Severe muscle disease-causing desmin mutations interfere with in vitro filament assembly at distinct stages

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Desmin is the major intermediate filament (IF) protein of muscle. Recently, mutations of the desmin gene have been reported to cause familial or sporadic forms of human skeletal, as well as cardiac, myopathy, termed desmin-related myopathy (DRM). The impact of any of these mutations on filament assembly and integration into the cytoskeletal network of myocytes is currently not understood, despite the fact that all cause the same histopathological defect, i.e., desmin aggregation. To gain more insight into the molecular basis of this process, we investigated how mutations within the α-helical rod domain of desmin affect both the assembly of the recombinant protein in vitro as well as the filament-forming capacity in cDNA-transfected cells. Whereas 6 of 14 mutants assemble into seemingly normal IFs in the test tube, the other mutants interfere with the assembly process at distinct stages, i.e., tetramer formation, unit-length filament (ULF) formation, filament elongation, and IF maturation. Correspondingly, the mutants with in vitro assembly defects yield dot-like aggregates in transfected cells, whereas the mutants that form IFs constitute a seemingly normal IF cytoskeleton in the cellular context. At present, it is entirely unclear why the latter mutant proteins also lead to aggregate formation in myocytes. Hence, these findings may be a starting point to dissect the contribution of the individual subdomains for desmin pathology and, eventually, the development of therapeutic interventions.

Materials and Methods

Cloning and Mutagenesis. The full-length clone of the complete mouse desmin WT cDNA was generously provided by Y. Capetanaki (Center of Basic Research, Academy of Athens, Athens, Greece). For protein expression, full-length WT or mutant cDNA were subcloned into the prokaryotic expression vector pDS5 as described in ref. 12. The mutations were introduced by site-directed mutagenesis (QuickChange, Stratagene) and verified by sequencing.

Protein Chemical Methods. Recombinant desmin proteins were produced in E. coli TG1 (Amersham Pharmacia) and purified from inclusion bodies as described in refs. 13 and 14. For in vitro reconstitution into IFs, 0.5–1.0 mg of purified recombinant protein was dialyzed at a concentration of 0.5–1.0 mg/ml overnight into a Tris-buffer (5 mM Tris-HCl, pH 8.4/1 mM EDTA/0.1 mM EGTA/1 mM DTT) by using regenerated cellulose dialysis tubing (molecular weight cut off of 50,000; Spectrapor,

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2Freely available online through the PNAS open access option.
3Abbreviations: IF, intermediate filament; DRM, desmin-related myopathy; ULF, unit-length filament.
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1 intermediate myopathy | intermediate filaments

Intermediate filament (IF) proteins constitute a large multi-gene family, which form extended filamentous networks in metazoan cells (1). In myocytes, desmin IFs constitute the principal structural component of the extrasarcomeric cytoskeleton, forming a three-dimensional scaffold around myofibrillar Z-discs (2). This scaffold, in turn, structurally integrates neighboring myofibrils and connects the myofibrillar apparatus with nuclei, as well as cell–cell attachment sites and mitochondria (3). Moreover, proteins such as the chaperone αB-crystallin have been demonstrated to be associated with desmin IFs (4).

In line with the functional importance of IFs for tissue integrity, mutations within IF proteins have been demonstrated to cause severe human diseases (5). Accordingly, an increasing number of mutations of desmin have recently been described to cause familial or sporadic forms of skeletal and cardiac myopathies, termed desmin-related myopathy (DRM) (6–8). Histologically, DRM is characterized by sub- and intrasarcolemmal aggregates of granulofilamentous material containing predominantly desmin. After the initial observations that mutations in the desmin gene can lead to DRM (9, 10), mutations in genes encoding other components of the extrasarcomeric cytoskeleton, such as αB-crystallin (CRYAB (11)), were found to give rise to the same disease. In fact, ~60% of all cases of DRM are due to mutations of yet unidentified genes (3).

Like all IF proteins, desmin (53 kDa) exhibits a tripartite structure consisting of an amphipathic central α-helical coiled-coil domain (“rod”) flanked by a non-α-helical “head”-and “tail”-domain (Fig. 1A). With the exception of a few missense mutations residing in the head or tail domain, most pathogenic desmin mutations were found in the evolutionarily highly conserved rod domain (3). Most of these mutations are located within coil 2B and cause the exchange of a single amino acid. The first mutation to be investigated in molecular detail, however, was a deletion of seven amino acids in coil 1B, causing a severe skeletal and cardiac myopathy with associated smooth muscle defects (10).

To date, the effects of distinct mutations on the structural and functional properties of desmin are largely unknown. Therefore, we examined the consequences of different desmin rod mutations on in vitro and in vivo IF assembly. More specifically, we focused our analysis on the distinct phases of IF assembly by a hybrid experimental approach involving analytical ultracentrifugation, viscometry, and “time-lapse” electron microscopy. As a result, desmin mutations were classified with regard to their effect on the progression of IF assembly from the elementary dimer building block all the way to the mature filament. Surprisingly, several of the disease-causing mutant desmin variants do behave seemingly normal in terms of filament formation in vitro and in cultured cells. This discrepancy indicates that these mutant amino acids give rise to the disease phenotype only in the physiological context of cytoskeletal organization and function.
Spectrum Laboratories, Rancho Dominguez, CA). Assembly was initiated by addition of an equal amount of assembly buffer (45 mM Tris-HCl, pH 7.0/100 mM NaCl). Viscosity measurements were performed at a protein concentration of 0.3 mg/ml in an Ostwald viscometer (Cannon–Nanning Semiviscisometer, Zematra, Rotterdam-Hoogvliet, The Netherlands) in 50 mM NaCl/25 mM Tris-HCl (pH 7.5) at 37°C (15). Time-lapse electron microscopy of negatively stained samples were performed as described in refs. 12 and 13.

Cell Culture and Fluorescence Microscopy Procedures. For transfection studies, we used desmin- and vimentin-free human adrenocortical carcinoma cells (SW13) and vimentin-positive 3T3 cells. Transfection with FuGENE 6 (Roche) and immunofluorescence microscopy were done according to standard protocols (13). Primary antibodies were the monoclonal anti-desmin antibody Vim 3B4 (both from Progen, Heidelberg). Secondary antibodies were a Cy-3-labeled donkey-anti-mouse antibody (Dianova) and an Alexa 488-labeled donkey-anti-rabbit antibody (Invitrogen). Nuclear DNA was stained with DAPI (4',6-diamidino-2-phenylindole) (Roche Molecular Biochemicals). Cells were viewed by confocal laser fluorescence microscopy (DMIRES 2, Leica).

Analytical Ultracentrifugation. Analytical ultracentrifugation was carried out in Tris-buffer by using an Optima XLA Beckman analytical ultracentrifuge equipped with UV absorption optics. Data analysis was performed by using the program DCDT+ [Version 1.13 (16)] as described in ref. 17.

Results

Complex Formation of Desmin Mutants. We have synthesized 14 mutant desmin variants and purified them according to procedures developed for WT desmin (see Fig. 1 and Table 1) (12).

Table 1. Effect of myopathic mutations located in the rod domain of desmin on filament assembly in vitro and in vivo

<table>
<thead>
<tr>
<th>Mutation*</th>
<th>Affected muscle</th>
<th>Age at onset, years</th>
<th>Type of inheritance</th>
<th>In vitro assembly</th>
<th>cDNA transfection</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Coil 1B</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DesA213V (f)†</td>
<td>Skeletal, cardiac</td>
<td>42</td>
<td>de novo</td>
<td>Filaments</td>
<td>Filaments</td>
</tr>
<tr>
<td>DesE245D (c)</td>
<td>Skeletal, cardiac</td>
<td>20–46</td>
<td>de novo</td>
<td>Filaments</td>
<td>Filaments</td>
</tr>
<tr>
<td><strong>Coil 2B</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DesA337P (c)</td>
<td>Skeletal</td>
<td>20–42</td>
<td>AD</td>
<td>Filamentous aggregates</td>
<td>Aggregates</td>
</tr>
<tr>
<td>DesA342D (a)</td>
<td>Skeletal</td>
<td>24–30</td>
<td>AD</td>
<td>Filamentous aggregates</td>
<td>Aggregates</td>
</tr>
<tr>
<td>DesA345P (d)</td>
<td>Skeletal, cardiac</td>
<td>24–46</td>
<td>AD</td>
<td>Small aggregates</td>
<td>Aggregates</td>
</tr>
<tr>
<td>DesR350P (b)</td>
<td>Skeletal, respiratory, cardiac</td>
<td>31–45</td>
<td>AD</td>
<td>Small aggregates</td>
<td>Aggregates</td>
</tr>
<tr>
<td>DesR357P (e, stutter)</td>
<td>Skeletal, respiratory</td>
<td>35–45</td>
<td>AD</td>
<td>Filamentous aggregates</td>
<td>Aggregates</td>
</tr>
<tr>
<td>DesA360P (a)‡</td>
<td>Skeletal, cardiac</td>
<td>2–10</td>
<td>AR</td>
<td>Filaments</td>
<td>Filaments</td>
</tr>
<tr>
<td>DesN393I (f)†</td>
<td>Skeletal, cardiac</td>
<td>2–10</td>
<td>AR</td>
<td>Filaments</td>
<td>Filaments</td>
</tr>
<tr>
<td>DesL370P (d)</td>
<td>Skeletal, respiratory</td>
<td>28</td>
<td>AD</td>
<td>Small aggregates</td>
<td>Aggregates</td>
</tr>
<tr>
<td>DesL385P (e)</td>
<td>Skeletal, cardiac</td>
<td>&lt;21</td>
<td>de novo</td>
<td>ULFs, short filaments</td>
<td>Aggregates</td>
</tr>
<tr>
<td>DesQ389P (b)</td>
<td>Skeletal, cardiac</td>
<td>42</td>
<td>de novo</td>
<td>Filaments</td>
<td>Filaments</td>
</tr>
<tr>
<td>DesD399Y (e)‡</td>
<td>Skeletal, cardiac</td>
<td>15–24</td>
<td>de novo</td>
<td>ULFs, short filaments</td>
<td>Aggregates</td>
</tr>
<tr>
<td>DesR406W (e)</td>
<td>Skeletal, cardiac</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The clinical features have been adapted from ref. 6; AD, autosomal dominant pattern of inheritance; AR, autosomal recessive.

*The heptad position of the mutated amino acid is given in brackets.

†P. Vicart, unpublished information.

‡Only family members carrying either allele develop desmin-related myopathy, possibly explaining the early onset of the disease.

Fig. 1. Desmin mutations found in the α-helical rod of human desmin. (a) Schematic view of the organization of the human desmin molecule. The α-helical central rod domain is interrupted by the three non-α-helical linker regions L1, L2, and L2, which results in the formation of four α-helical segments, termed coils 1A, 1B, 2A, and 2B. The N-terminal “head” and C-terminal “tail” segments are non-α-helical. PCD, precoiled-coil domain; red, region of the molecule where most mutations are located, i.e., coil 2B. Vertical bars depict the localization of all mutations investigated. (b) Comparison of the amino acid sequence of coil 2B of various desmin proteins (Hs, Homo sapiens; Mm, Mus musculus; Gg, Gallus gallus; Xl, Xenopus laevis; Om, Oncorhynchus mykiss; Ss, Sylorhinus stellars; the alignment was performed by using CLUSTAL). Missense mutations of desmin are depicted in the bottom line. Green, all amino acids of a column are identical; purple, amino acid in one column differs from the corresponding amino acid in Hs. Note that the sequence of murine desmin is virtually identical to the human sequence (>99%), and that most mutations reside in highly conserved regions of the last two-thirds of coil 2B. Data for mutations highlighted red are presented in Fig. 2.
Purified recombinant proteins were reconstituted into soluble oligomeric complexes by dialysis into low ionic strength buffers after denaturation with chaotropic chemicals (see ref. 14 and references therein). It has been documented previously that such distinct complexes, when made from WT proteins, are productive IF precursors (for desmin, see ref. 18). To compare mutant vs. WT desmin complex formation, we performed sedimentation velocity ultracentrifugation as described for WT and vimentin fragments (17). Accordingly, WT and 12 of the 14 mutant desmin variants yielded sedimentation curves with sedimentation coefficients, s, ranging between 4.8 S and 5.7 S (mean = 5.2 S, SD ± 0.2), indicating tetramer formation. In contrast, the two mutant desmin variants, DesQ389P and DesD399Y, exhibited much broader sedimentation curves with S-values in the range of 9.5–12.7 S, indicating the formation of a heterogeneous population of higher-order complexes.

**In Vitro Assembly.** Under standard in vitro assembly conditions, WT desmin assembled into ultrastructurally normal-looking filaments (Fig. 2). Under the very same conditions, the 14 mutant desmin variants assembled into various IF-like as well as non-IF structures and hence could be grouped into four distinct assembly modes (Table 1).

(i) *Seemingly normal filament formation* (DesA213V, DesE245D, DesA360P, DesQ389P, DesN393I, and DesD399Y). To our surprise, judged by electron microscopy, the two desmin variants DesA213V and DesE245D assembled into seemingly normal-looking filaments (Fig. 2; DesE245D). In contrast, the four mutants with amino acid substitutions in coil 2B yielded filaments of somewhat irregular diameter (open arrow in Fig. 2; DesA360P). These filaments exhibited a tendency to stick to one another and often coalesced onto a neighboring filament. Moreover, they appeared more flexible, with frequent “kinks,” and appeared to “branch” (filled arrow in Fig. 2; DesA360P). Such morphologies were never depicted with WT desmin.

IF assembly can be monitored effectively by viscometric analysis, which is a very sensitive tool to detect aggregation of filaments. For desmin assembly, best results were obtained at a protein concentration of 0.3 mg/ml in 25 mM Tris-HCl buffer (pH 7.5) with 50 mM NaCl. Accordingly, in these measurements, a normal increase in the relative viscosity was revealed for DesE245D, DesQ389P, and DesD399Y (Fig. 2; DesE245D). In contrast, for DesA213V, DesA360P, and DesN393I, the viscosity increased normally only during the first 30 min. Afterward, it increased dramatically, being indicative of the formation of highly viscous gels. Notably, for these mutants, we had to employ a much lower protein concentration, i.e., 0.1 mg/ml, to avoid clogging of the viscometer capillary (Fig. 2; DesA360P). Note that the corresponding control experiment with WT desmin was performed at 0.3 mg/ml protein.

(ii) *Disturbed longitudinal annealing and radial compaction* (DesL385P and DesR406W). With these two mutant desmin variants, apparently normal unit-length filament (ULF) formation was observed after 10 sec. At later time points, however, when WT ULFs start to anneal longitudinally, single ULF-like precursors could still be delineated within the short filamentous structures formed, indicating a major elongation defect (arrow in Fig. 2; DesL385P).

These data make it clear that the longitudinal annealing process of ULFs involves consecutive large-scale structural rearrangements. Moreover, not only longitudinal annealing but also radial compaction was compromised with these mutants, because the resulting relatively short filaments retained their ULF diameter. This disturbance of the longitudinal annealing process was confirmed by the viscometric analysis because, after the initial rise of the relative viscosity during the first 5 min, no further increase was observed (Fig. 2; DesL385P).

(iii) *Enhanced filament adhesiveness and aggregate formation* (DesA337P, DesN342D, and DesA357P). These three mutant desmin variants seemed to lead to enhanced stickiness of the assemblies formed, eventually yielding large aggregates. Nevertheless, these proteinaceous masses appeared to be composed of filamentous material because frequently single filaments protruded from these aggregates (filled arrow in Fig. 2; DesA337P). By viscometry, aggregation events were revealed by a sudden drop of the relative viscosity after an initial normal rise (Fig. 2). Such attenuation of the relative viscosity is consistent with a reduction in the number of free filaments concomitantly with aggregate formation. Often, aggregates became so huge that they clogged the viscometer capillary.

(iv) *Conserved ULF formation but deterioration of assembly and breakdown into small aggregates* (DesL345P, DesR350P, and DesL370P). With these three mutant desmin variants, IF assembly already stalled at the ULF state, and after 10 sec, only a few assemblies resembled bona fide ULFs. Most notably, however, ULF-like structures that initially formed slowly but definitely disintegrated into small fibrous to roundish aggregates. After 5 min, only spherical aggregates with a diameter of up to 50 nm could be detected. The DesR350P and DesL370P mutants often seemed to retain the outline of earlier filamentous precursors within the short polymers formed. With DesL345P however, the aggregates formed on disintegration of the ULF-like assemblies were smaller and appeared well separated from each other (Fig. 2; DesL345P). In agreement with the lack of significant filament formation, the apparent viscosity exhibited only a slight initial increase after the onset of assembly with no further change within 1 hr of observation (Fig. 2; DesL345P).

**Transfection of Desmin- and Vimentin-Free Cells.** To assess whether the various mutant desmin variants were able to form IF networks on their own, we transfected human SW13 cells, devoid of cytoplasmic IFs, with the cDNAs cloned into an expression vector carrying a human MHC H2 promoter. This procedure yielded moderate synthesis of the transgenic protein also during transient expression experiments. Accordingly, WT desmin formed an IF network similar to that observed in SW13 subclones reexpressing authentic vimentin (Fig. 3). Notably, mutants such as DesE245D and DesA360P that formed bona fide desmin-type IFs in the test tube also assembled into seemingly normal IF networks in vivo. In contrast, all of the other mutants that failed to assemble into IFs in vitro produced dot-like structures or short fibers in transfected cells (Fig. 3 and Table 1). Hence, our in vitro assembly studies faithfully mirrored the behavior of these desmin mutants in transfected cells.

**Discussion**

At present, our knowledge about the pathogenic events leading to desmin-related myopathies is limited to the identification of an increasing number of mutations in the desmin gene and functional analyses of these mutations by cDNA transfection into cultured cells. From these investigations, it has been concluded that all mutant desmin proteins are unable to assemble into bona fide IFs (7, 19). In contrast, it has recently been demonstrated that even a mutation in an IF-consensus motif in the epidermal keratin, K14, does not necessarily inhibit the formation of bona fide IFs in the test tube (20, 21); although, in affected patient epidermal cells, the protein assembles into huge aggregates causing severe epidermolysis (22, 23). Therefore, we initiated a systematic in vitro assembly analysis for all known missense mutations located within the desmin rod domain. The results obtained yield a complex picture of desmin mutations and their effect on the IF cytoskeleton in DRM.

Characteristically, the four separate α-helical segments of the central rod domain, termed 1A, 1B, 2A, and 2B, engage in the formation of a parallel, in-register dimeric coiled-coil (3). Dur-
Fig. 2. Assembly properties of various desmin mutants. Electron microscopic analysis of negatively stained structures obtained from WT desmin and from representative desmin mutants after assembly in 50 mM NaCl/25 mM Tris-HCl (pH 7.5) for 10 sec, 5 min, and 60 min as indicated in comparison to viscometric profiles of assembly (Right). (Scale bar: 100 nm.) Viscosity changes were measured after 1 min and then at 5-min intervals for 60 min. Abscissa, time (minutes); ordinate, relative viscosity. The profile obtained for DesWT (■) depicts the normal increase of relative viscosity after addition of filament forming buffer. △, the profiles obtained for corresponding mutant proteins. The protein concentration was 0.3 mg/ml except for DesA360P, which measured 0.1 mg/ml.

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ing renaturation from urea-containing into low ionic strength buffers, desmin dimers associate laterally to form distinct tetrameric complexes. Nearly all mutated proteins, when dialyzed into a low ionic strength buffer, form tetrameric complexes as determined by analytical ultracentrifugation. Only two mutations, DesQ389P and DesD399Y, appear to associate into a heterogeneous population of larger aggregates with distinctly higher sedimentation velocities. After increasing the ionic strength, tetramers rapidly associate laterally to yield so-called ULFs. In a next step, these ULFs further anneal longitudinally to form extended “open” filaments. With time, most filaments undergo a “radial compaction” process by a series of as yet undefined internal rearrangements. An extension of the original three-step scheme is depicted in Fig. 4 (see also ref. 24). Notably, some filaments may be in an unraveled state for their entire length even 1 hr after initiation of assembly (25). In vivo, stability and dynamics of extended filament networks is likely to involve additional proteins, such as IF-associated proteins and chaperones (ref. 26 and references therein), or crossbridging proteins, including plectin and synemin (27, 28). Nevertheless, the principal interactions for assembly are well represented by our in vitro experiments (see ref. 1).

The finding that point mutations of desmin can interfere with the distinct steps of filament formation, thereby leading to compromised assembly of ULFs, individual IFs, or disturbed interactions within the resulting filament network, indicates that different amino acids and subdomains are critically involved in distinct, well defined intra- and interfilament interactions. Interestingly, most point mutations of the desmin rod domain found to date are located in coil 2B (Fig. 1). Likewise, in human hereditary blistering skin diseases, such as epidermolysis bullosa simplex (EBS), mutations of the keratin gene that lead to the most severe pathology are often located at either end of the rod domain, i.e., in the IF-consensus motifs of coil 1A and in coil 2B (29, 30). In contrast, mutations reported to cause moderate forms of EBS reside in the head domain, the tail domain, or more central parts of the rod domain. In all IF proteins, the consensus motifs at either end of the central α-helical rod domain (1, 31) are known to be involved in dimer–dimer interactions within the mature filament (32, 33). To date, the molecular sites responsible for these associations have only partially been mapped (1).

Moreover, in the heterozygous situation of patients suffering from DRM, these interactions become even more complex because the presence of WT protein will result in the formation of mixed oligomers with varying numbers of mutant and WT protein. Nevertheless, all desmin mutants revealing defective assembly properties were dominant because, in 1:1 mixtures with WT desmin, they led to aberrant assembly as demonstrated by viscometry and cDNA-transfection into vimentin-containing 3T3 fibroblasts (data not shown). Consistent with these data, the disruption of the desmin cytoskeleton in cultured rat neonatal cardiac myocytes by overexpression of an engineered desmin mutant with an arginine to cysteine change in coil 1B has demonstrated that also in an authentic desmin environment such mutants exhibit a dominant effect (34).

Impact of Proline Substitutions. Notably, most mutations of the desmin rod domain leading to human DRM involve the replacement of alanine, leucine, or glutamine by proline, which is believed to act as a “helix breaker” (3). It has been demonstrated for transmembrane proteins that proline induces a distortion of \(\approx 20^\circ\) in the direction of the helix axis because of steric hindrance arising from its cyclic side chain (35). Furthermore, its side chain blocks the central nitrogen atom thus preventing it from forming a stabilizing hydrogen bond (36). Such structural alterations within the coiled-coil can destabilize its local conformation and exert deleterious effects on filament assembly (37, 38). For an acidic keratin, K14, synthetic experimental proline substitutions within the rod domain have been shown to have a milder effect on assembly than those within the conserved end domains (39). However, a proline residue does naturally occur in keratin 16 and appears to destabilize heterotrimer formation, although it still allows filament formation (40). Because acidic and neutral/basic keratins form obligate heterodimers, the effect of a respective proline substitution in the α-helical rod domain may be modulated by the nonproline residue at the corresponding position of the heterotrimer partner molecule and hence be not principally detrimental for IF assembly. Moreover, the impact of a mutation within a coiled-coil may not be exhibited under a certain chosen “standard” condition but may only become apparent when assembly conditions are varied and additional

Fig. 3. Forced expression of desmin mutants in human cultured cells. Indirect immunofluorescence microscopy of human desmin- and vimentin-free SW13 cells cDNA-transfected with WT desmin (A), DesE245D (B), DesA360P (C), DesL385P (D), DesA357P (E), and DesL345P (F). Green, immunostaining; blue, DAPI staining. Note that DesE245D and DesA360P form an apparently normal IF network in the transfected cells shown, whereas all other mutations, which exhibit a compromised in vitro filament assembly, only show intracytoplasmic aggregates. (Scale bar: 10 \(\mu m\).)

Fig. 4. Hypothetical scheme of the in vitro assembly of desmin. Mutations are indicated at the stage when first divergences from the normal assembly path become apparent. This scheme provides further support for the occurrence of distinct assembly intermediates as proposed by this model. DesQ389P and DesD399Y associate already under nonassembly conditions to complexes with sedimentation coefficients, \(s\), higher than the normal 5.2 S. Nonetheless, they still assemble into elongated filamentous structures. Mutations depicted in bold form filamentous structures during the early phase of assembly but tend to aggregate at later time points.
functional tests are used (21). These considerations now gain substantial support by the assembly studies we performed because proline mutations (DesAS377P, DesL345P, DesAS377P, DesR350P, DesL370P, and DesL385P) exhibited quite distinct effects and disrupted filament assembly at various stages. Nevertheless, we demonstrated that some proline mutations (DesAS606P and DesQ389P), even when present in both molecules of a desmin homodimer, do allow bona fide filament formation to occur in the test tube and even in transfected cells. Evidently, although insignificant in an in vitro environment, in myocytes these mutations give rise to filament properties that interfere with tissue homeostasis.

**Impact of Nonproline Substitutions.** The two mutations residing in coil 1B of desmin, DesA213V and DesE245D, do enable filament formation in vitro. Nevertheless, for DesA213V, we found a significant difference to the WT protein in one of our assays: in later stages of assembly, i.e., in the maturation phase of IFs, it aggregated in the viscometer. Therefore, the pathomechanisms of these mutations probably involve subtle but critical interactions with non-IF components in muscle cells. The amino acid substitutions in DesN342D and DesN393I gave rise to new properties of the mutated proteins during assembly that cannot be explained from first principles. In DesD399Y, the potential intrahelical salt bridge between Lys-395 and Asp-399 is abolished (20). Similarly, the interhelical salt bridge between Arg-406 of one strand and Glu-401 of the second strand is destroyed by the mutation in DesR406W (31). How these intra- and interhelical salt bridges are involved in the molecular rearrangements during any of the phases of assembly will have to await further biophysical analysis.

**Conclusions**

Taken together, mutations of the desmin rod domain can compromise in vitro filament assembly at various stages. Surprisingly, whereas some mutant variants that cause DRM do assemble into bona fide IFs in vitro, others with the same disease phenotype exhibit severely compromised in vitro filament formation. Hence, the severity of disease and the degree of the assembly defect of a mutant desmin variant cannot be correlated directly. In addition, one has to consider the different genetic background in affected patients and the low overall incidence of the disease with only small numbers of affected patients. Furthermore, altered biophysical properties of the filaments, which were not assessed in this study, as well as disturbed interaction with important cellular binding partners, including kinases and phosphatases, may lead to accelerated breakdown of the desmin IF network and/or aggregate formation.

In fact, this complex interdependence resembles the situation found with other intermediate filament diseases such as the laminopathies (5, 41). The >200 mutations described for lamin A give rise to 10 distinct disease phenotypes, which cannot be explained from first structural principles. Instead, their complex phenotype and onset at distinct stages of development suggests interference with the execution of genetic programs and/or metabolic as well as signaling processes during aging. Some of these disturbances of cell and tissue homeostasis may also apply for desmin mutations. Hence, further investigations on the influence of distinct mutations on these processes and their interaction with tissue-specific IF-associated proteins will ultimately lead to a more rational understanding of the pathomechanisms leading to DRM and other devastating IF-based illnesses. This understanding, in turn, will be a means for producing effective therapeutic interventions.

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