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*Original Article*

# Expression patterns of dystrophin products, especially of apodystrophin-1/Dp71, in the neural retina of Amphibian urodele *Pleurodeles waltl*

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**ABSTRACT The expression patterns of the DMD (Duchenne Muscular Dystrophy) gene products, especially of Dp71 (apodystrophin-1) were investigated by immunofluorescence and immunoblotting in the retina of the Amphibian urodele *Pleurodeles waltl.* H-5A3 monoclonal antibody (mAb), directed against the C-terminal region of dystrophin/utrophin, and 5F3 mAb, directed against the last 31 amino acids of dystrophin and specific of Dp71, were used. Western blot analyses with H-5A3 mAb revealed distinct dystrophin-family isoforms in adult newt retinal extracts: a doublet 400-420 kDa, Dp260 isoform, a protein at about 120 kDa, and a diffuse zone at 70-80 kDa, which might correspond to Dp71. Reactivity with H-5A3 mAb appeared nearly restricted to the outer plexiform synaptic layer. On the other hand, Dp71-specific 5F3 mAb recognized trhee polypeptide bands at 70-80, 60-65 and 50-55 kDa in adult newt retina corresponding most probably to alternative spliced isoforms of Dp71. In immunohistochemistry by conventional epifluorescence microscopy, 5F3 labeling was mainly observed in the plexiform layers, the outer nuclear layer, and the photoreceptor inner segments, especially at the myoid regions. Analysis by confocal scanning laser microscopy (CSLM) revealed that 5F3 labeling was, in addition, present in the pigmented epithelium and the inner nuclear layer. Furthermore, CSLM showed that 5F3 staining at the myoids was concentrated at discrete domains underneath the plasma membrane. Our findings raised the question concerning the functional significance of Dp71 isoforms, especially at the myoid where Dp71 was detected for the first time, although it occurred here highly expressed. Putative role(s) played in this retinal compartment and other ones by Dp71 and/or other dystrophin isoforms were discussed.**

KEY WORDS: *dystrophin isoforms, Dp71/apodystrophin-1, retina, Amphibian urodeles, confocal scanning laser microscopy*

## Introduction

Mutations in the *DMD* gene, located on the X chromosome, encoding for dystrophin, a membrane-associated cytoskeletal protein of muscle and several non-muscle cells, give rise to the hereditary muscle wasting disorders known as Duchenne and Becker muscular dystrophy (Hoffman *et al.*, 1987; Ahn and Kunkel, 1993, for review). Furthermore, abnormal or deficient dystrophin expression has been involved in mental retardation (e.g., Lenk *et al.*, 1993) and in a form of nightblindness (Pillers *et al.*, 1993). Dystrophin consists of four structural domains: (i) a N-terminal actin-binding region with homology to α-actinin, (ii) a rod structure with 24 spectrin-like repeats, (iii) a cysteine-rich domain with calcium-binding domain, and (iv) a C-terminal extremity which interacts with the plasma membrane via a glycoprotein complex (see e.g., Matsumura and Campbell, 1994 and Suzuki *et al.*, 1994, for review).

Studies in the last decade have revealed the existence of different dystrophin isoforms produced either by alternative splicing (Feener *et al.*, 1989) or by alternative promoter utilization (at

*Abbreviations used in this paper:* DMD, Duchenne Muscular Dystrophy; IPL, inner plexiform layer; OPL, outer plexiform layer; PhR, photoreceptor; mAb, monoclonal antibody.

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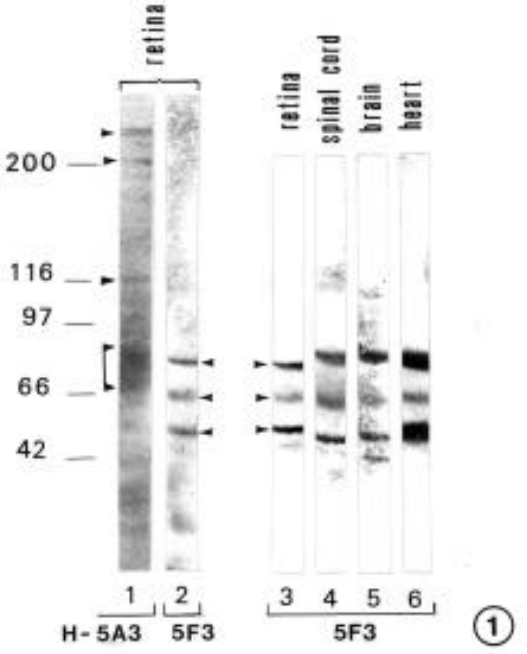


**Fig. 1. Immunoblot analysis of tissue extracts from adult newt.** *Protein samples (20-40 µg) of retina (lanes 1,2,3), spinal cord (lane 4), brain (lane 5) and heart (lane 6) were resolved in a 5-13% polyacrylamide/ SDS gradient gel, transferred onto a nitrocellulose sheet and immunoblotted with H-5A3 (lane 1) or 5F3 mAb (lanes 2-6).* **Lane 1:** *In retina, H-5A3 recognizes a doublet at about 400-420 kDa, a band most probably corresponding to Dp260 isoform, a band whose size seems to identify Dp116/apodystrophin-2, and a diffuse material with a range of 70-80 kDa, which may correspond to Dp71.* **Lane 2:** *In retina, 5F3 reactivity appears as three bands, at 75-80, 60-65 and 50-55 kDa.* **Lane 3:** *Another sample from adult newt retina. Like in lane 2, 5F3 recognizes three polypeptides at the same molecular weights. Note however that the first and the third components clearly appear here as more important bands than the intermediate one.* **Lanes 4-5:** *In spinal cord (lane 4) and brain (lane 5), 5F3 gives very similar staining patterns, i.e., a 80-85 kDa band, a 60-65 kDa band and a 45-50 kDa band. An additional band around 40 kDa can be however observed in brain.* **Lane 6:** *In the cardiac muscle, 5F3 reactivity appears as 75-80, 60-65 and 50-55 kDa bands. Note also here the high staining of the first and third components. Molecular mass standards: myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (42 kDa).*

least seven promoters), thus conferring cell-type specificity (Barnea *et al.,* 1990). The three full length alternative promoters are employed in the transcription of gene encoding for the 427 kDa dystrophin isoforms (see e.g., Sadoulet-Puccio and Kunkel, 1996, for review). The four other promoters initiate transcription from novel first exons and give rise to smaller dystrophin products, named according to their predicted molecular weight: Dp71 (or apodystrophin-1) for dystrophin protein 71 (Lederfein e*t al.*, 1992), Dp116 (or apodystrophin-2, a peripheral nerve-specific transcript: Byers *et al.*, 1993; Schofield *et al.*, 1994; Rivier *et al.*, 1996), Dp140 (Lidov *et al.*, 1995; Durbeej *et al.*, 1997) and Dp260 (D'Souza *et al.*, 1995). Dp71 consists of a subfamily of isoforms generated by alternative splicing (Lederfein *et al.*, 1992; Austin *et al.*, 1995; Ceccarini *et al.*, 1997). Dp71 has been first identified in liver, hepatoma cells and cardiac muscle (Bar *et al.*, 1990), and shown then to be the major DMD product in a wide variety of tissues, especially in the neural tissue (Blake *et al.*, 1992; Hugnot *et al.*, 1992; Lederfein *et al.*, 1992; Rapaport *et al.*, 1992a,b; Fabbrizio *et al.*, 1994; Schofield *et al.*, 1994; Ceccarini *et al.*, 1997). In addition to muscle, dystrophin has also been found in the nervous tissue, including brain (Lidov *et al.*, 1990, 1993; Jung *et al.*, 1991; Huard *et al.*, 1992) and cochlear hair cells (Dodson *et al.*, 1995). On the other hand, screening with antibodies indicated the existence of dystrophin or dystrophin-like proteins in the outer plexiform synaptic layer (OPL) of the neural retina of mammals such as mouse, rat (Miike *et al.*, 1989; Miyatake *et al.*, 1991; Zhao *et al.*, 1991; Yoshioka *et al.*, 1992; Schmitz *et al.*, 1993) and human (Schmitz *et al.*, 1993; Pillers *et al.*, 1993), but also bovine (Schmitz *et al.*, 1993) and amphibian urodeles (Arsanto *et al.,* 1991). It has been reported that dystrophin is absent from the OPL of mdx mouse retina (Miike *et al.*, 1989; Miyatake *et al.*, 1991; Pillers *et al.*, 1993). On the other hand, the localization of dystrophin to the OPL of the retina is consistent with clinical studies which showed the existence of an ocular phenotype associated with DMD and BMD (Becker Muscular Dystrophy) patients characterized by an abnormal electroretinogram (ERG) (Pillers *et al.*, 1993). Analysis of the expression of dystrophin proteins and transcripts enabled workers to demonstrate that different splice variants of dystrophin are present in human (Pillers *et al.*, 1993), mouse (Tamura *et al.*, 1993; D'Souza *et al.,* 1995) and rat (Rodius *et al.*, 1997) retina. D'Souza *et al.* (1995) identified and localized to the OPL of normal mouse retina the two dystrophin isoforms 427 kDa and Dp71, and a new isoform they referred to as Dp260. Furthermore, D'Souza *et al*. (1995) showed that Dp260 was required as well as full length dystrophin for normal signal transmission across the OPL. More recently, using Western blots and semi-quantitative RT-PCR, Rodius *et al.* (1997) demonstrated that the variety of proteins of the dystrophin superfamily expressed in retina was greater than previously described. Indeed, they identified in the adult rat retina not only four dystrophin isoforms (Dp427, Dp260, Dp140, Dp71), but also utrophin/DRP1 (an autosomal homolog of dystrophin: Love *et al.*, 1989) and G-utrophin and/or DRP2. Furthermore, Rodius *et al.* (1997) reported that Dp260 protein and mRNA expression increased progressively during retinal development, suggesting that Dp260 could be required for synaptic maturation.

Using H-5A3 mAb directed to the C-terminal domain of dystrophin/ utrophin, some of us (Arsanto *et al.*, 1991) were among the first workers who detected the presence of dystrophin family protein in the OPL of retina by immunolocalizing the protein in larval retina of *Pleurodeles waltl.* On the other hand, we subsequently showed (Mitashov *et al.*, 1995) that the adult newt retina provided a good model for an immunohistochemical survey, given its clear multilayered organization pattern and presence of conspicuous photoreceptors. This is the reason why we were interested in analyzing the expression patterns of dystrophin isoforms in the developing and adult newt retina using Dp71-specific 5F3 mAb in addition to H-5A3 mAb. Western blot analysis showed the presence in adult newt retina of dystrophin family isoforms, i.e., 420-400 kDa, Dp260, Dp116 (or Dp140) and Dp71, which could be mainly distributed in the OPL, as suggested immunohistochemistry. Furthermore, using 5F3 mAb, we were led to the idea that Dp71 isoforms might be present in urodeles in different retinal compartments, especially the photoreceptor myoids and the pigmented epithelium.

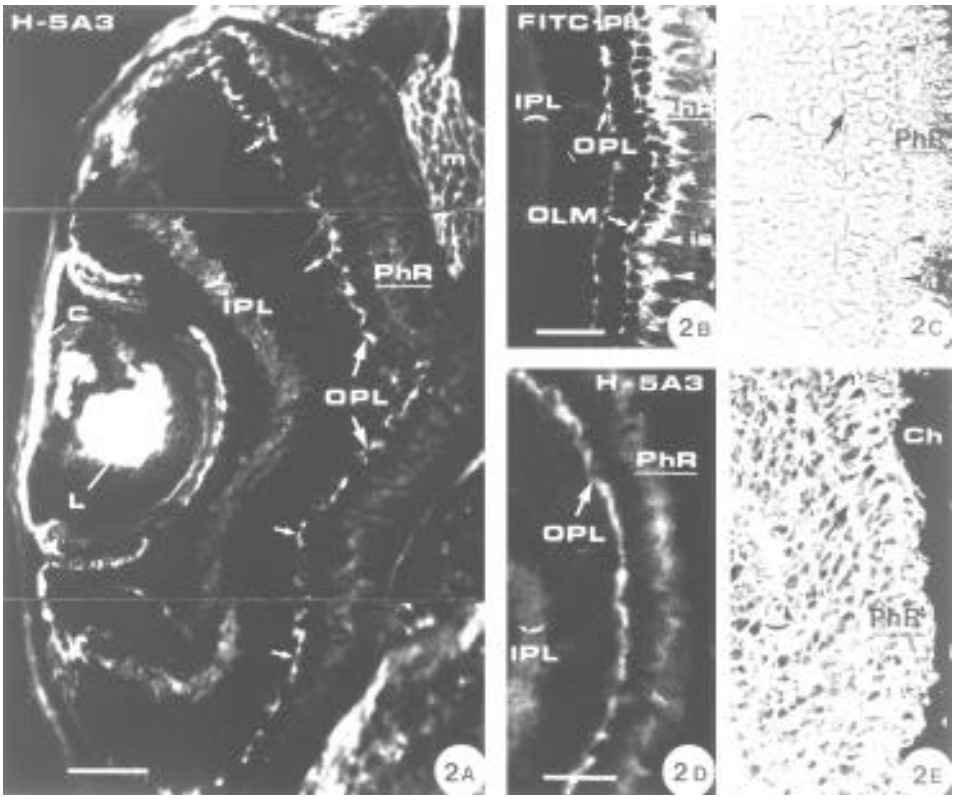


**Fig. 2. H-5A3 and phalloidin staining in retina of larval and adult newts. (A)** *Sagittal section through the eye of a larva. H-5A3 reactivity can be clearly seen in the outer plexiform layer (OPL, arrows), which shows a discontinuous staining pattern. Other retinal layers are unstained, except the inner plexiform layer (IPL) which appears lightly stained. Note also the intense labeling around the myofibers (m). Staining over the photoreceptors (PhR), lens (L) and cornea (C) is due to autofluorescence. Bar, 50 μm.* **(B)** *FITC-phalloidin labeling in radial section through retina. F-actin is detected in the OPL and the IPL, and at the basis of PhR, i.e., at the outer limiting membrane (OLM) and in structures (arrowheads) corresponding most probably to the microvilli of Müller cells interposed between the inner segments (is).* **(C)** *Phase-contrast image of (B). Bar, 50 μm.* **(D)** *Part of section through adult newt retina. Intense reactivity with H-5A3 is observed in the OPL. Note also a slight staining of the IPL. Labeling at the level of PhR corresponds to autofluorescence of the outer segments.* **(E)** *Phase-contrast image of (D). Ch, choroid. Bar, 50 μm.*

## Results

### *Western blot analysis*

In the adult newt retinal extracts (Fig. 1, lane 1), analyzed on SDS 5-13% gradient PAGE and immunoblotted onto nitrocellulose membrane, H-5A3 mAb detected a doublet around 400-420 kDa, the retinal Dp260 isoform, and a diffuse zone at about 70-80 kDa, which might correspond to Dp71. On the other hand, in retinal samples from adult newts (Fig. 1, lanes 2-3), 5F3 mAb detected a band of apparent molecular weight 75-77 kDa and two additional reactive bands, i.e., a band at 60-65 kDa and a band at 50-55 kDa. It is noteworthy that 5F3 gave a similar immunoblotting pattern in cardiac muscle extract from adult animal (Fig. 1, lane 6), since a 75-80 kDa band, a 60-65 kDa band and a 50-55 kDa band were observed. Interestingly, three bands of about the same molecular weights, i.e., 80-85, 60-65 and 45-50 kDa, were also observed in brain (Fig. 1, lane 5) and spinal cord (Fig. 1, lane 4) samples from adult newt. Furthermore, an additional reactive band at 40-45 kDa was detected in brain extracts from adult newts (Fig. 1, lane 5).

### *Immunofluorescence and phalloidin labeling*

The cellular and subcellular location of dystrophin products was examined by immunofluorescence microscopy in cryosections through retinae of larval and adult newts using H-5A3 mAb and Dp71-specific 5F3 mAb. In tissue sections through the neural retina of 3-week-old larval (Fig. 2A) or adult newts (Fig. 2D,E), showing a multilayered organization pattern, intense H-5A3 reactivity seemed to be restricted to the outer plexiform layer, although a slight staining could be seen in the inner plexiform layer. Labeling of the other eye structures (i.e., cornea, nucleus of the lens and PhR outer segments: Fig. 2A,D) has been shown to be due to background autofluorescence. Furthermore, it is noteworthy that the outer plexiform layer showed a discontinuous staining pattern (Fig. 2A,D) similar to that observed after FITC-phalloidin treatment for F-actin (Fig. 2B). However, no significant H-5A3 labeling was seen over the photoreceptor layer whereas phalloidin staining was observed in its basal part (Fig. 2B), i.e., at the outer limiting membrane, and in structures corresponding most probably to the F-actin cores present in microvilli of Müller cells close to the PhR.

In sections through the eye of a 3-week-old larva (Fig. 3A,B), although labeling with Dp71-specific 5F3 mAb was weak, it was seen in most of the retinal layers, especially as punctate areas at the basis of the PhR. In meridional sections through the retina of adult newt (Fig. 3C,D), 5F3 staining occurred in the wide inner and the narrow outer plexiform layers, but also as regularly-spaced bright spots in the PhR inner segments, and just underneath them as a dim, diffuse fluorescence outlining the outer nuclear layer somata. In favorable radial sections through adult newt retina (Fig. 3E/F), enlarged views of parts of the PhR layer revealed that intensely 5F3-stained areas in the inner segments were located in the supranuclear cytoplasmic regions of rods and cones, corresponding to the myoids. Moreover, it is noteworthy that 5F3 staining often showed a punctate pattern at the periphery of the myoids (Fig. 3E).

By offering improved optical resolution, analysis in confocal scanning laser microscopy suggested that 5F3 labeling was not only present in the inner segment myoids (Fig. 4A,B,C: arrowheads), the outer nuclear layer (Fig. 4A,B,H) and the plexiform layers (4A,H), but also in the pigmented epithelium (Fig. 4I) and even in the inner nuclear layer (Fig. 4A,H), which exhibited no or scarcely staining in conventional epifluorescence microscopy (Fig. 3C). Furthermore, confocal ima-ges showed that 5F3 staining was

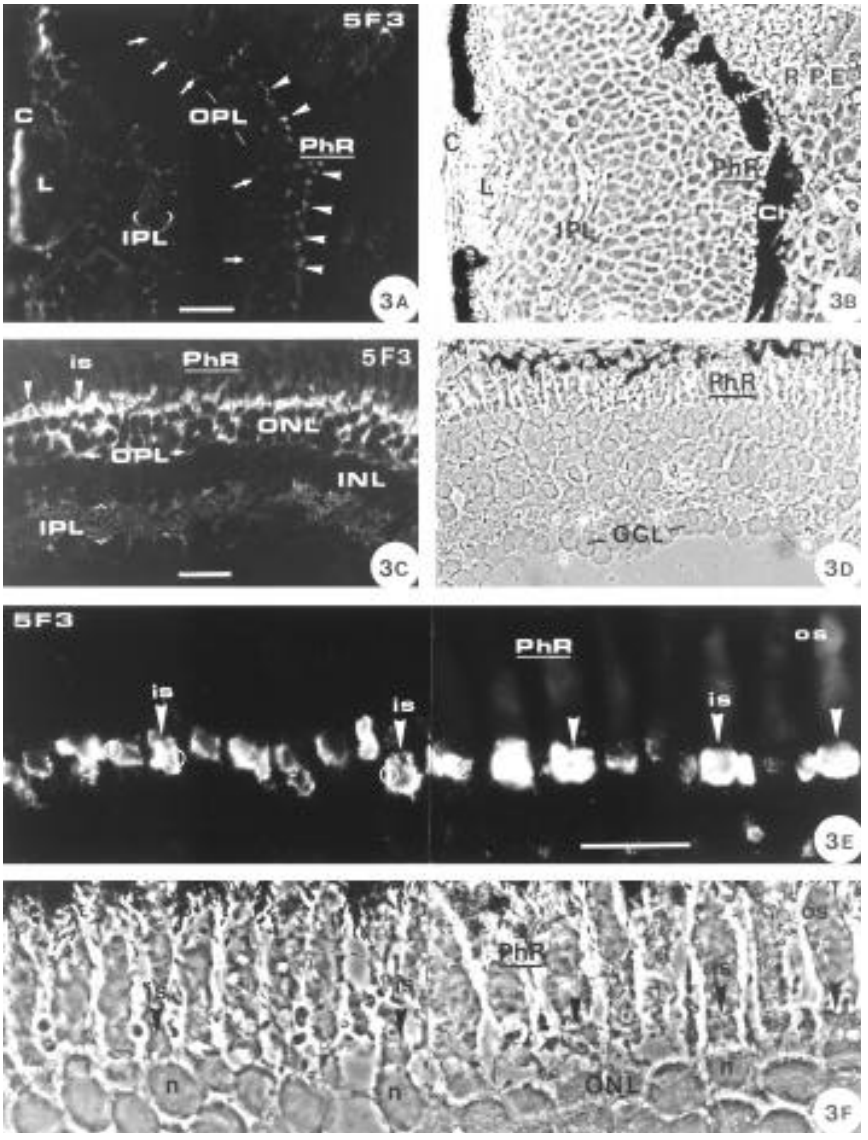


**Fig. 3. Dp71-specific 5F3 labeling in retina of larval and adult newts. (A-B)** *Vertical, meridional section through the eye of a larva. Staining seems to be present throughout the retina, especially in the inner plexiform layer (IPL) and at the basis of photoreceptors (PhR) as fluorescent punctate areas (arrowheads). The thin outer plexiform layer (OPL, small arrows) occurs discretely stained. C, cornea; L, lens.* **(B)** *Phase-contrast image of (A). RPE, retinal pigmented epithelium; Ch, choroid. Bar, 50 μm.* **(C)** *Part of radial section through retina of adult newt. In addition to the inner (IPL) and outer (OPL) plexiform layers, staining is seen as regularly-spaced bright punctate areas (arrowheads) at the inner segments (is) of photoreceptors (PhR), and as fine fluorescent profiles in the cells of the outer nuclear layer (ONL). INL, inner nuclear layer.* **(D)** *Phase-contrast image of (C). GCL, ganglion cell layer. Bar, 50 μm.* **(E)** *Enlarged part of the PhR layer. 5F3 reactivity occurs prominent in the inner segments (is), more precisely in the myoid regions (arrowheads), where label appears here and there (circles) lining the plasma membrane. PhR outer segments (os) show dim autofluorescence.* **(F)** *Phase-contrast of (E). n, nuclei of PhR cells; ONL, outer nuclear layer. Bar, 15 μm.*

especially prominent at the myoids (Fig. 4A,B) where it occurred clearly located underneath the plasma membrane (Fig. 4B,C). In magnified views of radial sections through myoids, 5F3 labeling appeared not uniformly distributed beneath the plasma membrane, but concentrated as densely patched clusters (Fig. 4C). Optical sections in a plane perpendicular to myoids confirmed such a discontinuous staining pattern (Fig. 4D).

On the other hand, in order to further locate the distribution of specific expression patterns, phalloidin staining was also analyzed either alone (Fig. 4E,F) or in combination with 5F3 staining (Fig. 4H,I,J,K). In these double staining studies, it is to note that DMSO present in phalloidin solution generally extracted a part of Dp71, e.g., from myoids, resulting in enhancing background autofluorescence on the outer segments (Fig. 4H). Nevertheless, these experiments enabled to show that Dp71 (Fig. 4A,H), as well as F-actin (Fig. 4E,G,H), were not only present in the plexiform layers, but also, in the pigmented epithelium (Fig. 4H,I,J,K) where Dp71 and F-actin appeared relatively co-localized, at least at the light microscopic level, as shown by sequential sections through the retinal slice at 1 μm intervals in the Z-plane (data not shown). On the other hand, although a direct interaction between Dp71 and F-actin was excluded, the strategic localization of Dp71 under the plasma membrane, i.e., at a cytoskeleton-plasmalemmal interface, raised the question about possibilities of interactions of Dp71 with underlying cytoskeletal components. In conventional fluorescence microscopy, double staining studies with FITC-phalloidin and 5F3 mAb gave pictures (results not shown) suggesting that actin filament bundles were present in myoids displaying 5F3 reactivity. Confocal laser microscopic analysis, especially that of side views generated by optically sectioning the samples in a plane perpendicular to the myoid layer, provided sharper images showing the presence of prominent actin filament bundles regularly distributed around the myoids (Fig. 4G). Such observations suggested that FITC-phalloidin visualized the cores of bundled actin filaments of long apical Müller cell microvilli closely associated to the inner segments in a basket-weave pattern rather than microfilament bundles confined to the periphery of the myoids. However, we cannot rule out the possibility that discrete actin filament bundles close to the plasma membrane are present in the myoids of the newt retina. Electron microscopical investigations are needed to specify this point.

## Discussion

This paper reports Western blot and immunofluorescence analysis of the expression patterns of dystrophin isoforms, especially of Dp71 in the retina of newt *Pleurodeles waltl.* For this study, two antibodies providing complementary data were used. Given that antibodies directed against utrophin/DRP-1 also showed reaction in the retina (Rodius et *al.*, 1997), H-5A3 mAb was useful to examine expression of dystrophin family products despite this antibody did not discriminate between dystrophin from utrophin (Fabbrizio *et al.*, 1993, 1994; Pons *et al.*, 1993). On the other hand, 5F3 mAb enabled to specify these data by showing in newt retina

the presence of Dp71 isoforms. Findings on the nature and distribution of dystrophin isoforms in the newt retina, and their putative role(s) in the retinal compartments may be summarized and discussed as follows:

***Dystrophin isoforms present in the adult newt retina***

Using H-5A3 mAb in newt retinal samples, a dystrophin doublet, a 260 kDa band, a band around 120 kDa and a diffuse material with a range at 70-75 kDa were detected. Dystrophin bands as a doublet were estimated to be around 420 and 410 kDa while the antibody detected full length dystrophin and/or utrophin at the expected size of 427 kDa in skeletal or smooth muscle of adult newt (Arsanto *et al.*, 1992). These data are very similar to those reported by Pillers *et al.* (1993) for the normal human retina, since the three antisera they used detected dystrophin as a doublet at 420 and 407 kDa. Concerning the 260 kDa band, it most probably corresponds to Dp260 that D'Souza *et al.* (1995) first identified in mouse retina and showed to be required as well as full length dystrophin (Pillers *et al.*, 1993) for normal retinal function. Importance played by Dp260 in retina was also strongly suggested by the study of Rodius *et al.* (1997), which demonstrated that during retinal development, Dp260 expression correlated with synaptic maturation. Although D'Souza *et al.* (1995) showed that Dp260 lacked the actin-binding domain, Rybakova *et al*. (1996) identified a new F-actin binding domain localized in the spectrin-like rod region of Dp260 and Dp427, suggesting that the two dystrophin isoforms were able to function in a similar manner (Rodius *et al.*, 1997). On the other hand, the protein whose size was estimated to be around 120 kDa may correspond to Dp116/apodystrophin-2. This dystrophin product has been found in peripheral nerves and cultured Schwann cells (Byers *et al*., 1993; Matsumura *et al.,* 1993; Schofield *et al*., 1994; Rivier *et al.*, 1996), brain (Finn and Ohlendieck, 1997), the olfactory bulb system (Lidov *et al*., 1993) and the vestibular (Dechesne *et al*., 1995) or cochlear hair cells (Dodson *et al*., 1995). However, until now the presence of Dp116 has never been reported for retina, thus raising the question of a possible contamination of retinal samples by surrounding tissues, especially peripheral nerves. This protein may also correspond to Dp140 detected by Rodius *et al*. (1997) in the adult rat retina. On the other hand, since H-5A3 mAb has been prepared against a common domain of utrophin and dystrophin, the band around 120 kDa may also correspond to $DRP_2$ protein, whose transcript has been described to be present in the mouse eye (Dixon *et al*., 1997). Further experiments are therefore needed to specify this point. Finally, the material extending

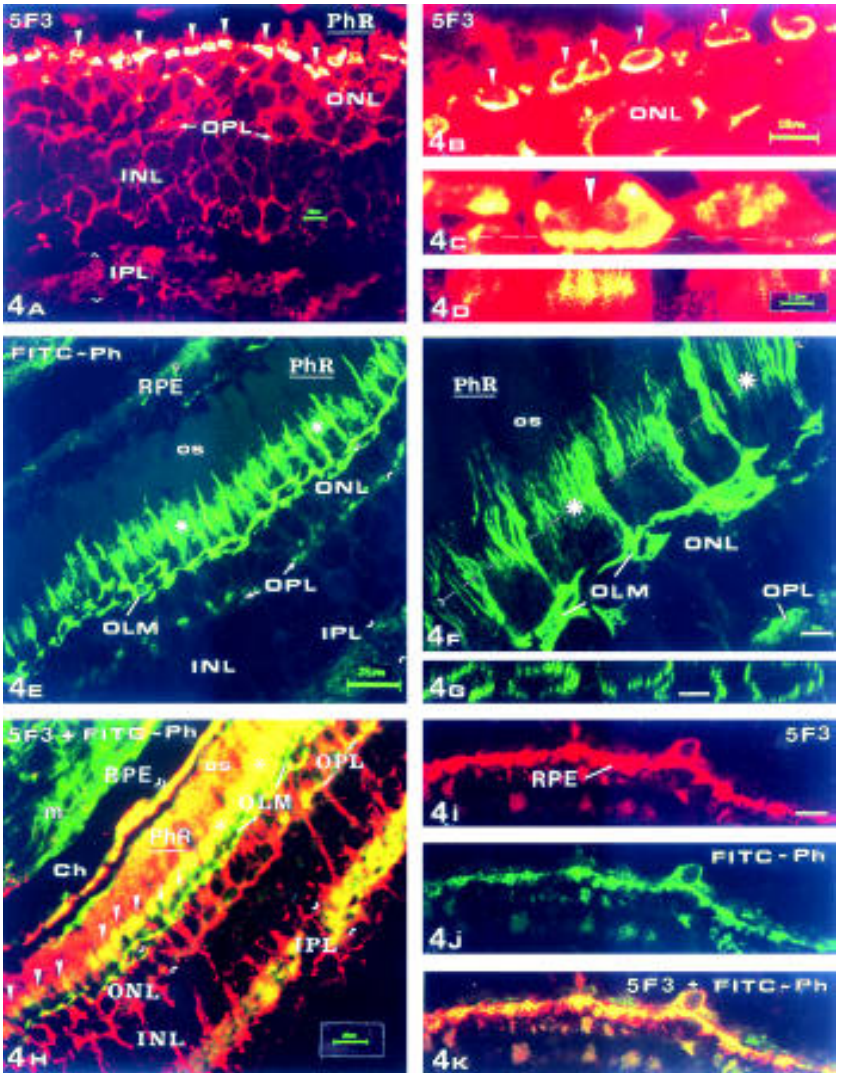


**Fig. 4. Laser confocal microscopical analysis of 5F3 and/or phalloidin staining patterns in adult newt retinal sections. (A)** *5F3 reactivity. Staining is observed here in the inner segment myoids (arrowheads) of PhR, in the outer (ONL) and inner (INL) nuclear layers and in the outer (OPL) and inner (IPL) plexiform layers. The most intense labeling is however seen under the plasma membrane of myoids and cells of the ONL. Bar, 10 μm.* **(B)** *Enlarged detail of PhR layer showing that label in myoids (arrowheads) is located underneath the plasma membrane. Bar, 10 μm.* **(C)** *Magnified view of 5F3-stained myoids. In one myoid (arrowhead), label appears not uniformly distributed beneath the plasmalemma, but concentrated as densely patched clusters.* **(D)** *Optical section in a plane perpendicular to myoids seen in (C). Discontinuous staining pattern appears clearly. Bar, 2.5 μm.* **(E-G)** *Phalloidin staining.* **(E)** *Strong labeling is seen at the outer limiting membrane (OLM) and as a basket-weave pattern at the level of myoids (asterisks). Note also staining of the OPL and IPL, and that of the pigmented epithelium (RPE). Bar, 25 μm.* **(F)** *Enlarged part of the myoid layer. Bar, 5 μm.* **(G)** *Optical section in a plane perpendicular to phalloidin-stained baskets shown in (F). Cross-sectioned bundles occur regularly distributed around areas corresponding to myoids, suggesting that they probably correspond to the F-actin cores of Müller cell microvilli close to the inner segments. Bar, 5 μm.* **(H)** *Confocal image of retina after double staining with 5F3 and phalloidin. The plexiform layers (OPL, IPL) and the RPE appear largely as yellow-stained structures because of some superposition of red (TRITC-5F3) and green (FITC-phalloidin) staining patterns. Note that it is also the case for parts (asterisks) of the inner segments because of superposition of F-actin-containing microvilli (small arrows) of Müller cells and 5F3-containing myoids (arrowheads). Orange labeling in the outer segments (os) is due to autofluorescence, enhanced here by diffusion of Dp71 from myoids after DMSO treatment. Ch, choroid; m, muscle. Bar, 25 μm.* **(I-J-K)** *Confocal images of RPE showing 5F3 (I), phalloidin (J) or double staining (K). Bar, 5 μm.*

from 70 to 80 kDa revealed by H-5A3 is most probably due to Dp71 since, using this antibody, Fabbrizio *et al.* (1994) could detect Dp71 as a band around 77 kDa in crude extracts from chicken and rat tissues. Western blot analyses with 5F3, specific of Dp71 (Lederfein *et al*., 1992; Fabbrizio *et al*., 1994), enabled us to confirm the presence of Dp71 in adult newt retina by revealing a major band at 75-77 kDa. However, Dp71 consists of a subfamily of alternatively spliced forms (Lederfein *et al.*, 1992; Austin *et al*., 1995) which are regulated during neural development (Ceccarini *et al.,* 1997). Between the two transcripts of Dp71 spliced out of exons 71 and 78, the shorter transcript to which Ceccarini *et al.* (1997) referred to as Dp60 was also alternatively spliced of exons 72-74, a region containing binding site to syntrophin. Furthermore, polypeptides around 50 kDa could result from splicing of exons 71-78 or correspond to Dp45/apodystrophin-3 (Tinsley *et al*., 1993). Therefore, the different bands detected with 5F3 in newt retina, but also brain and spinal cord, correspond most probably to spliced variants of Dp71.

***Localization and putative role(s) of retinal dystrophin isoforms***

*Dystrophin isoforms in the outer plexiform layer (OPL)*

In sections through retinae of larval and adult newts, immunoreactivity with H-5A3 was shown to be localized to the OPL. These findings suggest that the different dystrophin family proteins isoforms (i.e., full length dystrophin, Dp260, Dp71) detected by Western blotting in newt retinal samples are all present in the OPL. This is consistent with studies which showed the presence of dystrophin family proteins in the OPL of mouse and rat (Miike *et al*., 1989; Miyatake *et al*., 1991; Zhao *et al*., 1991; Yoshioka *et al*., 1992; Schmitz *et al*., 1993; D'Souza *et al*., 1995; Kameya *et al*., 1997), human (Schmitz *et al*., 1993; Pillers *et al.,* 1993) and bovine (Schimitz et al., 1993). Although the role played by dystrophin isoforms in retina remains unclear, the presence of full length dystrophin (Pillers *et al.,* 1993) as well as Dp260 (D'Souza *et al*., 1995; Kameya *et al*., 1997) has been shown to be crucial for normal retinal ERG. Schmitz *et al.* (1993) speculated that dystrophin may be important for the generation of synaptic microdomains and control of synaptic plasticity. Rodius *et al.* (1997) suggested that Dp260 could play an important role in synaptogenesis.

*Dp71 in retinal areas, including inner segments, especially myoid regions*

Using 5F3 mAb, specific for Dp71 (Lederfein *et al.,* 1992; Fabbrizio *et al*., 1994), we showed that Dp71 isoforms are not only present in the plexiform layers but also in the outer and inner nuclear layers, the pigmented epithelium, and the PhR inner segments, especially the myoid regions. However, because of their high density of 5F3 labeling, we focused here our attention on the myoids, which have been shown, by electron microscopy in the retina of *Triturus* (Keefe, 1971), to contain in particular a prominent Golgi apparatus complex and a glycogen-rich paraboloid. By providing sharp images with greater resolution than conventional imaging, confocal laser microscope enabled to reveal that 5F3 staining was confined to discrete domains beneath the plasma membrane. These observations are consistent with data from subcellular fractionation (Rapaport *et al.,* 1993) or extraction of Dp71 by saponin (Fabbrizio *et al.*, 1994) which indicated an association of Dp71 with the plasma membrane. The role that Dp71 isoforms play in the retina is unknown. However, Dp71 splicing variants retain domains for binding to the dystrophin-associated glycoproteins (DAG), especially β-dystroglycan (Rosa *et al*., 1996) and syntrophin (Kramarcy *et al*., 1994), except Dp71 isoform expressed in early neural development, which lacks the syntrophin-binding site (Ceccarini *et al*., 1997). Other studies provided evidence for co-localization of dystrophin and dystroglycan in the OPL (Montanaro *et al*., 1995; Drenckhahn *et al.,* 1996), indicated that Dp260 was required for normal formation of the dystrophin-dystroglycan complex in retina (Kameya *et al*., 1997) or showed that dystroglycan played a key role during early embryonic development (Williamson *et al*., 1997). Moreover, the absence of Dp71 in the brain of mutant mice was correlated with a reduced level of the DAG, suggesting that Dp71 is important for the formation and/or stabilization of a DAG complex in the brain (Greenberg *et al*., 1996). To our acknowledge, colocalization of dystroglycan(s) and Dp71 in the retina has not yet been reported in the same work, but β-dystroglycan has been recently detected in the OPL (Blank *et al*., 1997; Koulen *et al*., 1998), which may also contain Dp71. In the hypothesis that Dp71 isoform(s) and β-dystroglycan are codistributed in the retinal compartments, it is possible that Dp71 may play a part in their maturation and/or stability by interacting with the DAG. On the other hand, although a direct interaction between Dp71 and F-actin is excluded since Dp71 lacks actin-binding domain (Lederfein *et al*., 1992), the two proteins may indirectly interact via syntrophin which links both Dp71 (Kramarcy *et al*., 1994) and actin (Iwata *et al*., 1998). Apparent codistribution of Dp71and F-actin in the pigmented epithelium and the plexiform layers is consistent with this view but does not prove that it is the case in retina. Likewise, in the hypothesis that subcortical actin filament bundles exist in the myoids of the newt retina, as it was reported for non mammalian species, including amphibians (Ali, 1971), it is possible to postulate that Dp71 may indirectly interact with cytoskeletal components (e.g., actin, α-spectrin, protein 4-1: Madreperla *et al*., 1989; Spencer *et al*., 1991) involved in the maintenance of the polarized phenotype and function of the inner segment and/or retinomotor responses.

Finally another hypothesis is that Dp71 may contribute to intracellular calcium regulation since it contains two potential calcium-binding sites. This poses the question whether Dp71 could contribute to $Ca^{2+}$ homeostasis, similarly as it was suggested for dystrophin in skeletal muscle fibers (Kargacin and Kargacin, 1996). Indeed, it is not excluded that Dp71 may be involved in some regulation of calcium in PhR, especially in the myoid, in which calcium metabolism is important and $Ca^{2+}$-sequestering sites are prominent (Ungar *et al*., 1984).

Further studies including ultrastructural immunocytochemistry are now needed to specify our findings, especially by precisely localizing Dp71 in specific subcellular domains or structures of the adult newt retina.

## Material and Methods

***Animal surgical procedures***

The Urodelan amphibia used in this study were adults and larvae of the European newt, *Pleurodeles waltl.* These animals were obtained from the CNRS's Amphibian Farm, Centre de Biologie du Développement, Université Paul Sabatier, Toulouse, France. Newts were reared in groups of 10 and maintained in circulating tap water at 18-20°C; the water was completely renewed twice a week. Newts were fed twice a week with beef heart or liver. Larvae were obtained from egg layings in the laboratory. For immunostaining experiments of this study, six adult animals and five 2-to-3 week-old larvae were used.

Before surgery, animals were anesthetized with a 1% aqueous solution of MS 222 (tricaine methane sulfonate, Sigma Chemical Co., St. Louis, MO) for 10-15 min. For immunohistochemistry on tissue cryosections, the eyes

were enucleated before sacrifice. For immunoblotting experiments, retinae were removed by cutting through the dorsal part of the eye as reported in a previous paper on adult newt retinal regeneration (Mitashov *et al.*, 1995). The cut was made in the contact area between the pigmented epithelium and the root of the dorsal part of the iris. A physiological solution was injected between the pigmented epithelium layer and retina to obtain the detachment of one tissue from the other. The whole retina was removed by forceps after cutting the optic nerve.

### *Antibodies*

*H-5A3 mAb*

This monoclonal antibody was raised against the last 303 residues of the chicken skeletal dystrophin. It was obtained from the amino acid sequence 3357-3660 of the C-terminal region of dystrophin, expressed as a recombinant protein in *Escherichia coli.* It was shown to react with dystrophin, utrophin, and short dystrophin and/or utrophin products (Pons *et al.*, 1993; Fabbrizio *et al.*, 1993; 1994), especially Dp71 (Fabbrizio *et al.*, 1993).

*5F3 mAb*

This monoclonal antibody was obtained after injection of a fusion protein expressing the previously defined last 31 residues of Dp71 (Lederfein *et al.*, 1992). It is therefore specific for Dp71 (Lederfein *et al.*, 1992; Fabbrizio *et al.*, 1994).

### *SDS/PAGE and immunoblot analysis*

Freshly isolated adult newt samples (retina, brain, spinal cord, cardiac muscle) were homogenized in 7 volumes of 20 mM, Tris-HCl, 10 mM EDTA, 0.2% Triton X100, 1 mM Phenyl Methyl Sulfonyl Fluoride (PMSF), 1 μg/ml antipain and pepstatin 15 μg/ml, benzamidine pH 8 (antipain, pepstatin and benzamidine were first solubilized in dimethylsulfoxide (DMSO). The homogenization was carried out on ice in a Dounce homogenizer with a glass pestle for retina, brain and spinal cord. Cardial muscle tissues were crushed in an Ultra-Turrax T25 tissue grinder for 15-20s before being homogenized. Samples were then sonicated and centrifuged at 10,000g for 10 min at 4°C. Protein samples (20-40 μg) present in the supernatant were resolved on 5-13% sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The proteins were then transferred onto nitrocellulose filters. The nitrocellulose sheets were sequentially reacted with primary antibodies (used without dilution or at a 1:10), and then with alkaline phosphatase-coupled second antibody (at 1:500). BCIP (5-Bromo-4-Chloro-3-Indolyl Phosphate) and NBT (Nitroblue Tetrazolium) were used for development of phosphatase activity.

### *Immunohistochemistry*

Tissues were embedded unfixed in OCT Compound (Tissue Tek, Miles Lab. Inc., Naperville, IL). However, to prevent artifacts of freezing, before embedding, samples, especially larvae, were generally immersed in 15% sucrose-PBS solution for 3 h or overnight at 4°C. Pieces were then rapidly frozen in liquid nitrogen and stored at -20°C. Serial sections of 15 μm were obtained in a cryostat at -22°C, collected on gelatin-covered slides and stored at -20°C until immunofluorescent staining was performed. They were washed 1 h in PBS + 1% bovine serum albumin (BSA), then incubated for 1h in a humid chamber in the dark with primary antibodies (5F3 or H-5A3 mAb) used without dilution or at 1:5 dilution in PBS+BSA. Fluorescein isothiocyanate (FITC)- or tetramethyl rhodamine (TRITC)-conjugated anti-mouse IgG were used at a 1: 200 dilution as secondary antibodies.

Controls were made by omitting the first antibody or by replacing it with preimmune serum.

### *Phalloidin labeling*

Phalloidin is known to bind to filamentous actin (Wieland, 1977) and its use provided a means to visualize actin cytoskeleton (Wulf *et al.*, 1979). FITC-phalloidin staining was performed on cryosections from OCT-embedded tissue pieces. FITC-Phalloidin was applied to tissue sections at a concentration of 0.5-1 μg/ml in PBS containing 1% dimethylsulfoxide (DMSO) and allowed to react for 40-60 min at room temperature in a moist chamber. Slides were then washed thoroughly in PBS and prepared for microscopical examination. The procedure for staining was carried out in the dark. The specificity of staining was tested by treatment with unlabeled phalloidin.

### *Fluorescence analysis*

Washed slides were mounted in moviol, and examined either with a conventional epifluorescence Zeiss microscope or with a Zeiss LSM confocal system (Zeiss, Germany) and a Zeiss Axiovert microscope 135M, equipped with x10, x40 N.A.1.2, x63 N.A.1.4, x100 N.A.1.3 objectives. Confocal images were collected using a argon laser and a He-Ne laser with attenuating filters as excitation sources at 488 nm or 543 nm, for FITC or TRITC, respectively. For simultaneous excitations of FITC and TRITC, a double-banded beam splitter DBSP 488/543 was used. Excitation filters FT 510 nm or LP560 nm, and emission filters BP515/565 nm or LP 570 nm were used to separate acquisition between FITC and TRITC. Series of images were processed with Zeiss software to enhance contrast and to produce extended focus, rotation and stereopairs. Averaging of four images recorded with the lowest speed (1s for 1024x1024) was a good compromise to obtain images with good signal to noise ratio and to avoid bleaching. For color slides, Fujichrome 100 films were used. Digitized images were stored on an optical disk.

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