G-substrate and Phosphatase Activation in Retinitis Pigmentosa

Bachelor’s Degree Project, 15 hp

Supervisor:
Abstract

Retinitis pigmentosa is an inherited disease that leads to blindness. The cause of this disease is not yet identified and no cure is available. Many genetic factors are found, though, and animal models are available. Retinitis pigmentosa leads to photoreceptor degeneration. First the rod photoreceptors die, followed by the cone photoreceptors. It is known that cGMP-dependent protein kinase in Retinitis pigmentosa is elevated. The aim of this study is to follow up the signal cascade after the activation of the cGMP-dependent protein kinase. One molecule that can be phosphorylated by cGMP-dependent protein kinase is G-substrate. In this report we use an antibody against phospho-G-Substrate plus an assay for phosphatase activity. The focus of the study was to see if there is a difference between the retinitis pigmentosa 1 mouse model and the wildtype mouse in this respect.
Introduction
Retinitis pigmentosa (RP) is a disease that approximately affects 1.5 million people worldwide. It is an inherited disease that leads to selective degeneration of photoreceptors and irreversible loss of sight. Patients often develop tunnel vision by the age 40 and then lose central vision by the age of 60 (Berson, 2008). To understand the basics about RP you need to have a clear picture of the eye and how the machinery of vision works. Therefore this paper will start with a summary of the entire retina and its function.

Retinal morphology and mechanism of vision
The retina absorbs light and transforms it to electrical impulses so the brain can interpret it to a picture. The retina is the place were light energy is converted into action potentials. The retina contains 7 different types of cells of which almost all are neurons. The outermost layer of the retina is the pigment epithelium (RPE), a single layer of darkly pigmented hexagonal cells that are firmly attached to the underlying choroid and integrated with the photoreceptor cells. The role of the pigment cells is to absorb light that has not been absorbed first by the photoreceptors, they act like the blackened inside of a camera to reduce reflection. To be able to absorb that much light the RPE contains a lot of granules with melanin. The RPE also has a role in the phagocytosis of the outer segment of the photoreceptor cells which involves restoring trans-retinal to 11-cis-retinal (see below) with the help of vitamin A. The RPE controls the homeostasis in the outer part of the retina (the cells facing the RPE) by means of exchange with the blood. The RPE cells transport ions, water, metabolic end products and pick up nutrients for the photoreceptors.

The outer nuclear layer (ONL) of the retina contains the photoreceptors; rods and cones. The ONL cells are the most posterior neurons in the retina because the pigment cells are not neurons. The photoreceptors are cells that absorb light and generate a chemical signal, that is passed through to next layer of retinal neurons. The photoreceptors, rods and cones, are responsible for dim-light vision and for colour and day light vision, respectively. The retinal photoreceptors consist of approximately 90 % rods and 10 % cones, in mammals (Cote & Schordetet, 2004).The rods and cones have an outer segment that point towards the wall of the eye and an inner segment closest to the ONL, i.e. the layer were the nucleus and the rest of the photoreceptors are. The inner segments contain factors that may help in refining the vision process. The outer segments are different between the rods and cones in both shape and function. The outer segment of the rod is cylindrical and contains a thousand or so infolding membranous discs, which each contains visual pigments. In the discs the initial events of the visual transduction pathway occur. This involves a protein called opsin and retinal (related to vitamin A), which are different for the two photoreceptors. The mechanism and process of the vision cycle will be explained later, but it leads to hyperpolarization of rods and cones in response to light. In fact, the photoreceptors behave as if darkness is the stimulus, because they are relatively depolarized in the dark and are hyperpolarized by light, and thus end their signaling when hit by light. On the other hand, the signal in the dark is inhibitory for the next neuron in the chain, so once this signal is ended the bipolar cells can then transmit the signal further.

The outer plexiform layer (OPL) is the region were cones and rods are connected with dendritic processes from bipolar cells and horizontal cells. Three different types of neurotransmitters are used here in the synapse between the cells; L-glutamate, L-aspartate and N-methyl-D-aspartate (Dowling, 1987).

The OPL is connected to the inner nuclear layer (INL), which is post-synaptic to the photoreceptors and consists mostly of bipolar cell. The bipolar cells are the first order of neurons in the transduction of the signal further to the brain. Retinal bipolar cells can be divided into two different categories, cone bipolar cells and rod bipolar cell (Reese & Keelely, 2010). All cones release glutamate in the
synapses, but the responses in bipolar cells differ. The cone bipolar cell makes two different kinds of synaptic connections, which seem to be connected to the generation of either ON- or OFF-responses to light in the retina. The axonal terminals of the cone bipolar cells end in different parts of the next layer, which is the inner plexiform layer (IPL). Under dark conditions the photoreceptors will release glutamate (or a glutamate like molecule) which inhibits the on bipolar cells and excites (or activates) the off bipolar cells.

The rods also send the signals further to the horizontal cells. There are two kinds of horizontal cells (Dowling, 1987), which are depolarized by different neurotransmitters. The main function of horizontal cells, which have the ability to give feedback to the photoreceptors, is to enhance the contrast between adjacent light and dark regions. Both cones and rods have different horizontal cells that they transmit to, and the signals are isolated from each other. If the horizontal cells are given two signals transmitted from two different cones, one inhibitory and one excitatory, the horizontal cells adjust the system’s response to the overall level of light exposure. In other words they reduce the signals transmitted to the inner retina. The proportion of horizontal cells in the retina is very small, normally less than 5 % of the cells of the INL (Dowling, 1987).

The INL also contains the amacrine cells, which connect almost all of the bipolar cells to ganglion cells, but there is probably some communication directly between bipolar and ganglion cells too. There are about 29 different kinds of amacrine cells (Masland, 2001) and they are classified by the width of their receptive field and by the neurotransmitter type they release. The synapses occur in the IPL and some neurotransmitters used include GABA, acetylcholine and dopamine. As earlier mentioned neurotransmitters have different functions, GABA is inhibitory and often involved in sensitivity to moving stimuli. A significant difference between horizontal cells and amacrine cells is

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Figure 1. 11 day after birth, Wild type mouse retina. The uppermost layer consists of the pigment cells (RPE). In the ONL photoreceptors, nuclease and synaptic terminal are placed. Above the ONL layer the outer segments and inner segments of photoreceptors are placed. The bipolar, horizontal, amacrine and Müller cells all lie in the INL layer and at last the ganglion cells are placed in the ganglion cell layer.

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that horizontal cells may have a single general role while amacrine cells have more specific controlling functions for the signal transmission.

The largest retinal neurons are the ganglion cells, that are arranged in a single layer closest to the lens in the GCL (ganglion cell layer). Their main task is to transmit the signals through the optic nerve to the brain. The ganglion cells receive visual information from bipolar cells and from amacrine cells. In numbers there are less ganglion cells than photoreceptors, because one ganglion cell receives inputs from more than one photoreceptor. This makes the ganglion cell signals more complex and creates a more assembled response for further transmission the different parts of the brain (Reese & Keeley, 2010).

The two major macroglia cells in the mammalian retina are astrocytes and Müller cells. Müller cells extend from the inner retinal layer to the photoreceptors and astrocytes are established in the inner retinal layer. The roles of macroglia cells are to function as support cells and maintain the homeostasis around the other retinal cells. The Müller cells are radial shaped and they assist the retinal neurons to survive.

- General visual cycle

The electrochemical signal cascades in rods and cones are basically the same. The molecules used are often the same but of different isoforms. The process in rods starts in the disks with the three associated proteins, the visual pigment rhodopsin, the GTP-binding protein transducin (G-protein), and a phosphodiesterase (PDE6). Rhodopsin consists of opsin and retinal, and when light hits the retina the retinal undergoes a conformational change from 11-cis-retinal to trans-retinal (Ripps, 2010). This reaction leads to an activation of the rhodopsin molecule, which in turn activates the GTP binding regulatory protein transducin (G-proteins) to initiate the transduction cascade. In darkness the transducin is a stable heterotrimer that, after activation, binds to GDP and converts it to GTP. The activated G-protein (with GTP) binds and stimulates the phosphodiesterase (PDE6), which then is free to cleave cGMP (3.5 cyclic guanosine monophosphate), resulting in decreased intracellular concentration of cGMP.

The decreased concentration of cGMP causes closure of cyclic-nucleotide-gated cation channels (CNG), which effectively blocks the influx of millions of cations into the cell resulting in membrane hyperpolarization. However, intracellular cGMP also targets cGMP-dependent protein kinases (PKG) (Paquet-Durand, et al, 2009). This will be further discussed in the part about cGMP, PKG and G-substrate.

The freely diffusible rhodopsin in the disk membrane makes it possible to produce a cascade signal, which is amplified because one rhodopsin molecule can activate hundreds of transducins (G-protein). Even PDE6 can increase the signal because it can hydrolyze many molecules of cGMP to GMP. In order to maintain its function the rod must regenerate rhodopsin. Trans-retinal dissociates from opsin, is transported to the pigment cells were it is converted back to cis-retinal and returned to the rod outer segment.
- **Retinitis pigmentosa**

In many parts of the world retinal degenerations is a leading cause of blindness (Berson, 2008), with Retinitis pigmentosa (RP) as the perhaps most well known example. RP is a heterogeneous inherited condition with more than 100 genes or loci implicated so far (Cottet & Schorderet, 2009), with a worldwide frequency of 1 in 5000 (Jensen & Poonam, 2005). RP often follows a two-step process. In the first step the rod photoreceptors degenerate; this is noticed as a loss of night vision. The second step is when the patient loses his or her vision due to degeneration of the cone photoreceptors. It is inherited as autosomal dominant (30-40 %), autosomal recessive (50-60 %) or X-linked (5-15 %) (Cottet & Schorderet, 2009). As a result of studying patients and their families many genes and loci that are involved in the disease have been found, for instance by direct sequencing. The many different mutations in RP makes it hard to develop a cure. For example, more than 120 different mutations in rhodopsin are described and these constitute the most common cause of autosomal dominant RP. Other mutations can be in the genes that are involved in the visual cycle or genes for retina-specific transcription factors, and genes involved in cytoskeleton organization, intracellular trafficking, synaptic interaction and mRNA processing are also targets of disease-causing mutation. In all forms of RP the final outcome is loss of photoreceptors. Today we have detected mutations in 56 % of patients with autosomal dominant and 30 % of patients with recessive RP.

Retinal degeneration also includes other diseases than RP, which are categorized based on their clinical characteristics. These include; Leber’s congenital amaurosis (LCA, loss of vision in early childhood), Stargardt Disease (STGD, loss of vision in early adult) and choroideremia (CHM).

- **Animal models**

For many years RP animal models have been studied to get a better understanding about the photoreceptor cell death. There are a lot of different mouse models; the most common are rd1 and rd2. All of the different mutations in the mouse models result in photoreceptor degeneration as in humans, which gives rich opportunities for studying and setting up experiments (Bo Chang, 2007). More than 80 years ago the rd1 mouse was discovered by Keller (Keller, 1924). This mouse model has a mutation in chromosome 5 in the rod photoreceptor PDE6 gene, which gives a nonfunctional PDE6 that in turn leads to accumulation of cGMP. In 4-5 % RP patients with mutation in chromosome 4q16 the defective gene encodes a nonfunctional β-subunit of cGMP-PDE (McLaughlin et al, 1995), which should make the rd1 mouse a relevant model for the human disease.

Another common mouse model is rds, called rd-slow or rd2. It has a slower progression compared to rd1. rd2/rds is caused by a mutation in the gene coding for peripherin, which may function as a multifunctional building protein essential for the formation of rod photoreceptor outer segments. The rds mutation in mice was found in chromosome 17 and its human homolog is found on chromosome 6, which is the location of human peripherin.

- **Considerable differences between wildtype and rd1**

Postnatal (PN) means after birth and the day the mice are born counts as day zero. In the rd1 mouse the RP shows a fast rod degeneration that starts around PN9. After approximately PN10 the outer segment becomes disorganized and the rod photoreceptors initiate cell death. The degeneration of rods in RP peaks at PN12-14 and leads to subsequent damage of the cones through unknown mechanisms. At PN21 only one layer of photoreceptors is left (figure 3). All of the photoreceptors are in principle degenerated at PN28.
Figure 2. PN11, wild type at right and retinitis pigmentosa mice at left. Note that there is not much structural difference between them. The difference between rd1 and wt at this time-point still lays inside the photoreceptors metabolic cascades.

- cGMP, PKG, G-substrate

As already mentioned the RP photoreceptors show an accumulation of cGMP, which leads to activation of cyclic-nucleotide-gated cation channels (CNG), that in turn results in influx of calcium ions and finally photoreceptor degeneration. However, cGMP have many roles in the intracellular regulation and not only the function of opening cation channels, and may also affect gene expression through different cascade signals. In this context it targets cGMP-dependent protein kinases (PKG [or cGK]), a family of serine/threonine kinases, which are a 100-fold more sensitive than CNG channels to cGMP (Paquet-Durand, et al, 2009). Importantly, PKG can be one of the triggers in rd (Paquet-Durand, et al, 2009).

There are three different isoforms of PKG-I alpha, beta and PKG-II. Prkg1 and prkg2 are two PKG genes in mammals that encode PKG-I and PKG-II. The NH₂ terminal of PKG-I is encoded by two alternative exons that produce the isoforms PKGⅠα and PKGⅠβ (Hofmann et al, 2006). PKGⅠβ is activated at 10-fold higher cGMP concentrations than PKGⅠα. The distribution of PKG in the eye is not well studied. mRNA for PKG-I and PKG-II has been detected in GCL, INL and ONL, whereas PKG-I protein expression was highest in IPL, although it could also be seen in GCL, INL, OPL and inner segments but not in ONL (Gamm et al. 2000). Staining for phosphorylated PKG-substrate showed that such substrates were present in GCL and INL and, in the case of rd1, also in the ONL (Paquet-Durand, et al, 2009).

Figure 3, PN21, rd1. The remaining photoreceptor cells make up 1-2 rows in the ONL.
et al, 2009). This indirectly shows that PKG, which is responsible for the phosphorylation of these substrates, should also be present in the retina.

The staining for phosphorylated PKG substrates does not reveal the identities of the substrates in the ONL. Overall, there are more than 10 different substrates known to be phosphorylated by PKG, for example Vasp (Vasodilator-stimulated phosphoprotein, in smooth muscle), PDE5 (the most closely related protein to PDE6) (Cote, 2004), septin-3 (vesicle trafficking in brain) and a protein called G-substrate (Gamm et al, 2000, Endo et al, Nakazawa et al, 2005). Of these Vasp is one of those substrates that could be involved in the rd1 degeneration, since it was demonstrated in the ONL of this model (Paquet-Durand et al, 2009). However, also other substrates could be related to this disease.

Cellular activities are dependent on the phosphorylation status, which is the balance between protein kinases and protein phosphatases. In other words this means that both phosphorylation and dephosphorylation are important for cellular events. Two of the protein phosphatases known (PP1 and PP-2A) have major roles in regulation of cell function in different tissues (Sim, 1992), but they have not been well investigated in the eye. PKG is able to phosphorylate G-substrate, and phosphorylated G-substrate acts as an inhibitor of both PP1 and PP-2A (Endo et al, 2003).

Since PKG was found to have a role in the photoreceptor degeneration of the rd1 model (Paquet-Durand et al, 2009), it is of interest to better understand how the enzyme and the degeneration connect. G-substrate has been detected in brain (human) and eye (mouse or rat) (Hall et al, 1998, Nakazawa et al 2005) and maybe there is a relationship between the increased PKG activity in the degenerating rd1 photoreceptors and the control of phosphatase activity via the G-substrate. My goal with this study was therefore to observe if G-substrate and phosphatase activity correlate and if there is a difference between rd1 and wt in this context.

Material and Methods

The use of the animals was approved by the local ethics committee (M242-07, M221-09). Wild type mice and retinal degeneration 1 mutant mice are from C3H strain and homozygous and hereafter referred to as wt and rd1, respectively. The animals were stored in cages with free access to water, food and a light cycle in 12 hours.

Day of birth was considered as postnatal day 0 (PN0). Up to PN5 the pups were sacrificed by decapitation and older mouse above PN5 were sacrificed by asphyxiation on dry ice. PN11 is a good time-point for comparisons between rd1 and wt, since the rd1 and wt retinas have same thickness at this stage, and that is why the largest part of the comparisons used PN11 tissue. For this age, at least five different retinas of each type were analysed. All assays were made to minimize the number of animals used.

To make rd1 and wt comparisons more relevant, all photos were taken from the central part of the retina.

- Fixation and Embedding

Immediately after the eyes were removed they were fixed in 4 % paraformaldehyde in 0.1M Sörensen’s phosphate buffer (pH 7.2) for two hours. Later the eyes were washed in Sörensen’s phosphate buffer (BPS 0,1M) 4x15 minutes each. After washing the eyes they were transferred to Sörensen’s phosphate buffer 10 % sucrose and kept overnight at 4°C. The next day the eyes were moved to sörensen phosphate buffer with 25 % sucrose for cryoprotection. The eyes were embedded with Yazulla (consisting of egg albumin, gelatin and distilled water). Then they were mounted in the cryostat with TissueTek and the cut at 12µm. The sections were stored at -20°C.
- Histochemistry; ATPases, Phosphatases and Hematoxylin&Eosin

Hematoxylin and Eosin
This staining was performed to see the morphology of the entire retina and to observe the differences between rd1 and wt, in different ages. The sections were first air dried and then stained in hematoxylin (Fluka) for 3 minutes then washed in flowing tap-water for 5 minutes and rinsed in distilled water. Next the slides were stained in eosin for 1 minute and dehydrated with alcohol. At last the sections were washed in tissue-clear (HistoLab) and mounted with Pertex (HistoLab).

ATPases
To investigate how the phosphatase activity is distributed in the retina, I started to study the distribution of an enzyme which also removes phosphate groups, namely ATPase (ATPase histochemistry). The cleaved phosphate group is made to precipitate and to become visible by other components in the developing buffer (soluble lead nitrate is converted to insoluble lead phosphate).

The sections were produced as previously described. The sections were air dried for 45 minutes, then washed 3x5 minute with 0.2 M Tris-maleate buffer (pH 7.2). The slides were incubated with reaction buffer consisting of Tris-maleate buffer (pH 7.2 and 0.2M), MgSO₄ (0.1 M), Pb(NO₃)₂ (2 %), dextrose and disodium salt ATP between 30-60 minutes. The slides were then washed again with distilled water and the product from the incubation reaction was developed with (NH₄)₂S (1 %) solution between 30-120 seconds, thereafter the slides were washed again. This was then followed by mounting the sections; with glycerol or with Vectashield. The negative controls were incubated in the same medium without the substrate (ATP) (Pérez-Torres et al, 2002).

Phosphatases
To analyse the activity of phosphatases in retina, slides were washed in distilled water 3x5 minutes. Equal amounts of two different phosphoamines (with different pH and concentration of Tris-base in the buffer) were used in the incubation buffer. The sections were incubate with the incubation solution containing lead-maleate buffer and phosphoamine. One of the phosphoamines was o-phospho-L-tyrosine containing 20 mM o-phospho-L-tyrosine and 33 mM Tris base in a volume of 20 ml. The lead-maleate solution was prepared with 100 mM Tris-maleate (pH 6.0), 3.75 g of sucrose and at last a drop-wise addition of leadnitrate (1 ml, 100 mM). The two different solutions, incubation solution and lead-maleate solution, were mixed in proportion 1:1 and 100 µl was applied to each slide. The slides were incubated in 37°C in one, two, four and six hours respectively. Finally the slides were washed 3x5 minutes and then developed using ammonium sulfide solution (NH₄)₂S (0.3 % or 1 %). The slides were mounted with glycergel or Vectashield and stored in freezer (-20°C).

The other phosphoamine (o-phospho-L-serine) was used in the same way, but a different solution in the incubation buffer was used with pH 7.2 (82 mM Tris and 20 mM of o-phospho-L-serine). Another difference was that the Tris-maleate buffer had pH 7.2. Control slides were incubated in the same medium but without the substrate (o-phospho-L-serine or o-phospho-L-tyrosine) (Partanen, 2008).

-Immunofluorescence
The sections were first air dried in room temperature for 15-30 minutes, then rinsed in phosphate buffer saline (PBS) for 3x5 minutes. The sections were pre-incubated with 5 % blocking serum (donkey or goat, where appropriate) for 45 minutes.

The antibodies used in the assays are referred in table 1 and 2. The primary antibodies were diluted in PBS containing 1 % Bovin Serum Albumin (BSA) and 0.25 % Triton X-100. The slides were applied with 100 µl each. For the negative controls, sections were incubated without the primary antibodies. The sections were incubated over night at 4°C. The next day the slides were washed with PBS 3x5 minutes and incubated with fluorescent secondary antibodies for 45 minutes at room temperature. The secondary antibody (table 2) was also diluted in PBS with 1 % BSA and with 0.25 % triton. After incubation the slides were rinsed in PBS 3x5 minutes and mounted with the anti-fading mounting medium Vectashield containing DAPI (Vector) or with glycergel containing Hoechst.

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Both DAPI and Hoechst stain the nuclear in the cells. The staining was analysed with fluorescence microscope equipped with a digital camera.

Slides from at least five mice (rd1 and wt) of PN11 was stained and analysed. For other types of preparations 1-3 independent animals were studied.

### Table 1

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<th>Primary antibody</th>
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### Table 2

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<td>Goat anti-Mouse:HRPO (Immunoblot analysis)</td>
<td>1:40000</td>
<td>BioSite, Täby, Sweden</td>
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- Immunoblot analysis

Eyes were collected from mice at PN11; rd1 and wt; three retinas of each phenotypes were pooled for a sample. The retinas were homogenized in sample buffer (2 % SDS, 10 % glycerol, 0.0625 M Tris-HCL pH 6.8) and the samples were centrifuged at 10000xg for 10 minutes. The supernatants were collected and either stored in the freezer (-20° C) or quantified with respect to protein concentration according to the BIO-DC protein assay kit (BioRad). The samples were boiled for five minutes and loaded in the gels. Electrophoresis was run with samples of 10 µg protein, which was then separated in a SDS-polyacrylamide gel (6.8 % stacking gel, 11 % separation gel). The ladder “PageRuler™ Prestained Protein Ladder” (Fermentas GmbH,) was used. The electrophoresis running conditions was 100 minutes and 150 V. The gels were transferred onto 0.2 µm PVDF membranes (Polyvinylidene Fluoride membrane) (Millipore). The membrane was incubated for 2 hours in blocking solution (5 % dry milk in Tris buffer with 0.1 % Tween-100, TBS-T) and then incubated over night (4°C) with primary antibody (Table 1, Mouse Anti-Phospho-G-substrate (phospho T68/119). The primary antibody, phospho-G-substrate, was diluted with blocking buffer in four different concentrations 1:500, 1:1000, 1:2000 and 1:4000. The membranes were washed 1x15 minutes and 3x5 minutes each in TBS-T. The secondary antibody (Goat-anti mouse HRPO (Horseradish Peroxidase), table 2) was also diluted in blocking buffer, incubated for one hour and then the washed as before. For development the 50 % HRP substrate peroxydase was mixed with 50 % HRP substrate luminol and then incubated for five minutes. For development Hyperfilm from GE healthcare bioscience was used.

The antibodies were removed by incubating the membrane in stripping buffer (15 g glycine, 1 g SDS, 10 ml Tween, 1000 ml Millipore water, pH 2.2) for 20 minutes. The membranes were rinsed, blocked and then reused with mouse alfa-tubulin (loading control) as earlier described.

Results

The purpose of doing a hematoxylin and eosin staining was to receive a histological picture of the different stages of photoreceptor degeneration in the rd1 model (figure 1, 2 and 3).

The ATPase method worked well when modified and suited for the different sections. The used sections were in vivo PN9, PN11, PN13, as well as from in vitro cultures (with PN11 as the end-point) and in every case retina from both wt and rd1 were used. Incubation time and development time were all individually modified to get the best results for the different ages and for the cultures, respectively. This method, ATPase histochemistry, had to my knowledge never been used in order to study possible differences between wt and rd1 before.

A microscope analysis of the sections did not reveal any direct differences between rd1 and wt. Interestingly, however, there was a consistent difference in the colour tones of the staining within the photoreceptor layer in both rd1 and wt samples, so that the outer and inner borders of this layer were more stained than the rest of the ONL (Fig. 4). The tone difference was seen also in culture, both in rd1 and wt. Another finding was that in the INL and the GCL certain sub-nuclear structures had significantly stronger staining than the rest of the nucleus. The ATPase results were not examined further.
In the retina phosphatase histochemistry, using phospho-amino acids as phosphate sources, the negative control did give the same results as the samples, so that in both cases you could see some kind of pale staining. It is not clear whether this staining represented some specific structures or not. The use of o-phospho-L-tyrosine on the section did not result in any staining and with o-phospho-L-serine the sections was partly stained. For this reason only o-phospho-L-serine was used in further examinations. The outcome of the phosphatase staining was the same in *in vitro* preparations and in those from different *in vivo* ages (PN9-PN13). Furthermore there was no direct difference between rd1 and wt.

In order to verify the method (phosphatase histochemistry) and see if it at all can work correctly other tissues were tested. Previous studies have shown that phosphatases of the kidney, but not liver, can be visualized by this type of histochemistry (Partanen, 2008). In my hands the staining of liver and kidney correlated with the results from the referred article, with staining of the kidney (figure 5) but no staining on the liver (figure 6). In the negative control for the kidney the same pale staining as in the wt and rd1 retina could be seen.

Figure 4 ATPases, PN11, left wt and right rd1. The upper layer, ONL, consist of photoreceptors. INL is in the middle layer consist of bipolar cells, amacrine cells and horizontal cells. The last layer consists of gangliie cells. In ONL (upper layer) there is a colour difference; the outer and inner parts are more brownish than the central parts.

Figure 5 Phosphatase activities in kidney, left negative control and right sample. Some small pale staining in the negative control can be seen. The staining in the right picture is relatively clear, which should indicate true phosphatase activity.
Immunofluorescence for phospho-G-substrate revealed staining in several layers, including ONL, INL and GCL (Figure 7). The negative control (omission of the primary antibody) did not give any staining other than of blood vessels, which comes from the secondary antibody. Importantly, there was a difference in the ONL between the rd1 and wt. In comparison, the ONL in the rd1 retinas were more intensely stained than that of the wt retinas (Figure 7). Furthermore, the ONL staining in rd1 seemed less concentrated/organized in the nuclear structures (had a smoother appearance) than in the wt. At the same time the intensity of INL staining in rd1 was less than in wt, suggesting that the increased staining in the rd1 ONL is not the result of a generally elevated immunoreaction in the rd1 sections compared to wt.

Different ages (PN9 to PN13) were examined but the results were not different from the age of PN11, indicating that the rd1 ONL has more phospho-G-substrate also in these situations.

In vitro preparations representing PN11 were also examined but results were difficult to interpret. The immunoblot analysis did not give any results; the antibody (Anti-phospho-G-substrate) did not work in any of the concentrations tested (table 1). To confirm that the electrophoresis and the blotting had worked, immunodetection of α-tubulin was done on the same membrane afterwards. The result from α-tubulin was correct, and showed a band in the right region.
Discussion

The mechanism for the ATPase histochemistry works by adding ATP to the sections and if there is ATPases in the tissue the phosphate will be cleaved off and then precipitated. This principle may be modified through exchanging the ATP for some other substrate that contains phosphate. If so the phosphate can be cleaved off by an enzyme of interest, like in the present study were phosphatases were investigated. Here I used o-phospho-L-tyrosine and o-phospho-L-serine since these were supposed to act as ATP and give a precipitate in the same way when there were tyrosine- or serine-phosphatases present, respectively.

No proper retinal phosphatase staining could be obtained. The facts that the kidney samples gave precipitates similar to what had previously been shown (Partanen, 2008) suggest that the method actually works but that there are only very low levels of phosphatase activity in the retina. The Partanen study (2008) used also other variants of this method for the staining of phosphatase activity and it is possible that these would have worked better here, but they were not tried.

In the retinal assays (both rd1 and wt) there was some pale staining which was not the case in the liver. The reaction solution contain lead nitrate and it cannot be excluded that the retinal preparations contained both a phosphate donor and an enzyme that could cleave it and therefore a precipitate. Perhaps these molecules were absent from the liver. Regardless of the reason for the lack of suitable staining, it is not possible to here conclude whether there is a difference or not in phosphatase activity in rd1 and wt retinas.

The results from the immunofluorescence are compatible with a higher PKG phosphorylation of G-substrate in the ONL cells of the rd1 mouse, which is a new finding. Such an elevation in rd1 photoreceptors could mean that the inhibition of PP1 and PP2 is here larger than normal. This would then suggest that the normal phosphatase activity by PP1 and PP2a is lower in these cells. In other words the balance between phosphorylation and dephosphorylation would be disturbed so that proteins are more phosphorylated. Interestingly, an increased level of phosphorylation has previously been shown in the rd1 photoreceptors for several proteins (Johnson et al 2005, Hauck et al 2006, Azadi et al 2006)

With the immunoblot analyses I wanted to confirm the presence of G-substrate within the retina, but this assay did not give any results. However, there is evidence that G-substrate is expressed in the retina (Nakazawa T, 2005), and in that case two other antibodies were used. Furthermore, the phospho-G-substrate antibody gave results in immunofluorescence staining, which suggest that the G-substrate can be found in the retina. Maybe the antibody used here is not well suited for Western blot and there was no information from the provider about this. There are still things that can be changed, and perhaps improved, by using the phospho-G-substrate in Western blot, but this needs more evaluation.

In conclusion, my results suggest that photoreceptor cells that have high PKG activity (Paquet-Durand et al., 2009) phosphorylate more G-substrate, since the phospho-G-substrate appeared to be elevated in the ONL of the rd1 compared to the wt. Possibly, this contributes to a disturbed balance between phosphorylation and dephosphorylation disturbing, by the G-substrate’s influence on protein phosphatases (PP1 and PP2A). The main task of phosphorylation lies in the control and regulation of functions in the cell and an interference with this might be a contributing reason for the degeneration of rods in retinitis pigmentosa.

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