Expression of Dp71 in Müller Glial Cells: A Comparison with Utrophin- and Dystrophin-Associated Proteins

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Purpose. The abnormal retinal electrophysiology observed in patients with Duchenne muscular dystrophy (DMD) has been attributed to an altered expression of C-terminal products of the dystrophin gene. It has been shown that Dp260 is expressed by photoreceptor cells, whereas Dp71 is present in glial cells. The present study was intended to identify all known members of the dystrophin superfamily and their associated proteins expressed in Müller glial cells (MGC).

Methods. The expression of the proteins and of their messengers was studied in MGC cultures from 2-week-old rats, by polymerase chain reaction amplification, Western blot analysis, and immunocytochemistry. An immunocytochemical localization of the proteins was also performed on enzymatically dissociated Müller cells from adult rat retinas.

Results. MGCs expressed a spliced isoform of Dp71 called Dp71f, as well as utrophin, β-dystroglycan, δ- and γ-sarcoglycans, and α1-syntrophin. In morphologically preserved differentiated Müller cells, Dp71f was localized in clusters, utrophin was diffusely distributed in the cytoplasm, and dystrophin-associated proteins (DAPs) were membrane-bound. Most of these proteins were preferentially expressed in the vitread portion of the cells. Dp71f and utrophin expression was restricted to MGCs, whereas all DAPs were also present in other retinal cell types.

Conclusions. The exclusive localization of Dp71f and utrophin in MGCs suggests that these proteins, together with DAPs, play a specific role in these cells. Further knowledge of possible interactions of these proteins within a functional complex may provide new insights into the molecular basis of the electroretinogram phenotype in DMD. (*Invest Ophthalmol Vis Sci.* 2000;41: 294–304)

Duchenne muscular dystrophy (DMD) is a severe and lethal X-linked myodegenerative disease caused by the absence of the *DMD* gene product called dystrophin.[1] In the muscle, dystrophin is a submembranous cytoskeletal protein[2] that links actin[3] to a complex of dystrophin-associated proteins (DAPs) composed of transmembranous and submembranous proteins, such as dystroglycans, sarcoglycans, and syntrophins.[4] The mechanism(s) by which the absence of dystrophin causes the myodegenerative disorder is still unclear, although it has been suggested that dystrophin may be responsible for maintaining the cell shape in skeletal fibers and for the clustering of acetylcholine receptor molecules at the neuromuscular junction.[5] In addition to full-length dystrophin, shorter *DMD* gene products have been identified and denominated, depending on their apparent molecular mass: Dp260, Dp140, Dp116, and Dp71.[6–10] Alternative splicing of Dp71 has been described in the exons 71 and 78.[11] This late splicing generates a specific hydrophobic C-terminal sequence of 31 amino-acids called the founder sequence; thus, this Dp71 isoform was named Dp71f. In addition, different autosomal and X-linked genes express proteins highly homologous to *DMD* gene products such as utrophin, G-utrophin, and DRP2.[12–14]

The expression of different dystrophins is not restricted to skeletal muscle but also occurs in other tissues, including the central nervous system (CNS). Accordingly, DMD involves nonprogressive deficiencies that may be cognitive and/or visual,[15–19] consistent with an important role of the *DMD* gene products in normal CNS function.

Abnormal retinal electrophysiology is present in 80% of patients with DMD.[20] It is characterized by a delayed implicit time and by an amplitude reduction of the b-wave of the electroretinogram (ERG) recorded under scotopic conditions. The b-wave is the sum of the electrical activities of more than one cell type but is believed primarily to reflect the activation of depolarizing bipolar cells.[21] This bipolar depolarization is

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Supported by a grant from the Association Française contre les Myopathies (ARN DM), Grant CI1.CT93.0098MX from the Commission of the European Communities, the Fédération Nationale des Aveugles et Handicapés Visuels de France and La Fondation de l'Avenir, and Grant 01KS9504, project C5, from the Bundesministerium für Bildung und Forschung, Interdisciplinary Center for Clinical Research at the University of Leipzig. TC was the recipient of a PhD studentship from the Region Alsace and from the Association pour l'Aide au Developpement de la Recherche sur la Retine et sa Transplantation.

Submitted for publication June 22, 1999; revised August 18, 1999; accepted September 8, 1999.

Commercial relationships policy: N.

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initiated by a photoreceptor hyperpolarization that results in the a-wave of the ERG. It is also known that the Müller glial cells (MGCs) amplify the b-wave signal of the ERG generated by the bipolar cell. At the cellular level, photoreceptors are depolarized in the dark and continuously release glutamate, the major excitatory transmitter of the CNS. The metabotropic glutamate receptor mGluR6[22] causes depolarized bipolar cells to be hyperpolarized in the dark. When light shines on the retina, photoreceptors hyperpolarize, stopping the Ca^{2+}-dependent release of glutamate and, in consequence, the bipolar cell depolarizes. When the glutamate is washed away from the synaptic cleft, depolarizing bipolar cells depolarize in response to light. Müller cells are crucial in this process of reuptake of glutamate and K^+ released by depolarized neurons. Nevertheless, it remains controversial whether the elicited K^+ buffering currents through MGCs cause much of the b-wave. In toad retina, the spatial buffering fluxes of K^+ can be abolished by blocking Müller cell K^+ conductance with Ba^{2+}, and the K^+-evoked Müller cell depolarization and the b-wave are decreased in amplitude.[23,24] On the contrary, in rabbit retina, Müller cells generate the M-wave, and a portion of the b-wave, but a stronger direct contribution from depolarizing bipolar cells is suggested.[25,26] Thus, any search for the causes of the ERG phenotype in DMD must involve both neuronal and glial cells of the retina.

Patients with DMD with deletions in the central region of the *DMD* gene display the most severe ERG changes.[20,27] Further information has come from studies on animal models with selective gene defects. Whereas *mdx* mice do not express dystrophin and have a normal ERG, *mdx*^{3cv} mice are characterized by an impaired expression of all *DMD* gene products[28] and exhibit an ERG phenotype similar to that of patients with DMD.[29] Thus, as clearly shown by Pillers et al.,[30] and in contrast to the muscle degeneration, the ERG phenotype is not due to dysfunction of the full-length dystrophin but rather to a failure of the shorter *DMD* gene products. In addition to dystrophin, three short *DMD* gene products are present in the retina: Dp260, Dp140, and Dp71.[10,31] Kameya et al.[32] reported that exon 52 knockout mice, characterized by impaired expression of full-length dystrophin, Dp260, and Dp140, displayed a prolonged implicit time but no significant amplitude reduction of the ERG b-wave.[32] This suggests that a more dramatic decrease of the ERG b-wave may involve a dysfunction of the remaining member of the family, Dp71.

An elucidation of the functional role(s) of Dp260 and Dp71 in the retina requires a precise knowledge of their expression at the cellular level. It has already been shown that Dp260 is localized at the outer plexiform layer[32,33] where it is expressed by photoreceptor cells[34] and that the Dp71f isoform, spliced for exon 78, was localized at the inner limiting membrane (ILM),[34] probably in Müller cells. Furthermore, the DAP β-dystroglycan was shown to be present in both photoreceptor terminals and glial cell end feet.[35] Because retinal cells are densely packed and most neuronal cell somata are ensheathed by the Müller cell process, glial versus neuronal expression of a given protein may not be easily distinguished. Thus, to complete these data and to establish a "map" of all known members of the dystrophin superfamily and the DAPs that are expressed in Müller cells, we analyzed their expression by polymerase chain reaction (PCR), Western blot, and immunofluorescence techniques in both cultured and freshly dissociated cells. As a result of these studies, we clearly confirmed that Dp71f is the only short *DMD* gene product expressed in Müller cells, and we identified and localized utrophin, dystroglycan, δ- and γ-sarcoglycans, and α1-syntrophin in these cells. Our results support the hypothesis that the Dp71-DAP complex in Müller cells is a prerequisite for their normal functioning. In particular, inherited disturbances of this complex may be involved in the molecular basis of the ERG phenotype of patients with DMD.

METHODS

Müller Cell Isolation

All animals used in this study were cared for and handled in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Wistar rats were anesthetized by dry ice inhalation and then killed by decapitation, the eyes were enucleated, and the retinas were isolated in phosphate-buffered saline (PBS; Ca^{2+}-Mg^{2+}–free). The retinas were then incubated in PBS containing 0.3 mg/ml papain (Boehringer–Mannheim, Mannheim, Germany) at 37°C for 30 minutes, as described by Reichenbach and Birkenmeyer.[36] Thereafter, the tissue was washed several times with PBS, and fixed in 4% paraformaldehyde (4 minutes at room temperature). After washout of paraformaldehyde, the cells were isolated by trituration with a 1-ml pipette tip. The resultant Müller cell–rich suspension was spread on glass slides and air dried.

Müller Cell Culture

Primary retinal glial cell cultures were prepared from 2-week-old Wistar rats. Pure glial cell cultures were prepared as described by Hicks and Courtois.[37] After enucleation, eyeballs were stored at room temperature in the dark overnight. Intact eyeballs were incubated in Dulbecco's modified Eagle's medium (DMEM; Gibco, Cergy–Pontoise, France) containing 0.1% trypsin and 70 U/ml collagenase (Sigma–Aldrich, Saint-Quentin Fallavier, France), 0.5 ml per eye at 37°C for 50 minutes. The eyes were subsequently placed in a petri dish containing DMEM supplemented with 10% fetal calf serum (FCS; Gibco) The retinas were removed, chopped into small fragments, and seeded into 10-cm culture dishes (Falcon, Becton Dickinson Labware, Bedford, MA), approximately six to eight retinas per dish. The culture medium was left unchanged for 5 to 6 days, the aggregates were removed by vigorous rinsing, and fresh medium was added. The cells proliferated rapidly, achieving confluence within 3 to 4 days when a complete monolayer of epithelioid-like cells was established. A first passage was made by trypsinization after rinsing twice with calcium-free PBS, followed by a brief incubation (2–3 minutes at 37°C) in PBS containing 0.05% trypsin, 1 mM EDTA, and 1 mg/ml glucose. The suspension was pelleted at 800 g for 5 minutes, and the cells were resuspended and seeded in fresh DMEM + 10% FCS into 24-well plates containing 12-mm coverslips. They were then maintained at 37°C in a humidified atmosphere of 5% carbon dioxide-95% air. Under the described conditions, no neuronal, fibroblastic, or other glial cells (such as astrocytes or microglia) contaminated the MGC cultures, the identity of which was confirmed immunocytochemically, as previously published.[37,38]

cDNA Synthesis

Total RNA was extracted from rat retinas by the guanidium thiocyanate method established by Chomczynski and Sacchi.[39]

TABLE 1. Oligonucleotides Used for PCR Reaction

mRNA	Primers	Sequence (5′ to 3′)	Cycles	Product Size
Dp427	427.F	CTTTCAGGAAGATGACAGAATCAG	35	314 bp
	427.R	TTGTTCAGGGATGAATTCTTGTA		
Dp260	260.F	ATAAGCAAAGCTGAATGAGTGCT	35	289 bp
	260.R	TTCTTCATTTCTTCTAAACT		
Dp140	140.F	GCATTGCTGACTGTTCTGAGC	35	129 bp
	140.R	CCCAGTTGCATTCAGTGTTCTG		
Dp71	71.F	ATGAGGGAACAGCTCAAAGG	35	183 bp
	71.R	TGCAGCTGACAGGCTCAAGA		
Utrophin	DRP1.F	AGGTTTTACATCAGAACAATGT	35	335 bp
	DRP1.R	CTCAATTGGGGACATTTTGAC		
Dystroglycan	DGLYF	TTCACAACATGAAGTTGGTGCC	35	308 bp
	DGLYR	AATGGCAGTAACAGGTGTAG		
α-Sarcoglycan	AsarcoF1	CCATACCAAGCCGAGTTCC	35	419 bp
	AsarcoR1	TCATGCTCCAGGATCCCATC		
β-Sarcoglycan	betaSG-F	TCGTCCTCCTGTTTATCC	35	311 bp
	betaSG-R	GTCAAAAAACTGCATCCC		
δ-Sarcoglycan	DsarcoF	CTCACCACAGGAGCACCATGC	35	423 bp
	DsarcoR1	GCAATTTTCCAGAAACAGTTTTTAC		
γ-Sarcoglycan	GsarcoF1	AAAGTGATGTGGTTTTCTCCA	35	377 bp
	GsarcoR1	ACAAGGGGTGTCTCCACT		
α1-Syntrophin	alpha1F	TGGGATCCAGGACATCAAGCAGATTGGCT	35	250 bp
	alpha1R	GTGAATTCCCGTGCGCAGGGCAAAGGAGA		
β-Actin	BA.F	AAGAGAGGCATCCTCACCCT	25	215 bp
	BA.R	TACATGGCTGGGGTGTTGAA		

Oligonucleotides were chosen according to the sequences available in GenBank. Accession number is X03765 for β-actin. Dystrophin primers correspond to the brain isoform (accession number X14182); 260.F, 140.F, and 71.F were chosen in the promoter sequences. The other dystrophin primers were designed in the mouse dystrophin mRNA sequence (accession number M68859). Utrophin and DAP primers were chosen in their respective mRNA sequences (Y12229, X86073, D83651, AA611466, X95191, D83653, and U00678). F, forward; R, reverse.

One microgram of RNA was primed with random hexadeoxynucleotides and reverse transcribed using MuLV reverse transcriptase according to the recommendations of the manufacturer (Perkin–Elmer, Norwalk, CT). To check the integrity and the concentration of the messengers, 1-μg/ml aliquots of RNAs were loaded on a 1% agarose gel.

PCR Reactions

PCR amplifications were performed on 1 μg cDNA with 200 μM dNTP, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.25 μM of each primer, and 2 units *Taq* polymerase (Extra-Pol; Eurobio, Les Ulis, France). Each cycle consisted of a heat-denaturation step at 94°C for 30 seconds, annealing of primers for 30 seconds, and polymerization at 72°C for 30 seconds. PCR conditions were adjusted so that the reactions were performed in the linear range of amplification, and β-actin was also amplified as a control for the amount of starting RNAs. We have previously shown that the expression level of β-actin remains constant in the developing rat retina and can be used as internal control.[31] The sequences of the primers used for PCR, the number of cycles of amplification, and the predicted size of the amplified products are given in Table 1.

Antibodies

Monoclonal antibodies were used in the characterization of the DMD gene products. These antibodies were directed against different domains of the full-length dystrophin (Table 2). H5A3 and 5F3 antibodies have been produced and characterized in detail by Fabbrizio et al.[40] Dys2 antibody was from Novocastra (Newcastle-on-Tyne, UK). Cut polyclonal antibody was used to identify utrophin, as characterized in detail previously.[40] Rabbit polyclonal anti-actin (A2066) was from Sigma–Aldrich. Anti-

TABLE 2. Characteristics of the Different Antibodies Used in This Study

Antibody	Position of Antigen	Nature	Specificity
H5A3	Peptide 3357–3660	Monoclonal	Dystrophin, utrophin, Dp260, Dp140, Dp116, Dp71, G-utrophin, DRP2
Dys2	Peptide 3669–3685	Monoclonal	Dystrophin, Dp260, Dp140, Dp116, Dp71
5F3	Alternative 31-aa C-terminal end (founder sequence)	Monoclonal	Dp71f*
Cut	C-terminal 6 aa	Polyclonal	Utrophin
JAF	C-terminal 7 aa	Polyclonal	β-Dystroglycan
Nini	C-terminal 84–290	Polyclonal	δ-Sarcoglycan
P1	Peptide 42–52	Polyclonal	γ-Sarcoglycan
C4	Peptide 191–206	Polyclonal	α1-Syntrophin

* Dp71 founder sequence; aa, amino acid.

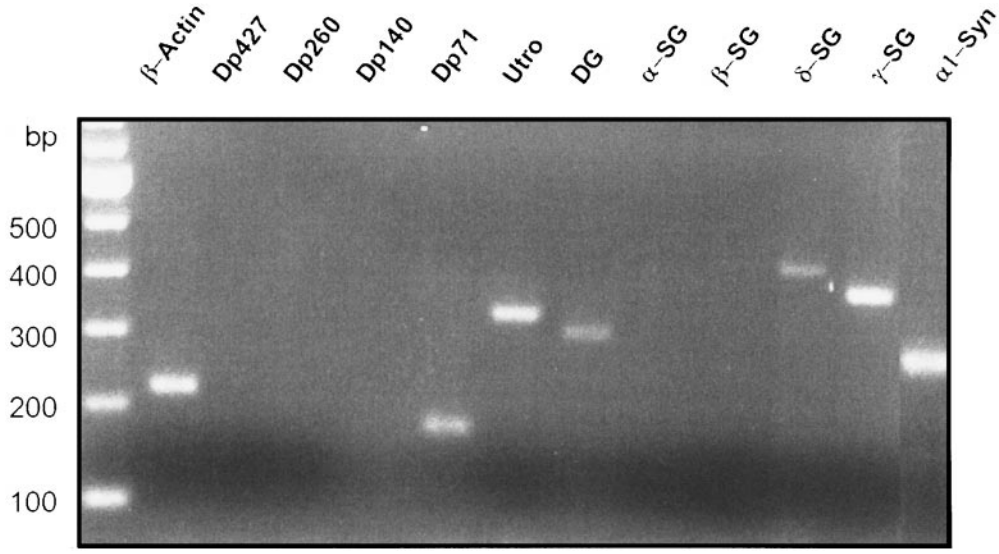

FIGURE 1. RT-PCR on cultured MGCs, performed with specific primers for each of the DMD gene products, utrophin (Utro) and DAP. The β-actin messenger was amplified as a control for the amount of starting RNA. Dp427, Dp260, and Dp140 mRNAs were absent in the cultures (positive controls were performed on total retina extracts). Neither α- nor β-sarcoglycan (SG) mRNA was detected (positive control was performed on muscle extracts). Dp71, utrophin, dystroglycan (DG), δ- and γ-sarcoglycans, and α1-syntrophin (Syn) mRNAs were expressed in the culture.

vimentin antibodies V9 and V4630 (Sigma–Aldrich) were used to identify glial cells. Antibodies against DAPs were all rabbit polyclonal antibodies and were produced and characterized by Rivier et al.[41] (see Table 2).

Electrophoresis and Western Blot

Cells were homogenized at 4°C in 10 volumes (wt/vol) of extraction buffer (0.32 M sucrose, 10 mM Tris-HCl, 1 mM EDTA [pH 7.4]), containing a mixture of protease inhibitors,[31] and were centrifuged at 1000*g* for 5 minutes. Supernatants were recovered and centrifuged at 17,000*g* for 30 minutes at 4°C. Pellets contained a crude membrane preparation and were recovered in the extraction buffer. Protein concentrations were determined using bovine serum albumin (BSA) as a standard.[42] Protein extracts were resolved by 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), as described previously[43]; 20 μg of proteins were loaded per well. Proteins were electrotransferred to nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) according to Towbin et al.[44] The efficiency of protein transfer was controlled by both ponceau red staining of the blot and Coomassie blue staining of the remaining gel. Western blot analyses were blocked with 1% BSA and 3% dry milk (Bio-Rad, Herts, UK) in PBS overnight at 4°C and then probed with primary antibodies diluted 1:100 in PBS and with horseradish peroxidase–labeled secondary antibody developed in goat (Interchim, Montluçon, France) diluted 1:10,000 in PBS and 0.1% Tween 20. Chemiluminescence detection was performed (Super Signal Kit; Pierce, Rockford, IL) and documented on film (Hyperfilm; Amersham, Amersham, UK).

Immunocytochemistry

Immunocytochemical labeling was performed by using the indirect fluorescence method. Cells were cultured on glass coverslips or freshly dissociated and collected on slices and then fixed 5 minutes in 4% paraformaldehyde at room temperature. After a 5-minute permeabilization with 0.1% Triton X-100 in PBS and blocking in PBS (0.01 M, pH 7.4) containing 0.1% BSA, cells were incubated with primary antibodies, diluted 1:50 for the polyclonal antibodies and 1:10 for the monoclonal antibodies. Secondary antibodies (Interchim) coupled to either Bodipy or Texas red were used, diluted 1:200 in PBS containing 0.1% Tween-20. Cell nuclei were stained using diamidino phenylindole (DAPi) 1:200 in PBS (Molecular Probes, Eugene, OR). Coverslips were mounted in 50% glycerol in PBS and examined with a photomicroscope (Optiphot 2; Nikon, Tokyo, Japan) using a ×40 objective.

RESULTS

Dp71, Utrophin, and DAP mRNAs Expressed in Cultured Müller Cells

DMD gene products (dystrophin, Dp260, Dp140, and Dp71) and utrophin are expressed in the whole rat retina.[31] We postulated that Dp71 and utrophin are the only members of the dystrophin superfamily expressed in Müller cells.[34] To test this hypothesis, we studied the mRNA expression of each of the *DMD* gene products in Müller cell cultures from 2-week-old Wistar rats, by RT-PCR. We also performed amplification of mRNAs of utrophin, dystroglycan, α-, β-, δ-, and γ-sarcoglycans, and α1-syntrophin (Fig. 1). Positive controls were performed with rat whole-retina extracts for *DMD* gene products and utrophin mRNAs and with muscle extracts for DAP mRNAs (data not shown). All amplification products had the expected size (see Table 1). In cultured Müller cells, we failed to detect any signal for dystrophin, Dp260, and Dp140 mRNAs. Thus, the only *DMD* gene mRNA amplified was Dp71 mRNA. Utrophin mRNA was also present in the Müller cell culture. As expected from previous observations,[35,45,46] we were also able to detect dystroglycan mRNA. Sarcoglycan mRNA amplification revealed that δ- and γ-sarcoglycan mRNAs were expressed in

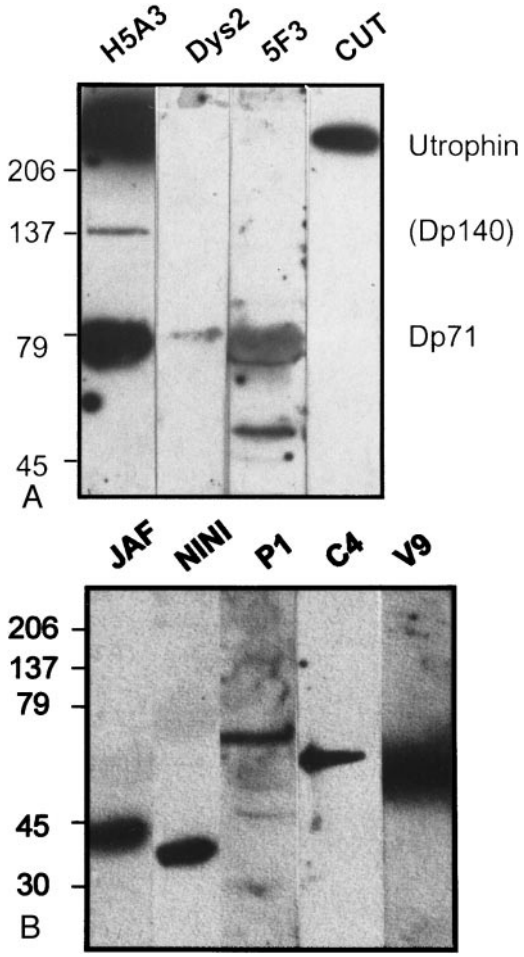

Figure 2. Immunoblots on cultured MGCs. (**A**) Characterization of the DMD gene products using antibodies: H5A3 (dystrophins and utrophins), Dys2 (dystrophins); 5F3 (Dp71f) and Cut (utrophin). Molecular weight markers are indicated on the left. Müller cells contain utrophin and Dp71f. The weak band at approximately 140 kDa may correspond to Dp140 expressed in a few contaminating astrocytes (<1% of cells). (**B**) Characterization of the expressed DAPs using the following antibodies: JAF (β-dystroglycan), NINI (δ-sarcoglycan), P1 (γ-sarcoglycan), and C4 (α1-syntrophin). Molecular weight markers are indicated at the *left*. The V9 antibody (vimentin) revealed a band at approximately 55 kDa. All proteins had the expected size except γ-sarcoglycan (65 kDa instead of 35kDa).

Müller cells, whereas no signal was obtained for α- or β-sarcoglycan mRNA. Finally, we identified α1-syntrophin mRNA expression.

Dp71f, Utrophin, and Associated Proteins Expressed in Cultured Müller Cells

At the protein level, we examined extracts of cultured Müller cells with several antibodies against different regions of dystrophins, utrophins, and DAPs by Western blot (see Table 2). H5A3 antibodies, known to recognize both dystrophins and utrophins, revealed two major bands at approximately 400 and 71 kDa, respectively (Fig. 2A). Because Müller cells do not express full-length dystrophin mRNA, the 400-kDa band probably corresponds to utrophin. In addition, a thin band appeared at approximately 137 kDa, which may correspond to Dp140, suggested to be expressed in astrocytes[34] which represent less than 1% of the cultured cells.[37] The Dys2 antibody, which does not cross-react with utrophin, revealed only a weak single band that apparently corresponds to Dp71, not spliced for exon 78, also suggested to be expressed in astrocytes.[34] The 5F3 antibody raised against the last 31 residues (founder sequence) of Dp71 spliced for the exon 78, revealed a doublet band at approximately 70 kDa and a minor band at approximately 60 kDa. The Cut antibody, which is utrophin specific, revealed only a single band at approximately 400 kDa. Thus, we confirmed that cultured Müller cells express Dp71, identified as Dp71f, and utrophin.

DAPs were revealed with specific antibodies against β-dystroglycan, δ- and γ-sarcoglycans, and α1-syntrophin (JAF, NINI, P1, and C4 antibodies, respectively; Fig. 2B). The β-dystroglycan was revealed as a single 43-kDa band. We also obtained a single band at the predicted molecular mass for the δ-sarcoglycan (35 kDa). However, the P1 antibody revealed γ-sarcoglycan as a band of approximately 65 kDa instead of the predicted size of 35 kDa. A similar result has been reported in the brain by Jung et al.,[47] suggesting that γ-sarcoglycan may be subject to major posttranscriptional modifications in the CNS. We identified the expression of α1-syntrophin in the Müller cells. A single band of 59-kDa was stained with the C4 antibody. Finally, using the V9 antibody, we detected a 55-kDa band that corresponds to vimentin, a well-established Müller cell marker.

Thus, we clearly determined the expression of β-dystroglycan, δ- and γ-sarcoglycan, and α1-syntrophin in the Müller cells. No signal was obtained by using specific antibodies against α-sarcoglycan or β-sarcoglycan (data not shown). These results corroborated the mRNA studies and established that cultured Müller cells express Dp71f, utrophin, and several distinct members of the DAP complex. We further examined the subcellular localization of these proteins by immunofluorescence techniques, using the same antibodies.

Immunocytochemical Localization of Dp71f, Utrophin, and DAPs in Cultured Müller Cells

In cultured Müller cells, the 5F3 antibody revealed very strong Dp71f immunoreactivity within the cytoplasm surrounding the nucleus, which itself was devoid of label (Fig. 3A). In close examination the Dp71f signal was seen to be concentrated in clusters (arrow in Fig. 3A). Utrophin staining by the Cut antibody was less strong and appeared to be diffusely distributed inside and surrounding the nucleus and in rays within the cytoplasm (Fig. 3B). All antibodies against the various DAPs also stained the cells in a rather diffuse manner, but the cellular margins were more intensely labeled (Figs. 3C through 3F). In particular, membranous folds were strongly fluorescent (arrows in Figs. 3C through 3F); thus, the cell contours were clearly outlined. These observations suggest that all DAPs expressed by Müller cells may have a similar subcellular localization. Thus, depending on the protein analyzed, three different patterns of subcellular localization were identified in cultured Müller cells: 1) a clustered intracellular localization of Dp71f, 2) a diffuse cytoplasmic localization of utrophin, and 3) a predominantly membranous localization in the case of β-dystroglycan, δ- and γ-sarcoglycans, and α1-syntrophin.

Double Labeling of Dp71f and Actin Filaments

To explore a possible alignment of Dp71f clusters along the actin cytoskeleton, double-labeling experiments with 5F3 and

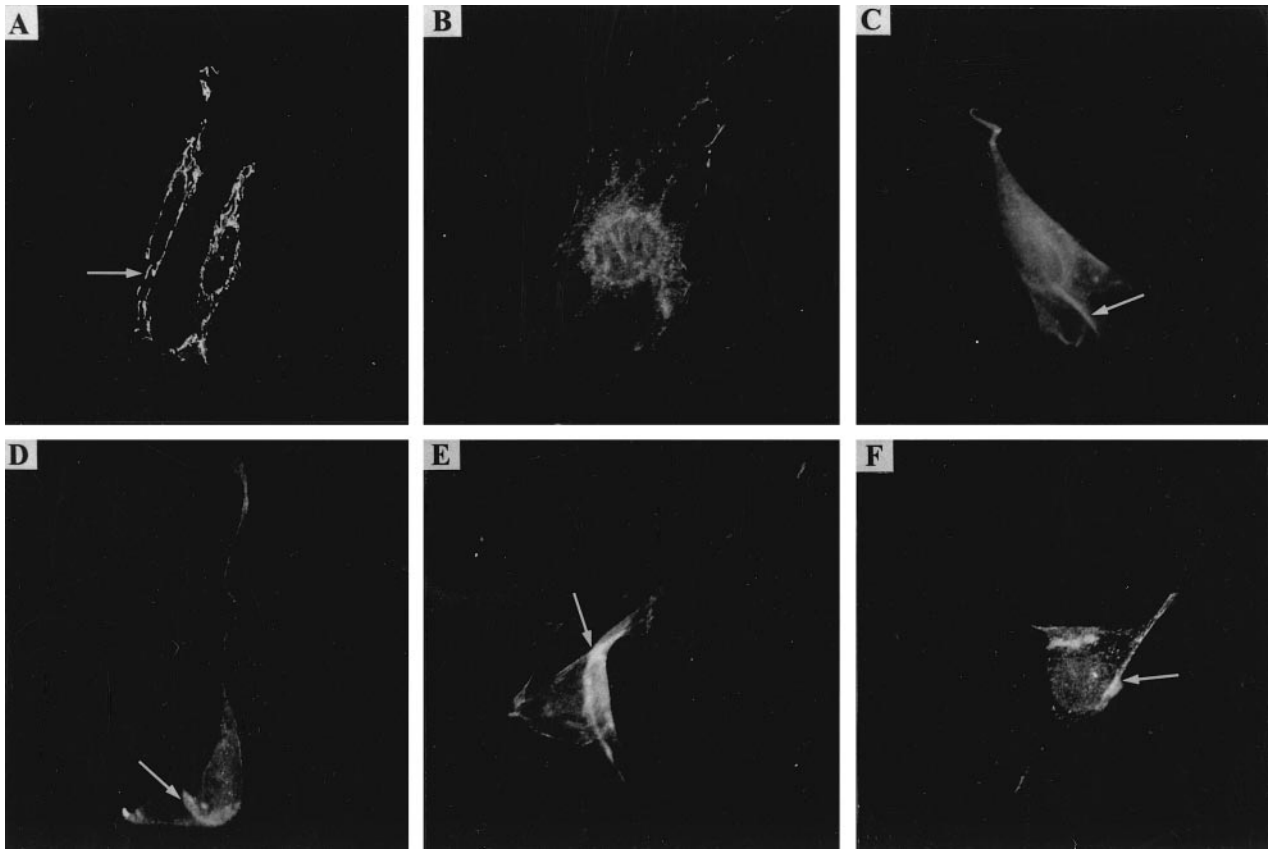

FIGURE 3. Immunocytochemical demonstration of Dp71f, utrophin, and DAPs in cultured MGCs. Cells from 14-day-old Wistar rats were immunolabeled with the following antibodies: (**A**) 5F3 (Dp71f), (**B**) Cut (utrophin), (**C**) JAF (β-Dystroglycan), (**D**) NINI (δ-sarcoglycan), (**E**) P1 (γ-sarcoglycan), and (**F**) C4 (α1-syntrophin). Dp71f was localized in clusters along a network (*arrow* in **A**). Utrophin staining was diffuse in the cytoplasm (**B**). All DAPs were membranous, as shown by enhanced fluorescence at the membrane folds (*arrows* in **C** through **F**). Magnification, ×400.

actin antibodies were performed. As shown in Figure 4, the antibody 5F3 immunolabeled clusters of Dp71f within the cytoplasm surrounding the nucleus and within the proximal parts of the soma (arrow in Fig. 4A). Whereas actin immunoreactivity was also concentrated around the cell nucleus (Fig. 4B, arrow), actin filaments were widely distributed throughout the cell body up to the distal margins (Fig. 4B, arrowheads). It is apparent from Figure 4C that the Dp71f clusters were distributed along with the actin filaments in cytoplasmic regions where the density of actin filaments was high (Fig. 4C, arrow). However, no Dp71f signal was observed in the distal cytoplasm where the density of actin filaments was lower. It seems as through Dp71f deposition is prevented in regions in which the cytoplasm (and thus, the cytoskeletal framework) is particularly flat, such as in the periphery and overlying the nucleus of the cultured cells. In addition, by treating MGCs for 1 hour with 100 nM cytochalasin D (Sigma–Aldrich), we observed a redistribution of Dp71f that became randomly expressed in the whole cytoplasm (Fig. 4D). This event paralleled the disorganization of the actin filaments (Fig. 4E) induced by the treatment.[48] The double-labeling showed that Dp71f and actin was not codistributed anymore (Fig. 4F). Thus, even if the Müller cell shape was modified after the treatment in comparison with control cells, our observation suggests that actin filaments are required for the organization of Dp71f in clusters of MGCs.

Subcellular Localization of Dp71f, Utrophin, and DAPs in Freshly Dissociated Müller Cells

Müller cells freshly dissociated from adult rabbit retinas have been shown to be morphologically intact and virtually free of adhering membranes or organelles from neighboring cells.[36,49] Because maintenance in cell culture may evoke significant alterations of Müller cells,[50] we wanted to confirm the localization of proteins in freshly dissociated Müller cells. The cells were identified by their unique bipolar morphology and large size, as well as by vimentin immunocytochemistry, which was distributed along the entire cell length (visualized by green fluorescence in Fig. 5; single-fluorescence data not shown). Dp71f, utrophin, and DAPs were visualized by red fluorescence; thus, double exposure of both images resulted in yellow staining of structures where vimentin and one of the other proteins were codistributed (Fig. 5). Control experiments were performed by omitting the first antibodies; there was not any visible fluorescence in these experiments (data not shown). Additionally, all cell nuclei, including those of dissociated retinal neurons, were visualized by DAPI fluorescence (Figs. 5B, 5D, 5F, 5H, 5J, 5L; each at the right side of the corresponding double-exposed microphotograph). All parts of Figure 5 are aligned so that the vitread end feet of the isolated Müller cells are directed toward the bottom of the figure.

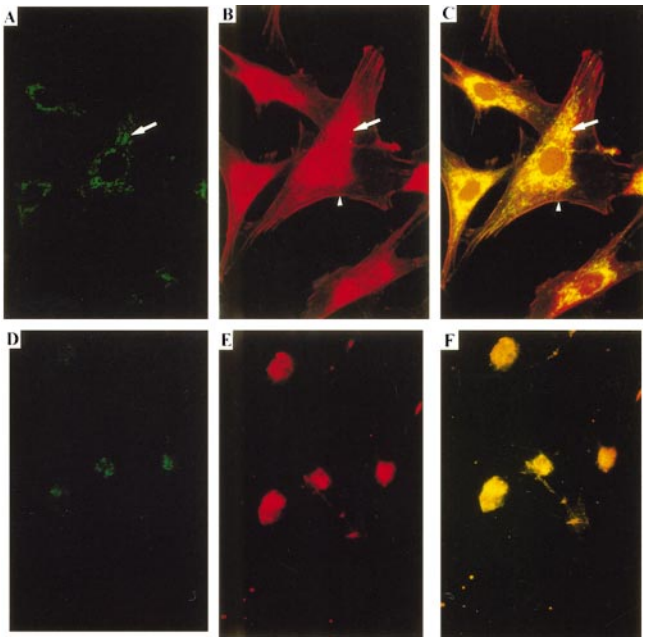

Figure 4. Immunocytochemical analysis on cultured MGCs with 5F3 (Dp71f-specific) and A2066 (actin-specific) antibodies. (**A**) Dp71f was localized in clusters that were distributed within the proximal somatic cytoplasm around the nucleus (*arrow*). (**B**) Actin was concentrated around the nucleus (*arrow*) and formed filaments extending into the distal cell processes (*arrowheads*). Double labeling (**C**) revealed that the Dp71f clusters were aligned along the actin filaments (*arrow*). Treatment with 100 nM cytochalasin D disorganized both the Dp71f cluster (**D**) and the actin cytoskeleton (**E**). Double labeling (**F**), using both 5F3 and anti-actin antibodies, suggested that disappearance of Dp71f clusters is due to actin cytoskeleton disorganization. Magnification, ×400.

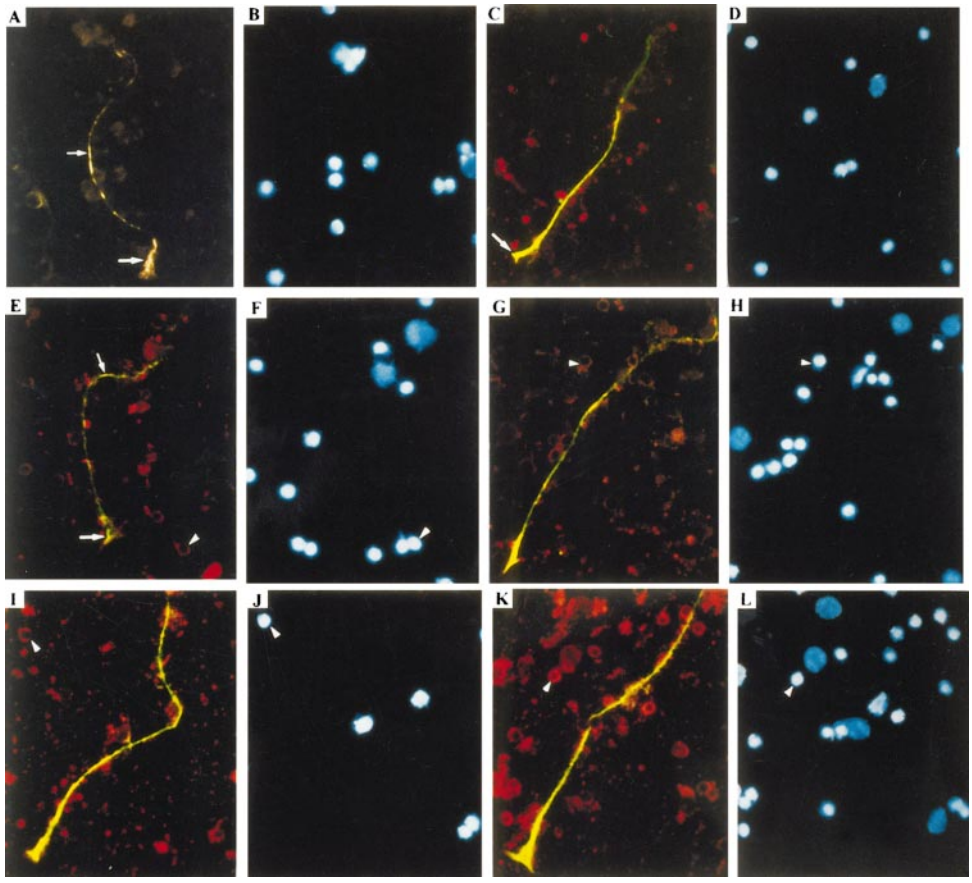

Figure 5. Immunocytochemical demonstration of Dp71f, utrophin, and DAPs (*red* fluorescence) in freshly dissociated Müller cells. Double labeling with vimentin antibodies (*green* fluorescence) resulted in a *yellow* color (**A**, **C**, **E**, **G**, **I**, and **K**). The nuclei of the dissociated cells were stained with DAPI; the same field of the preparations is shown at the right side of each of the immunolabels (**B**, **D**, **F**, **H**, **J**, and **L**). (**A**) Dp71f was localized in clusters in the vitread portion of the cell, in the end foot (*large arrow*) and along the cell body (*small arrows*). Dissociated neurons were not stained. (**C**) Utrophin was expressed diffusely in the vitread portion of the Müller cells, with the same intensity in the end foot (*large arrow*) and the vitread stem process (*small arrows*). In the sclerad part of the cell, only vimentin was detected (*green* fluorescence). No neurons but only cell debris were labeled. (**E**) β-Dystroglycan staining was intense in the end foot (*large arrow*) and in clusters along the entire cell body (*small arrows*). δ-Sarcoglycan (**G**), γ-sarcoglycan (**I**), and α1-syntrophin (**K**) were expressed throughout the Müller cells, with the same intensity in the vitread and the sclerad portions. These DAPs were also expressed by surrounding neurons (*arrowheads* in **E** through **I**). Magnification, ×400

The 5F3 antibody recognized a clustered Dp71f immunoreactivity within the vitread (i.e., downward-directed) portion of the Müller cells, whereas the sclerad (upward-directed) cell process was devoid of label (Fig. 5A). Particularly strong labeling was observed in the end foot (Fig. 5A, large arrow), and several clusters were found along the vitread stem process and the soma of the Müller cells (small arrows). This clustered pattern of expression was similar to what we observed in cultured Müller cells (cf. Figs. 3A, 4A). Dp71f immunoreactivity was virtually exclusively found in Müller cells; very weak background label, if any, was detectable in the surrounding cells (Figs. 5A, 5B).

Similar to that of Dp71f, utrophin immunoreactivity was confined in the vitread portion of the isolated Müller cells (Fig. 5C, arrows). In the sclerad part of the cell, only vimentin was present, causing a singular green fluorescence (Fig. 5B, arrowheads). As in the case of Dp71f, utrophin was specifically expressed by Müller cells; whereas some of the surrounding cell debris revealed red immunofluorescence, none of the adjacent cells (indicated by their DAPI-fluorescent nuclei) was stained (Fig. 5D). Although these features were similar to those of the Dp71f immunoreaction, there was also a clear difference: Utrophin was diffusely distributed in the vitread portion of the Müller cells without any traces of clustering (Fig. 5C).

Immunostaining of β-dystroglycan (Fig. 5E) was present throughout the length of the Müller cells, although the signal was particularly strong in the end feet (Fig. 5E, large arrow). Generally, the label was concentrated in clusters (Fig. 5E, small arrows) similar to that of Dp71f (cf. Fig. 5A), suggesting that in freshly dissociated cells, β-dystroglycan may be in close association with Dp71f. In contrast to Dp71f and utrophin, however, β-dystroglycan seemed also to be expressed by surrounding small round cells (Fig. 5E, 5F, arrowheads) probably representing photoreceptor cells which constitute more than 90% of the neurons of this preparation (Reichenbach and Birkenmeyer, 1984).[36]

There was a rather uniform subcellular distribution of the remaining three DAPs studied. Both δ- and γ-sarcoglycan (Figs. 5G, 5I, respectively) and α1-syntrophin (Fig. 5K) were labeled throughout the length of Müller cells. There was no indication of clustering (although the fluorescence intensity varied with the local density of side branches). Similar to β-dystroglycan, these DAPs were also strongly expressed by surrounding photoreceptor cells (Figs. 5G through 5L, arrowheads). Thus, we showed that in freshly dissociated, morphologically preserved Müller cells, Dp71f, utrophin, and individual DAPs display distinct patterns of subcellular distribution, with Dp71f and utrophin showing a marked longitudinal asymmetry of expression.

Discussion

Although the work of the past few years increased our knowledge on the large family of structurally diverse and differentially regulated proteins generated by the DMD gene, their functional roles within the CNS are still largely unknown. The ERG phenotype observed in patients with DMD and *mdx*^{3cv} mice provides a useful model of neural dysfunctions apparently caused by mutations of the short *DMD* gene products (i.e., Dp260 and/or Dp71). Still, one of the major problems the exact cellular and subcellular localization of these proteins. Thus, the purpose of the present study was to verify earlier results on Dp71f and utrophin[34] in pure Müller cell preparations (cultured and freshly dissociated), and to add data about the expression of DAPs in Müller cells.

Dp71f in Müller Cells

Dp71 is the major *DMD* gene product expressed in the retina.[31] It has been hypothesized that Dp71 plays an important role in the generation of the ERG b-wave.[32] Here, we show unequivocally that the Dp71f isoform is expressed by Müller cells. Thus, in rat retina, Dp260 and Dp71f are located in different cell types. The 5F3 antibody revealed three bands, indicating that all Dp71 isoforms in Müller cells were spliced for exon 78. The reasons for an exclusive Müller cell localization of Dp71 isoforms spliced for exon 78, as well as the functional significance of the Dp71 splicing variants, are still unknown. It may be speculated that the addition of a hydrophobic amino acid-sequence to the Dp71 protein results in distinct functional properties.

Two peculiarities were observed in the subcellular distribution of Dp71f in Müller cells: a distinct clustering of the protein and a longitudinal asymmetry of expression. In a recent study Howard et al.[51] observed a similar clustered pattern of Dp71f localization in myogenic cells. The above-mentioned localization of Dp71f is consistent with earlier immunohistochemical observations on rodent retinal sections locating Dp71f at the ILM[33] and in the Müller cell end feet,[34] respectively. Moreover, the Müller cell was the only cell type stained with 5F3 in freshly dissociated retinal cell preparations, indicating that in the retinal sections, no other (e.g., neuronal) cells contributed to the immunoreactivity observed at the ILM. Our observation provides a new hypothesis of the molecular basis of the ERG phenotype of DMD animal models. If Dp71f is crucial for the b-wave genesis,[32] if Müller cells are involved in the generation of the b-wave,[21] and if Dp71f is expressed exclusively by Müller cells (present study), it is highly probable that disturbances of the (yet unknown) functions of Dp71f in Müller cells are involved in the ERG phenotype of DMD animal models. As discussed later, it is interesting to note that Dp71f accumulates in the Müller cell end feet where the cells are attached to the vitreous and where large K^+ ion fluxes into the vitreous occur.[52,53]

Utrophin in Müller Cells

Immunofluorescence analysis of utrophin expression in rat retinal sections have shown that this protein is expressed in the vitread retinal layers, probably in the end feet of the Müller cells.[34] This observation has been confirmed and extended by the present study. Utrophin was found in cultured and freshly dissociated Müller cells but not in other retinal cell types. This is in apparent contradiction to the work of Ueda et al. [54] who localized utrophin in ganglion cells by immunostaining of rat retinal sections. Although we cannot rule out a binding of our anti-utrophin antibody to an unrelated epitope in Müller cells, the detection of only a single band in Western blot of cultured Müller cells, together with the specific mRNA expression, provides strong evidence that utrophin is indeed expressed by Müller cells, also in situ. As mentioned earlier, Müller cell end feet may be mistaken in retinal sections for ganglion cells, because both structures are located in the same retinal layer, display a similar size, and are closely intermingled with each

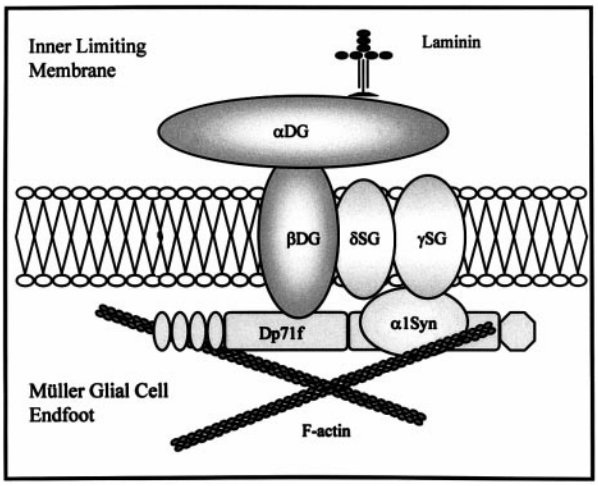

FIGURE 6. Possible structural model of the Dp71f/DAPs complex in the MGC. DG, dystroglycan; SG, sarcoglycan; Syn, syntrophin.

other. It is for this reason that the present study was performed on cultured and dissociated Müller cell preparations.

In both types of preparations, utrophin was diffusely expressed within the cytoplasm, rather than in clusters as observed for Dp71f. Similar observations have been made in brain astrocytes[55] and Schwann cells.[56]

DAPS in MGCs, Evidence for a Complex

We have established in the present study that Müller cells expressed β-dystroglycan, δ- and γ-sarcoglycans, and α1-syntrophin. To our knowledge, this is the first experimental evidence that a defined CNS cell type expresses several components of the DAP complex. This observation would be further strengthened if CNS phenotypes (such as mental retardation or visual perturbations) were identified in limb–girdle muscular dystrophies, which are characterized by the absence of δ- or γ-sarcoglycan.

Taken together, our results strongly support the assumption that Dp71f, utrophin, and several DAPs do not merely coexist in Müller cells but form structural and/or functional complexes. In skeletal muscle cells, β-dystroglycan is associated with utrophin at the neuromuscular junction. Because from the present and previous studies β-dystroglycan is known to be expressed in Müller cells,[35,45,46,57] it is plausible to postulate that utrophin is associated with β-dystroglycan in the vitread part of Müller cells. Also, there is ample experimental evidence for an interaction of Dp71 with β-dystroglycan.[55,58–61] In total extracts from cultured Müller cells, we coimmunoprecipitated Dp71f in the course of immunoprecipitation with a β-dystroglycan antibody (Claudepierre et al., unpublished results, 1999). This result further supports the possible existence of a Dp71–β-dystroglycan complex in Müller cells. Thus, even if further experiments are required, we propose in Figure 6 a possible model of the Dp71f-DAP complex in MGCs, based on our results and in accordance with the literature we have described. As in muscle cells[62] this complex could be involved in maintaining the Müller cell shape, by mediating the contact with the extracellular matrix. Laminin[63] and agrin[64] are components of the ILM and are known to link α-dystroglycan.[56,65,66] Thus, a possible mechanical function may cause the Müller cell end feet (i.e., the retina as such) to adhere to the basal lamina of the ILM and to the (fibrillar cortex of the) vitreous body, as has been suggested by Schmitz and Drenckhahn.[57] Such a function would be deleted in cases of retinal detachment or macular hole formation resulting from shrinkage processes in the vitreous body.

It is also tempting to speculate that this complex may contribute to the clustering of receptor or ion channel molecules in the Müller cell membrane. In particular, the proposed function of Müller cells in b-wave generation[21] is dependent on their ability to carry large K^+ ion currents through specific K^+ channels.[53] It is noteworthy in this context that the anchoring proteins PSD-95 and PSD-93 have been shown to regulate the distribution and function of inwardly rectifying K^+ channels (Kir4.1) in Müller cells,[67] the inwardly rectifying channels are crucially involved in retinal K^+ siphoning,[53] an interaction between proteins of the PSD-95 protein family and the Dp71/DAP complex is very likely because of, among other reasons, similar subcellular localization and clustering (Claudepierre et al. unpublished results; Horio et al.[67] their Fig. 4).

Acknowledgment

The Authors wish to thank Gérald Hugon for skillful technical assistance in the antibodies preparation.

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