



The Target of the Antiproliferative Antibody (TAPA) in the Normal and Injured Rat Retina

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Purpose: The target of the antiproliferative antibody (TAPA, CD81) is a member of the tetramembrane spanning superfamily of proteins and appears to be involved in the regulation of mitotic activity and the stabilization of cellular contacts [J Neurosci 1996; 16:5478-5487]. The present study examines the distribution of this protein in the normal rat retina and its role in reactive gliosis occurring after retinal injury.

Methods: An immunoblot was used to define the relative level of TAPA in the normal rat retina. The distribution of the protein was examined using indirect immunohistochemical methods. Both of these methods were used to define the upregulation of TAPA in the rat retina injured with a needle scrape.

Results: The immunohistochemical analysis of the retina shows that TAPA is found in all layers of the normal retina with a distinct lack of labeling in the inner and outer segments of the photoreceptors. After retinal injury, a dramatic upregulation of TAPA was observed. The immunohistochemistry also revealed a pattern of expression similar to that observed in the normal retina with two notable exceptions: (1) small finger-like projections extending down into the outer segments are immunopositive, and (2) the elevated levels of TAPA can be seen outlining cell bodies in the outer nuclear layer and the ganglion cell layer.

Conclusions: TAPA is found in the normal rat retina and there is a dramatic upregulation of this protein following injury. The distribution of the protein within the retina is consistent with its expression in retinal glia, the Müller cells which span the thickness of the retina, and astrocytes found in the ganglion cell layer. These data suggest that TAPA may play a role in the proliferative response of non-neuronal cells that occurs following a mechanical injury to the retina.

The response of the mammalian retina to injury is similar to that occurring in other parts of the central nervous system. Like the brain and spinal cord, the retina contains glial cells: astrocytes in the ganglion cell layer, and Müller cells that span the cellular layers. When the retina is injured, one of the most striking features is the change occurring in astrocytes and Müller cells [1,2]. These cells hypertrophy and increase expression of the intermediate filament protein, glial fibrillary acidic protein (GFAP) [3-6]. Although this reactive response is very similar to that observed in astrocytes within the brain and spinal cord, in the retina, the glial cells also proliferate and migrate throughout the retina [7]. Once the retina is detached, the Müller cells send their processes into the spaces between the photoreceptors and retinal pigment epithelium, causing a subretinal fibrosis or scar [8]. This glial scar appears to contribute to the lack of retinal reattachment and the death of photoreceptors [9]. The retinal glial cells also can migrate into the vitreal space, proliferate, and form cellular membranes [7]. This response is known as proliferative vitreoretinopathy and can result in retinal detachment [10]. Thus, reactive glial responses can have serious consequences, potentially resulting in the loss of sight. Understanding molecular mechanisms associated with these changes may provide insights into interventions that may stop or reverse the detrimental effects of reactive gliosis in the retina.

Our laboratory [11] has recently identified the expression of the Target of the Antiproliferative Antibody (TAPA) [12,13] by glial cells in the rat brain. TAPA is a member of the

tetramembrane superfamily of proteins and plays a major role in the maturation of CNS glia, the regulation of their mitotic activity, and the process of reactive gliosis [11]. After an injury to the brain or spinal cord, a glial scar is formed and high levels of TAPA are found in association with these reactive astrocytes [11]. The present study is designed to: (1) determine if TAPA is expressed in the mammalian retina, (2) define the distribution of the protein in the retina, and (3) determine if TAPA is upregulated following retinal injury as it is in other regions of the CNS.

METHODS

Ten adult Sprague-Dawley (Albino) rats were used in the present study: five for the immunohistochemical analysis and five for the immunoblot analysis. To examine the effects of retinal injury, six of the animals received a scrape injury to the right retina. The animals were anesthetized with an intraperitoneal injection of a mixture of xyaline (13 mg/kg, Rompun), and ketamine (87 mg/kg, Ketalar). A 22 gauge needle was used to penetrate the lateral sclera and to scrape along the inner surface of the nasal retina. The injury was allowed to heal for fourteen days, after which all of the rats were euthanized with a mixture of xyaline (26 mg/kg, Rompun), and ketamine (174 mg/kg, Ketalar) by intraperitoneal injection. For immunohistochemistry the animals were perfused as described and for biochemistry the eyes were removed and the tissues placed in sample buffer (see below).

Antibodies— Three monoclonal antibodies and one polyclonal antibody were used to stain the sections and protein samples of the normal and injured retinas. In the rat retina, the AMP1 monoclonal antibody recognizes only TAPA (CD 81) in tissue sections or on immunoblots of retinal proteins. This is not the case for the brain, where AMP1 antibody rec-

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ognizes TAPA in tissues, but labels two proteins on immunoblots: alpha actinin and TAPA. The AMP1 monoclonal antibody only recognized TAPA in formalin fixed tissue or in cultured cells [11]. The AMP1 antibody will only immunoprecipitate TAPA, not alpha actinin [11]. Furthermore, the AMP1 antibody recognizes alpha actinin on immunoblots of protein from tissues it will not stain. For example, on immunoblots of C6 glioma, human glioma (U373), or human brain, the AMP1 antibody recognizes a single protein at 106 kDa (our antibody does not recognize human TAPA-1). When these glioma or sections of human brain are stained using immunohistochemical methods no immunoreaction product is observed (data not shown), demonstrating that the antibody can see alpha actinin on immunoblots but not in tissues. Taken together these data demonstrate that the AMP1 antibody recognizes only TAPA in the rat retina. The anti-vimentin monoclonal antibody was purchased from Boehringer Mannheim (Catalog Number 814318). To identify glial fibrillary acidic protein (GFAP) two antibodies were used: a polyclonal antibody purchased from Lipshaw (Catalog Number 460740) and a monoclonal antibody TED1 produced in our laboratory [14]. The peroxidase conjugated secondary antibodies were: goat anti-mouse IgG and goat anti-rabbit IgG. Fluorescein labeled antibodies included a goat anti-mouse IgG antiserum and a goat anti-rabbit IgG antibody. All secondary antibodies were purchased from Jackson ImmunoResearch Laboratories.

Immunohistochemistry— For the immunohistochemical analysis, the rats were euthanized with a mixture of xyaline (26 mg/kg, Rompun), and ketamine (174 mg/kg, Ketalar) by intraperitoneal injection. The rats were perfused through the heart with a 0.1 M phosphate buffered saline (PBS, pH 7.5), followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Both eyes were removed from the normal animals (2 rats, 4 eyes) and the injured eye was removed from the other three rats (3 eyes). The eyes were placed in 4% paraformaldehyde in 0.1 M phosphate buffer overnight at 4 °C. The cornea and lens were removed from each eye and the globes were placed in 30% sucrose in phosphate buffer. A cryostat was used to make 15 µm sections of the globe. The sections were mounted on poly-L-lysine coated slides and stored at 4 °C. The sections were blocked for 1 h in borate buffered saline containing 4% BSA with 0.1% DMSO. They were then incubated in primary antibody overnight at 4 °C. The sections were rinsed extensively and placed in the secondary antibody overnight at 4 °C. The sections were then rinsed three times. When fluorescein labeled antibodies were used, the sections were examined directly in phosphate buffer using a water immersion, 25x objective (Zeiss). When peroxidase-conjugated secondary antibodies were used, the sections were reacted with diaminobenzidine and hydrogen peroxide, mounted on glass slides and coverslipped.

SDS-PAGE/Immunoblotting— To analyze changes in expression levels of TAPA in the retina following injury, a quantitative immunoblot method was used as previously described in Geisert et al. [11]. Protein samples were taken from four normal eyes (2 rats) and three injured eyes (3 rats). The rats were euthanized with a mixture of xyaline (26 mg/kg,

Rompun), and ketamine (174 mg/kg, Ketalar) by intraperitoneal injection and the eyes removed. Using a dissecting microscope the retinae were removed and placed in 200 µl of non-reducing sample buffer (2% SDS, 10% glycerol in 0.05 M Tris-HCl buffer, pH 6.8). The protein samples were balanced and equal loads of proteins were run on 4-16% acrylamide gels on a Bio-Rad Mini Protein II Gel apparatus (Bio-Rad, Hercules, CA). The proteins were then transferred to nitrocellulose, using a Bio-Rad mini-transblotter. These blots were blocked in borate buffer (pH 8.4) containing 5% non-fat dry milk, and probed with the primary antibody. After rinsing in borate buffer, the blots were incubated in peroxidase-labeled secondary antibody, rinsed extensively, and reacted with 0.05% diaminobenzidine and 0.01% hydrogen peroxide.

To define the level of proteins on immunoblots, the immunoreaction product was quantified by scanning the blots and analyzing these scans with the NIH Image software. All protein samples were balanced. The samples were then diluted in two fold increments and run on 4% to 16% gradient gels and transferred to nitrocellulose. The immunoblots were stained with antibodies directed against one of three proteins (TAPA, GFAP, or vimentin). Blots were scanned using a Macintosh computer with a high resolution desktop scanner (Hewlett Packard ScanJet II CX). NIH Image software was then used to compare the intensity of the immunolabeling, defining the dilution factor that produced a similar level of reaction product. The relative level of each protein band was defined in relative absorbance units. For this analysis only, samples run on the same immunoblots were used to define changes in the levels of proteins.

RESULTS

Normal Retina— When sections through normal retina are stained using the AMP1 antibody, a distinct laminar pattern of labeling is seen (Figure 1A). In the ganglion cell layer the large cell bodies of the ganglion cells are outlined by the antibody. The AMP1 antibody also appears to label structures surrounding blood vessels in the ganglion cell layer. A dense reticular pattern of staining is observed in the inner plexiform layer with very intense labeling throughout the layer. The cell bodies in the inner nuclear layer are outlined by the AMP1 labeling. The labeling in the outer plexiform layer is similar to that seen in the inner plexiform layer with high level of staining throughout the layer in a dense reticular pattern. In the outer nuclear layer the cell bodies of the photoreceptors are outlined by the immunolabeling. In the layer of photoreceptors (inner and outer segments), no antibody-dependent immunolabeling was observed. There were high levels of autofluorescence in this layer. This autofluorescence is also observed in sections stained for vimentin (Figure 1B), for GFAP (Figure 1C), or when the sections were exposed to the secondary antibody only (data not shown). True immunolabeling in the retina was limited to cellular processes that extend from the inner limiting membrane to the external limiting membrane and filled the two plexiform layers. This

pattern of antibody localization suggests that the Müller cells are labeled by the AMP1 antibody.

To allow us to compare the pattern of AMP1 labeling to the major glial cell types in the retina, sections through normal retina were stained using anti-vimentin antibodies to label Müller cells (Figure 1B) and anti-GFAP to label astrocytes in the ganglion cell layer (Figure 1C). In the sections stained for vimentin, an array of processes is observed extending from the internal limiting membrane through the cell bodies in the inner and outer nuclear layers to end at the external limiting membrane. The level of immunoreactivity is highest in the inner nuclear layer where the Müller cell bodies are located. In some instances there is a hint of cell body labeling in this layer. As with other samples, a high level of autofluorescence is observed in the layer of outer segments. When normal retinal sections are stained for GFAP (Figure 1C), the label was restricted to astrocytes in the ganglion cell layer. In the normal retina, there are no indications of labeling in Müller cell bodies or their processes.

Retinal Response to Injury— The retinal injuries were made by scraping the retinal surface with a 22 gauge needle. Nissl stained sections showed deep enfoldings of the retina 14 days after the lesion (data not shown). In sections stained with the AMP1 antibody, the level of immunoreactivity appeared to be dramatically increased compared to normal retina (Figure 2A). The ganglion cell bodies in the ganglion cell layer are outlined and the inner plexiform layer also shows a high

level of AMP1 reactivity. The cell bodies in the inner nuclear layer become even more distinctly outlined due to the high levels of TAPA. The high levels of immunoreactivity are evident through the outer plexiform layer and into the outer nuclear layer. In the outer nuclear layer, the cell bodies of the photoreceptors are distinctly outlined. A distinct separation between the outer nuclear layer and photoreceptor segments is more apparent in injured retina compared with normal retina. A dramatic increase in the immunolabeling at the external limiting membrane is observed. In addition, small labeled processes can now be seen extending down into the layer of outer segments (Figure 2A). When sections through the injured retina are stained for GFAP (Figure 2B), a dramatic pattern of vertical processes is observed. This pattern is characteristic of reactive Müller cells within the injured retina.

Immunoblot Analysis— An immunoblot analysis of retinal tissues was conducted to confirm that TAPA is expressed in the retina. The samples of retinal proteins were blotted and probed with antibodies directed against TAPA (Figure 3, Lane A), GFAP (Figure 3, Lane C) and vimentin (Figure 3, Lane E). On immunoblots of protein samples from the normal retina, the AMP1 antibody recognized only TAPA at 27 kDa (Figure 3, Lane A). When reducing agents were added to the sample buffer, the band at 27 kDa was no longer recognized (data not shown), demonstrating that the epitope recognized by the AMP1 antibody is destroyed by reduction. The sensitivity of TAPA to reduction is believed to be due to the presence of the

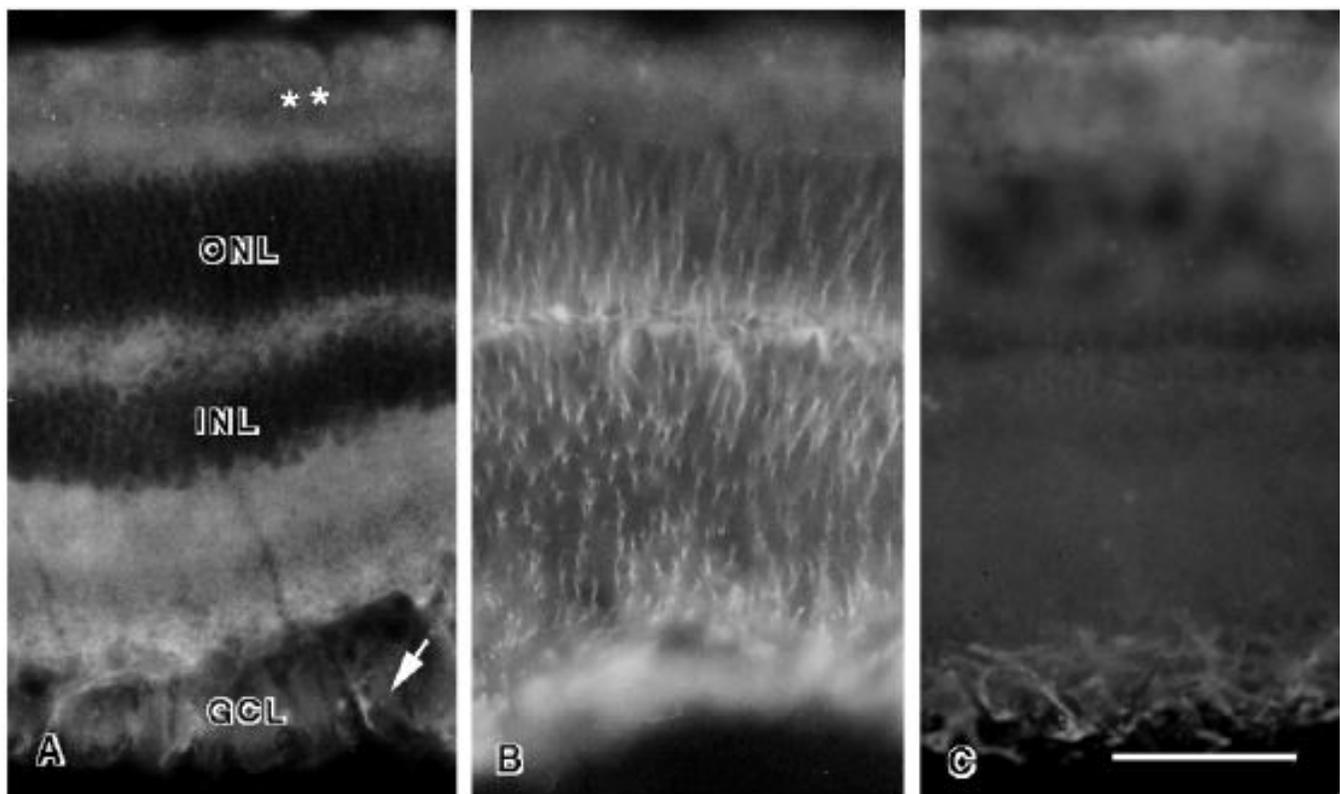


Figure 1. TAPA in the Normal Rat Retina. Normal retina stained with the AMP1 antibody (A) shows distinct laminar patterns of staining. Blood vessels in the ganglion cell layer are distinctly outlined by the AMP1 antibody (arrow). In the inner nuclear layer and the outer nuclear layer the cell bodies are outlined by the AMP1 antibody. The high level of staining in the photoreceptor layer is due to autofluorescence (double asterisk). The sections of normal retina were stained with antibodies directed against vimentin (B) or GFAP (C). The cellular layers of the retina are indicated by: ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. The scale bar in C represents 50 μ m.

disulfide bonds between cysteines in the second extracellular loop of the protein [11]. Taken together, these data demonstrate that the AMP1 antibody recognizes TAPA in the adult rat retina.

The upregulation of TAPA that occurs following retinal injury was quantified using an immunoblot analysis. Protein samples from four normal retinas and three injured retinas were run out on gels and transferred to nitrocellulose. These immunoblots were then probed with antibodies directed against TAPA, vimentin, and GFAP (Figure 3). The blots were scanned and the relative intensities of immunolabeling of the 27 kDa protein were examined (Figure 3A and B). There was no significant difference in the intensity of TAPA in the samples from the normal retinas, indicating that the levels of this protein were similar in all samples. There were elevated levels of TAPA in all of the protein samples from the injured retinas relative to the samples of the normal retinas. To determine the extent of TAPA upregulation, limiting dilutions of each sample were prepared, run on immunoblots, scanned and analyzed to define the level of immunoreaction product. This analysis revealed an average 1.7-fold increase in TAPA in the protein samples from the injured retinas (mean = 7.2, standard deviation = 1.4) relative to the samples of the normal rat retinas (mean = 4.2, standard deviation 0.6). All of the samples from the injured retinas contained levels of TAPA that were higher than the maximum level observed in the protein samples from the normal retinas. Using the conservative non-parametric statistic the Mann-Whitney U test, the increase in the overall levels of TAPA in the samples from the injured retinas is significant

($p = 0.028$). The same protein samples also were probed with antibodies directed against GFAP (Figure 3C and D) and vimentin (Figure 3E and F). There was an elevation in the levels of both of these intermediate filament proteins associated with retinal injury. For vimentin there was an approximate 2-fold increase in the injured retinas (mean = 2.1, standard deviation = 0.4) relative to the normal retinas (mean = 1.1, standard deviation = 0.1). The most dramatic upregulation was observed in the levels of GFAP, with a 6.7-fold increase in the injured retinas (mean = 10.5, standard deviation = 1.3) relative to the normal retinas (mean = 1.6, standard deviation = 0.5).

DISCUSSION

This is the first study to demonstrate that TAPA is present in the retina. Based on the distribution of TAPA in the retina, it appears to be expressed by astrocytes and Müller cells. However, we cannot exclude the possibility that TAPA is also expressed by other retinal cells. At the present time, we have only examined the retina at one time point after injury. With only a single time point in these initial studies, we cannot make a precise correlation with the upregulation of TAPA and the

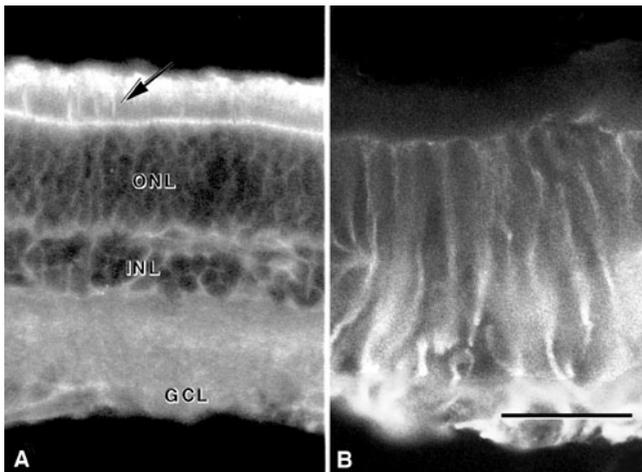


Figure 2. TAPA in the Injured Rat Retina. Sections of injured retina stained with the AMP1 antibody (A) demonstrate higher levels of immunoreactivity than seen in normal retina. A very distinct line of separation is apparent at the junction between the outer nuclear layer and the photoreceptor layer. High levels of immunoreactivity are apparent throughout the layers of the retina. Notice the fine AMP1 labeled projections extending through the outer segments of the photoreceptors (arrow). Sections of the injured retina stained for GFAP (B) show high levels of immunoreactivity in the ganglion cell layer as well as in the filaments extending throughout the layers of the retina. The cellular layers of the retina are indicated: ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. The scale bar in B represents 20 μm .

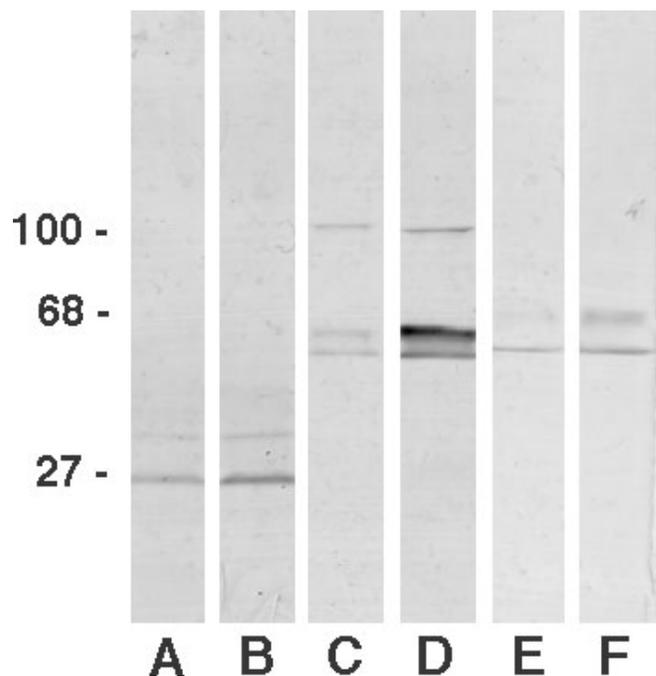


Figure 3. Immunoblots from Normal and Injured Retinae. Immunoblots of protein samples from normal (Lanes A, C, and E) and injured (B, D, and F) rat retina. The blots were probed with the antibodies directed against TAPA (A and B), GFAP (C and D), and vimentin (E and F). The presence of TAPA in the retina is confirmed by the presence of a single dark band at 27 kDa in Lane A on the immunoblot. Following injury there is an increase in the intensity of the TAPA band (B). There is also a dramatic increase in the levels of GFAP when the protein sample from the normal retina (C) is compared to that of the injured retinal sample (D). Molecular weights are indicated to the left in kDa.

time-course of the reactive response of the retinal glia [7]. Nonetheless, this upregulation of TAPA indicates it is a critical component of this injury response.

Although the specific role of this protein remains to be defined, it belongs to a family of proteins that appears to regulate cell behavior based on adhesive interactions. The tetramembrane spanning superfamily of proteins is characterized by the presence of four hydrophobic membrane spanning domains with two extracellular domains and intracellular C- and N- termini [15-18] sharing a high level of homology in their amino acid sequence [19]. This family of proteins appears to regulate cell adhesion, mitotic activity, and second messenger systems [21-26]. For example, KAI1 (the metastatic suppressor for prostate cancer) is necessary for prostate cells to form stable cell contacts [15]. In the absence of this protein, the prostate cells are metastatic. When the levels of KAI1 are elevated by gene transfection, there is a dramatic decrease in the metastatic behavior of these cells [15]. This same theme of stabilizing cellular contacts is demonstrated by TAPA [11,16] and other members of the tetramembrane spanning family, Late Bloomer [27]. This type of cell contact dependent signaling is similar but distinguishable from paracrine signaling (growth factor and cytokines). An additional example of this contact dependent, "juxtacrine" signaling is in the development of the compound eye, where the membrane receptor Boss binds to the tyrosine kinase receptor Sevenless to regulate cell fate [12]. The data from the current study suggest that TAPA may be playing a similar role in the stabilization of cellular contacts in retinal glial cells.

The effects of injury in the retina are similar to those observed elsewhere in the CNS. This is especially the case for the non-neuronal cells. In the retina, astrocytes and Müller cells become reactive, expressing high levels of GFAP, becoming hypertrophied and proliferating [2,3,7,9]. Elsewhere in the central nervous system, astrocytes are the main participants in the gliotic response [28,29]. Following an injury to the retina, we have confirmed the dramatic increase in the levels of GFAP and have demonstrated a significant upregulation in the levels of TAPA. This increase in TAPA is reminiscent of the upregulation observed following a cortical stab wound [11,30]. The elevation of TAPA following retinal injury is associated with the reactive response of glial cells, similar to that seen in injury-induced glial scar formation in the brain [30]. In the present study we demonstrated a 1.7-fold upregulation of a glial membrane protein that is associated with the regulation of mitotic activity and stabilization of cellular contacts [11]. This may appear to be a minimal increase in the levels of TAPA; however if this protein is only expressed in retinal glia this upregulation may represent a substantial increase per glial cell. In the rat retina there are approximately 31 neurons per Müller glial cell [31]. Thus, the 1.7 increase in TAPA relative to total retinal proteins would represent an approximate 20-fold increase in the levels of TAPA per Müller glial cell. These findings provide a key insight into the molecular mechanisms regulating the proliferative response of non-neuronal cells in the injured retina by raising the possibility that TAPA may be a molecular switch through which reactive gliosis and retinal scarring can be controlled.

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