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Research Article

Forced expression of desmin and desmin mutants in cultured cells: Impact of myopathic missense mutations in the central coiled-coil domain on network formation

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ABSTRACT

We recently demonstrated that inherited disease-causing mutations clustered in the α-helical coiled-coil "rod" domain of the muscle-specific intermediate filament (IF) protein desmin display a wide range of inhibitory effects on regular in vitro assembly. In these studies, we showed that individual mutations exhibited phenotypes that were not, with respect to the severity of interference, predictable by our current knowledge of the structural design of IF proteins. Moreover, the behavior of some mutated proteins in a standard tissue culture cell expression system was found to be even more complex. Here, we systematically investigate the behavior of these disease mutants in four different cell types: three not containing desmin or the related IF protein vimentin and the standard fibroblast line 3T3, which has an extensive vimentin system. The ability of the mutants to form filaments in the vimentin-free cells varies considerably, and only the mutants forming IFs in vitro generate extended filamentous networks. Furthermore, these latter mutants integrate into the 3T3 vimentin network but all the others do not. Instead, they cause the endogenous network of 3T3 vimentin to reorganize into perinuclear bundles. In addition, most of these assembly-deficient mutant desmins completely segregate from the vimentin system. Instead, the small round to fibrillar particles formed distribute independently throughout the cytoplasm as well as between the collapsed vimentin filament arrays in the perinuclear area.

Introduction

In the past 15 years, an increasing number of mutations in intermediate filament (IF) proteins has been found to lead to distinct human disease phenotypes [1,2]. As nearly every mammalian cell type expresses at least one of the more than 70 different IF proteins, these disorders can affect almost any organ. Normal IF expression follows distinct programs of embryonic development leading to a strictly regulated tissue-specific expression pattern [3]. For skeletal and cardiac muscle, mutations in the muscle-specific IF protein desmin were demonstrated to cause severe forms of myopathy [4–8].

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Analogous to the situation in other IF diseases, desmin-related myopathy is characterized by the formation of intracellular aggregates containing desmin and associated proteins.

We recently characterized the details of in vitro assembly defects caused by desmin mutations which lead to severe myopathies in humans [9]. We grouped these missense mutations located in the coiled-coil forming rod domain of desmin into 4 different classes according to the observed assembly defects. Most remarkably, we demonstrated that the in vitro filament-forming potential in 6 out of 14 mutations investigated was preserved. Upon closer examination, however, most of the filamentous structures differed from wild-type desmin (*Des*WT) in several respects [8,9]. Even so, the preserved filament-formation capacity of some mutated desmins was surprising because it has generally been assumed that a severe disease-causing mutant will also exhibit a major assembly defect in in vitro assembly. This has been illustrated by forced expression of mutated keratins in cultured cells and transgenic mice, where an inability to form IFs in vitro correlated with poisoning of assembly in vivo (for review see [10]).

In contrast to the in vitro situation, the assembly process for IFs in vivo is not yet understood. Thus, we wanted to investigate how these various mutants assemble in living cells. Cellular chaperones and factors that scan proteins for correct folding may influence filament formation and stability, and thus modulate IF network assembly, turnover and function. Hence, individual mutations may indeed be processed in different ways. Moreover, in cells devoid of cytoplasmic IFs such as SW13, wild-type desmin (*Des*WT) was shown to assemble only into "inferior" networks after cDNA-transfection employing standard expression plasmids [4]. This indicates that the establishment of well-spread, extended desmin arrays is apparently dependent on factors only present in cells naturally expressing type III IF proteins.

To further assess the assembly properties of *Des*WT and the mutant desmins in the chosen cells, we systematically performed transient transfection experiments of these desmin constructs into different well-established mammalian cell lines. In pioneering studies on the forced expression of desmin both in cultured cells and in transgenic mice, it was demonstrated that the coexpression of vimentin and desmin did not result in any detectable abnormalities [11,12].

In order to relate the results obtained by our in vitro assembly studies with the fate of mutated desmins in transfected cells, we employed human SW13, bovine BMGE + H and human MCF 7 cells, all not expressing endogenous vimentin or desmin, and murine 3T3 fibroblasts, which express vimentin [9]. We chose this latter cell line because it has been employed in many studies on type III IF proteins and displays a very elaborate vimentin network [13,14]. Our experiments clearly revealed that point mutations in desmin, which inhibit proper in vitro assembly of filaments, also do not organize into filaments in vimentin-free cells. Furthermore, these mutants caused a drastic reorganization of the endogenous vimentin network in 3T3 fibroblasts and segregated from vimentin filaments.

Materials and methods

Cloning and mutagenesis

The full-length clone of the mouse desmin cDNA was generously provided by Y. Capetanaki (Athens, Greece). For protein expression, the wild-type (*Des*WT) or mutant cDNAs were subcloned into the prokaryotic expression vector pDS5 as described previously [15,11]. Mutations were introduced by site-directed mutagenesis (Quickchange®, Stratagene,

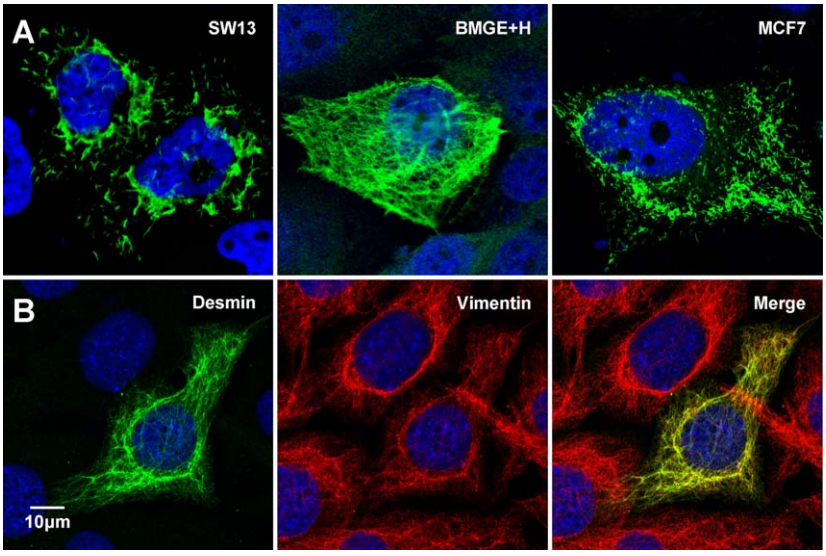


Fig. 1 – Indirect immunofluorescence microscopy of (A) adrenocortical carcinoma cells (SW13), bovine mammary gland epithelial cells (BMGE + H), mamma carcinoma cells (MCF 7) and (B) 3T3 fibroblasts transiently expressing *Des*WT. Green, desmin; red, vimentin; blue, DAPI staining. Note that *Des*WT forms only short filamentous structures in some SW13 cells, whereas others demonstrate predominantly perinuclear aggregate formation. Note that the cytoplasmic IF network is more elaborate in BMGE + H cells. In 3T3 cells, desmin and vimentin filaments colocalize as indicated by the yellow color in the merge image. Scale bar, 10 µm.

Germany) and verified by sequencing. For transfection studies, the full-length clones were inserted into the unique *Eco*RI site of the eukaryotic expression vector p163/7, which drives expression with a MHC promoter [16].

Protein chemical methods

Recombinant desmin and mutated proteins were produced in *E. coli* (TG 1, Amersham, Germany) and purified from inclusion bodies as described previously [8,17]. Vimentin wild-type (*Vim*WT) protein was purified as described previously [18]. 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 buffer containing 5 mM Tris–HCl (pH 8.4), 1 mM EDTA, 0.1 mM EGTA and 1 mM DTT ("Tris-buffer") using regenerated cellulose dialysis tubing (Spectra/Por®, MWCO 50.000; Roth, Germany). Mixing experiments were performed by addition of equal amounts of *Vim*WT and the respective mutant desmin prior to dialysis. Assembly was initiated by addition of an equal amount of "assembly buffer" (45 mM Tris–HCl, pH 7.0, 100 mM NaCl). Electron microscopy of negatively stained samples were performed as previously described [8,15].

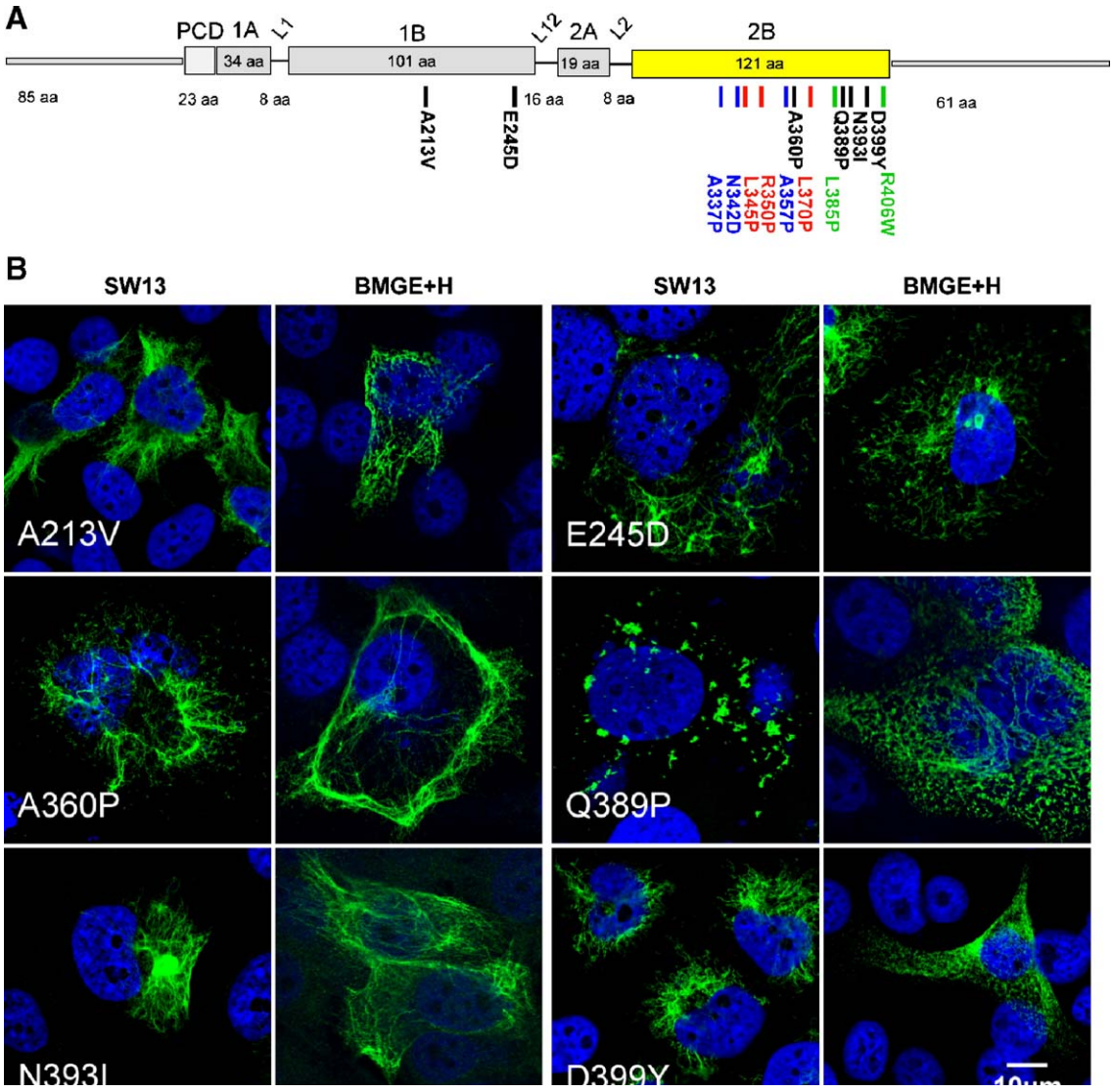


Fig. 2 – (A) Schematic view of the organization of the desmin molecule (adapted from [9]). The α-helical central rod domain is interrupted by three non-α-helical linker regions (L1, L12 and L2), which results in the formation of four α-helical segments, termed coil 1A, 1B, 2A and 2B. The N-terminal head and the C-terminal tail segments are non-α-helical. PCD, precoiled-coil domain; yellow, region of the desmin molecule where most mutations have been described to date. Vertical bars depict the relative localization of all mutations investigated. The mutations have been color-coded according to their observed in vitro assembly defect: black, preserved filament formation; green, disturbed longitudinal annealing; blue, formation of filamentous aggregates; red, disassembly of filamentous precursors. (B) Desmin mutants capable of filament formation in in vitro assembly studies are expressed in SW13 and BMGE + H cells. Mutant desmin variants are indicated in the lower left of the respective frames. Green, desmin; blue, DAPI staining. Scale bar, 10 μm.

Cell culture and microscopic procedures

For transfection studies, we used human adrenocortical carcinoma cells completely devoid of cytoplasmic intermediate filaments (SW13), bovine mammary gland epithelial cells (BMGE + H), human breast cancer cells (MCF 7) and murine 3T3 fibroblast-derived cells [14,19–22]. Cells were grown on glass coverslips and transiently transfected with 5 μg plasmid DNA per 5 ml plate using Fugene 6® according to the manufacturer's protocol (Roche, Germany). 48 h after transfection, cells were processed for immunocytochemistry. Briefly, cells were fixed in methanol for 5 min followed by permeabilization in acetone for 3 min at – 20°C. After rehydration, specimens were blocked in 10% donkey serum in phosphate-buffered saline (PBS) for 30 min. The coverslips were incubated with the monoclonal anti-desmin antibody RD301 (dianova, Germany) or the polyclonal rabbit anti-desmin serum (Progen, Germany) together with the monoclonal anti-vimentin antibody Vim3B4 (Progen, Germany) for 60 min at room temperature. After thoroughly rinsing in PBS, a Cy-3-labeled donkey-anti-mouse antibody (dianova, Germany) and Alexa 488-labeled donkey-anti-rabbit antibody (Invitrogen, Germany) were applied simultaneously for 30 min together with DAPI (4,6-diamidino-2-phenylindole; Roche Diagnostics, Germany) for nuclear staining. The coverslips were finally mounted on glass slides in Fluoromount G (Southern Biotechnology Associates, USA). Cells were viewed by confocal laser scanning fluorescence microscopy (DMIRE 2, Leica, Germany).

Results

We have investigated how hallmark desmin mutations, identified in patients with severe myopathies, affect assembly and impact on network formation by forced expression in cultured cells [9]. Based on the in vitro assembly properties of the recombinant proteins, we have grouped these mutations into four major categories: (1) six mutations allow the formation of extended filamentous networks; (2) two mutations lead to disturbed longitudinal annealing and loss of radial compaction; (3) three mutations cause the formation of filaments with enhanced adhesiveness eventually leading to the formation of filamentous aggregates and (4) three mutations lead to transient association into short filaments that rapidly disintegrate into small aggregates later in the assembly process (Fig. 2A).

Here, we study the effects of these mutations in four different cell types. In the three vimentin- and desmin-free

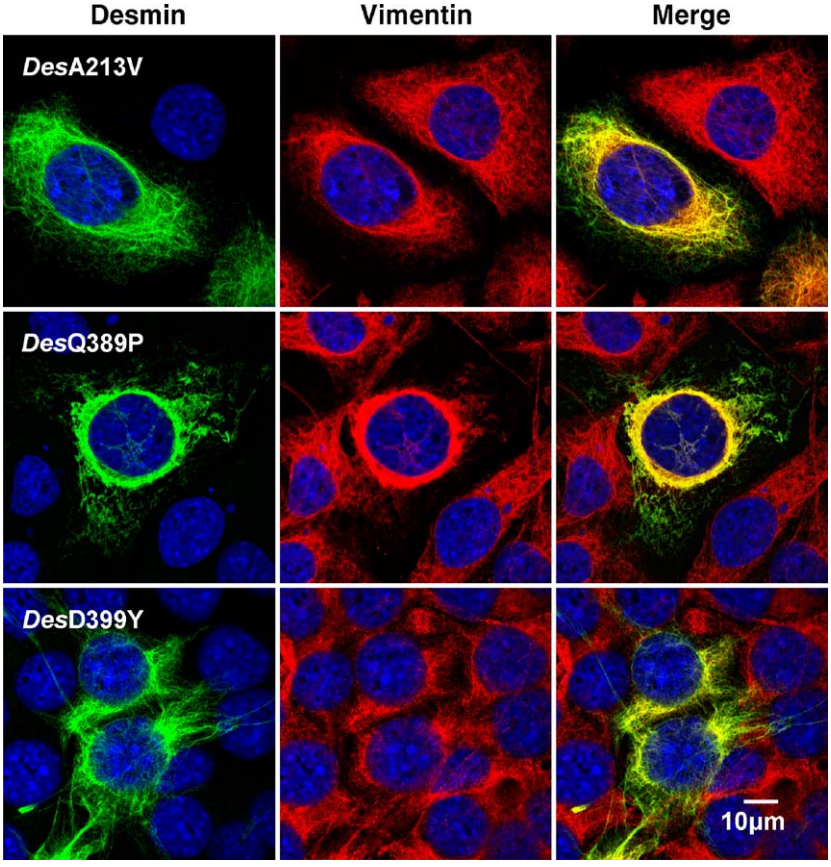


Fig. 3 – Transfection studies of murine 3T3 fibroblast cells with constructs coding for the desmin mutants indicated in the respective upper left frame of each row of images. Note that *Des*Q389P forms irregular fibrillar structures and the endogenous vimentin cytoskeleton in transfected cells is collapsed and exhibits perinuclear aggregation. Colocalization of desmin and vimentin staining is indicated by the yellow color in the merge in the right column. Left column, transfected desmin variants (green); middle column, endogenous vimentin (red); right column, merge. Blue, DAPI. Scale bar, 10 μm.

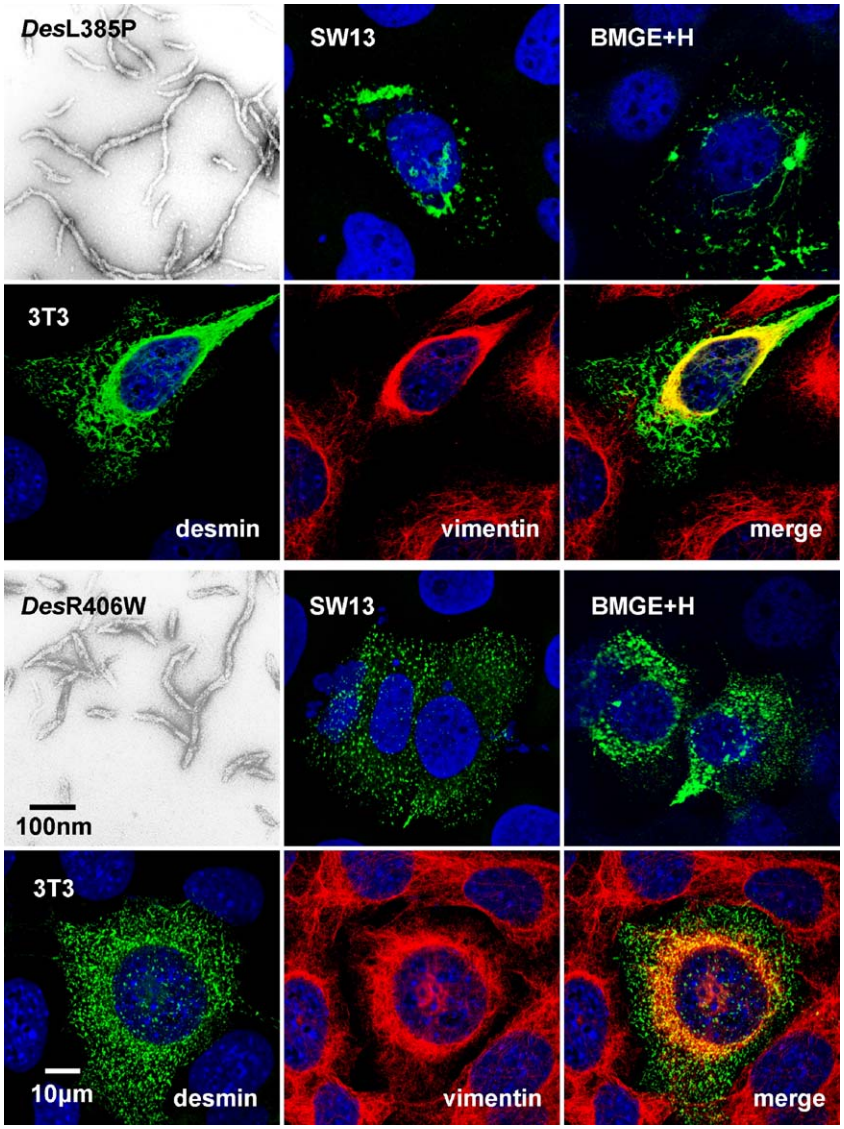
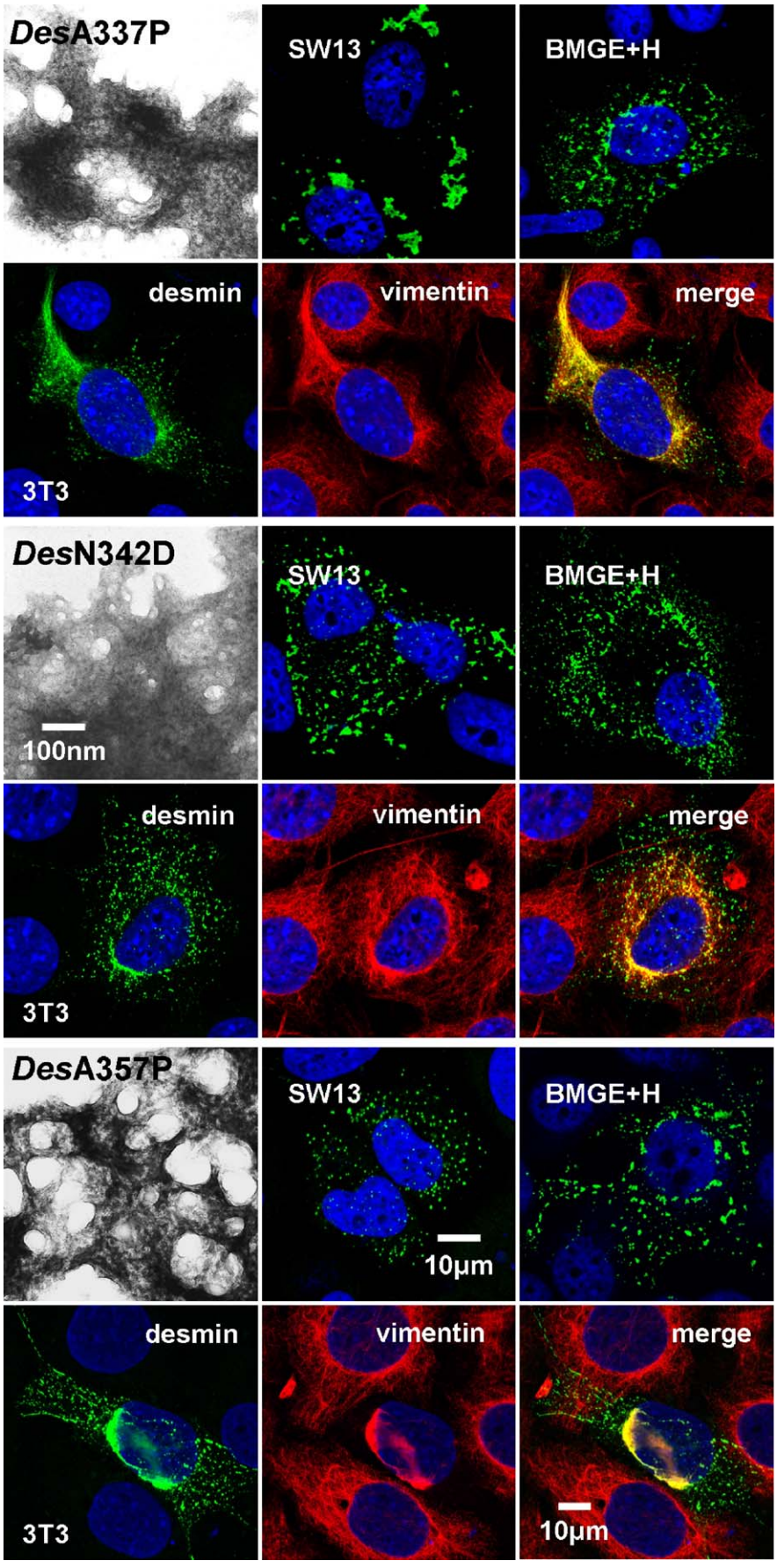


Fig. 4 – Electron microscopy of negatively stained assembly products obtained with desmin mutants *Des*L385P and *Des*R406W after in vitro assembly for 60 min. Note that only short filamentous structures are formed, indicating severely compromised longitudinal elongation properties of these mutants. Scale bar, 100 nm. Cells transiently expressing these mutants are imaged by confocal laser scanning micrographs. Green, desmin immunostaining; red, vimentin staining; blue, DAPI staining. For 3T3 fibroblasts, desmin and vimentin immunofluorescence staining as well as the merge image of both stainings is shown. Scale bar, 10 µm.

cell systems employed, *Des*WT formed long, individual filaments 48 h after transfection. In BMGE + H cells, this was seen in nearly every transfected cell (Fig. 1A). In SW13, which are devoid of cytoplasmic IFs, and MCF 7 cells, which express endogenous keratins, desmin IFs exhibited short, less regular filamentous structures. In some SW13 cells, sporadic dot-like aggregates

Fig. 5 – Electron microscopy of negatively stained assembly products obtained with desmin mutants *Des*A337P, *Des*N342D and *Des*A357P after in vitro assembly for 60 min. Note the formation of filamentous aggregates, indicating the adhesiveness of these mutants. Scale bar, 100 nm. Corresponding representative transfection results are shown in the confocal laser scanning images. Note that *Des*A337P is still capable of forming short filamentous structures in SW13 cells. In all other transfection studies, coarse aggregates can be documented throughout the cytoplasm. A dominant negative effect is exerted on the endogenous vimentin cytoskeleton in 3T3 cells by these mutants, leading to a perinuclear collapse of the cytoplasmic IF network. Desmin-positive aggregates predominate in the cellular periphery of transfected cells, as indicated by the green fluorescence in the merge image. Green, desmin immunostaining; red, vimentin staining; blue, DAPI staining. For 3T3 fibroblasts, desmin and vimentin immunofluorescence staining as well as the merge image of both stainings shown. Scale bar, 10 µm.


DesA337P
SW13
BMGE+H
desmin
vimentin
merge
3T3
DesN342D
SW13
BMGE+H
100nm
desmin
vimentin
merge
3T3
DesA357P
SW13
BMGE+H
10µm
desmin
vimentin
merge
3T3
10µm

were observed (Fig. 1A). However, this is a well recognized feature with this latter cell type that is even seen with cells that spontaneously begin to re-express endogenous vimentin [23].

In 3T3 fibroblast cells, desmin integrated homogeneously into vimentin filaments, as judged by the extended networks in merged images (Fig. 1B). Hence, the additional synthesis of *Des*WT in these cells did not cause any visible alterations of the fibroblast cytoskeleton.

Category 1: mutants exhibiting filament formation in vitro

The mutants located in coil 1B (Fig. 2A), *Des*A213V and *Des*E245D, formed IFs comparable to *Des*WT in SW13 cells. In BMGE + H cells, however, *Des*A213V filaments were less regular and bundle-like, appearing throughout the cytoplasm. This type of network has not previously been observed for any of the other mutant desmin variants (Fig. 2B). In contrast to the other mutants studied, *Des*A360P formed pronounced filamentous "rings" in the cytoplasmic periphery of SW13 and BMGE + H cells (Fig. 2B). In SW13 cells, *Des*Q389P displayed only short filament arrays, which often collapsed into aggregates at the nuclear periphery, whereas in BMGE + H cells, individual short filaments were displayed throughout the cell. Notably, close to the cell border, filaments were very short, but exhibit increased length with increasing distance from the cell periphery. Filamentous networks comparable to *Des*WT were found for both *Des*N393I and *Des*D399Y in SW13 cells. In BMGE + H cells, *Des*N393I displayed normal *Des*WT-type arrays, whereas *Des*D399Yfilaments were on average very short, especially at the cell borders, resembling the network formed by the *Des*Q389P mutant (Fig. 2B). An additional feature, only observed with *Des*N393I in SW13 cells, was the formation of huge aggresome-type single associations of mutant protein in the nuclear vicinity.

In 3T3 fibroblasts, all mutant desmin variants apart from *Des*Q389P were capable of integrating into vimentin filaments, shown for *Des*A213V and *Des*D399Y in Fig. 3. The *Des*Q389P mutant variant led to the formation of thick and irregularly oriented fibrillar structures. The most striking difference was that the mutant desmin *Des*Q389P segregated completely from vimentin in the cell periphery and, in addition, caused the "collapse" or reorganization of the endogenous vimentin cytoskeleton into the perinuclear region (Fig. 3).

Category 2: mutants exhibiting disturbed longitudinal annealing and radial compaction

When engineered into bacterial expression systems, the longitudinal annealing of mutant desmins of this category was severely compromised in in vitro assembly (Fig. 4, left panels). Even after 1 h of assembly, no regular, extended IFs were observed. Correspondingly, all of them exhibited severe assembly defects in transfected SW13 and BMGE + H cells. *Des*L385P formed coarse granular cytoplasmic aggregates both in SW13 and in BMGE + H cells as well as filamentous structures located predominantly in the perinuclear region (Fig. 4, upper row, middle panels). In stark contrast, *Des*R406W induced appearance of numerous and very small, dot-like aggregates, which were distributed throughout the cytoplasm (Fig. 4, lower row, middle panels).

The results obtained with 3T3 cells were similar in that *Des*L385P formed many individual fibers of varying length throughout the cell and that *Des*R406W localized to a multitude of dots and short filaments. Most remarkably, both mutant desmins exerted a dominant negative effect on the vimentin network, leading – like *Des*Q389P – to a reorganization of the entire IF cytoskeleton in the perinuclear region of 3T3 cells (Fig. 4, right panels).

Category 3: mutants exhibiting enhanced filament adhesiveness and aggregate formation

The three mutant desmins that form huge network-type aggregates of fused fibers in vitro (Fig. 2A) exhibit similar localization patterns of the mutant protein in vimentin-free cells. With each mutant, variously shaped and sized aggregates were distributed throughout the cytoplasm. Especially for DesA337P, these aggregates were quite large.

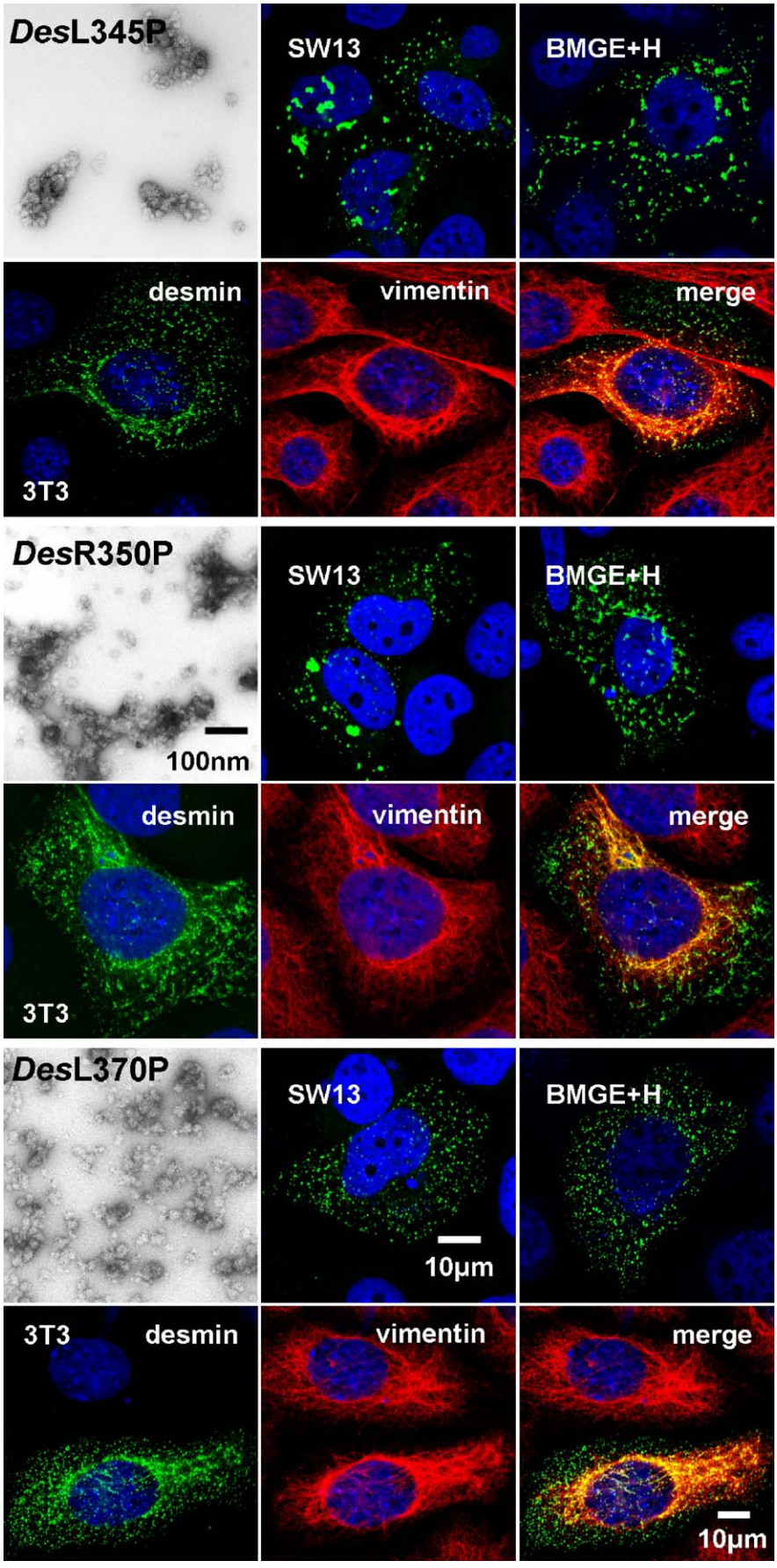
In 3T3 cells, the effect was more complex. The *Des*A337P mutant segregated from the vimentin filaments, but left the network mostly unaffected. Similarly, *Des*N342D hardly affected the vimentin system and did not coassemble with vimentin. In contrast, *Des*A357P had a pronounced deleterious effect on the endogenous vimentin cytoskeleton (Fig. 5). In this case, the vimentin IF system was heavily concentrated at the nuclear periphery colocalizing with numerous desmin mutant aggregates. This is particularly evident when both channels are inspected separately (Fig. 5).

Category 4: mutants that form filamentous assembly precursors but subsequently disintegrate into small aggregates

These mutants (Fig. 2A) form very peculiar associations in vitro in that they associate into elongated fibers immediately after initiation of assembly [9]. Within the first minute, however, these fibers disintegrate into round aggregates. After 1 h of assembly, they form, on average, spherical, approximately 20 to 40 nm in diameter, particles that do not, even after prolonged incubation, associate into larger aggregates. After transfection into vimentin-free cells, they form structures very similar to members of category 3 (Fig. 6).

In 3T3 cells, all three mutants lead to a collapse of the endogenous vimentin in the perinuclear region (Fig. 6). This collapse, however, is not as drastic as with mutants described above.

Fig. 6 – Electron microscopy of negatively stained assembly products obtained from desmin mutants *Des*L345P, *Des*R350P and *Des*L370P after in vitro assembly for 60 min. Note that only ball-like aggregates are visible. Scale bar, 100 nm. Corresponding representative transfection results are depicted in the confocal laser scanning micrographs. Neither mutant is capable of filament formation in each of the cell type studied. A dominant negative effect on the endogenous vimentin cytoskeleton can be appreciated in the right column. For 3T3 fibroblasts, desmin and vimentin immunofluorescence staining as well as the merge image of both stainings shown. Scale bar, 10 μm.


DesL345P
SW13
BMGE+H
desmin
vimentin
merge
3T3
DesR350P
SW13
BMGE+H
100nm
desmin
vimentin
merge
3T3
DesL370P
SW13
BMGE+H
10μm
3T3
desmin
vimentin
merge
10μm

High-resolution imaging of the desmin mutant-induced perinuclear vimentin network

The vimentin arrays concentrated around the nucleus are on close inspection largely devoid of copolymerized desmin mutant (Fig. 7). The *Des*L345P protein (upper left panel) exhibited mostly small, round aggregates at the cell periphery that are often observed to fuse into slightly longer stumps closer to the nucleus. The vimentin staining pattern around the nucleus instead consists of thick bundles interspersed with the desmin aggregates; the yellow signal in the merged image shows that these structures are close together but not within the same polymer (upper panel). This is even more evident with those structures documented above the nucleus, where small green dots are seen in proximity with the red vimentin bundles but are not part of the same structure (arrowheads, upper right panel). Note that the transfected mutant causes a rearrangement of the endogenous vimentin filaments. Similarly, with *Des*L385P, a cap consisting of collapsed vimentin filaments is formed on the left side of the nucleus (arrows), which harbors both proteins (see merged images). However, again, the staining pattern of both proteins indicates that they segregated more or less completely. Note that also the many vimentin particles and "squiggles" – the red dots and short fibers in the middle and the right panel – do not light up in the green channel, indicating that they do not contain the desmin mutant. Hence, the yellow color in the merged images indicates the close neighborhood of both the desmin and the vimentin structures. The details of their distribution as well as the non-merged appearance of the smaller particles nevertheless indicate that the mutant desmins segregated from vimentin. A minor degree of coassociation of the two proteins, however, cannot be formally excluded. In the cap formed by the collapsed vimentin filaments, neither did short fibers (lower panels) nor did more extended aggregates (upper

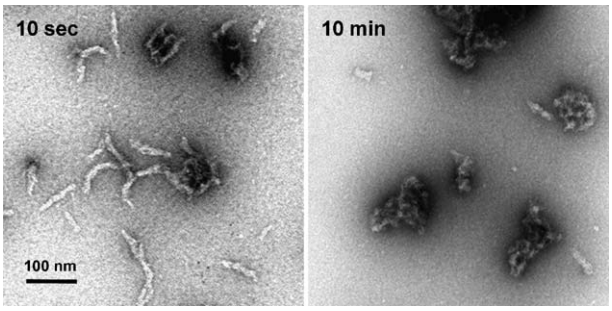


Fig. 8 – Electron microscopy of negatively stained assembly intermediates obtained by mixing equal amounts of *Des*L345P and vimentin wild-type (*Vim*WT) prior to dialysis. After assembly in "Tris-buffer" for 10 s (left panel) and 10 min (right panel), the reaction was stopped by adding 0.1% glutaraldehyde in filament buffer. Note that the desmin mutant exerts a dominant negative effect on vimentin assembly, leading to the formation of irregular proteinaceous aggregates within minutes. At 10 s, some "unit-length filament (ULF)"-like assembly precursors can readily be observed. In control experiments, vimentin formed extended, smooth filaments (data not shown). Scale bar, 100 nm.

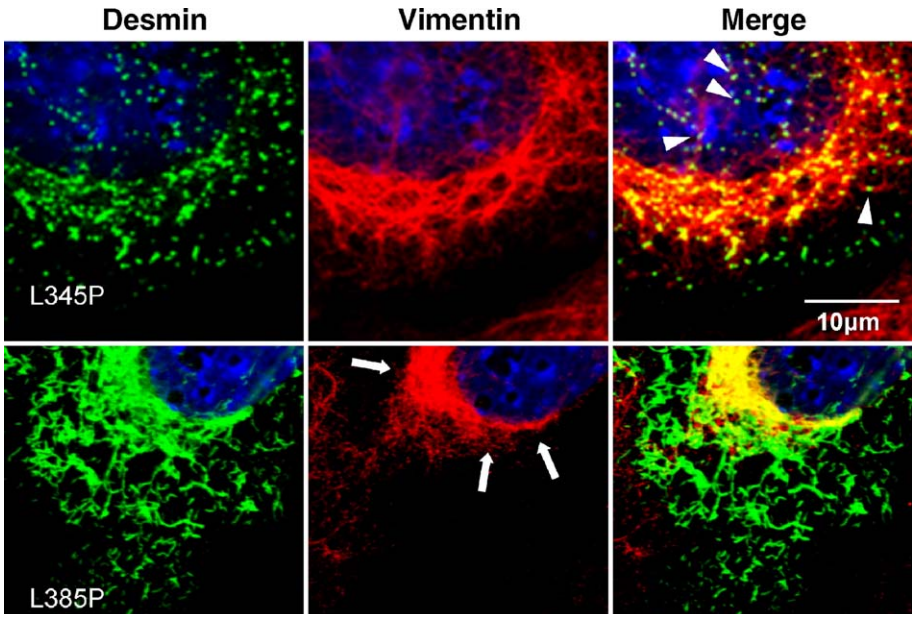


Fig. 7 – Detailed immunofluorescence visualization of the endogenous vimentin system and the transfected mutant desmins *Des*L345P (upper row) and *Des*L385P (lower row). Detection was with the mouse monoclonal antibody 3B4 for vimentin and a rabbit anti-desmin serum against desmin. Arrowheads: desmin particles (green) in close proximity with vimentin bundles (red). *Des*L385P causes a rearrangement of the endogenous vimentin cytoskeleton. A cap (arrows) consisting of collapsed vimentin filaments is formed on the left side of the nucleus. Green, desmin immunostaining; red, vimentin staining; blue, DAPI staining. Scale bar, 10 μm.

panels) of desmin exactly codistribute with the filamentous vimentin arrays. Nevertheless, the structures formed by the desmin mutants were not excluded but rather trapped within vimentin caps.

In vitro assembly of mutant desmin DesL345P with wild-type vimentin

This obvious segregation of desmin and vimentin in transfected fibroblast cells prompted us to perform in vitro assembly studies where both proteins were mixed in 9.5 M urea and hence were exposed to conditions that may allow heterodimer formation upon dialysis [24]. Indeed, as exemplified here for the mutant desmin variant *Des*L345P, both proteins do coassemble into a distinct non-IF-like structure. Notably, no residual *Vim*WT filaments were observed, indicating that the mutant protein exerted a pronounced dominant negative effect on filament formation of WT vimentin (Fig. 8).

Discussion

Mutations in the desmin gene can lead to a severe and devastating human disease characterized by the presence of intracellular aggregates in myocytes containing desmin and associated proteins such as plectin and αB-crystallin. In order to understand the molecular pathogenesis of the ever rising number of various missense mutations leading to this distinct but uniform histological picture in affected patients, we have performed an extensive in vitro assembly study to characterize most of the known mutant desmin variants [9]. To our surprise, 6 out of 14 desmin proteins harboring single amino acid exchanges were able to form filaments, although in human, they all lead to myopathy with intrasarcoplasmatic desmin aggregation. In addition to this finding, we were able to group the mutants studied into four principal categories according to the assembly properties: those exhibiting (1) preserved filament formation; (2) disturbed longitudinal annealing; (3) formation of filamentous aggregates; and (4) disassembly of filamentous precursors into proteinaceous masses.

In order to investigate the physiological relevance of our in vitro assembly model and to further improve the classification of desmin mutations with respect to the four in vitro categories, we performed transfection studies in different cell types.

Transfection of vimentin-free cells—generation of desmin networks

In particular, we used SW13 cells, which are completely devoid of cytoplasmic IF proteins. Many groups that have described assembly properties of novel desmin mutations have used this cell line [4,5,25]. However, these cells, most likely due to the fact that they lack factors required for the generation or maintenance of extended filament arrays, exhibit the formation of only rudimentary IF networks. In addition, SW13 cells may spontaneously begin to express vimentin, and, with increasing passage number, the number of vimentin-positive cells present in the culture increases. This, of course, severely interferes with the mutant analysis, because vimentin and desmin coassemble and endogenous vimentin may rescue the effect of the mutation. Therefore, we also employed BMGE + H cells, an epithelial cell line that expresses keratins but does not synthesize vimentin [19]. In the past, we have used this cell line successfully to characterize the assembly properties of vimentin mutants [26]. In these cells, *Des*WT assembled into more elaborated desmin networks than in SW13 cells. Interestingly, nearly all desmin mutants that were able to assemble into IF-like structures in vitro were also able to generate extended filamentous systems in the absence of vimentin. The members of the other three categories, however, formed aggregates, only with the exception of *Des*L385P, which also generated extended filaments to some extend.

Transfection of vimentin-containing cells—integration into existing vimentin networks

The fibroblast cell line 3T3 provides a valuable tool to study the interaction and coassembly of two type III IF proteins, endogenous vimentin and transfected desmin. Such coassembly is of biological relevance, because both proteins are transiently coexpressed during early embryonic differentiation of myocytes (for review, see [27]). Using in vitro analyses including analytical ultracentrifugation, we have demonstrated that both proteins interact at all stages of assembly from the elementary dimer to the mature filament [18]. Furthermore, this in vivo system mimics heterozygosis and enabled us to investigate how a distinct mutant desmin variant influences a preexisting endogenous filamentous network. Moreover, because we could rely on protein-specific antibodies, we avoided potentially harmful labels such as the green fluorescent protein (GFP), which would have to be used in cells expressing endogenous desmin in order to discriminate the mutant from the wild-type protein. In our opinion, this would have been a serious disadvantage, because the comparatively large GFP-tag has been demonstrated to convert vimentin into a strong dominant negative entity that abolishes in vitro filament formation of wild-type vimentin when present in amounts as low as 5% [28]. In addition, we used a well-established vector containing a mouse MHC H2 promoter and a 5′-UTR beta-globin sequence to avoid overexpression of the respective mutant desmin as often observed with the very strong viral promoters of SV40 or CMV origin [12]. In this way, we were able to obtain meaningful levels of protein synthesis through transient transfection. In most cells, transient expression was estimated to be below or in the range of endogenous vimentin.

Mutants that fail to incorporate into the vimentin network cause its collapse

A very notable result of the experiments with 3T3 cells was that some point-mutated desmins (1) segregated from vimentin and (2) caused a drastic reorganization of the vimentin system into the perinuclear region. This reorganization resembles the effect of microtubule-disrupting drugs such as colcemid [14]. Part of the mutant desmin colocalized with

reorganized vimentin filament bundles. However, our data strongly indicate that it was not – or only to a very low extend – integrated into these filaments, since it did not follow the outline of vimentin fibers as revealed by double-immunofluorescence labeling of both proteins and confocal microscopy. Nevertheless, desmin aggregates may physically associate with vimentin IFs, for example via crossbridging factors such as plectin or motor protein complexes residing nearby on microtubules or microfilaments. For this reason, desmin aggregates are also found to be distributed along the vimentin bundles. This observation supports the hypothesis that filamentous subunits of vimentin and desmin may be assembled and further elongated on moving motor complexes [29,30]. Desmin mutants, which are non-functional for proper assembly, may occupy such associated proteins or protein complexes and thereby disturb their interaction with vimentin IFs. Another possibility is that these mutant desmin proteins fail to interact with other cellular desmin-binding proteins necessary for targeting to their final cellular destination. Why the point mutants that are not able to form IFs in vitro segregate from vimentin, in contrast to the ones that form filaments in vitro, is not clear. Future work is aimed at understanding the molecular details underlying the fact that only certain mutations completely abolish filament assembly whereas others do not. At present, neither does the position nor the type of amino acid alteration gives a clue why a certain mutation is "toxic" for IF assembly. This is exemplified by both *Des*A357P and *Des*A360P, where a proline is substituted for the same amino acid, alanine. The former alanine is in an "*e*" position of the heptad repeat, the latter in an "*a*" position. Similarly, *Des*A337P is disastrous for filament assembly and here the substitution is in a "*c*" position. Why a proline in the "*c*" and the "*e*" position is deleterious both in vitro and in vivo, whereas a proline in the "*a*" position three amino acids downstream from *Des*A357P is not, remains completely elusive. At first structural consideration, a proline in an "*a*" or "*d*" position of the heptad pattern, mediating coiled coil formation, appears to be much more significant. However, our in vitro assembly and the transfection studies demonstrate that the reverse is true.

Conclusions and perspectives

Our studies indeed provide evidence for a segregation of single mutant desmins from the vimentin polymer in the living cell, whereas in vitro vimentin and the respective desmin mutants do form copolymers. Hence, one may speculate that coiled-coil dimer formation in vivo is strictly controlled. As a result, some of the mutant desmins are excluded from associating into coiled-coils with vimentin and others are not. The rules controlling this process may reflect cellular mechanisms that have been established during evolution to keep different assembly groups, i.e. the keratin, the lamin and the desmin/vimentin system, separated from each other. This is important, because in the case that monomers were able to associate into dimers in an uncontrolled, diffusion-based "collision" mode, also keratins might, for instance, interact with desmin/vimentin or the lamins, leading to the generation of unproductive or even harmful dimeric complexes. Clearly, more work is needed in order to understand the principles underlying the generation of cytoplasmic IF precursors in vivo and the way in which changes in single amino acids impact network formation and structure.

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