C. R. Biologies 337 (2014) 207–213

Contents lists available at ScienceDirect

Comptes Rendus Biologies

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Spotlight on vision

Therapeutic strategy for handling inherited retinal degenerations in a gene-independent manner using rod-derived cone viability factors

Une stratégie thérapeutique des dégénérescences rétiniennes héréditaires indépendante du gène causal : la voie des facteurs de survie des cônes

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ARTICLE INFO

Article history:
Received 2 December 2013
Accepted after revision 2 December 2013
Available online 20 February 2014

Keywords:
Vision
Cone photoreceptors
Nucleoredoxin-like
TAU
Thioredoxin
Redox signalling

Mots clés :
Vision
Photorécepteurs à cône
Nucleoredoxin-like
TAU
Thiorédoxine
Signalisation redox

ABSTRACT

The most common hereditary retinal degeneration, retinitis pigmentosa (RP), leads to blindness by degeneration of cone photoreceptors. Meanwhile, genetic studies have shown that a significant proportion of *RP* genes is expressed only by rods, which raises the question of the mechanism leading to the degeneration of cones. Following the concept of sustainability factor cones, rods secrete survival factors that are necessary to maintain the cones, named Rod-derived Cone Viability Factors (RdCVFs). In patients suffering from RP, loss of rods results in the loss of RdCVFs expression and followed by cone degeneration. We have identified the bifunctional genes nucleoredoxin-like 1 and 2 that encode for, by differential splicing, a thioredoxin enzyme and a cone survival factor, respectively RdCVF and RdCVF2. The administration of these survival factors would maintain cones and central vision in most patients suffering from RP.

RÉSUMÉ

La dégénérescence héréditaire rétinienne la plus fréquente, la rétinopathie pigmentaire (RP), conduit à la cécité par dégénérescence des photorécepteurs à cônes. Pourtant, les études génétiques ont montré qu'une proportion significative des gènes de *RP* n'est exprimée que par les bâtonnets, ce qui soulève la question du mécanisme entraînant la dégénérescence des cônes. Suivant le concept de facteur de viabilité des cônes, les bâtonnets sécrètent des facteurs de survie qui sont nécessaires au maintien des cônes. Chez les patients souffrant de RP, de la perte des bâtonnets résulte celle de ces facteurs et donc la dégénérescence des cônes. Nous avons identifié les gènes bi-fonctionnels

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1631-0691/$ – see front matter
http://dx.doi.org/10.1016/j.crvi.2013.12.002

nucleoredoxin-like 1 et *2*, qui codent chacun par épissage différentiel pour une enzyme de type thiorédoxine et un facteur de survie des cônes. L'administration de ces facteurs de survie permettrait de maintenir les cônes et la vision centrale chez la plupart des patients souffrant de RP.

1. Introduction

In patients suffering from retinitis pigmentosa (RP), the most common form of inherited retinal degeneration, the vision loss develops in two successive steps. Early in their adult life, these patients lose their ability to see in dim light conditions, which refers to a night vision lost, and corresponds to the loss of function and degeneration of rod photoreceptors. This is felt as a minor handicap, especially in individuals affected by congenital stationary night blindness, another type of inherited retinal disease characterized exclusively by lack of rod function; in our current well-illuminated environment, these people retain an almost normal way of life [1]. For patients with RP, the disease then progresses through another debilitating step resulting from loss of function and degeneration of the second class of photoreceptors, the cones, which dominate at the centre of the retina. Cones represent only 3–5% of all photoreceptors in most mammals, but their role for vision is essential. This secondary event leads to central vision loss and potentially complete blindness. Because the cones underlie all visual functions in lighted environment, cone rescue was deemed to be a clinically relevant target. Cone death being widespread cone death in the naturally occurring *rd1* mutant mouse, a model of RP, has been well described [2]. The degeneration does not arise in this model through a mutation within cone photoreceptor cells, but as a result of a recessive mutation in the rod photoreceptor-specific cGMP phosphodiesterase-β subunit, and is consequently non-cell autonomous [3]. This mutation also leads to rod-cone degeneration in humans [4]. Various hypotheses have been proposed to explain the secondary loss of cones: toxic by-products of rod cell death, structural alterations in the microenvironment, abnormal synaptogenesis, secondary changes at the level of the retinal pigmented epithelial or the glial cells, and loss of trophic interaction, reviewed in [5]. To discriminate among the possibilities, we demonstrated that grafting normal photoreceptors (97% rods) into the eye of the rodless *rd1* mouse before the cones degenerate exerts a positive effect on the host retina cones [6]. Subsequent work *in vitro* showed that this paracrine protective activity was carried by molecules, most likely proteins, secreted in the presence of rods [7,8]. Globally, this part of our work revealed the existence of proteins that we designated as Rod-derived Cone Viability Factors (RdCVFs) [9] and was recently further supported by the work of Punzo et al. [10]. If so, following rod death in the first phase of the disease, the degeneration of cones would be triggered by a mechanism reminiscent of the loss of trophic support. This cone degeneration mechanism is also likely to occur in human retinas presenting RP, when most rods have degenerated but before central vision is affected [11].

Since the cones represent only 3–5% of the photoreceptors in most mammals, we studied the survival of cones in cone-enriched cultures prepared from the retina of chicken embryos [12,13]. When plate at low density and chemically defined medium, the progenitor cells isolated from chicken embryos at embryonic day 6, stages 29 [14] differentiate into mostly photoreceptors in the absence of cell fade cues. Since the retinas of birds, contrarily to mammals, are dominated by cones, these primary post-mitotic cultures are enriched (60–80%) in cones. These cones develop *in vitro* a complex set of photoreceptor-specific properties, as polarized structural and opsin immunoreactivity. Interestingly, they are also capable of responding to light [15]. When maintained on a 12-h-light/12-h-dark cycle, approximately half of the cultured photoreceptors elongate in response to light, and contract in response to darkness [16]. These primary post-mitotic neurons degenerate over a period of a few days. We observed an increase in cell survival when cultured in the presence of fractions of secreted proteins isolated from the retina of wild-type mice [8]. The active molecule(s) are heat labile, with an apparent molecular weight of 25 kDa. Because of the simplicity of getting fertilized eggs, the chicken embryo retinal culture system is an easy, reproducible, and high-throughput cone viability assay. We made the hypothesis that RdCVF protein is encoded by a messenger RNA expressed in the normal retina and the encoded polypeptide would be secreted by any cell types. Based on this minimal two-term definition, we constructed an expression library from wild-type mouse retina and used expression cloning methods to screen genes for their potential to promote chicken cone survival. Briefly, pools of 100 clones from the expression library were used to transfect a cell line (COS-1). The conditioned media from the transfected COS-1 cells were added to primary chicken cone cells seeded into 96 well-plates. After seven days, viable cells from the cone-enriched cultures were counted using an in-house high content screening method and were compared to counts from the empty library vector. We screened 2100 pools, corresponding to 210,000 individual clones. Pool number 939 contained twice as many living cells as the negative controls. Using limiting dilution, we isolated clone 939.09.08 and found that it contained a 502 base pairs insert with an open reading frame encoding a putative 109 amino acids polypeptide, Rod-derived Cone Viability Factor [17]. This unbiased approach let us to identify a novel gene called now *nucleoredoxin-like 1* (*Nxnl1*). RdCVF is translation product made from an unspliced mRNA encoding the exon 1 with an in-frame stop codon of the nucleoredoxin-like gene (*Nxnl1*) and corresponds to a truncated thioredoxin-like protein with no thiol-oxidoreductase activity (Fig. 1a). The other product (RdCVFL),

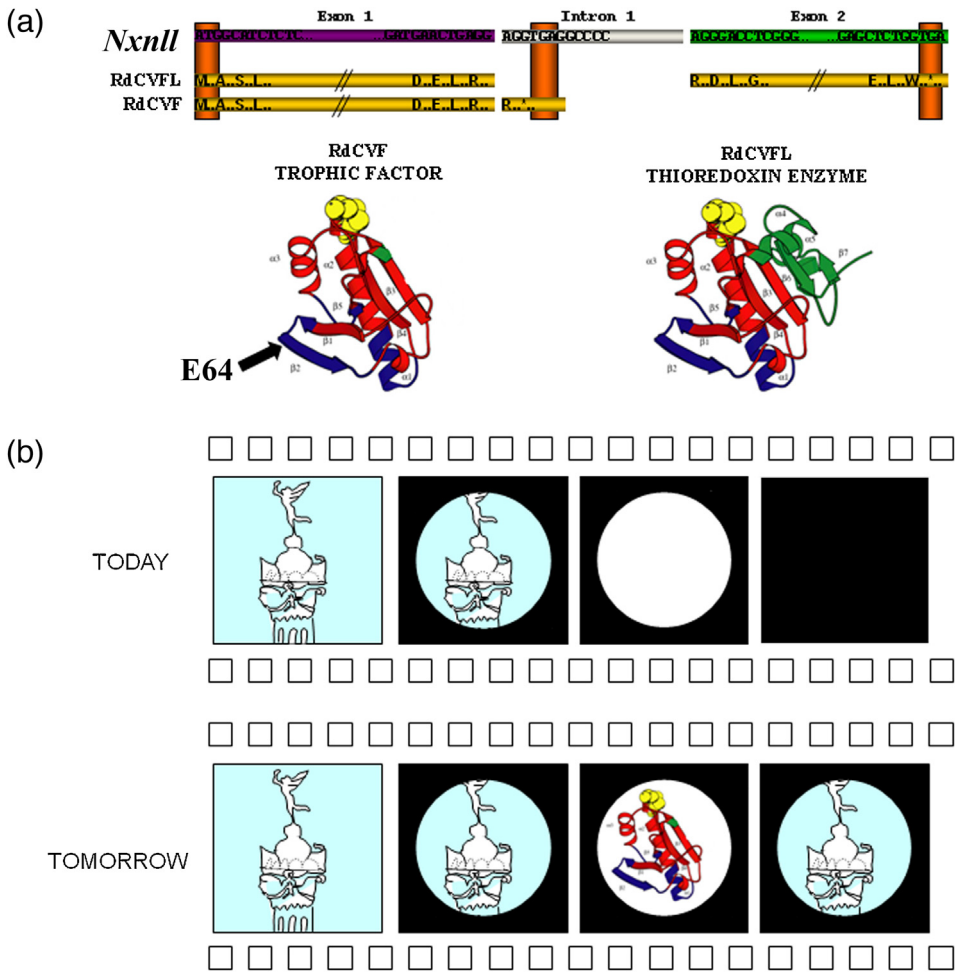

Fig. 1. (Color online) RdCVF redox signalling: **a**: schematic representation of the *Nxnl1* gene encoding for the trophic factor RdCVF and the thioredoxin enzyme RdCVFL by differential splicing. Ribbon colours: red matching tryparedoxin structure; green, only present is RdCVFL; blue, specific to *Nxnl1*. Yellow balls, the thioredoxin active site CPQC. The conserved glutamic acid residue at position 64 is indicated by an arrow; **b**: today: schematic representation of RP, with the secondary loss of central vision proceeding over 20 to 30 years. Tomorrow: preservation of central vision loss by administration of RdCVF. For interpretation of references to colour, see the online version of this article.

made by splicing intron 1 of the *Nxnl1* gene, is an active thioredoxin enzyme [18–20]. *Nxnl2*, the paralog of *Nxnl1*, also encodes for at least two proteins: RdCVF2, a trophic factor produced by the rods and active on cones, and thioredoxin protein RdCVF2L by differential splicing [21,22]. In the mouse, the *Nxnl1* is expressed by cells in the layer of photoreceptors and also by bipolar cells (interneurons located between the photoreceptors and the ganglion cells that project axones through the optic nerve). The *Nxnl1* promoter is activated by VSX2, CRX, SP3, and PAX4 [23,24]. Surprisingly, *Nxnl1* expression by bipolar cells is lost after rods have degenerated in the *rd1* retina, even if the viability of these cells is not compromised. In human retina, *NXNL1* expression is reduced in conjunction with that of *RHO*, a marker of rods following retinal detachment [25]. The injection of purified GSTRdCVF protein into the eye of the *rd1* (*Pde6b* mutant) slows down the secondary degeneration of cones [17]. The RdCVF protein is particularly well suited for preventing the secondary degeneration of cones and for transforming RP in a type of night blindness associated with moderate visual impairment, almost independent of the causative mutations [26] (Fig. 1b). *PDE6B* mutations are the cause of only 4% of autosomal recessive RP in man, while mutations in the rhodopsin gene (*RHO*) are involved in ∼25% of autosomal RP (http://www.sph.uth.tmc.edu/Retnet/) [27]. We proceeded to the sub-retinal injection of a synthetic RdCVF protein into the eye of the transgenic P23H rat. The P23H rat is a model of autosomal dominant RP, which overexpressed under the control of the rhodopsin promoter a *Rho* transgene carrying the most prevalent mutation (P23H) in the United States. The secondary degeneration of cones in that model could be partially blocked over a period of three months by three monthly injections of RdCVF [28]. This supports the fact that the therapy using RdCVF is gene-independent and could be effective for the vast majority of patients carrying mutation in any of the 43 known RP genes. With a prevalence of 1/5000, RP is the

most common form of inherited retinal degeneration affecting about 1.5 million people worldwide [1]. The Center for Clinical Investigation (INSERM) of the "Centre national ophtalmologique des Quinze-Vingts", the largest eye clinic in continental Europe, which is located in the direct vicinity of the "Institut de la vision", is following genotyped RP patients for already more than 10 years. It will enroll patients that will enter a future RdCVF clinical trial based on genotypic and clinical data (http://www.institut-vision.org).

Because in the P23H rat model, the degeneration of the cones proceeds over a period of several months, contrarily to few weeks for the *rd1* mouse, it was possible to test the functionality of the cones by electroretinography (ERG). We could demonstrate that the injection of RdCVF prevents the loss of visual function of the cones [28]. The amplitude of the protective effect recorded by photopic ERG was found five orders of magnitude higher than the survival effect measured by the viability of the cones. This intriguing observation led us to analyse the morphology of the outer segment of the cones, a specialized cytostructure containing the visual opsin molecules. We showed that the cone outer segment is significantly preserved by RdCVF, suggesting that the survival effect might be an indirect consequence of the maintenance of a photoreceptor structure essential for vision. This finding further enhances the therapeutic significance of RdCVF as, for example, the only factor currently tested in clinical trials, CNTF, induces in animal models an increase in the number of cells and a decrease in the ERG amplitudes [29–31]. The secondary degeneration of cones in RP patients occurs over a period of more than a decade. The strategy aims at preventing their loss through the use of RdCVF is medically rational, since most patients that the ophthalmologists see have already loss most of the rods. The chance of success of this therapy is great, since in human, the loss of 50% of the cones does not reduce visual acuity, and even with 90% cone loss a patient retains visual performance [32]. Nevertheless, the deliverance of RdCVF through protein injection is not satisfactory over extended periods of time. Consequently, we use adeno-associated viral vector (AAV2/8) to deliver RdCVF by sub-retinal injection. One single injection is sufficient to slow the secondary cone degeneration in the P23H rat or even to block completely the degeneration of cones in the *Nxnl2*−/− mouse [22].

At the cutting edge of the approach aimed at translating biological research into clinical practice is the successful ongoing gene therapy trial using the *RPE65* gene for treating patients suffering from a severe form of inherited retinal degeneration, Leber Congenital Amaurosis (LCA) [33]. In 2001, the disease was successfully cured in a dog model of LCA by delivering a normal copy of the gene by an AAV [34]. The efficacy of this corrective gene therapy approach was confirmed in subsequent clinical trials conducted by three independent groups in 2008 [35–37]. This was celebrated as a success story, biblical in impact [38]. Even though it resuscitates the interest in gene therapy, it has to be positioned in its medical context, i.e. the *RPE65* mutations do represent only 2% of the autosomal inherited retinal degenerations, and consequently this approach is applicable only to a limited number of patients, contrarily to RdCVF. The protective effect of RdCVF on cone and their function was also demonstrated in the *rd10* mouse, a RP model carrying a hypomorphic *Pde6b* mutation. Expression of RdCVF was accomplished via intravitreal injection of a novel engineered AAV variant vector (7m8). 7m8 was selected for its ability to transduce all retinal layers, including photoreceptors, from the vitreous with near-endogenous levels of protein expression and secretion. The panretinal transduction achieved by 7m8 obviates the need for penetrating subretinal injections, which requires retinotomy and retinal detachment, to deliver high levels of transgene to photoreceptors. The LCA clinical trials revealed that adverse effects, including retinal thinning and decrease in visual acuity were associated with subfoveal injections (the fovea is the centre of the primates retina enriched in cone photoreceptors and responsible of visual acuity), indicating that the human fovea is not able to tolerate this approach [39].

The strategy used to identify RdCVF by high content screening has revealed the existence of a novel class of trophic factors (RdCVF and RdCVF2) homologous to thioredoxin proteins. Bioinformatics analyses show that the *NXNL* genes were originally coding for tryparedoxin-like enzymes have acquired a trophic role during evolution by a mechanism involving inhibition of splicing of intron 1 [21] leading to the production of a protein truncated within thioredoxin structural domain. This novel ancestral protein has acquired its trophic role by binding to a surface receptor expressed by target cells. The mechanism leading to the inhibition of splicing therefore constitutes the initial event to the origin of the new signalling during evolution.

Interestingly, thioredoxins are proteins whose role in protection against oxidative stress has been described in great details [40]. In fact, treatment of antioxidants reduces cone cell death in the *rd1* mouse [41]. The inverse correlation between the rate of oxygen consumption and the life-span of eutherian mammals has directed the attention of aging research onto oxygen metabolism. It was discovered that reactive oxygen species (ROS), which are too reactive to exist in biological systems, are formed *in situ* in response to radiation and oxygen poisoning and are responsible for associated toxicity. Free radicals are continuously produced in the cell as products of aerobic life. In order to avoid the damage of macromolecules by ROS, proper redox conditions must be maintained within the intracellular environment. Therefore, aerobic organisms have developed several antioxidant systems, including superoxide dismutase, catalase, and thioredoxin (TXN) systems. The prototype of the thioredoxin proteins, TXN, is a 12 kDa protein with a redox active disulphide/dithiol group within the conserved active-site sequence CGPC. Reduced TXN catalyses the reduction of disulphide bounds in many proteins, and oxidized TXN is reversibly reduced by the action of the thioredoxin reductase and NADPH [42]. Secretion of TXN by a hitherto unknown mechanism that is not dependent on a signal peptide has been observed under conditions of oxidative stress [43].

Cysteine is a rarely used amino acid that accounts for about 2% of the amino acids in eukaryotic proteins. ROS as

well as reactive nitrogen species (RNS) can induce redox signals by means of oxidative modifications of cysteine residues. ROS and RNS, including hydrogen peroxide (H_2O_2), superoxide ion ($O_2^{\bullet -}$), nitric oxide ($NO^{\bullet}$), and hydroxyl radical ($OH^{\bullet}$), can be produced *in vivo* from a wide range of cellular processes, such as metabolism, photo-oxidation, inflammation, and senescence. The large, polarizable sulphur atom in a thiol group is electron-rich and highly nucleophilic; hence, cysteines can undergo a broad range of chemical reactions. C–SH is in equilibrium with C–$S^-$and to the disulphide S–S, can be oxidized by ROS to C–SOH, –SO_2H and SO_3H, or S-nitrosylated by RNS to C-SNO and finally, in the presence of glutathion (GSH), S-thiolated to –S–SG. Cysteines differ in their reactivity properties depending on the protein microenvironment, and not all cysteines are susceptible to modification. As many of these modifications are reversible through reduction catalysed by oxidoreductases, such as thioredoxins (TXNs), glutaredoxins (GRXs) sulphiredoxin (SRX), and sestrins (SESs), the protein thiol redox state can respond to the redox environment. Post-translationally modified cysteines are not necessarily directly involved in the catalytic activities of enzymes, but may function at an allosteric site and, thus, regulate the enzymatic activities or other protein functions through structural changes. Redox-sensitive cysteines have been identified in a wide spectrum of proteins including transcription factors: AP-1, NF-κB, p53, and HIF-1; signal transducers: cAMP-dependent protein kinase, mitogen-activated protein kinases, protein tyrosine phosphatase, apoptosis signal-regulating kinase 1, and stress response proteins, such as peroxiredoxins, superoxide dismutase, thioredoxin, and heat shock proteins. ROS have been shown to be toxic but also function as signalling molecules. This biological paradox underlies mechanisms that are important for the integrity and fitness of living organisms and their ageing. These pathways generally make use of ROS sensors that "measure" the intracellular concentration of ROS by a redox-based mechanism and proportionally set the expression of ROS-specific scavengers, thereby maintaining the concentration of ROS below a toxic threshold.

The disruption of the rod-derived cone viability gene leads to photoreceptor dysfunction and susceptibility to oxidative stress [9]. Cone function is normal in young *Nxnl1*−/− mice, but deteriorates month by month as the mice age, indicating that the gene is involved in the later (but not early) developmental stages, or aging. Furthermore, cones of the mice lacking *Nxnl1* are more sensitive to oxidative stress with a reduction of 65% in the cone flicker ERG amplitude measured under hyperoxic conditions. Interestingly, the rods are also affected over time, a fact that we attribute to the enzymatic function of RdCVFL more than to the trophic activity of RdCVF. This progressive retinal degeneration involves increasing oxidative damage, showing a relation between RdCVF signalling and redox signalling. We postulate that RdCVF is not only a therapeutic molecule but also a physiological signal involved in the maintenance of photoreceptors during aging and exposure to oxidative stress. This observation is also supported by results demonstrating higher susceptibility of the *Nxnl1*−/− retina to light-induced damage and in agreement with the fact that there is an attenuation of retinal photo-oxidative damage in thioredoxin transgenic mice [44]. Consequently, the degeneration of cones in RP may result from both the loss of a protective mechanism (i.e. RdCVFs), and the increased exposure to light and oxygen from the choriocapillaris resulting from the rod loss rather than a canonical loss of trophic support.

Oxidative stress has been suggested to be part of the initial pathogenic mechanism leading to age-related macular degeneration (AMD), the leading cause of blindness in the developed world. AMD is a genetically complex disease characterized by a progressive degeneration of cone photoreceptors and retinal pigmented epithelium in the macula, the centre of the retina around the fovea. The macula is subject to the highest levels of cumulative irradiation, also has the highest level of oxygen consumption of any tissue. AMD is genetically associated with the complement factor H gene *CFH*. Malondialdehyde (MDA) is a common lipid peroxidation product that accumulates in many physiopathological processes, including AMD. Recently, complement factor H (CFH) was identified as a major MDA-binding protein that can block both the uptake of MDA-modified proteins by macrophages and MDA-induced pro-inflammatory effects *in vivo* in mice. The CFH allele H402, which is strongly associated with AMD, markedly reduces the ability of CFH to bind MDA [45]. One should notice that we have observed signs of oxidative damage and elevated level of MDA associated with microglial cell activation in the retina of the *Nxnl1*−/− mouse, making the *Nxnl1*−/− a possible model of some aspect of AMD [9]. When we compared the effect of delivering through an AAV the two protein products of the *Nxnl1* gene in the *rd10* mouse, we observed that only RdCVF, but nor RdCVFL is able to protect the cones, even if the amino acid sequence of RdCVF is entirely included in that of RdCVFL. Conversely, only the thioredoxin RdCVFL, but not the trophic factor RdCVF, reduced lipid peroxidation (MDA concentration) triggered by rod degeneration.

Since many proteins possess a TXN-related domain, it is expected that each thioredoxin-like protein will act on a limited subset of specific substrates. In addition, proteome-wide identification of oxidized protein thiols shows that H_2O_2 does not cause random protein thiol oxidation [46]. Consequently, the identification of proteins for which the redox status is regulated by the thioredoxin RdCVFL produced by the *Nxnl1* gene through its interaction with O_2 is a prerequisite to understand the role of RdCVF and RdCVFL in the physiology of the retina. It is tempting to speculate that the two isoforms of the *Nxnl1* gene, RdCVF and RdCVFL work in the same regulatory system: the RdCVFL enzyme would be the sensor for oxidative conditions, coupling to the trophic protein RdCVF for an environmentally adapted response. We searched for RdCVFL targets by screening for interacting proteins using a proteomic approach [47]. We identified components interacting with the splicing machinery, ribosomal proteins and actin and microtubule binding proteins. The interaction with the splicosome is perhaps related to a possible regulation of *Nxnl1* splicing by its protein product RdCVFL. Among the actin and microtubule-binding proteins, we were particularly interested in the interaction of

RdCVFL with the microtubule-associated protein TAU (a protein that also interacts with microtubule), which is involved in the formation of neurotoxic aggregates in the brain of patients suffering from Alzheimer's disease. We found that TAU is hyperphosphorylated in the retina of *Nxnl1*−/− mice and, furthermore, that rod degeneration in these mice is associated with TAU aggregation in the retina [9]. *In vitro*, RdCVFL inhibits TAU phosphorylation and prevents oxidative damage by H_2O_2 on TAU [47].

While the mechanism of action of the thioredoxin likely requires its thiol-oxidoreductase activity, the way RdCVF is acting on cones is not mediated by an enzymatic reaction. We were unable to demonstrate any thiol-oxidoreductase activity for purified RdCVF [17]. It should be noticed, in parallel, that the founder member of the thioredoxin family, TNX, was originally isolated as a secreted protein, the adult T-cell leukaemia-derived factor [48]. TNX, as many thioredoxins including RdCVF, is secreted by cells by an unknown mechanism that does not require a signal peptide sequence [49]. TRX80, the only other example of truncated thioredoxin, is also secreted and has mitogenic activity toward cells of the immune system [50,51]. Extracellular TXN was shown to regulate cell surface tumour necrosis factor receptor superfamily member CD30 (TNFRSF8) on infected and transformed lymphocytes [52]. The catalytic activity of TXN modulates CD30-dependent changes in cellular behaviour and function. The kinetic trapping technique used to discover this interaction requires the presence of enzymatic activity and cannot be applied to RdCVF receptor identification. It nevertheless demonstrates non-ambiguously for the first time that the extracellular function of a thioredoxin member is mediated through the interaction with a cell surface receptor expressed by the targeted cells. The absence of RdCVF thioredoxin activity is most likely due to truncation within the thioredoxin fold, a motif comprising five beta sheets surrounded by four alpha helices and not to the absence of a thioredoxin catalytic (CPQC) that is included in the sequence of RdCVF (Fig. 1a). Among thioredoxins, *Nxnl1* is most closely related to nucleoredoxin and tryparedoxin. We used a homology modelling package to build a 1.7-Å resolution structural model of RdCVF using TryX-I from *Crithidia fasciculata* as a template [21]. In the model, the amino acid sequences that do not align to Tryparedoxin show a clear spatial proximity of the three RdCVF-specific insertions forming a putative interaction on the surface of RdCVF at the opposite pole of the catalytic site (Fig. 1a). This suggests the existence of a specific interaction involving this putative interaction domain with an RdCVF receptor expressed at the surface of cones. This putative interaction surface contains the epitope recognized by polyclonal antibodies that neutralize the trophic effect on cones in the *rd1* mouse retina mediated by the conditioned media of the wild-type retina. The neutralization may be due to steric hindrance of the antibodies when the RdCVF-specific surface interacts with an RdCVF receptor at the surface of cones.

The absence of thioredoxin activity and the existence of a putative interaction surface suggest that RdCVF acts on cones by activating a cell surface receptor. We directly demonstrated *in vitro* that there is a specific binding site on cones. We used a binding protocol with the cone-enriched culture system to look for a high-affinity binding of RdCVF on target cells. Human RdCVF (hRdCVF) and mouse RdCVF (mRdCVF) were synthesised and refolded (>90% purity). The chloramine T method was used to label hRdCVF with ^{125}I with a specific activity of 2130 Ci/mmol. The cells were incubated with [^{125}I]-hRdCVF. The specific binding was measured by competition assay with excess unlabelled recombinant mouse RdCVF. The level of radioactivity was partially reduced by 300 nM unlabelled mRdCVF, suggesting the presence of specific sites, despite the fact saturation is not reached in this range of [^{125}I]-hRdCVF concentrations (0.05–0.5 nM). This binding is specific to the target cells since no specific binding could be detected on pig retinal pigmented epithelial (RPE) or COS-1 cells. The identity of the RdCVF cell surface receptor would permit the development of pharmacological approaches to support cones, their role in maintaining central vision in patients suffering from RP.

Acknowledgements

We thank Emmanuel Moyse (Laboratoire de physiologie neurovégétative, case 352, Faculté St-Jérôme, avenue Escadrille-Normandie-Niemen, 13397 Marseille cedex 20, France) for its contribution to the binding experiments.

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