Pathogenic effects of a novel heterozygous R350P desmin mutation on the assembly of desmin intermediate filaments in vivo and in vitro

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Mutations of the human desmin gene on chromosome 2q35 cause a familial or sporadic form of skeletal myopathy frequently associated with cardiac abnormalities. Here, we report the pathogenic effects of a novel heterozygous R350P desmin missense mutation, which resides in the evolutionary highly conserved coil 2B domain of the α-helical coiled-coil desmin rod domain, on the assembly of desmin intermediate filaments (IF) in cultured cells and in vitro. By transfection experiments, we show that R350P desmin is incapable of de novo formation of a desmin IF network in vimentin-free BMGE1H, MCF7 and SW13 cells and that it disrupts the endogenous vimentin cytoskeleton in 3T3 fibroblast cells. Hence, transfected cells displayed abnormal cytoplasmic protein aggregates reminiscent of desmin-positive protein deposits seen in the immunohistochemical and ultrastructural analysis of skeletal muscle derived from the index patient of the affected family. To study the functional effects of the R350P desmin mutation at the protein level, we performed in vitro assembly studies with wild-type (WT) and mutant desmin protein. Our analysis revealed that the in vitro assembly process of R350P desmin is already disturbed at the unit length filament level and that further association reactions generate huge, tightly packed protein aggregates. On assessing the pathogenic effects of R350P desmin in various mixtures with WT desmin, we show that a ratio of 1:3 (R350P desmin/WT desmin) is sufficient to effectively block the normal polymerization process of desmin IFs. Our findings indicate that the heterozygous R350P desmin mutation exerts a dominant negative effect on the ordered lateral arrangement of desmin subunits. This disturbance of the lateral packing taking place in the first phase of assembly is ultimately leading to abnormal protein aggregation.

INTRODUCTION

Mutations in several cytoskeletal proteins of muscle have been shown to cause various types of severe muscle dystrophies (1–4). One of the more recently elucidated inherited mutations affects the muscle-specific protein desmin. The human desmin gene is located on chromosome 2q35, and several distinct mutations have been demonstrated to cause familial or sporadic forms of skeletal myopathy frequently associated with cardiac abnormalities (OMIM no. 125660). The disease usually manifests in the second or third decade of life with distal weakness of the lower extremities. The majority of cases exhibits an autosomal-dominant inheritance. However, rare autosomal-recessive cases as well as an increasing number of sporadic cases have been reported (5–12). Desmin is an intermediate filament (IF) protein that is characteristically expressed in myoblasts early during embryogenesis of vertebrates (13). The 53 kDa protein has a

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tripartite structure comprising a central $\alpha$-helical coiled-coil rod domain flanked by non-$\alpha$-helical head and tail domains. The central rod domain, formed by four $\alpha$-helical segments [1A, 1B, 2A, 2B] separated by three short polypeptide linkers [L1, L12, L2], has been shown to play a critical role in the dimerization and further assembly of desmin polypeptides (14). This assembly process is the structural basis for the formation of the three-dimensional desmin IF network in all mature muscle cells (15). Apart from a few cases of missense mutations residing in the head or tail domain, all pathogenic desmin mutations were found in the evolutionary, highly conserved $\alpha$-helical coiled-coil rod domain (15–17). Desmin is the major IF protein in skeletal and cardiac muscle cells and is a structural component of the extrasarcomeric cytoskeleton which forms a three-dimensional scaffold around myofibrillar Z-discs, thereby interlinking neighboring myofibrils and connecting the myofibrillar apparatus to nuclei, the subsarcolemmal cytoskeleton and cytoplasmic organelles such as mitochondria (18,19–24). Studies of desmin ($−/−$) mice showed progressive muscle weakness and dystrophic alterations in both cardiac and skeletal muscle. As severe structural changes were most prominent in highly used striated and cardiac muscle, it was concluded that the lack of desmin results in an increased susceptibility of muscle fibers to physical strain during muscle contraction (24,25). However, even in the absence of desmin, interconnecting filamentous structures were observed between neighboring myofibrils and from the Z-discs of most peripheral myofibrils to the overlying sarcolemma (19). In normal muscle, the extrasarcomeric cytoskeleton is composed of a network of various components comprising the IF proteins desmin, synemin, paramemin and syncoilin, the molecular chaperone $\alpha$B-crystallin and the multifunctional cytoskeletal linker plectin (18,26,27–30). The pivotal role of this extrasarcomeric cytoskeleton in human skeletal muscle is further highlighted by the observation that mutations in desmin, $\alpha$B-crystallin or plectin genes give rise to a progressive skeletal and/or cardiac myopathy (5,7,31–33). This indicates that these essential members of the extrasarcomeric cytoskeleton have complementary but not interchangeable roles in the structural and functional maintenance of striated muscle fibers in response to physical stress. All three disorders, which are morphologically characterized by myofibrillar abnormalities and abnormal cytoplasmic accumulation of desmin-immunoreactive material, share their structural myofibrillar and intermyofibrillar abnormalities with the large group of the so-called myofibrillar myopathies. These disorders comprise sporadic and familial neuromuscular conditions of considerable clinical and genetic heterogeneity. In a recent study, mutations of the myotilin gene on chromosome 5q31 have been shown to cause an autosomal dominant form of myofibrillar myopathy (34). However, the vast majority of myofibrillar myopathies are due to so far unidentified gene defects. Accordingly, familial myopathies mapping to chromosomes 12q, 10q23 and 2q24–31 are not yet genetically defined (16,35,36).

The primary event in the pathogenesis of myopathies caused by desmin, $\alpha$-crystallin or plectin mutations seems clearly related to structural as well as functional defects of the three-dimensional extrasarcomeric IF cytoskeleton. The corroborative data derived from transfection studies indicate that various desmin mutants are incapable of forming a de novo desmin IF network. Furthermore, the expression of mutant desmin protein induces a collapse of a preexisting desmin cytoskeleton, thereby leading to abnormal cytoplasmic protein aggregation reminiscent of desmin-positive protein deposits seen in the immunohistochemical and ultrastructural analysis of skeletal muscle biopsies from patients harboring pathogenic desmin mutations. This is also the case with experimentally introduced desmin mutations which have been demonstrated to disrupt the endogenous desmin system in cultured rat neonatal myocytes (37). However, the direct molecular effects of individual desmin mutants and their mechanical interference with WT desmin on the assembly of the desmin filament system are not known.

Here, we report on the genetic, clinical and myopathological findings in a German family harboring a novel heterozygous R350P missense mutation in exon 6 of the desmin gene. In addition, we analyzed the functional consequences of the novel R350P mutation by means of transfection studies and desmin IF assembly studies in vitro.

RESULTS
Clinical phenotype
The pedigree of the reported family is shown in Figure 1A. The index patient (II.3) is a 55-year-old Caucasian male who presented with a history of increasing dyspnea on exertion starting in his mid-forties. A chest X-ray examination performed in 1997 showed evidence of a bilateral paralysis of the diaphragm, necessitating a bi-level positive airway pressure ventilation treatment because of nocturnal hypoventilation. Muscular problems with difficulty in lifting his arms over the level of his shoulders were first noted at the age of 48. Neurological examination in 2004 showed mild bilateral weakness of proximal arm and shoulder girdle muscles and moderate weakness of both pelvic and proximal leg muscles. In addition, he exhibited mild weakness of distal leg muscles. Respiratory and cardiac work-up showed evidence of a restrictive ventilation disorder and hypertrophy of the left cardiac ventricle. A 24 h-holter-ECG at the age of 53 revealed basal sinus rhythm with multiple arrhythmias: first-degree AV block, couplets and ventricular bigemini/trigemini, a high number of isolated supraventricular and ventricular extrasystolic beats and some short lasting supraventricular tachycardias. Needle electromyography of various muscles showed myogenic motor unit potentials. Nerve conduction studies of phrenical nerves gave normal results. The family history indicated that several other members of the family of the index patient were affected by the same disorder. His mother (I.2), who died at the age of 44 because of acute cardiac failure, was reported to have suffered from a slowly progressive leg weakness. His 50-year-old sister (II.5) presented with a history of increasing dyspnea on exertion, starting at the age of 44. Her neurological examination demonstrated moderate weakness of distal and proximal leg muscles. His brother (II.1), who died at the age of 46 because of progressive cardio-respiratory insufficiency, was also reported to have suffered from proximal leg weakness beginning at age 31. Furthermore, three out of
four offsprings of patient (II.1) underwent clinical examination. Although patient III.1 showed mild weakness of foot extensor muscles, the neurological examination of patients III.2 and III.3 was normal.

**Mutation analysis**

DNA mutation analysis of the index patient (II.3) revealed a novel heterozygous R350P missense mutation (CGG → CCG) in codon 350 (exon 6) of the desmin gene (GenBank ID 181539, accession no. M63391) (Fig. 1B). This missense mutation, which resides in the evolutionary, highly conserved coil 2B domain of the α-helical coiled-coil desmin rod domain, causes an amino acid change from arginine to proline (R350P) (Fig. 1C). This novel R350P mutation was also found in the clinically affected patients II.5 and III.1 as well as in the as yet unaffected patients III.2 and III.3. This mutation was not detected in individuals II.2, II.4 and III.5 and in 100 normal control patients.

**Morphological analysis**

Histological analysis of a muscle biopsy taken from the index patient at the age of 52 showed a myopathic pattern with rounding of muscle fibers, increased fiber size variability with diameters ranging from 8 to 175 μm, fiber splitting, many necrotic and regenerating fibers, internalization of nuclei in 60% of the fibers and an increase of endomysial and perimysial connective tissue. In addition, haematoxylin–eosin and Gomori trichrome stains revealed multiple fibers with cytoplasmic and subsarcolemmal basophilic inclusions (Fig. 2A). Succinate dehydrogenase (SDH) and cytochrome c oxidase (COX) staining revealed multiple muscle fibers displaying focal areas of attenuated or even absent SDH- and COX-staining indicating a focal depletion of mitochondria (Fig. 2B and C). Furthermore, immunofluorescence analysis revealed intense labeling of pathological aggregates with antibodies against desmin, αB-crystallin and plectin (Fig. 2D–I). Ultrastructural analysis showed multiple fibers displaying large areas of intermyofibrillar and subsarcolemmal desmin-immunoreactive material (Fig. 3).

**Desmin protein expression**

To address the issue whether the increased desmin immunostaining is paralleled by an altered desmin protein expression, we performed western blotting of total protein extracts from normal and diseased muscle samples. For one- and two-dimensional gel electrophoresis, equal amounts (1D: 60 μg, 2D: 30 μg) of protein were loaded. GAPDH staining was used as an internal loading control. Desmin immunoreaction (Fig. 4A) revealed a single band corresponding in size to 53 kDa in both normal and diseased muscle. However, the signal intensity in the muscle biopsy from the index patient was far more intense, indicating an increase in the total amount of desmin protein in diseased muscle.

Two-dimensional gel electrophoresis and immunoblotting analysis of total protein extracts from normal human skeletal muscle detected desmin isoforms in a range of pH 5.3–5.5, which is close to the calculated pI of 5.2. In contrast, the
analysis of diseased muscle indicated a broader spectrum with a range of pH 5.1–5.6, which mainly increased by additional acidic desmin variants (Fig. 4B).

**In vitro assembly studies**

After renaturation of both recombinant WT and R350P desmin protein by dialysis from 8 M urea to a buffer containing 5 mM Tris–HCl (pH 8.5), assembly was initiated by increasing the ionic strength to 50 mM NaCl and 25 mM Tris–HCl (pH 7.5). Under these conditions WT desmin assembled into ultrastructurally normal filaments, whereas, R350P desmin exhibited a completely different assembly pattern (Fig. 5). Within 10 s, rather heterogeneous and irregular unit length filament (ULF)-type structures were formed. During further incubation, they failed to assemble into extended filaments but instead associated into tightly packed, roundish masses. These aggregates, like the initially observed ULFs, exhibited the ability to stick to each other. However, within these aggregates, the arrangement of the initially formed filamentous precursors was retained.

**Viscometric measurements**

Viscometry is a versatile tool to monitor IF assembly (38). We therefore investigated the effects of the R350P mutation on desmin’s ability to assemble into filaments, both of the mutant protein alone and of the various mixtures of WT and R350P desmin. In contrast to the normal increase of the relative viscosity of WT desmin, R350P desmin exhibited only a slight increase upon addition of salt with no further increase with time. When various mixtures of WT and R350P desmin were assessed, a drastic drop in viscosity was found 10–15 min after assembly was started. To observe this effect to full extent, addition of only 25% of R350P desmin was sufficient. Hence, R350P desmin is indeed a dominant negative mutant form of desmin. With 10% of R350P desmin, little effect on the viscosity profile was observed (Fig. 6A). This is reflected by ultrastructural analysis of corresponding samples by electron microscopy (Fig. 6B). Increasing amounts of R350P in mixtures with WT protein apparently lead to enhanced stickiness of formed filaments, ultimately leading to tightly interconnected filamentous structures forming large bulky aggregates.
Transfection studies

In contrast to WT desmin (Fig. 7A and C), R350P desmin failed to develop an extended filamentous network in vimentin-free MCF7 (Fig. 7B), SW13 (Fig. 7D) and bovine mammary gland epithelial cells (BMGE + H, data not shown) after transient transfection. For these experiments, we employed a vector system with a non-viral, i.e. a MHC H2 promoter, that yields moderate amounts of the transgene product (39). However, independent of the expression level, desmin-positive aggregates could readily be observed throughout the cytoplasm of transfected cells. This result is in full agreement with our in vitro assembly data and the fact that R350P desmin is not capable to form filamentous IF structures.

In vimentin-containing fibroblast cells, WT desmin forms an elaborate filamentous network that colocalises with the endogenous vimentin cytoskeleton (Fig. 8A–C). In contrast, R350P desmin causes the break-down of the endogenous vimentin cytoskeleton and the formation of cytoplasmic aggregates. As a result, the endogenous vimentin cytoskeleton is completely removed from the periphery of the cell and is found exclusively in the form of distinct small aggregates at the nuclear periphery (Fig. 8D–F). In a low percentage of cells (~5%), in which the expression of R350P desmin is apparently low as judged by immunofluorescence microscopy, the cytoskeleton remains intact. In cells grossly overexpressing R350P desmin, dense aggregates can be observed throughout the cytoplasm, finally leading to premature death of the affected cell. This finding corresponds well to the observed dominant negative effect of the R350P mutation with respect to desmin in vitro assembly.

DISCUSSION

Mutation analysis in the reported family with the clinical phenotype of a distal myopathy and cardiomyopathy revealed a novel heterozygous R350P missense mutation residing in the evolutionarily highly conserved coil 2B segment of the α-helical coiled-coil desmin rod domain.

Characteristically, the α-helical organization of the central rod domain of desmin, like in all other IF proteins, is interrupted at three sites, i.e. at the so called ‘linkers’ L1, L12 and L2, thereby leading to the formation of four separate α-helical segments, termed 1A, 1B, 2A and 2B (14). IF proteins form amphipathic helices that wrap around each other thereby forming a dimeric coiled-coil (40). The amino acid sequence of each such helix contains a characteristic seven-residue heptad repeat, (abcdefg)n, with positions ‘a’ and ‘d’ being mostly occupied by hydrophobic amino acids (41,42). Notably, most mutations of desmin leading to human desmin-related myopathy and cardiomyopathy identified so far are located within the 2B segment and lead to the replacement of different amino acids by proline (15). In the reported family, the pathogenic R350P desmin leads to a single amino
acid change from arginine to proline in the ‘b’ position of the heptad repeat. This missense mutation is likely to exert a dual pathogenic effect on the desmin protein conformation. Proline, because of fixed angles about its C bond, is likely to kink or break the backbone of an α-helix. In addition to these conformational constrains, proline lacks an amide nitrogen to make a stabilizing hydrogen bond. As a consequence, the substitution of arginine by proline is likely to decrease the local flexibility of a coiled-coil rod domain (43), and this may obstruct the proper de novo assembly of desmin IFs.

That these theoretical assumptions truly reflect the pathogenic effects of R350P desmin is strongly supported by our in vitro assembly studies and transfection of cultured cells. In contrast to results obtained with WT desmin, the analysis of R350P desmin revealed that the resulting filaments are quite irregular and depict an increased ‘stickiness’ which is likely to mirror serious disturbances in the ordered lateral IF assembly. Although the formation of ULFs is not severely disturbed, they obviously are not properly organized because the following longitudinal elongation is indeed compromised. Instead, ULFs break down into tightly packed aggregates which still tend to attach to each other and thus reveal a ‘pearl on the string’ appearance. This effect is as drastic as that observed for the ‘synthetic’ mutation R117C in the coil 1A ‘IF consensus’ motif, which in keratins is the most disruptive (37). These findings are in perfect agreement with our studies in IF-free MCF7, SW13 and BMGE + H cells, where R350P desmin is not capable to form a bona fide IF network.

However, in skeletal muscle of patients with heterozygous desmin mutations, the pathophysiological situation is far more complex. In each myonuclear domain of every single muscle fiber, the normal allele produces WT desmin whereas the mutated allele gives rise to a certain amount of mutated desmin protein. To address this quantitative aspect in more detail, we performed in vitro assembly studies using the R350P desmin in various mixtures with WT desmin. Here, we were able to show that a ratio of 1:3 (R350P desmin/WT desmin) is sufficient to effectively block the normal polymerization process of desmin IFs. These results suggest that the heterozygous R350P desmin mutation exerts a dominant negative effect on the ordered arrangement of desmin subunits into full-width IFs. Again, these findings are mirrored by our transient transfection studies in 3T3 cells, in which a dosage-dependent effect of R350P desmin leads to a breakdown of the endogenous vimentin cytoskeleton and the formation of cytoplasmic aggregates, which are the morphological equivalent to pathological desmin-positive protein aggregates seen in the immunohistochemical and ultrastructural analysis of skeletal muscle from patients suffering from primary desminopathies. If the same concept holds true for human skeletal muscle, our findings would indicate that a ratio of 1:3 or higher (R350P desmin/WT desmin) in a certain myonuclear domain would produce a disruption of the desmin IF cytoskeleton and pathological protein aggregation, whereas a smaller R350P desmin/WT desmin ratio in another myonuclear domain of the same muscle fiber would lead to the formation of a normal appearing desmin extrasarcomeric cytoskeleton. Though the ratio causing cytoskeletal pathology may be different with each individual desmin mutant, this hypothesis is further corroborated by two previous studies on the focal cytoskeletal derangements in a patient with a heterozygous L345P desmin missense mutation and a patient with heterozygous K239fsX242 desmin insertion mutation (30,44). In both cases, the immunohistochemical analysis of either longitudinal muscle sections or isolated muscle fibers showed pathological protein aggregates in some areas, whereas other regions within the same muscle fiber displayed a normal, transverse cross-striated desmin-staining pattern of the intermyofibrillar cytoskeleton. Focal disturbances in the assembly of desmin IF may inhibit the proper interaction of desmin with other cellular binding partners like the IF proteins synemin and syncoilin, the molecular
chaperone αB-crystallin and cytoskeletal linker proteins like plectin (30,44). As a consequence, all these essential components of the extrasarcomeric cytoskeleton are enriched in focal pathological protein aggregates, which in turn are likely to interfere with a wide variety of structural and signaling functions of these molecules.

In summary, we identified a novel heterozygous R350P mutation of desmin in a German family with distal myopathy and cardiomyopathy. Our in vitro and in vivo assembly studies demonstrate that the substitution of arginine with proline (R350P) in coil 2B exerts a dominant negative effect on the ordered lateral arrangement of desmin subunits. This disturbance of the lateral packing taking place in the first phase of assembly ultimately leads to abnormal protein aggregation.

**MATERIALS AND METHODS**

**Mutation analysis**

Genomic DNA of the patient was isolated from peripheral lymphocytes by standard procedures. The complete coding region and intron–exon boundaries of the desmin gene were screened for variations by direct sequencing of PCR products as described previously (44). Information on primers and PCR conditions can be obtained from the authors upon request.

**Muscle biopsy**

A diagnostic biopsy was taken from the left vastus lateralis muscle of the index patient. Normal control muscle was obtained from a patient who underwent muscle biopsy for diagnosis of neuromuscular symptoms but was ultimately deemed to be normal by means of combined clinical, electrophysiological and histological criteria.

**Morphological analysis**

Cryostat sections (9 μm) of snap-frozen, unfixed muscle were stained with hematoxylin and eosin, oil red O, periodic acid-Schiff, Gomori trichrome, nicotin-adenine dinucleotide tetrazolium-H reductase, myofibrillar adenosine triphosphatase at pH 4.2, 4.6 and 9.4, COX and SDH. These experiments were performed by standard procedures.

**Antibodies**

The following primary antibodies were used in this study: (1) mouse monoclonal anti-desmin antibody D33 (Dako, Germany), (2) rabbit polyclonal anti-αB-crystallin antiserum (Chemicon, USA), (3) P2 guinea pig serum, an antiserum directed against the C-terminal repeat domain 6 of human plectin (22) and (4) mouse monoclonal anti-GAPDH antibody (ACRIS, Germany). Isotype specific secondary antibodies conjugated with fluorescein isothiocyanate, Cy2 or Texas red were applied according to the recommendations of the manufacturers (Southern Biotechnology Associates Inc., Birmingham, AL, USA; Jackson Immunoresearch Laboratories Inc., Pennsylvania, PA, USA; Molecular Probes Inc., Eugene, OR, USA).

**Immmunoelectron microscopy**

Desmin immunogold electron microscopy of skeletal muscle was performed as described previously (22). The primary...
antibody against desmin was diluted 1:20. A secondary anti-rabbit antibody coupled to 10 nm colloidal gold particles was purchased from Amersham Pharmacia (Freiburg, Germany).

Gel electrophoresis and western blotting
For one-dimensional gel electrophoresis, preparation of total protein extracts, protein quantification, SDS–PAGE on 12% polyacrylamide gels, protein transfer and visualization of proteins on membranes were carried out as described previously (22). Two-dimensional gel electrophoresis of protein extracts from human skeletal muscle was performed as described in Schröder et al. [44].

Cloning and mutagenesis
The full-length clone of human WT desmin in the prokaryotic vector pDS5 (Quiagen, Germany) was described previously (45). The G1049C mutation was introduced using a site directed mutagenesis kit (Quickchange®, Stratagene, Germany) and specifically designed oligonucleotide primers. For transfection studies, both WT desmin and R350P desmin were cloned into the eukaryotic expression vector p163.7 using the unique EcoRI site (39). The accuracy of all clones was checked by sequencing.

Protein chemical methods
The Escherichia coli strain TG1 (Amersham) was transformed with both WT desmin and R350P desmin plasmids. Recombinant desmin was purified from inclusion bodies as described previously (38,46,47). For in vitro reconstitution of purified recombinant desmin protein, 0.5 mg of protein was dialyzed overnight into a buffer containing 5 mM Tris–HCl (pH 8.5), 1 mM EDTA, 0.1 mM EGTA and 1 mM DTT using a regenerated cellulose dialysis tubing (Spectra/Por®, MWCO 50,000, Roth, Germany). Viscosity measurements were performed at a protein concentration of 0.3 mg/ml in an Ostwald viscometer at 50 mM NaCl and 25 mM Tris-HCl (pH 7.5) at 37°C. Assembly studies and negative staining experiments were performed as described (45).

Cell culture and microscopic procedures
For transfection studies, we then employed vimentin-free BMGE + H, the human adrenocortical carcinoma cells (SW13), the human breast cancer cells (MCF7) and vimentin-positive murine fibroblast-derived cells (3T3-L1), which were cultured as described (48–51). Cells were grown on glass cover slips and transiently transfected (Fugene 6® according to the manufacturer’s protocol; Roche, Germany) with WT desmin or R350P desmin plasmids. Forty-eight hours after cDNA transfection, cells were processed for immunocytochemistry. Briefly, cells were fixed in methanol for 5 min, permeabilized in acetone for 3 min and blocked in 10% donkey serum in phosphate-buffered saline (PBS) for 30 min. The cover slips were incubated with the monoclonal anti-desmin antibody RD301 or the polyclonal rabbit anti-desmin serum together with the monoclonal anti-vimentin antibody Vim 3B4 (all reagents from Progen, Germany, unless specified otherwise) for 30 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 for 30 min together with 4,6-diamidino-2-phenylindole (DAPI).
for nuclear staining (Boehringer Mannheim, Germany). The cover slips were mounted on glass slides in Fluoromount G (Southern Biotechnology Associates). Cells were viewed by confocal laser fluorescence microscopy (DMIRE 2, Leica, Bensheim, Germany).

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