The EMBO Journal Vol.16 No.7 pp.1600–1609, 1997

Site-directed mutagenesis of highly conserved amino acids in the first cytoplasmic loop of *Drosophila* Rh1 opsin blocks rhodopsin synthesis in the nascent state

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The cytoplasmic surface of *Drosophila melanogaster* Rh1 rhodopsin (*ninaE*) harbours amino acids which are highly conserved among G-protein-coupled receptors. Site-directed mutations which cause Leu81Gln or Asn86Ile amino acid substitutions in the first cytoplasmic loop of the Rh1 opsin protein, are shown to block rhodopsin synthesis in the nascent, glycosylated state from which the mutant opsin is degraded rapidly. In mutants Leu81Gln and Asn86Ile, only 20–30% and <2% respectively, of functional rhodopsins are synthesized and transported to the photoreceptive membrane. Thus, conserved amino acids in opsin's cytoplasmic surface are a critical factor in the interaction of opsin with proteins of the rhodopsin processing machinery. Photoreceptor cells expressing mutant rhodopsins undergo age-dependent degeneration in a recessive manner.

Keywords: G-protein-coupled receptor/photoreceptor/protein processing/retinal degeneration/rhodopsin

Introduction

In visual transduction, the photopigment rhodopsin absorbs light and undergoes a series of conformational changes that leads to the activation of the transduction cascade. Rhodopsins belong to the family of G-protein-coupled receptors and related proteins, which now also includes a rhodopsin from the green alga *Chlamydomonas* (Deininger *et al.*, 1995). Except for chlamyrhodopsin, for which only four transmembrane helices can be identified, the characteristic structural feature of these receptor molecules are seven membrane-spanning helices which are connected by three loops on each side of the membrane. The cytoplasmic surface of the membrane protein rhodopsin, consisting of three helix-connecting loops (i1–i3) and the C-terminal peptide, is thought to provide the domain for interactions of rhodopsin with other proteins of the phototransduction machinery, e.g. the visual G-protein, arrestin, rhodopsin kinase and rhodopsin phosphatase. In vertebrate rhodopsin, the importance of particular sites for these interactions has been demonstrated by site-directed mutagenesis in loops i2 and i3 (Franke *et al.*, 1992) and by peptide inhibition (Krupnik *et al.*, 1994). In addition, it has been proposed that the cytoplasmic surface of the opsin molecule provides structural information for the post-translational modifications underlying rhodopsin maturation and for the targeting of rhodopsin to the photoreceptive membrane compartment (Deretic *et al.*, 1996). However, much less is known about rhodopsin processing and transport than about signal transduction. In vertebrates, Rab proteins are involved in the transport of newly synthesized rhodopsin (Deretic and Papermaster, 1995). In *Drosophila*, the transport of Rh1 rhodopsin, the major rhodopsin form (O'Tousa *et al.*, 1985; Zuker *et al.*, 1985), to its target membrane requires deglycosylation by an as yet unidentified enzyme (Ozaki *et al.*, 1993; Huber *et al.*, 1994) and is guided by the chaperone NinaA-cyclophilin (Schneuwly *et al.*, 1989; Shieh *et al.*, 1989; Colley *et al.*, 1991).

A need for the understanding of the structure–function relationship of rhodopsins comes from the finding that mutations in rhodopsin genes can lead to severe dysfunction and degeneration of photoreceptor cells. Since the early discovery that mutations of the *Drosophila* Rh1 gene induce photoreceptor cell degeneration (Leonard and Pak, 1984; O'Tousa *et al.*, 1989; Leonard *et al.*, 1992; O'Tousa, 1992), numerous mutations in the human rhodopsin gene have been discovered that cause inherited progressive retinal degeneration leading to the clinical symptoms of Retinitis Pigmentosa (RP) (for reviews see Nathans *et al.*, 1992; Berson, 1993). In *Drosophila*, rhodopsin mutant phenotypes are characterized by a reduced amount of visual pigment and a loss of the rhabdomere, the photoreceptive membrane compartment, in an age-dependent manner. Most of the *Drosophila* mutants isolated to date display a dominant degeneration phenotype (Colley *et al.*, 1995; Kurada and O'Tousa, 1995). Amazingly, except for a nonsense mutation which leads to a truncation in cytoplasmic loop i3 (ninaE^{ora} or ninaE^{JK84}; Washburn and O'Tousa, 1989), to date, no mutations have been analyzed which affect the cytoplasmic loops of Rh1 opsin. From studies in heterologous expression systems, two types of mechanism are proposed to underlie the retinal dysfunction and photoreceptor cell degeneration caused by mutations in the human rhodopsin gene (Sung *et al.*, 1991): in most cases, mutations cause incorrect folding or maturation of rhodopsin. As a result, rhodopsin transport to the photoreceptive membrane can be blocked, leading to disturbances in the balance of membrane turnover ('turnover defective mutants'). Few mutations represent 'transduction defective mutants'. In these cases, mutant rhodopsins are properly processed and targeted to the photoreceptor membrane but fail to correctly trigger the phototransduction cascade (Min *et al.*, 1993; Robinson *et al.*, 1994).

Within the cytoplasmic loop structure, rhodopsins show a high degree of conservation at the amino acid

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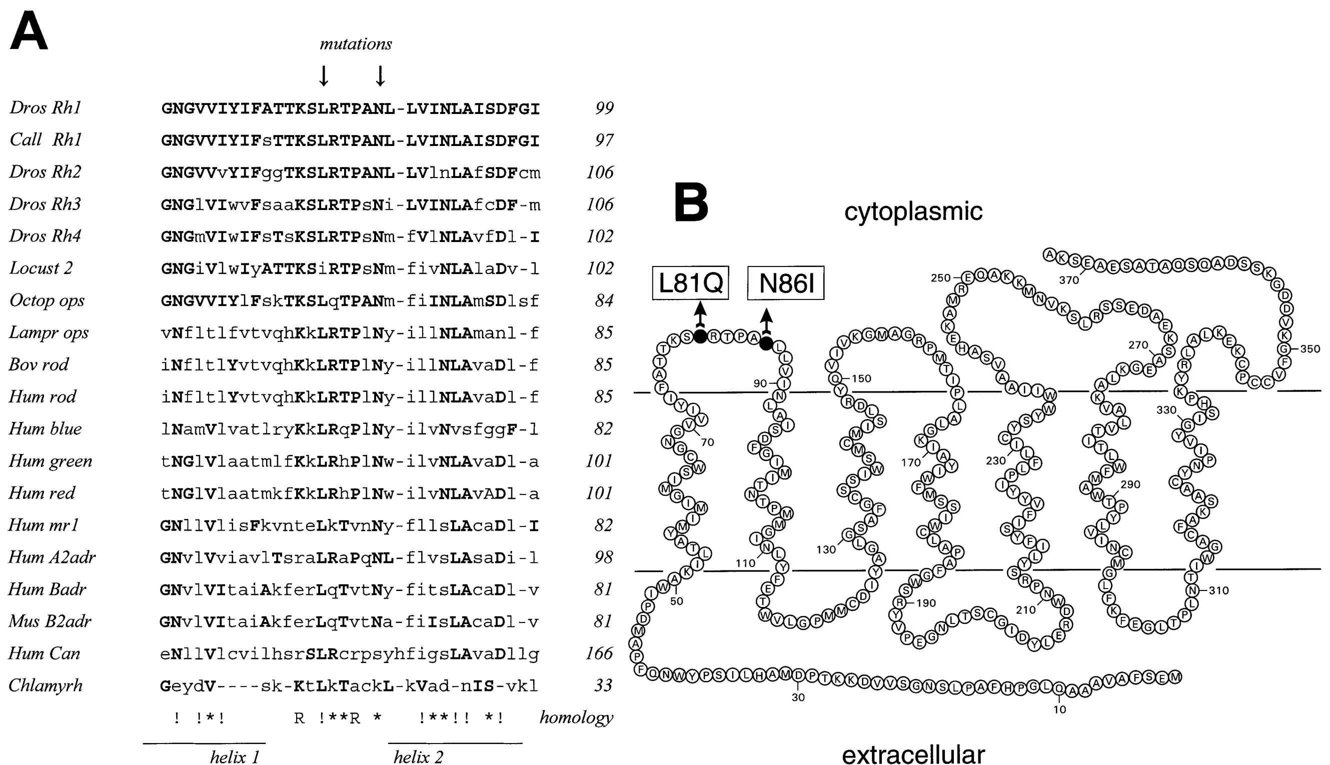

Fig. 1. Sequence comparison of G-protein-coupled receptors, and location of *Drosophila* Rh1 rhodopsin mutations. (**A**) Partial sequence alignment of receptors belonging to the G-protein-coupled receptor family, indicating amino acid conservation in cytoplasmic loop i1. The receptors chosen are representative of most of the general classes of G-protein-coupled receptors. Abbreviations: *Dros Rh1*, *Rh2*, *Rh3*, *Rh4*, *Drosophila melanogaster* rhodopsins (O'Tousa *et al.*, 1985; Zuker *et al.*, 1985, 1987; Cowman *et al.*, 1986; Montell *et al.*, 1987); *Call Rh1*, *Calliphora erythrocephala* R1–6 rhodopsin (Huber *et al.*, 1990); *Locust 2*, *Schistocerca gregaria* opsin 2 (Gärtner and Towner, 1995); *Ocotop ops*, *Octopus dofleini* opsin (Ovchinnikov *et al.*, 1988); *Lampr ops*, *Lampetra japonica* rhodopsin (Hisatomi *et al.*, 1991); *Bov rod*, Bovine rhodopsin (Ovchinnikov, 1982); *Hum rod*, human rod opsin (Nathans and Hogness, 1984); *Hum blue*, *green*, *red*; human rhodopsin of blue-, green-, or red-sensitive cones respectively (Nathans *et al.*, 1986); *Hum mr1*, human muscarinic receptor 1 (Peralta *et al.*, 1987); *Hum A2adr*, human kidney A_2-adrenergic receptor (Regan *et al.*, 1988); *Hum Badr*, human β-adrenergic receptor (Schofield *et al.*, 1987); *Mus B2adr*, mouse β_2-adrenergic receptor (Allen *et al.*, 1988); *Hum Can*, human cannabinoid receptor (Gerard *et al.*, 1991); *Chlamyrh*, *Chlamydomonas* rhodopsin (Deininger *et al.*, 1995). Amino acids identical to *Dros Rh1* are marked in upper case letters. (!) indicates only conservative exchanges in all sequences listed, as judged by exchange frequencies according to Schulz and Schirmer (1990). (*) denotes semiconserved amino acids, i.e. less then four non-conservative exchanges. (R) marks amino acids conserved among all rhodopsins listed, except for chlamyrhodopsin. (↓) indicates the amino acids mutated in the present study. (**B**) Proposed secondary structure model of *Drosophila* Rh1 opsin showing amino acid mutations in cytoplasmic loop 1, indicated by the original amino acid, location and mutation.

level among invertebrates, protozoans and vertebrates (Hargrave and McDowell, 1992; Deininger *et al.*, 1995; Gärtner and Towner, 1995). Cytoplasmic loop i1 in particular contains a stretch of amino acids highly conserved within the family of visual pigments, other G-protein-coupled receptors and related proteins, indicating that this region serves a function which is conserved in all receptor proteins and which is strongly dependent on the presence of specific amino acid residues. In the present study we assessed the functional importance of such conserved amino acids in *Drosophila melanogaster* Rh1 rhodopsin. Into the Rh1 gene (*ninaE* gene) we introduced point mutations leading to the exchange of single amino acids in cytoplasmic loop i1 of the Rh1 protein. Transgenic animals were generated in a Rh1-null mutant background, resulting in flies that only express the mutant Rh1 in the peripheral photoreceptor cells, R1–6. Photoreceptors R7 and R8 remain unaffected by this procedure. Flies expressing mutant Rh1 were investigated for alterations of rhodopsin processing, transport and function, as well as for ultrastructural changes of the photoreceptor cell.

Results

Site-directed mutagenesis and germ line transformation

Loop i1 of *Drosophila* Rh1 rhodopsin harbours several amino acids that are highly conserved among rhodopsins (Figure 1A) and other G-protein-coupled receptors. The sequence LRTPXN is not only conserved in the four *Drosophila* rhodopsins sequenced to date, but also in lamprey, bovine rod and human rod opsins. Most highly conserved of these amino acids are L81 (all but one receptor molecules listed) and N86 (all receptor molecules listed except for the human cannabinoid receptor and chlamyrhodopsin). To assess the functional importance of these two amino acids, we performed *in vitro* mutagenesis of the Rh1 gene, inducing point mutations that would result in substitutions L→Q and N→I respectively (Figure 1B). Both mutations lead to a change in hydrophilicity. Mutation Rh1 L81Q leads to the exchange of the hydrophobic amino acid leucine for a hydrophilic glutamine, Rh1 N86I replaces the hydrophilic amino acid asparagine with a hydrophobic isoleucine. The mutant genes were

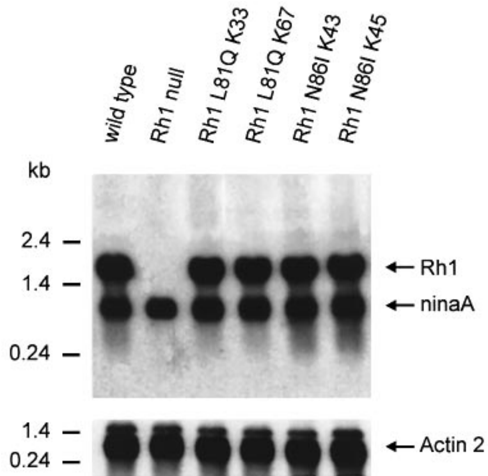

Fig. 2. Opsin gene expression in wild-type flies and Rh1 mutants. mRNA levels were examined by Northern blot analysis of total head RNA of the indicated fly strains. Upper panel: 5 µg RNA probed with Rh1 and *ninaA* antisense RNA probes; lower panel; 0.5 µg RNA probed with antisense Actin 2 RNA probe.

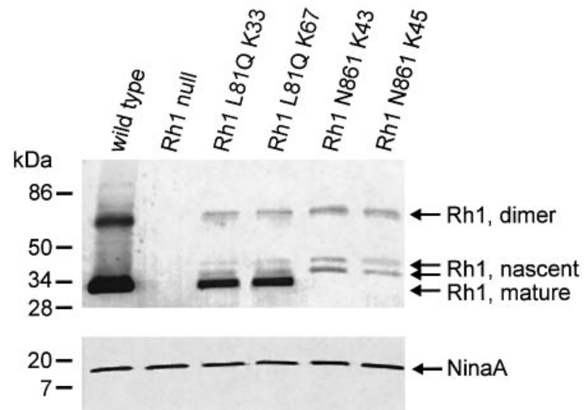

Fig. 3. Rh1 opsin levels in wild-type and Rh1 mutant flies. Immunoblots after separation by SDS–PAGE of protein extracts from eyes of the indicated fly strains. Upper panel: blot probed with antibodies directed against Rh1 opsin; lower panel: blot probed with antibodies against NinaA-cyclophilin.

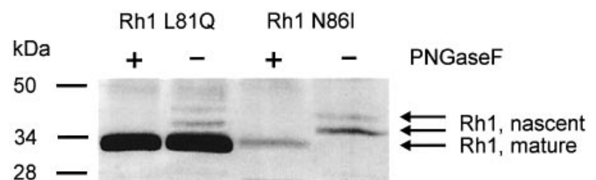

Fig. 4. Glycosylation state of Rh1 L81Q and N86I opsins. Protein extracts from eyes of the indicated fly strains were incubated with or without PNGaseF as indicated. The immunoblot after separation of proteins by SDS–PAGE was probed with antibodies directed against Rh1 opsin.

introduced, by P-element transformation, into the germ line of Rh1-null mutants, resulting in flies which express only the mutant rhodopsin in photoreceptor cells R1–6 of the compound eye. Several transformant lines were generated for each mutation, and the mutation was verified by sequencing the PCR-amplified Rh1 gene. Transformants were first checked by electroretinogram (ERG) measurements, and the lines showing the biggest ERG amplitude were chosen for further analysis. Initial ERG measurements had indicated that both mutations lead to a reduction in the level of functional rhodopsin, i.e. a reduction in the amount of rhodopsin capable of eliciting a normal ERG response. The present work focuses primarily on the steps in rhodopsin biogenesis and/or maturation that might be altered in these mutants.

Transcription of the opsin gene and opsin synthesis in mutant flies

Of each mutation, two independently generated transformant lines were tested. Northern blot analysis (Figure 2) shows that the abundance of Rh1 transcript in mutant flies is comparable with that of the wild type. To ascertain that all samples contained similar amounts of undegraded RNA, duplicate gels were run and probed with antisense actin RNA. These experiments show that the transcription of the opsin gene has not been altered as a result of the transformation procedure. Thus, the lowered amount of rhodopsin in the mutants is likely to result from disturbances of post-transcriptional steps of rhodopsin biogenesis. Next, at the post-transcriptional level, we investigated the content of opsin protein in the mutant photoreceptors by Western blot analysis, using a polyclonal antiserum directed against a loop i3-peptide of Rh1 opsin. Photoreceptors of Rh1 L81Q mutants contain an estimated one-fifth of wild-type opsin (Figure 3). In Rh1 N86I mutants, however, there is no evidence for the synthesis of a protein corresponding to mature opsin. Instead, in both mutants, two fainter bands of lower electrophoretic mobility are visible. These higher molecular weight species of opsin represent the nascent, glycosylated form of the protein (Huber *et al.*, 1994), as shown in Figure 4. Digestion of eye membrane proteins from Rh1 L81Q and N86I mutants with peptide-*N*-glycosidase F produces one protein band that co-migrates with mature, rhabdomeral opsin, which indicates that, in Rh1 N86I mutants, opsin accumulates in two glycosylated forms (Huber *et al.*, 1990; Ozaki *et al.*, 1993; Colley *et al.*, 1995), which are not processed to the mature, deglycosylated form. The amount of nascent opsin in photoreceptors of both these mutants is higher than in wild-type photoreceptors (all lanes in Figure 3 represent the same amount of protein). Thus, in both mutants, opsin tends to accumulate in the glycosylated form. Note that most of the opsin dimer which is formed after the membrane extraction procedure in the case of mutant flies is also in the glycosylated state, whereas the opsin dimer in wild type is in the mature, deglycosylated form (Figure 3). The NinaA-cyclophilin, a chaperone implicated in Rh1 opsin folding and required in the opsin secretory pathway, has been reported to form a stable complex with rhodopsin (Baker *et al.*, 1994). We, therefore, checked for possible up- or down-regulation of *ninaA* expression in the rhodopsin mutant flies and found that the *ninaA* mRNA and protein levels are unchanged, as compared with wild type (Figures 2 and 3). Transformant lines Rh1 L81Q^{K33} and Rh1 N86I^{K43} were chosen for further analysis.

Formation and functionality of the mutant rhodopsins

An important question is to what extent rhodopsin synthesis occurs in photoreceptors expressing mutant opsin genes, i.e. how much of the opsin detected by Western blot analysis is attached to the chromophore 11-*cis*-3-OH-

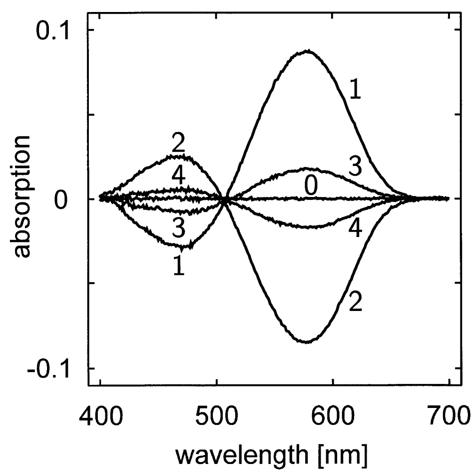

Fig. 5. Spectral properties of wild-type and mutant Rh1 rhodopsins. Light-induced absorbance changes of digitonin extracts of head membrane proteins from the indicated fly strains. Curve 1: wild type, conversion of rhodopsin to metarhodopsin with blue light, resulting in a net conversion of 69% of total visual pigment; curve 2: wild type, total conversion of metarhodopsin to rhodopsin by red light; Curve 3: Rh1 L81Q, conversion of rhodopsin to metarhodopsin with blue light; curve 4: Rh1 L81Q, total conversion of metarhodopsin to rhodopsin by red light; curve 0: baseline.

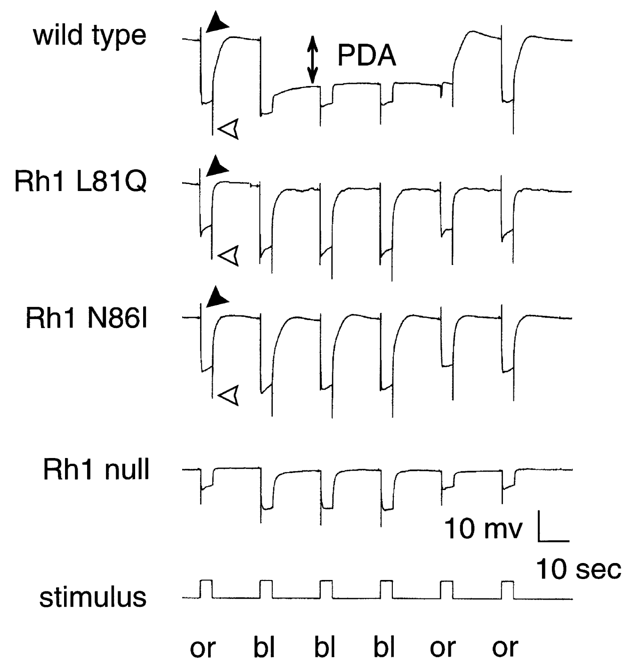

Fig. 6. Electroretinogram phenotypes of wild-type and Rh1 mutant flies. Responses of Rh1 mutants are compared with the wild type (top trace). The response in the Rh1-Null mutant results from photoreceptors R7 and R8. The stimulus (bottom trace) consists of a series of five 4 s light pulses; or, orange; bl, blue. The PDA is the potential that persists after a sufficiently bright blue stimulus in wild type, but not in the mutants. On-transients (filled arrowheads) and off-transients (open arrowheads) are labelled for first response of each trace in which they occur. R1–6 photoreceptors respond to blue light stimuli so rapidly that the amplitude of on-transients is lowered in those responses.

retinal. The analysis of the mutants was carried out by difference spectrophotometry as outlined by Paulsen (1984). Figure 5 shows the difference spectra obtained after illumination of visual pigment extracts with blue light (Figure 5, curves 1 and 3). From these spectra, the amount of rhodopsin in wild-type flies is calculated to be 0.55 ± 0.077 pmol per head (n = 7), while that in L81Q mutants is found to be 0.11 ± 0.008 pmol per head (n = 5), i.e. ~20% of that of wild-type flies. No visual pigment is detectable in Rh1 N86I flies (n = 5), indicating that the amount is below the sensitivity limits of the method, i.e. less than ~2% of the amount in wild type. The difference spectra show that mutation Rh1 L81Q does not detectably affect either the spectral characteristics or the thermal stability of rhodopsin and metarhodopsin. Mutant metarhodopsin, like wild-type metarhodopsin, formed by irradiation with blue light (Figure 5, curves 3 and 1 respectively) can be completely re-converted to rhodopsin by illumination with red light (Figure 5, curves 4 and 2).

The capacity of the small amount of (chromophore-bound) rhodopsin detectable in the mutant flies to trigger phototransduction was assessed by recording ERGs, the extracellularly recorded mass responses of the eye to light stimuli. Figure 6 compares ERGs of L81Q and N86I mutant flies with those of wild type and the Rh1-null mutant *ninaE*^{oI17}. The ERG amplitude of both L81Q and N86I mutants is significantly larger than that of the null mutant. In fact, except for the absence of the prolonged depolarizing afterpotential (PDA), the mutant responses are remarkably similar to the wild-type response both in amplitude and waveform. Moreover, the on- and off-transients are present in the ERGs of these mutants, whereas they are absent in the null-mutant ERG. Each ommatidium of the *Drosophila* compound eye contains three different classes of photoreceptor cells, R1–6, R7 and R8. R1–6 cells express Rh1 rhodopsin, encoded by the *ninaE* gene, while R7 and R8 cells express different rhodopsins encoded by other rhodopsin genes (eye structure reviewed by Pak, 1994). In wild type, photoreceptors R1–6 as well as R7 and R8 contribute to the ERG, while in the null mutant, only photoreceptors R7 and R8 contribute because the mutation has elimited Rh1 rhodopsin in R1–6 photoreceptors. Results of ERG recordings suggest that R1–6 photoreceptors in L81Q and N86I mutants respond to light nearly as well as those in wild type. The PDA, however, is absent in those mutants because the generation of the PDA requires the photoconversion of a large amount of rhodopsin to metarhodopsin (Hamdorf and Razmjoo, 1977; Pak, 1979; Minke, 1986). With a greatly reduced rhodopsin content, a sufficient amount of metarhodopsin cannot be photoconverted from rhodopsin to generate a PDA. Thus, the only obvious difference between the L81Q and N86I mutant ERGs and the wild-type ERG are those that can be readily explained solely through the reduced rhodopsin content in the mutants. The on- and off-transients of the ERG arise from the second order neurons in the lamina, as a result of synaptic inputs from photoreceptors R1–6 (Coombe, 1986), but not R7 and R8 photoreceptors, which make synaptic contacts with cells in another structure. They are absent in the null-mutant ERG because R1–6 do not respond and cannot make synaptic inputs to post-synaptic neurons. They are also absent in the wild-type ERG during the period of PDA because the PDA inactivates R1–6 photoreceptors. ERGs of both L81Q and N86I mutants show robust and normal on- and off-transients, indicating

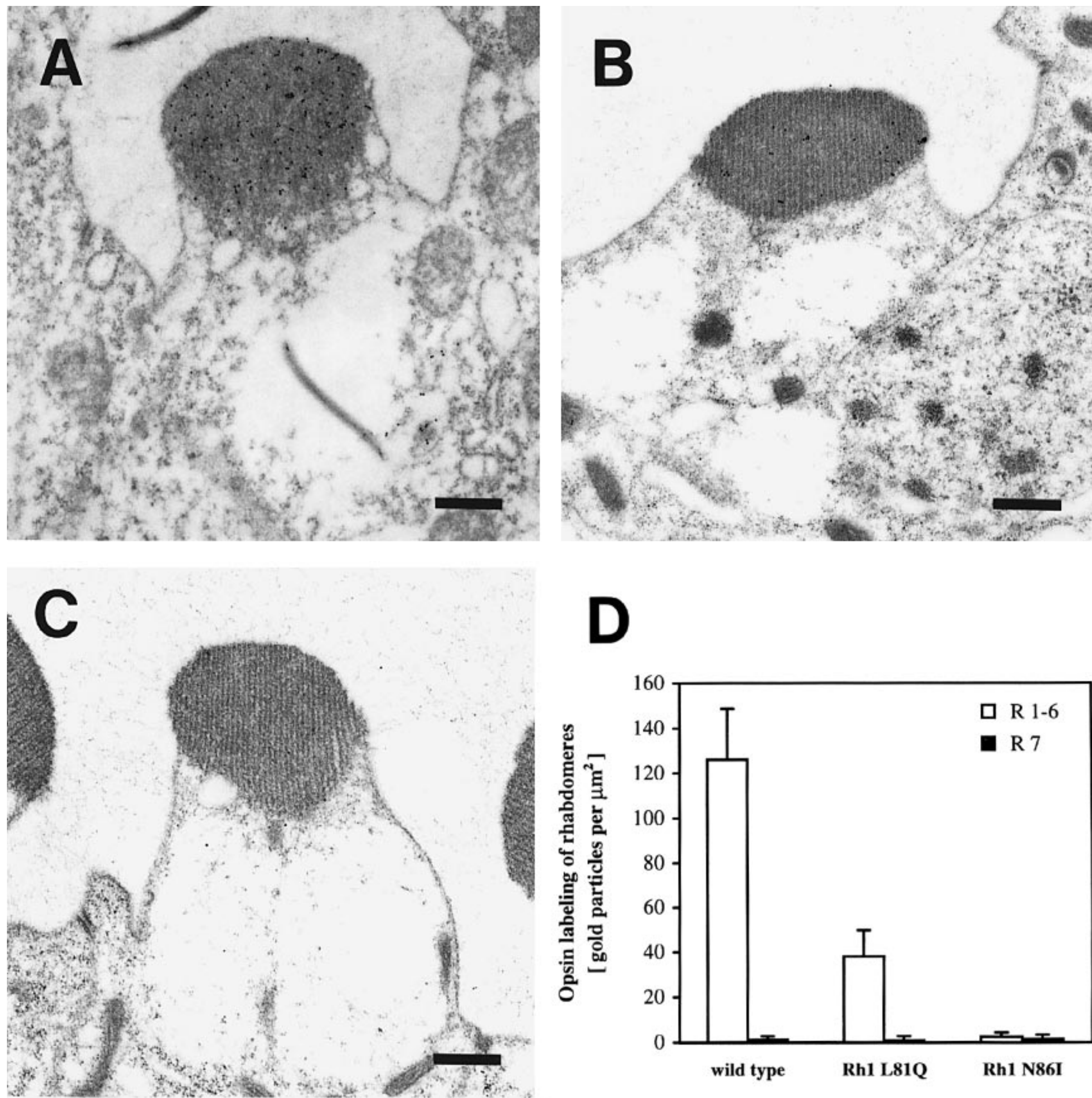

Fig. 7. Immunocytochemical localization and quantification of Rh1 opsin in wild-type and Rh1 mutant rhabdomeres. Ommatidia of wild-type or Rh1 mutant flies, as indicated, were transversely sectioned at 0 day post-eclosion. Binding of antibodies directed against Rh1 opsin was visualized by immunogold-staining. (A–C) rhabdomeric region of a representative R1–6 photoreceptor cell, (**A**) wild type, (**B**) Rh1 L81Q, (**C**) Rh1 N86I, Scale bar = 0.5 μm. (**D**) Opsin quantification was determined from the immunogold grain density in rhabdomeres (five ommatidia per eye, three eyes per mutant). Values are shown with standard deviation. The low labelling of R7 rhabdomeres demonstrates the high specificity of the antiserum used for Rh1 opsin.

that R1–6 photoreceptors make normal synaptic inputs to the post-synaptic laminar neurons. It may be noted that the small, spectrophotometrically undetectable amount of rhodopsin present in N86I mutants (<2% of wild type) is sufficient to generate nearly normal ERG responses (Figure 6), consistent with the previous observation that mutants with <1% of the normal amount of rhodopsin are capable of generating photoreceptor potentials of normal amplitude (Johnson and Pak, 1986).

Targeting of mutant opsin and photoreceptor degeneration

For an assessment of membrane turnover and vesicle transport in photoreceptors expressing mutant rhodopsin genes, an immunocytochemical analysis was carried out to subcellularly localize the opsin. Figure 7B shows that opsin molecules in Rh1 L81Q mutants are correctly targeted to the rhabdomeric microvilli of R1–6 photoreceptor cells. The difference in opsin labelling intensity in R1–6 rhabdomeres of Rh1 L81Q and wild-type rhabdomeres (38.01 ± 11.77 versus 126.04 ± 22.71, P <0.01, student's t-test, Figure 7D) roughly correlates to the 1:5 ratio in the opsin amounts detected by spectrophotometry (Figure 5). Compared with the wild-type level, <2% of opsin labelling can be detected in Rh1 N86I rhabdomeres (2.35 ± 2.02 versus 126.04 ± 22.71, P <0.01, student's t-test, Figure 7D).

Since point mutations L81Q and N86I drastically interfere with opsin maturation, it is of interest to determine whether these mutations also affect photoreceptor cell ultrastructure, in particular whether they induce photoreceptor cell degeneration. Therefore, we investigated photoreceptor morphology as a function of age. Figure 8 displays cross sections through ommatidia of mutant flies at different ages. Rhabdomeres of R8 photoreceptors are located below the level sectioned here. In wild-type flies,

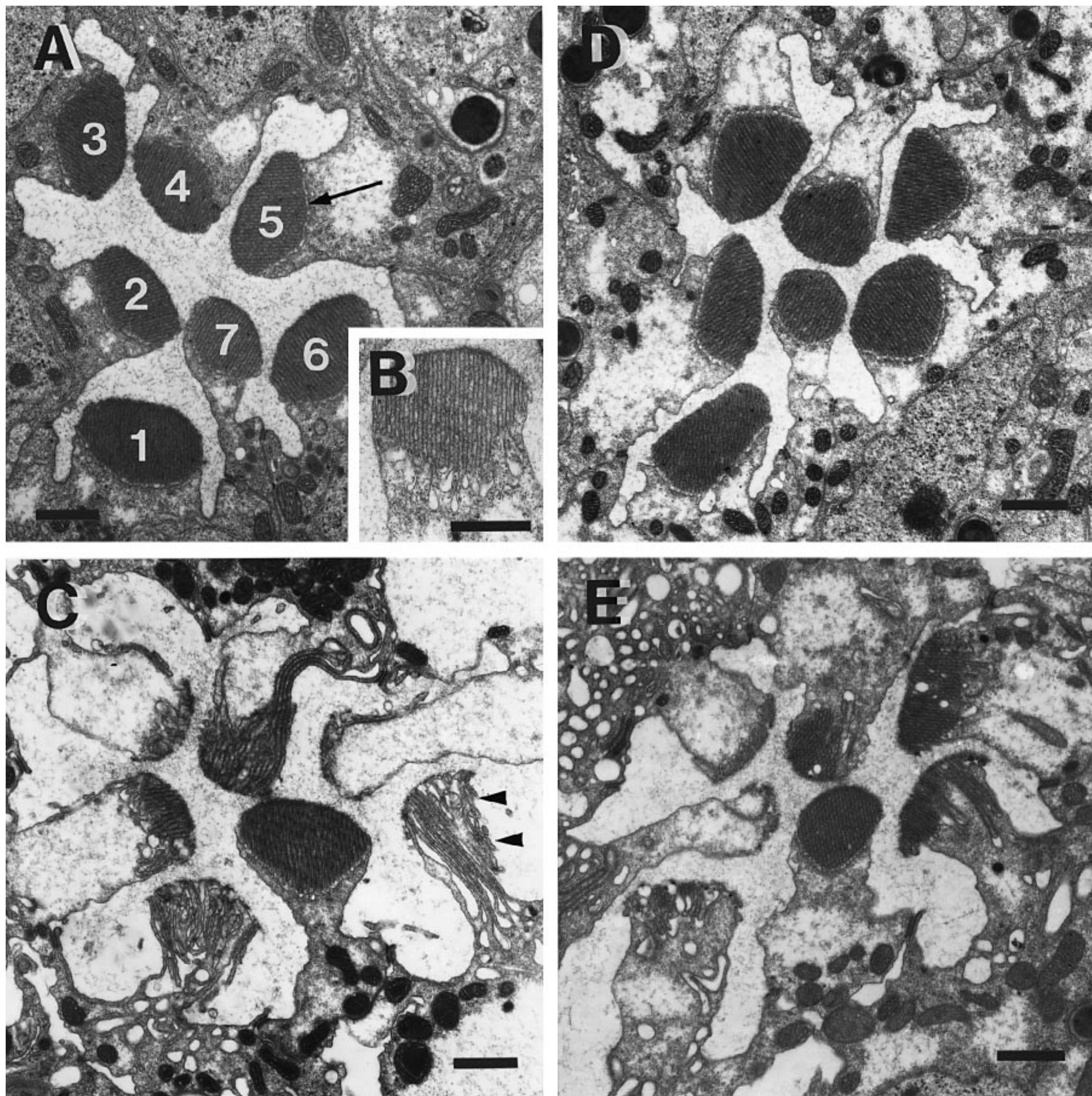

Fig. 8. Photoreceptor degeneration in Rh1 L81Q and Rh1 N86I mutant flies. Ommatidia were transversely sectioned at the nuclear level of R 1–6. (A–C) Rh1 L81Q: (**A**) day 0, (**B**) 4 weeks, representative R1–6 rhabdomere, (**C**) 8 weeks post-eclosion, (D and E) Rh1 N86I: (**D**) day 0, (**E**) 4 weeks post-eclosion. Numbers indicate the identity of photoreceptor cells; the arrow points to the subrhabdomeral catacombs; arrowheads indicate membrane shedding into the extracellular cavity. Scale bar = 1 μm.

rhabdomeres and photoreceptor cells remain intact over the 8 week period of investigation (not shown; see Leonard *et al.*, 1992). At eclosion, Rh1-null mutant flies display shortened microvilli, distortant catacomb-like structures at the microvillar base, and curtains of microvillar membranes involuting into the photoreceptor cell (Kumar and Ready, 1995). The remnant microvilli degenerate within one day after eclosion. L81Q mutant flies, on the other hand, have intact rhabdomeres (Figure 8A) and normal subrhabdomeral catacombs at eclosion. Microvilli remain largely intact until ~4 weeks post-eclosion, at which time, however, the catacomb-like structures deteriorate and the rhabdomere membrane starts filling the rhabdomeric stalk as vesicles or sheets of apposed membranes (Figure 8B). By 8 weeks post-eclosion, about half of the R1–6 rhabdomeres have degenerated (Figure 8C) and signs of membrane shedding into the extracellular cavity are visible, which may suggest a possible abnormality in membrane degradation, since such extracellular membrane whorls do not occur in the wild-type. Rhabdomere degeneration in Rh1 N86I mutants proceeds much faster than in L81Q. Although, at eclosion the rhabdomeres do not look much different from those of wild type (Figure 8D), by 4 weeks post-eclosion, most R1–6 rhabdomeres are absent, and vesiculation of the subrhabdomeric space is much more pronounced than in the L81Q mutant at comparable age (Figure 8E). All R1–6 rhabdomeres have disappeared by 6 weeks post-eclosion (not shown). All above descriptions refer to observations on sections seen at the R1–6 nuclear level. Even when no rhabdomeres are visible in these sections, it is possible that some remnant rhabdomeres may be present in more distal sections (cf. Leonard *et al.*, 1992). No evidence of rhabdomere degeneration is detected in 8-week-old heterozygotes, L81Q/+ and

N86I/+ (J.Bentrop, K.Schwab, W.L.Pak and R.Paulsen, in preparation), indicating that the phenotype is completely recessive.

Discussion

The cytoplasmic surface of *Drosophila* Rh1 opsin harbours amino acids which are highly conserved among visual pigments and other G-protein-coupled receptors. The current view has been that these domains are particularly important for the interaction of rhodopsin with other proteins in triggering the phototransduction cascade. This hypothesis was tested in the present study by combining site-directed mutagenesis and P-element-mediated germline transformation, which enabled us to generate transgenic flies expressing mutant Rh1 genes in an Rh1-null background. It was thereby possible, for the first time, to obtain information on the function of cytoplasmic loop i1 of Rh1 rhodopsin and on the importance of highly conserved amino acids in that region. We demonstrate that two point mutations which cause amino acid substitutions L81Q and N86I respectively, lead to a decrease in rhodopsin expression. Accordingly, a specific conformation of the cytoplasmic surface of rhodopsin is not only required for triggering the phototransduction cascade, but it is also of importance for protein interactions in the assembly of functional rhodopsin molecules.

In wild-type *Drosophila*, the major form of opsin present in photoreceptor membrane preparations is mature, deglycosylated opsin. Most of the opsin detected for mutant Rh1 L81Q is also in the mature, deglycosylated form (Figure 3), but a small amount—slightly more than in wild type—of nascent, glycosylated opsin is also detectable. In Rh1 N86I mutants, only the nascent opsin can be detected by immunoblotting in an amount distinctly higher than in L81Q. These results suggest that mutant opsin molecules are retained in the nascent, glycosylated state longer than those of wild type, much likely because they are processed more slowly. The fact that no drastic pile-up of nascent opsin occurs in the mutants (Figure 3) indicates that glycosylated opsin is degraded rapidly. This suggestion is consistent with the findings of Huber *et al.* (1994), who showed that flies, when deprived of sources of chromophore in the diet, express similar amounts of nascent opsin as undeprived flies, but the newly synthesized opsin is degraded instead of being processed to rhodopsin. These results led them to conclude that newly synthesized opsin is unstable and subjected to degradation unless it is rapidly processed to the chromophore-bound, deglycosylated, mature form of rhodopsin. In this latter process, chromophore binding is a prerequisite for deglycosylation (Ozaki *et al.*, 1993; Huber *et al.*, 1994). The above considerations suggest that mutations L81Q and N86I interfere with normal opsin processing at the chromophore binding and/or deglycosylation step. Which of these steps is blocked is still to be determined in future experiments. We conclude that cytoplasmic loop i1 of rhodopsin forms a binding surface for proteins involved in opsin processing, in which the conservation of single amino acids is of high importance for the correct formation of that interface. Alternatively, missense mutations in cytoplasmic loop i1 might lower the fraction of opsin that is able to fold correctly and thereby affect the amount of mature opsin formed. The fact that the two amino acid substitutions tested display a difference in severity suggests that some amino acids are more important than others. The high degree of conservation of these amino acids points to a general mechanism preserved throughout the family of G-protein-coupled receptors.

To date, just one protein is known which functions in Rh1 rhodopsin synthesis in rhabdomeric photoreceptors: NinaA-cyclophilin, a chaperone, which forms a stable complex with rhodopsin and which is required for proper passage of rhodopsin through the cell's secretory pathway (Colley *et al.*, 1991; Baker *et al.*, 1994). Due to the low opsin concentrations in Rh1 L81Q and Rh1 N86I flies, direct interactions of the mutant opsins with the NinaA-cyclophilin have not yet been studied. We have shown, however, that neither mutation affects the expression of the NinaA-cyclophilin. In view of the fact that L81 and N86 are also conserved in the minor *Drosophila* rhodopsins, which can be functionally expressed in R1–6 photoreceptor cells and do not require NinaA-cylophilin for their synthesis (Stamnes *et al.*, 1991), we conclude, that cytoplasmic loop i1 contains structural information for opsin processing involving the interaction with proteins other than the NinaA-cyclophilin. Interestingly, the blockage of opsin processing by either L81Q or N86I is not complete. A small fraction (20–30% and <2% for L81Q or N86I respectively) of mutant molecules are processed correctly to form spectrally intact rhodopsin, which is capable of triggering the visual transduction cascade. Moreover, the mutant molecules, once processed are transported to the rhabdomere, as could be shown for L81Q by immunohistochemistry (Figure 7). Evidently, the structural changes resulting from correct processing provide the rhodopsin molecule with a tag for proper targeting to the rhabdomeric membrane. The mutant molecules forming intact rhodopsin may account for molecules which are misread by the enzymes involved in opsin processing because of a structure similar enough to that of wild type.

Both mutations induce a slow degeneration of the photoreceptor cells, starting with the breakdown of microvillar membranes. Immediately upon eclosion, the rhabdomeral architecture is indistinguishable from that of wild type, with well-defined catacomb-like structures separating individual microvilli at their bases (Kumar and Ready, 1995). As a function of time, these catacombs disappear, microvilli deteriorate and involutions of microvillar membranes fill the cell body. Accordingly, there appear to be different requirements for the formation of rhabdomeric microvilli and for their long-term maintenance. As Kumar and Ready (1995) have shown, initiation of microvilli formation during photoreceptor morphogenesis in early pupal development is mostly independent of rhodopsin. Rhodopsin becomes important in later pupal development, the formation of normal rhabdomeres requires the rhodopsin-induced separation of membranes at the microvillar neck (Kumar and Ready, 1995), which defines the length of the microvilli. After eclosion, the amount and the structure of rhodopsin molecules in the rhabdomere become a crucial factor for maintaining rhabdomere structure. Even the low amount of rhodopsin formed in Rh1 N86I mutants is sufficient for the initial formation of intact microvilli; both mutations, however, eliminate some

structural feature required for the long-term integrity of the rhabdomere.

Two mechanisms can be largely excluded as the cause of degeneration in these mutants: first, the electrophysiological analysis shows no differences in the L81Q and N86I mutant ERGs from wild-type ERGs other than those resulting from a reduced rhodopsin content. These measurements give no indication that either amino acid substitution results in defects in the phototransduction and adaptation cascades. Secondly, as 1–5% of wild-type rhodopsins are sufficient to keep rhabdomeres intact for over 6 weeks (Leonard *et al.*, 1992; Kumar and Ready, 1995), the lowered amount of rhodopsin in the mutants is unlikely to be the cause of degeneration. Thus, we conclude that degeneration in both mutants results from structural changes of the rhodopsin molecule which might affect protein–protein interactions required for the stabilization of the microvillar architecture. Both mutations L81Q and N86I are recessive for the degeneration phenotype as no obvious photoreceptor degeneration is observed in heterozygotes (J.Bentrop, K.Schwab, W.L.Pak and R.Paulsen, in preparation). Many mutations have been isolated in the *ninaE* gene (Scavarda *et al.*, 1983; Colley *et al.*, 1995; Kurada and O'Tousa, 1995). A majority of these mutants are dominant for retinal degeneration, including those that were isolated in a mutagenesis scheme designated to isolate recessive mutants (Pak, 1979; Kurada and O'Tousa, 1995). The mutations generated in this study are distinct from these other mutations in that they are recessive for the degeneration phenotype and affect specific amino acids in cytoplasmic loop i1.

Taken together, we show for the first time that substitutions of single, highly conserved amino acids in cytoplasmic loop i1 of a seven transmembrane helix receptor protein, here exemplified by *D.melanogaster* Rh1 rhodopsin, induce blockage of the correct receptor protein processing and induce age-dependent degeneration of the receptor cell. In accordance with current classifications, both mutants belong to the group of 'turnover-defective mutants'. Since L81 and N86 are conserved in human rhodopsin, mutations of both amino acids must be regarded as possible candidates for recessive forms of RP in humans as well.

Material and methods

Construction of mutants, fly stocks

In vitro mutagenesis was carried out following the protocol described by O'Tousa (1992). A single stranded template was used, which consisted of M13mp18 containing a 2.3 kb *Bam*HI-fragment of the Rh1 gene. Primers CAAATCAC**A**GCGCACGC and GCCCGCTA**T**CCTGCTGG (bold type indicates the mutant nucleotide) were used to induce amino acid mutations L81Q or N86I respectively (listing indicates original amino acid, location, mutation). The mutant fragment was used to reconstruct a complete 5.5 kb *Kpn*I fragment of the Rh1 gene, which was then cloned into the unique *Kpn*I site of the P-element transformation vector Carnegie3 rosy2 (Rubin and Spradling, 1983). P-element-mediated transformation into host strain ninaE^{oI17} was carried out as described (O'Tousa, 1992), and transformant lines were made homozygous for the P-element insert and the X chromosome mutation *white*. The coding region of the Rh1 gene was amplified from transformant flies by polymerase chain reaction and sequenced to confirm successful mutagenesis. ninaE^{oI17} was used as Rh1-null mutant control, these flies contain a large deletion in the 5′-region of the gene and make no detectable Rh1 transcript (O'Tousa *et al.*, 1985). Flies were raised on a standard corn meal diet and were kept under a 12 h light/12 h dark cycle. To age the flies, they were collected at less than 24 h post-eclosion and maintained until use for the time indicated.

RNA analysis

Total RNA was isolated from *Drosophila* heads (1–2 days post-eclosion) by the method of Chirgwin *et al.* (1979). Five micrograms of RNA were run on 1% agarose, 2% formaldehyde gels and blotted onto nylon filter membranes (Hybond-N, Amersham, Braunschweig, Germany) following standard protocols (Sambrook *et al.*, 1989). Northern blot analyses were carried out according to Huber *et al.* (1994), using digoxigenin-labelled anti-sense cRNA-probes generated from *Drosophila Rh1*, *ninaA* (Schneuwly *et al.*, 1989; Shieh *et al.*, 1989) or, as a control for RNA quantity and quality, *Actin 2* (Fyrberg *et al.*, 1983) cDNA clones. Immunological detection of probes was performed with a digoxigenin detection kit (Boehringer Mannheim) using CDP-Star (Tropix, Bedford, MA) as luminescent dyes.

Western-blot analysis, PNGase F treatment

For the detection of opsin protein in mutant flies, 150 compound eyes (1–2 days post-eclosion) were dissected on ice and collected in 100 µl ice-cold/deionized H_2O, which was supplemented with 1 mM Phenylmethylsulfonyl-fluoride, 0.42 µg/µl leupeptin, 0.83 µg/µl pepstatin and 0.83 µg/µl aprotinin. Five times, the eyes were homogenized in 100 µl ice-cold/deionized H_2O with a plastic pestle. The resulting membrane suspensions were pooled, 1 ml 33 mM phosphate buffer (Na/K) pH 6.2; 100 mM NaCl was added, and eye membranes were collected by centrifugation (14 000 *g*, 10 min, 4°C). The membrane pellet was resuspended in 100 µl SDS–PAGE sample buffer (5% SDS, 65 mM Tris–HCl, pH 6.8). Protein concentrations were determined by the bicinchoninic acid procedure (Smith *et al.*, 1985). Samples containing 12 µg of protein (equivalent of 10 heads) were adjusted to a final concentration of 5% glycerol, 2% β-mercaptoethanol. In order to remove sugar side chains from the opsin protein, membrane proteins from fly heads were prepared and incubated with peptide-*N*-glycosydase F (Boehringer Mannheim) exactly as described by Ozaki *et al.* (1993). Samples were separated by SDS–PAGE according to Laemmli (1970) on 8–20% gradient gels. For immunoblotting, proteins were electrophoretically transferred onto PVDF membranes in 50 mM Tris, 20% methanol, 0.1% SDS. Membranes were incubated with polyclonal antibodies directed against peptide I237–K258 of *Drosophila* Rh1 opsin or with antibodies directed against peptide Y166–D177 of NinaA-cyclophilin, followed by binding of alkaline phosphatase-conjugated protein A and visualization through a chromogenic reaction with 5-bromo-4-chloro-3-indolyl phosphate/4-nitro-blue-tetrazolium chloride.

Spectrophotometry and ERG recordings

Heads of 150 flies were dissected under red light, and total head membranes were isolated as described for eye membranes above. Visual pigment extracts were obtained by incubating total head membranes in 40 µl 4% digitonin, 100 mM phosphate buffer (Na/K), pH 6.2 at room temperature for 10 min. Supernatants after two subsequent centrifugations (14 000 *g* for 10 min and 50 000 *g* for 10 min) were subjected to spectrophotometric measurements as described by Paulsen (1984). Electroretinograms were recorded as described previously (Larivee *et al.*, 1981).

Immunolabelling of ultrathin sections, quantification of labelling densities

Immunolabelling of ultrathin sections was carried out according to Wolfrum (1995). Basically, flies were fixed with 0.1% glutaraldehyde/3% paraformaldehyde in PB (0.1 M sodium phosphate buffer, pH 7.2) for 1 h at room temperature and for 2 h at 4°C, dehydrated in a graded ethanol series, infiltrated and then embedded in LR-White. Ultrathin sections were cut with a Reichert Ultracut microtome and were collected on formvar-coated nickel grids. Sections were first incubated with 0.01% Tween 20 in phosphate buffered saline (PBS) for 30 min, followed by 50 mM NH_4CL in PBS and a second blocking step with BB (blocking buffer: 0.5% coldwater fish gelatine/0.1% ovalbumin in PBS) for 45 min. The sections were incubated with the polyclonal primary antibody (diluted 1:20 in BB) for 24 h at 4°C. Grids were rinsed with BB twice, pre-incubated with 10 mM PB containing 0.5% coldwater fish gelatine/0.1% ovalbumin/0.5 M NaCl/0.01% Tween 20, and then incubated with goat anti-rabbit IgG, conjugated to 10 nm gold particles (Nanoprobes Inc.). Sections were post-fixed with 2.5% paraformaldehyde and then stained with 2% uranyl acetate. For controls, the primary antibody was replaced by PBS. Sections were examined with a Zeiss EM 912 electron microscope. Quantitative evaluation of opsin molecules localized to

rhabdomeres was performed by basically following the protocols of Sapp *et al.* (1991) and Arikawa and Matsushita (1994). Randomly selected ommatidia (four to five per eye, three eyes per mutant) were photographed, and the number of gold particles per rhabdomere was counted. The particle density was determined from the counts in individual rhabdomeres divided by the cross-sectional area of the rhabdomere, the latter was measured using the AnalySIS 2.1 software (Soft-Imaging Software). Opsin labelling in controls, which were performed by omitting the primary antibody, was <1 grain per 10 rhabdomeres.

Transmission electron microscopy

Before dissection, flies were prefixed by injection of 0.1 M sodium-cacodylat buffer, pH 7.3 containing 3.5% glutaraldehyde/4% paraformaldehyde according to Leonard *et al.* (1992). Eyes were then immersed in the same fixative for 3 h at room temperature, followed by a second step with 1% tannic acid overnight at 4°C. After washing several times with 1% Na_2SO_4 in 0.1 M sodium-cacodylate buffer, the eyes were post-fixed in 2% OsO_4, dehydrated in a graded ethanol series and stained *en bloc* with 2% uranyl acetate. Samples were infiltrated and embedded in Epon resin. Ultrathin sections were cut with a Reichert Ultracut microtome, collected on Formvar-coated copper grids and stained with 2% uranyl acetate and lead citrate. Sections were examined with a Zeiss EM 912 electron microscope.

Acknowledgements

The authors thank K.Weber for expert technical assistance and L.L.Randall for recording the ERGs. Supported by the Deutscher Akademischer Austauschdienst (Prog. 312, to J.B.), the Deutsche Forschungsgemeinschaft (Pa 274/4-1 to R.P. and J.B.) and NEI grant No. EY00033 (to W.L.P.).

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Received on September 2, 1996; revised on December 3, 1996