle homogenates were incubated with or without the protease inhibitors Z-LLal, E64D, calpeptin, PMSF, aprotinin, pepstatin A or leupeptin, or in the absence of Ca²⁺ for 30 mins at 30 °C. The calpain inhibitors Z-LLal, E64D, calpeptin and leupeptin inhibited the autoproteolysis of myotilin (Z-LLal, E64D, calpeptin and leupeptin; Figure 2C), whereas the serine protease inhibitors PMSF and aprotinin, and the aspartic protease inhibitor pepstatin,
Figure 2  Time-dependence and the effect of inhibitors on myotilin cleavage

(A) Rat muscle lysate was incubated in the presence of 1 or 5 units of calpain 1 with or without the calpain inhibitor Z-L-Lal (10 μM) or 3 mM Ca²⁺ for various times. Calpain and/or Ca²⁺ increase induces cleavage of myotilin. The molecular mass in kDa is indicated on the left-hand side. (B) Quantification of the myotilin in samples incubated for 30 min at 30 °C in calcium buffer compared with muscle lysate without calcium in the buffer. Arbitrary units +– S. D. of three experiments. *, significant difference from the control (no calcium) value at P < 0.01 in the unpaired Student’s t-test. (C) Rat muscle lysate was incubated with protease inhibitors. The calpain inhibitors Z-L-Lal (10 μM), E64D (75 μM) and calpeptin (500 μM), but not other inhibitors, prevented degradation of myotilin. In the top panel, a longer exposure of the Western blot is shown to visualize the degradation products. In the bottom panel, α-tubulin detection is shown as a loading control.

were ineffective (PMSF, aprotinin and pepstatin; Figure 2C). Calcium was required for the proteolytic activity (calcium buffer and no calcium; Figure 2C). This shows that myotilin is specifically degraded by calpains.

Analysis of calpain cleavage sites in myotilin

Various bacterially produced myotilin fragments were cleaved by calpain 1 and the cleavage products were analysed by Western blotting (Figures 3A–3C) or silver staining (Figure 3D). Several proteolytic fragments were detected from full-length myotilin and from the longer myotilin fragments (Figures 3A and 3B).

An even smaller myotilin fragment recognized by the C-terminal 231 polyclonal antibody was cleaved by calpain activation in transfected COS7 cells (Figure 3C). This amino acid 217–339 fragment was used to map the cleavage site by MS. Two calpain-cleavage sites were detected, one at the N-terminal side of Gln226 and the other at the N-terminal side of Ile253 (Figure 3E). The first cleavage site was also detected in a shorter fragment including GST and amino acids 217–250 of myotilin. The same degradation pattern was seen when identical samples were run on SDS/PAGE and detected by silver staining (Figure 3D).

Metabolic stabilization of myotilin by proteolysis inhibitors

The different patterns of myotilin degradation in vivo (no visible degradation products) and in vitro (several degradation products) after calpain activation suggest that additional proteolytic mechanisms contribute to the degradation of myotilin. Calpains cleave polypeptides at specific sites, but they do not cause protein degradation. We further characterized the turnover of myotilin in transfected COS7 cells and in C2C12 myotubes by inhibiting protein synthesis with cycloheximide and proteolytic pathways with MG132. In untreated COS7 cells, myotilin was degraded with a half-life of approximately 8 h (0 h and 8 h; Figures 4A and 4B). When the cells were treated with cycloheximide and MG132, the half-life was markedly prolonged (CHX 2 h and CHX 2 h + MG132, CHX 4 h and CHX 4 h + MG132; Figure 4C). The degradation rate of myotilin in C2C12 cells was lower than in COS7 cells. Myotilin degradation was seen only after 17 h of cycloheximide treatment (CHX 17 h; Figure 4D). When the sarcomeric structure was destabilized with Latrunculin B, which targets actin filaments, the degradation rate of myotilin was increased (CHX 7 h + Lat B 7 h and CHX 17 h + Lat B 17 h; Figure 4D), indicating that the myotilin turnover is slower in organized thin filament structures.
Myotilin turnover

Figure 3 Analysis of myotilin cleavage fragments

GST–myotilin or its fragments produced in bacteria (A, B, D and E) were incubated in the absence or presence of 0.4 units of calpain 1 and 10 μM Z-LLal and analysed with Western blotting (A and B) and silver staining (D). (C) COS7 cells were transfected with myotilin amino acids 217–339 and incubated with or without Ca2+ activation as described in Figure 1. Quantification of myotilin after Ca2+ activation compared with unstimulated cells as arbitrary units ± S.D. of three experiments. *, P < 0.05 (unpaired Student’s t test). Cleavage occurs in the C-terminal part of myotilin. MS analysis of fragments shown in (D) identified the residue Gln226 as a cleavage site (E).

We also treated myotilin-expressing COS7 cells with proteolysis inhibitors overnight in the absence of cycloheximide. MG132, lactacystin and bafilomycin A increased the amount of full-length myotilin, indicating the role of constitutive degradation of myotilin by the proteasome and lysosome systems (MG132, Bafilomycin A, Lactacystin; Figures 4E and 4F). In the MG132-treated cells, a degradation product of approximately 30 kDa was seen with an N-terminal antibody (Figure 4E, right-hand panel). The amount of myotilin in bafilomycin A-treated cells was, however, lower than in MG132- and lactacystin-treated cells, suggesting marked degradation of myotilin via the proteasome pathway. Ezrin, another actin-associated protein, was not affected, showing that the accumulation of proteins is not a general phenomenon for all proteins in MG132-, lactacystin- and bafilomycin A-treated cells (Figure 4E, middle panel).

Morphology of cells treated with proteolysis inhibitors

To study the morphological changes in GFP–myotilin-transfected COS7 cells, they were stained with fluorescent phalloidin to visualize F-actin. In untreated cells, myotilin induced thick actin cables as expected (Figures 5A–5C compared with 5J–5L). After overnight treatment with MG132, myotilin accumulated in large aggregates, which also contained F-actin (Figures 5D–5F). These cells were devoid of organized actin filaments, unlike MG132-treated cells expressing GFP alone (Figures 5M–5O). Treatment with lactacystin induced smaller aggregates or dots visible with GFP–myotilin and phalloidin (Figures 5G–5I). The dots concentrated around the membranes and the normal actin cytoskeleton was partially intact. This indicates that when the UPS is disturbed in cells, the turnover of myotilin is dysfunctional and the protein accumulates in the cells. This further leads to aggregation of actin filaments. Figure 5(S) shows the quantification of different actin phenotypes after MG132 and lactacystin treatment. The myotilinopathy mutations Ser55Phe, Thr57Ile, and Ser60Cys changed their subcellular localization similar to wt myotilin after inhibitor treatments (results not shown).

Stability of myotilin mutants

To test whether the myotilinopathy mutations affect protein turnover, HA-tagged wt or mutant myotilin was expressed in COS7 cells. After 4 h of cycloheximide treatment, cells were lysed and myotilin was quantified. All of the myotilinopathy mutations tested, Ser55Phe, Thr57Ile, and Ser60Cys, degraded more slowly than the wt myotilin, indicating that the mutated proteins accumulate in COS7 cells in a similar manner to mutated myotilin in human dystrophic muscle cells (Figure 6). For Ser55Phe and Ser60Cys, the difference was significant. As shown by Western blot analysis, the expression level of myotilin mutants in untreated cells did not differ significantly from the expression of wt protein.

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**DISCUSSION**

Mutations in myotilin, as well as other sarcomeric proteins, filamin C, ZASP, desmin and αB-crystallin, cause a muscle disease termed MFM which is associated with specific morphological changes. The typical findings include Z-disc alterations and aggregation of dense filamentous material [27]. The cause and mechanism of protein aggregation in these disorders has remained unknown. It has been suggested, however, that impaired degradation may explain in part the abnormal protein accumulation [28]. The results of the present study support this hypothesis.

To gain more information about the degradation of myofibrillar proteins, we explored the turnover of myotilin. Our results show that myotilin is a substrate for calpain at least at two cleavage sites. The interplay between myotilin and calpain is in line with their co-localization in the Z-band and under the plasma membrane in mouse skeletal muscle fibres [29].

Our findings add myotilin to the list of several myofibrillar calpain substrates [18,20,21]. Calpain activity is typically not restricted to a single site in a myofibrillar protein. For example, troponin I undergoes truncation in the post-ischaemic myocardium of rat heart in at least three steps [30]. Also proteolysis of myotilin by calpain yielded several fragments, of which the most prominent fragment was approximately 30 kDa. Calpain I cleaved several C-terminal fragments including amino acids 231–342 at the C-terminus of myotilin in vitro and in transfected cells. By MS we showed that the N-terminal side of Glu226 is a calpain-cleavage site in amino acids 217–250 and 217–339 fragments of myotilin. The absence of a consensus sequence at the cleavage sites indicates lack of primary structure specificity; in fact calpains usually cleave destructured regions [31]. The calpain cleavage site at amino acids 225–226 in myotilin resides at a destructured region before the first Ig domain.

The endogenous Ca²⁺-activated proteolytic alterations were reminiscent of those induced by in vitro test incubations with calpain. Our C-terminal anti-myotilin antibody identified two degradation products from muscle tissue after endogenous calpain activation and three degradation products after the addition of recombinant calpain 1. The difference in the amount of degradation products might be due to the higher concentration of active recombinant calpain. These results imply that the rat muscle myofibril has the potential to modulate its proteins via its own calpains. In mature sarcomeres, myotilin co-localizes with α-actinin and Z-disc titin, showing the striated pattern typical of sarcomeric proteins. In skeletal muscle, calpain cleavage of myotilin could be required for reorganization of muscular fibres after eccentric exercise. Myotilin is present in increased amounts in lesions related to Z-disc streaming and events leading to insertion of new sarcomeres in pre-existing myofibrils induced by eccentric exercise [32]. Myotilin is more associated with F-actin than with the core Z-disc protein α-actinin during these events, and might dissociate from α-actinin by calpain cleavage.
et al. [33] have suggested that calpain 1 may release α-actinin from the Z-line intact via the modulation of other interacting proteins. α-Actinin has also been shown to be least susceptible to calpain 1 proteolysis of several myofibrillar proteins [20]. Myotilin appears to the new sarcomeres already during initial steps of the remodelling process, well before α-actinin, titin and nebulin. The susceptibility to calpain 1 cleavage, leading to further degradation and release of new building blocks, could explain the rapid turnover of myotilin when the level of calcium in muscle cells is high during muscle contraction. For example, in cultures of quail myotubes and zebrafish skeletal muscle cells myotilin has a fast recovery rate as compared with six other Z-disc proteins [34,35].

Z-disc streaming and other myofibrillar abnormalities, are characteristic features of all forms of MFM. Accumulation of protein aggregates may be related to mutant ubiquitin-abnormal protein complexes, which resistant to proteasome degradation. Abnormal protein deposition, expression of proteasomal subunits and MHC class I antigens co-localize in MFM muscle fibres [28]. In MFM caused by myotilin mutations, the biologically dysfunctional UBB+ is related to the protein aggregates in myofibrillar inclusions shown by microscopical methods [13]. In transgenic mice and cultured cardiac myocytes, another mutant MFM protein, desmin, escapes proteolytic breakdown [36,37]. In the present paper, we have shown that in addition to calpain cleavage the proteasome pathway degrades myotilin. The turnover of myotilin in the myotubes with organized sarcomeres was slower than in transfected fibroblasts. However, destabilization of the sarcomeric actin increased the degradation rate. This is consistent with the idea that the myofibrillar proteins must be dissociated from the myofibril before they can be degraded by the proteasome and cellular peptidases [38]. It is estimated that 80–90% of all proteins in a cell are ultimately degraded via the proteasome pathway [39], and it is not surprising therefore that the turnover of myotilin is regulated by the proteasome. However, when misfolded and damaged proteins are ubiquitinated by the UPS, their destruction by the proteasome is not always possible because of their aggregated conformation. The results of the present study show that while myotilin is mainly degraded by the UPS, their destruction by the proteasome is not always possible because of their aggregated conformation. The results of the present study show that while myotilin is mainly degraded by the UPS, other pathways are also involved in degradation of the protein. This interpretation is based on the differential effects of MG132, a broad inhibitor affecting calpain-, proteasome-, lysosome- and autophagosome-dependent protein degradation, and the specific proteasome inhibitor lactacystin and lysosome inhibitor bafilomycin A. MG132 reduced myotilin degradation more effectively than lactacystin, and induced large myotilin aggregates and markedly distorted actin cytoskeleton, whereas the aggregates in lactacystin-treated cells were smaller and the changes in actin cytoskeleton less severe. This is consistent with the idea that when the degradative capacity of the proteasome
is exceeded, harmful proteins can be degraded via lysosomal-mediated autophagy [25].

Missense mutations in the unique serine-rich N-terminal domain of myotilin [10,11] lead to myopathy and result in myotilin-positive aggregates in the affected muscle. Previously, mutations in myotilin have been tested for their role in myotilin dimerization, interaction with α-actinin and actin, actin bundling and myotilin phosphorylation [5] (P. von Nandelstadh, unpublished work). These experiments have not revealed differences between wt and mutant myotilin. The results of the present study indicate for the first time a functional difference, as the patient mutations showed to be more resistant to degradation than wt protein. This is supported by the fact that the amount of myotilin in the patient’s muscle samples is increased [40,41]. The indication that mutant myotilin accumulates in cells and causes aggregates due to defective degradation is in agreement with findings from human dystrophic muscle cells. On the basis of these results, we propose a model on the pathogenic mechanism, by which myotilin mutations induce muscular dystrophy (Supplementary Figure S1 at http://www.BiochemJ.org/bj/436/bj4360113add.htm). It remains to be tested if a similar mechanism, by which myotilin mutations induce muscular dystrophy pathway, can be found in other diseases involving muscular dysfunctions.


Myotilin turnover


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Analysis of myotilin turnover provides mechanistic insight into the role of myotilinopathy-causing mutations

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Figure S1 Schematic picture of events leading to myotilin degradation in muscle cells

Myotilin turnover is initiated by calpain-cleavage at sites N-terminal to the first Ig domain. The cleavage is not affected by disease-causing mutations. Cleaved myotilin undergoes degradation via the ubiquitin–proteasome system (and additional mechanisms). Mutated myotilin is more resistant to degradation. This leads to accumulation of myotilin and induction of actin-containing protein aggregates.

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