Point mutations in bovine opsin can be classified in four groups with respect to their effect on the biosynthetic pathway of opsin

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Expression *in vitro* with the recombinant baculovirus expression system showed correct biosynthesis and post-translational processing of 'wild-type' bovine opsin with regard to translocation, glycosylation, palmitoylation and targeting. However, several of these processes were severely affected by point mutations. From the overall results of 16 mutants reported here, four groups were distinguished. One group significantly affected neither biosynthesis nor folding of opsin (D83N, P291A, A299C-V300A-P303G). A second group produced a truncated protein (R69H, Y301F), suggesting that these positions are essential for a correct translational process. A third group affected membrane trans-

location as well as glycosylation, which can be interpreted as interference with the function of a transfer signal. Substitutions at positions Glu-113, Glu-122, Glu-134, Arg-135 and Lys-248 belong to this category. A fourth group induced structural changes in the protein that led to heterogeneous distribution in the plasma membrane (E113Q/D, W265F, Y268S). Taking any functional consequences of these mutations into consideration, it seems that point mutations can have mosaic effects and therefore should be examined at several levels (folding, targeting, functional parameters).

INTRODUCTION

Rhodopsin is a complex integral membrane protein containing seven transmembrane domains, which harbour the chromophore 11-*cis* retinal covalently linked to Lys-296 via a protonated Schiff base [1]. The N-terminal region is glycosylated [2] and is of structural importance, together with the three intradiscal loops and the disulphide bridge between Cys-110 and Cys-187 [3,4]. Two cytosolic loops are thought to play a role in signal transduction [5] together with an additional surface loop generated by palmitoylation and membrane anchoring at Cys-322 and Cys-323 [6] (Figure 1). Evidently, rhodopsin requires intermittent membrane translocation and several post-translational modifications (glycosylation, palmitoylation, disulphide bridge formation, recombination with 11-*cis*-retinal) to fulfil all its structural and functional properties. Expression *in vitro* permits the exploration of properties such as wavelength regulation, receptor mechanism, biosynthesis and lipid-protein interactions in much detail through (site-specific) mutagenesis. For this purpose we have selected the recombinant *Autographa californica* nuclear polyhedrosis virus (AcNPV)-baculovirus system, as this is a eukaryotic expression system that performs all common eukaryotic post-translational modifications and has been successfully employed for the high-level expression of heterologous proteins (reviewed in [7,8]).

Bovine rhodopsin and its mutants have been expressed *in vitro* in many different systems: COS cell lines [9], insect cell lines with the use of recombinant baculovirus [10], *Xenopus* oocytes [11], human embryonic kidney cell line [12], the Chinese hamster ovary cell line [13] and a cell-free expression system [14]. Usually the effect of mutations on functional properties is examined; possible underlying effects on the level of biosynthesis and folding are rarely investigated. Here we examine the biosynthesis of opsin in insect cells under the control of the baculovirus polyhedrin promoter in more detail [15], and investigate the

influence of a panel of mutations on selected positions (Figure 1) on this process. For this panel we have selected highly conserved amino acid residues that are located in the transmembrane or interface regions, where effects on functional properties have been described and effects on biosynthesis and folding are indicated or might be expected. In this context we do not address functional properties such as the activation of transducin, but for most of the mutations described such parameters have been reported elsewhere.

We reported before that the mutation E134D, shown earlier to affect transducin activation [16,17], also affects rhodopsin biosynthesis in that a larger proportion of non-glycosylated product was generated and indirect evidence was obtained for incorrect folding of part of the protein population [18,19]. Our hypothesis is that Glu-134 is part of a stop-transfer signal, which shows slip when mutated. This would lead to incorrect folding of part of the total opsin population. Here we investigate these aspects further, along the following lines. We have identified several residues that affect functional as well as biosynthetic regulators of the protein, showing that this is not an isolated phenomenon. These results further support the concept that critically positioned polar residues contribute significantly to stop/start-transfer signals, whereas structurally important non-polar residues are less essential in this respect. To demonstrate this we concentrate here on several highly conserved polar (Arg, Lys, Glu, Asp) or structural (Tyr, Trp, Pro) residues in or near the second, third, sixth and seventh membrane domains (Figure 1). In two cases several mutations were analysed to address possible charge effects (E134D/R, E134R-R135E) or any direct relation with functional effects (E113D/Q/Y/C).

The picture emerges that the rhodopsin sequence is delicately tuned to correct membrane translocation and folding. A variety of substitutions affect this process to a variable extent, and some mutations even have an effect at an earlier stage and seem to abort the translational process.

Abbreviations used: AcNPV, *Autographa californica* nuclear polyhedrosis virus; DoM, dodecyl β -1-maltoside; dpi, days post-infection; TM, transmembrane domain; v-ops, opsin produced in insect cells by recombinant baculovirus.

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Figure 1 Model of the topography of rhodopsin [59]

The amino acids replaced in the present work are indicated in black. Abbreviation: Th, thermolysin proteolytic recognition site [44].

EXPERIMENTAL

Baculovirus expression, site-directed mutagenesis, SDS/PAGE, immunoblotting, ELISA and the immunofluorescence assay

These were performed as described [10,18,20–22]. For molecular mass calibration a low-molecular-mass protein electrophoresis calibration kit (Pharmacia) was used, containing phosphorylase *b* (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α -lactalbumin (14.4 kDa).

Some aspects, relevant to the present study, are explained here in more detail. IPLB-Sf9 cells were cultured on glass coverslips (immunofluorescence analysis) or in monolayer culture (1–2 ml; Gibco 6–12-well plates; all other analyses). Infection with baculovirus was performed at a density of 10^6 cells/ml and a multiplicity of infection of 10. Immunofluorescence analysis was performed at 2 days post-infection (dpi) because as the infection proceeded the cells tended to dissociate from the glass substrate. Although dissociated cells were still viable and impermeable to Trypan Blue, only attached cells were used for the analysis. The other studies were performed on attached cells at 3 dpi. At this stage over 95% of the cells were still viable as assessed by Trypan Blue exclusion, irrespective of the type of recombinant virus used. As reported before, in our hands at a multiplicity of infection of 10 or less, significant cell death does not occur until 5 dpi [15]. Even at that stage, attached cells are still viable. Because non-viable cells detach from the substrate, all analyses were performed on a cell population of which less than 1% was permeable to Trypan Blue or to the antibodies used. This excluded the possibility that the results presented were influenced by variability in the initial permeability of the cells.

Palmitoylation

Sf9 cells were incubated with [$1\text{-}^{14}\text{C}$]palmitic acid ($1\text{ }\mu\text{Ci}/10^6$ cells; specific radioactivity 50–60 mCi/mmol; Amersham) during

infection with recombinant virus. Cells were harvested at 3 dpi, lysed by freeze–thawing twice in distilled water with the addition of leupeptin ($200\text{ }\mu\text{M}$; Sigma) and EDTA (10 mM), sedimented by centrifugation (Eppendorf; 17000 g for 5 min at room temperature), and the precipitate was resuspended in buffer A [20 mM Pipes/ 130 mM NaCl/ 5 mM KCl/ 2 mM MgCl_2 / 2 mM CaCl_2 / 0.1 mM EDTA/ 5 mM 1,4-dithioerythritol (pH 6.5)]. Then dodecyl β -1-maltoside (DoM) was added to a final concentration of 20 mM and the suspension was rotated overnight at 4°C . The resulting DoM extract was used for immunoprecipitation of opsin produced in insect cells by recombinant baculovirus (v-ops).

Immunoprecipitation

Protein A–agarose ($10\text{ }\mu\text{l}$; Pierce) was loaded with the anti-rhodopsin antibody [23] CERNJS858 ($10\text{ }\mu\text{l}$) by rotation overnight at 4°C in 0.5 ml of buffer A containing 20 mM DoM. Unbound material was removed by several steps of centrifugation (Eppendorf; 500 g for 5 min at 4°C) and washing (buffer A/ 20 mM DoM). The DoM extract described above was freed from insoluble material by centrifugation (Eppendorf; 17000 g for 15 min at 4°C), applied to Protein A–agarose loaded with CERNJS858 ($100\text{ }\mu\text{l}/10\text{ }\mu\text{l}$ of gel) and rotated overnight at 4°C . Unbound material was removed by centrifugation (Eppendorf; 500 g for 5 min at 4°C), followed by two washing steps (0.5 ml of buffer A/ 20 mM DoM) and elution of bound material in SDS/PAGE sample buffer [4% (w/v) SDS/ 15 mM Tris/HCl (pH 6.8)/ 10% (v/v) glycerol/ 0.006% Bromophenol Blue/ 20 mM dithioerythritol; $100\text{ }\mu\text{l}/10\text{ }\mu\text{l}$ of gel].

Digestion by thermolysin

Sf9 cells were infected with recombinant virus and collected at 3 dpi by centrifugation (1000 g for 5 min at room temperature).

Lysed cells (prepared as above) and intact cells were gently resuspended in thermolysin digestion buffer [10 mM Tris/acetate (pH 7.2)/4 mM CaCl_2 /10 % thermolysin (w/w on protein basis); 50 μl per 10^6 cells] and incubated for 5 min at room temperature. Under these conditions, although intact cells slowly became permeable to the enzyme, less than 25 % of wild-type opsin in intact cells became susceptible to proteolysis, whereas over 90 % of the opsin in lysed cells was cleaved by the protease. Subsequently the reaction was stopped by the addition of EDTA solution (0.5 M, pH 8) to a final EDTA concentration of 0.2 M. Intact cells were then lysed and SDS/PAGE samples (150 μl per 10^6 cells) were prepared by the addition of SDS/PAGE sample buffer (for final concentrations see above).

Quantification

Opsin bands on immunoblots and autoradiograms were quantified with an imaging densitometer (Model GS-670; Bio-Rad) combined with Molecular Analyst Software for image analysis. For determination of the extent of palmitoylation, monomer opsin bands were used, as oligomers are artifacts of the SDS/PAGE analysis. Because expression levels also varied, the amount of monomeric opsin varied strongly per lane. Therefore the palmitoylation signal on the autoradiogram was normalized to the same amount of opsin by dividing by the monomeric band signal on a parallel Coomassie Blue-stained gel or immunoblot.

For quantification of proteolysis, two controls were incorporated. For all three conditions (intact cells without proteolysis, intact cells with proteolysis, or lysed cells with proteolysis) extracts of the same numbers of cells were applied on two parallel SDS/polyacrylamide gels. One gel (A) was immunoblotted with the polyclonal antiserum CERNJS858, the other (B) with the monoclonal antibody 1D4 [24], which recognizes residues 341–348 of opsin. Hence the two proteolytic products of opsin (1–327 and 1–336), which migrate just below the intact protein, appear in A but not in B. The combined monomeric bands (intact and fragments) should represent about the same amount of protein under the three conditions. Experiments were therefore accepted only if the combined intensity in A did not vary between the three conditions by more than 10 %. The intensity of the monomeric bands in B was used to determine the extent of proteolysis. Here, with the intensity in the intact cells without enzyme (1) set at 100 %, the intensity in the lysed cells treated with enzyme (3) should be less than 10 %. With all requirements met, the ratio between the band intensity in the intact cells treated with enzyme (2) and that in (1) presented a semi-quantitative measure of the percentage of protein accessible to proteolysis.

RESULTS

Incomplete translation

Two of the mutations investigated, R69H and Y301F, produced very little intact protein, but mainly truncated products of approx. 22 and 30 kDa respectively (Figure 2, lanes 1 and 15; Table 1). This pattern was identical in cells analysed earlier after infection (1 and 2 dpi). Both products still contained the N-terminus and the extracellular loop e2 but lacked the C-terminus (Figures 2 and 3). These mutants also produced bands that were less discrete, indicating that these two mutations induced aberrant interruption and abortion of the translational process. The truncated products had a much stronger tendency to aggregate in SDS solution than did the intact protein (Figure 2, lanes 1 and 15; Figure 3, lane 5).

Figure 2 Effect of selected mutations on v-ops glycosylation

Immunoblot analysis of opsin mutants, produced in Sf9 cells with the corresponding recombinant mutant opsin virus. Opsin was identified by incubation with antiserum CERNJS858 (A) or 1D4 (B). Lane 1, R69H; lane 2, D83N; lane 3, E113Q; lane 4, E113D; lane 5, E113Y; lane 6, E113C; lane 7, E122L; lane 8, E134D; lane 9, E134R; lane 10, E134R-R135E; lane 11, K248L; lane 12, W265F; lane 13, Y268S; lane 14, P291A; lane 15, Y301F; lane 16, A299C-V300A-P303G. The loading per lane was the extract of 2×10^4 cells (lane 7), 4×10^4 cells (lanes 2, 5, 6, 8 and 11), 10^5 cells (lanes 1, 9, 12, 13, 14 and 16) and 2×10^5 cells (lanes 3, 4, 10 and 15). The positions of glycosylated (arrow) and non-glycosylated (arrowhead) opsin are indicated. The positions of molecular mass markers (kD, kDa) are shown at the left.

Glycosylation

Previously we demonstrated that v-ops migrates on SDS/PAGE gels with about the same apparent molecular mass as fully processed native bovine rod opsin [15]. A positive immunoreaction with monoclonal antibodies against the N-terminus (R2-12N), loop e2 (T13-34L) [25] and the C-terminus (1D4) [24] indicates that the full-length protein is produced (Figure 3). That v-ops is glycosylated was confirmed by adding the N-glycosylation inhibitor tunicamycin on infection of Sf9 cells with recombinant virus. The resulting product still contained the complete opsin sequence [18] but migrated with a lower apparent molecular mass [15], corresponding to that of non-glycosylated native rhodopsin. Under high-resolution conditions the glycosylated v-ops seemed to consist of two species. Three different recombinant viruses producing wild-type opsin, constructed with different transfer vectors, produced the glycosylated species with no detectable or very low levels of non-glycosylated product (less than 10 %; Table 1). Several of the amino acid substitutions reported here affected opsin glycosylation to a much larger extent (Figure 2). First, the ratio between the upper and lower glycosylated bands (arrow) showed considerable variation. However, this depended partly on cellular factors, because some variation was also observed within different batches of one mutant. Hence it is currently not clear whether a mutation-dependent effect also operates here. Secondly, a number of mutations clearly enhance the relative amount of non-glycosylated product (Figure 2, arrowhead, E113C/Y; E122L; E134D/R; K248L). This pattern was reproduced in at least three

Table 1 Biosynthesis of opsin and selected mutant species in Sf9 cells infected with corresponding recombinant virus

The pAc transfer vector used is shown in parentheses in the first column. The expression level was measured by inhibition ELISA and/or by immunoblot analysis. The molecular masses shown are the apparent molecular masses by SDS/PAGE; 38 kDa represents the glycosylated and 31 kDa the non-glycosylated intact protein; 30 and 22 kDa represent a glycosylated but truncated protein. The ratings in parentheses in the third column indicate the amount of non-glycosylated as a percentage of total species: $\pm$, less than 10%; +, 20–30%; ++, 30–40%; + + +, 40–60%, determined by densitometer imaging of immunoblots analysed with CERNJS858.

Species	Expression level (pmol/ 10^6 cells)	Molecular mass of product(s) (kDa)
Wild-type (373)	20–25	38
Wild-type (RP23)	20–25	38
Wild-type (DZ1)	30–35	38, 31($\pm$)*
R69H (DZ1)	25–30	22
D83N (RP23)	25–35	38
E113Q (DZ1)	10–15	38
E113D (DZ1)	10–15	38
E113Y (DZ1)	15–25	38, 31 (+)
E113C (DZ1)	15–25	38, 31 (+)
E122L (DZ1)	25–35	38, 31 (+ + +)
E134D (RP23)	30–40	38, 31 (+ + +)
E134R (DZ1)	20–30	38, 31 (+)
E134R/R135E (DZ1)	10–20	38, 31 (+)
K248L (RP23)	35–45	38, 31 (+ + +)
W265F (DZ1)	10–15	38
Y268S (DZ1)	15–20	38
P291A (RP23)	15–20	38
Y301F (DZ1)	5–10	30
A299C/V300A/P303G (RP23)	15–20	38

experiments (Table 1). These non-glycosylated products do contain the full protein sequence because they all reacted with the C-terminal monoclonal antibody 1D4 (Figure 2B). The strongest effects were seen for E122L, K248L and E134D. Remarkably the production of non-glycosylated species varied for different mutations on one site (E113C > E113Y > E113Q $\approx$ E113D; E134D > E134R). No interference with glycosylation was observed with the other mutants described here (Figure 2): D83N (lane 2), E113Q (lane 3), E113D (lane 4), W265F (lane 12), Y268S (lane 13), P291A (lane 14) and A299C-V300A-P303G (lane 16). Expression levels and apparent molecular masses of the synthesized v-ops and mutant products are listed in Table 1. All mutants were expressed at average or below average levels and we conclude that the interference with glycosylation is due to the mutation, as already previously inferred for E134 mutants [18]. In addition, the mutations E113Q/D, E134R-R135E and W265F significantly decreased the expression level, indicating that these amino acid substitutions do affect the translation efficiency in this system.

Palmitoylation

Because insect cells are able to sustain thiopalmitoylation [26], we also examined whether v-ops and selected mutants were palmitoylated. Sf9 cells were incubated with [14 C]palmitic acid during infection with recombinant virus. Opsin was then solubilized with DoM, immunoprecipitated with CERNJS858 and, after SDS/PAGE, the opsin band was identified by immunoblot and palmitoylation was identified by autoradiography. Both bands were quantified by densitometer imaging and the extent

Figure 3 Product analysis of selected v-ops mutants with a panel of antibodies

Immunoblot analysis of Sf9 cells infected with wild-type (AcNPVDZ1/ops) and recombinant mutant opsin virus. Lane 1, v-ops; lane 2, E134D; lane 3, E134R; lane 4, E134R-R135E; lane 5, R69H; lane 6, K248L; lane R, bovine ROS membranes. Opsin was identified by incubation with (A) polyclonal antiserum CERNJS858; (B) monoclonal antiserum R2-12N against the N-terminus; (C) monoclonal antiserum T13-34L against loop e2; (D) monoclonal antiserum 1D4 against the C-terminus. Each lane contained the extract of 5×10^4 cells. The positions of glycosylated (arrow) and non-glycosylated (arrowhead) opsin are indicated. The positions of molecular mass markers (kDa, kDa) are shown at the left.

of palmitoylation was normalized with respect to the amount of protein in the same band, to permit a comparison of palmitoylation levels in wild-type and mutant proteins. A large population of proteins in infected cells was palmitoylated under these conditions (results not shown). Opsin was isolated by immunoprecipitation and clearly displayed label incorporation (Figure 4, lane 1). The label was quantitatively removed by incubation with hydroxylamine [1 M hydroxylamine/1 % BSA/PBS (pH 7.5) for 4 h at 37 °C], demonstrating that it was bound as a palmitoyl thioester [27]. Hence opsin was also palmitoylated in this expression system.

For all mutants investigated the intact glycosylated form behaved comparably to its wild-type equivalent: it was palmitoylated and was easily solubilized and immunoprecipitated from detergent solution (Figure 4). The extent of palmitoylation of glycosylated mutant opsins was estimated relative to the wild type by densitometer imaging of the immunoprecipitated bands on immunoblot and on the corresponding autoradiogram. All mutants showed a palmitoylation level within 30 % of the wild-type level, which is within the error range of this complex analysis, and hence should be considered normal, except for D83N and W265F, in which the extent of palmitoylation was decreased to respectively 35 ± 10 % and 21 ± 15 % of the wild-type level (values given $\pm$ S.D.).

Membrane translocation and targeting

As we have shown previously by immunocytochemical analysis [10,15], bovine opsin produced in Sf9 cells is targeted to the

Figure 4 Palmitoylation of mutant opsins

Autoradiographic analysis of immunoprecipitated wild-type and mutant opsins. Sf9 cells were infected with recombinant virus in the presence of labelled [14 C]palmitic acid. Lane 1, v-ops; lane 2, K248L; lane 3, E134R; lane 4, E134D; lane 5, E134R-R135E; lane 6, E113C; lane 7, E113D; lane 8, E113Q; lane 9, E113Y; lane 10, D83N; lane 11, W265F. Each lane contained the immunoprecipitated fraction of approx. 8×10^5 cells. R shows the position of glycosylated rhodopsin from bovine ROS membranes as detected by Coomassie R-250 and subsequent silver-staining of the SDS/PAGE gel. The positions of molecular mass markers (kD, kDa) are shown at the left.

Figure 5 Mutant opsins are also targeted to the plasma membrane

Indirect immunofluorescence assay of Sf9 cells infected with recombinant wild-type (AcNPVDZ1/ops) (A) or E113Q mutant (B) opsin virus. After fixation at 2 dpi, opsin was localized with the polyclonal antiserum CERNJS858. Immunoreactivity was detected by reaction with fluorescein-conjugated goat anti-rabbit IgG. The mutants E113D, E113Y, E113C, W265F and Y268S display a similar patch-like distribution pattern to that shown for E113Q (B). Scale bar, 1 μ m.

plasma membrane (Figure 5A). Because the opsin recombines to a large extent with added 11-*cis* retinal into rhodopsin it was assumed that this complex membrane protein had been correctly translocated and folded in the endoplasmic reticulum membrane of the Sf9 cell. However, this cannot be assumed for mutant proteins because the mutation might impair translocation and/or folding without affecting targeting. A dual assay to probe for correct translocation was developed, by using (1) limited proteolysis of intact or lysed cells and (2) immunofluorescence analysis of both intact and permeabilized cells with monoclonal antibodies directed against epitopes distributed over the opsin sequence [24,25]: antibodies against the N-terminal domain (R2-12N) and loop e2 (T13-34L) recognize extracellularly located sequences in correctly folded opsin. Antibodies against the C-terminal domain (1D4) or loop i3 (K42-41L) recognize sequences that should be located intracellularly. Only after permeabilization

Table 2 Immunohistochemical analysis of wild-type rhodopsin folding by monitoring the reactivity of monoclonal antibodies with intact and fixed Sf9 cells infected with recombinant opsin baculovirus

Immunoreactivity was detected by reaction with fluorescein-conjugated second antibodies, and is rated from — (negative: signal not above background) to + + + (very strong positive reaction).

Antibody	Intact cells	Fixed cells
R2-12N (N-terminus)	+ +	+ + +
T13-34L (loop e2)	+	+
K42-41L (loop i3)	—	+ +
1D4 (C-terminus)	—	+ + +
858 (polyclonal)	+ +	+ + +

Table 3 Membrane translocation of wild-type and mutant opsins analysed by double-label indirect immunofluorescence analysis (IFA) of intact cells and immunoblot analysis of intact thermolysin-treated cells

The immunohistochemical reactivity of rhodopsin monoclonal antibodies against the N-terminus (R2-12N) and C-terminus (1D4) on intact Sf9 cells infected with recombinant opsin or mutant virus was detected by reaction with fluorescein-conjugated goat anti-mouse IgG. Total reactivity was determined after fixation with the polyclonal antiserum CERNJS858 and rhodamine-conjugated pig anti-rabbit IgG. Immunoblot analysis of intact Sf9 cells after infection with recombinant virus and incubation with thermolysin was performed as described in Figure 6. Immunoreactivity (IFA) was rated as: —, less than 10%; +, 20–30%; + +, 40–60%; + + +, 70–80%; + + + +, more than 90%, measured by the number of reactive cells relative to total reactivity with the polyclonal antiserum CERNJS858 after fixation. Thermolysin digestion is rated as: —, less than 25%; +, 25–50%; + +, 50% or more, measured by digestion of the glycosylated (Glyc) or non-glycosylated (Nglyc) forms, in intact cells after 5 min of incubation with thermolysin. Abbreviations: n.a., not applicable; n.d., Nglyc not detectable.

Opsin species	IFA		Thermolysin digestion	
	R2-12N	1D4	Glyc	Nglyc
Wild-type	+ + + +	—	—	—
R69H	+ + + +	—	n.a.	n.d.
D83N	+ + + +	—	—	n.d.
E113Q	+ + + +	+ +	+	n.d.
E113D	+ + +	+ +	+	n.d.
E113Y	+ + + +	+ + + +	+	+
E113C	+ + + +	+ + + +	+	—
E122L	+ + + +	+ + + +	+	—
E134D	+ + +	+	+	—
E134R	+ + +	+ +	+ +	+ +
E134R/R135E	+ +	+ +	+ +	+ +
K248L	+ +	+ +	+	—
W265F	+ + +	+	—	n.d.
Y268S	+ + + +	—	—	n.d.
P291A	+ + +	+	—	n.d.
Y301F	+	—	n.a.	n.d.
A299C/V300A/P303G	+ + + +	—	—	n.d.

of the cells by mild fixation should the latter epitopes become accessible to antibodies. Because cells dissociate from the surface on or after 3 dpi, immunohistochemical analysis was performed at 2 dpi and the polyclonal antiserum CERNJS858 was used to estimate the relative number of positive cells and their average reactivity. This approach was tested on Sf9 cells producing wild-type opsin; the expected results were obtained (Table 2): epitopes that should have been located intracellularly became accessible only after permeabilization of the cells. From these results we conclude that Sf9 cells accomplish correct membrane insertion

Figure 6 Analysis of incorrect translocation of mutant opsins by a short digestion of intact or permeabilized cells with thermolysin

Immunoblot analysis of intact and lysed Sf9 cells infected with recombinant wild-type or mutant opsin virus. R, bovine ROS membranes. Lanes 1, total cell extracts of cells not incubated with thermolysin; lanes 2, total cell extracts of intact cells incubated with thermolysin [10% (w/w) for 5 min]; lanes 3, total cell extracts of lysed cells incubated with thermolysin. Opsin was identified with polyclonal antiserum CERNJS858 (**A**) or monoclonal antibody 1D4 (**B**). The positions of the intact glycosylated (arrow) and non-glycosylated (arrowhead) opsin are indicated. The positions of molecular mass markers (kD, kDa) are shown at the left.

and targeting of the opsin sequence. Subsequently this approach was used to analyse the effect of mutations on membrane translocation and targeting. Again, Sf9 cells were infected with recombinant mutant opsin virus, and the immunofluorescent reactivity of intact cells with monoclonal antibodies against the N-terminal domain (R2-12N) and the C-terminal domain (1D4) was estimated, relative to the total immunofluorescent reactivity with CERNJS858 observed after permeabilization of the cells by fixation. The results are given in Table 3. This type of analysis shows that all mutants are targeted to the plasma membrane (see Figure 5), but quite a number of mutations show appreciable reactivity with 1D4 in intact cells. This could have been due to a complete reversal of the opsin disposition (N-terminus cytosolic, C-terminus extracellular) or to incorrectly translocated opsin (both N- and C-termini extracellular). All available evidence supports the latter alternative. First, all cells in this group, immunopositive for the C-terminally directed antibody, also reacted with the N-terminally directed antibody. Secondly, a strong increase in immunolabelling intensity with 1D4 was not accompanied by a strong decrease for that with R2-12N. These results suggest strongly that the 1D4-reactive mutant proteins were produced by incorrect translocation across the endoplasmic reticulum membrane.

It seemed logical to assume that the non-glycosylated mutant form was responsible for this phenomenon. However, in contradiction with this assumption is the fact that a number of mutant proteins did not produce detectable amounts of a non-glycosylated form but did react with 1D4 in intact cells (E113Q, E113D, W265F). To distinguish between the glycosylated and non-glycosylated species the following strategy was developed. Sf9 cells were infected with recombinant virus and exposed after 3 dpi to thermolysin digestion (Figure 6; thermolysin cleavage sites in bovine rhodopsin are indicated in Figure 1). In correctly folded opsin these sites were only accessible in permeabilized cells, and proteolysis resulted in a shift to a lower molecular mass (Figure 6A, lanes 3) and a loss of reactivity with 1D4 (Figure 6B,

lanes 3). Proteolysis was allowed to proceed until in permeabilized cells 1D4 reactivity had been largely abolished, as determined by immunoblot analysis. During this incubation, intact cells were slowly permeabilized but most (more than 75%) wild-type opsin remained protected against proteolysis because it retained immunoreactivity with 1D4 (Table 3 and Figure 6B; compare v-ops, lanes 1 and 2). Glycosylated and non-glycosylated bands on immunoblots were quantified by densitometer imaging and the degree of proteolysis was determined by converting the amounts of intact protein to ratios (Figure 6B, lanes 2 to 1).

When applied to mutant opsins, the results support the immunofluorescence analysis. Because the immunofluorescence assay was more sensitive but much less quantifiable than the proteolytic approach, we compare the two approaches qualitatively only. Mutations that resulted in a positive reaction with 1D4 on intact cells showed a significant increase in susceptibility to proteolysis in intact cells (Table 3). For mutations that produced glycosylated as well as non-glycosylated species (E134D/R, E134R-R135E, K248L) the non-glycosylated forms sometimes seemed to be less susceptible on proteolysis of intact cells (Table 3). Currently we interpret this lower susceptibility as being due to retention in intracellular compartments, because nearly complete proteolysis was observed in permeabilized cells.

Our results indicate that the described mutations of residues Glu-113, Glu-122 and Glu-134 (third membrane domain) and Lys-248 (loop i3) enhanced the chance of incorrect translocation of the opsin sequence, which was also accompanied by a partial inhibition of glycosylation. The most severe effects were observed with the mutations E134R and E134R-R135E, which produced only 30–40% correctly folded glycosylated protein. This might explain the low yields of regenerated pigment (5–10% of total protein produced) obtained with these mutants [22].

Another remarkable observation was made on some mutants (Glu-113 mutants, W265F and Y268S) with the immunohistochemical analysis. These mutants presented a different pattern from that observed for the wild type. Several cells

showed a normal diffuse plasma membrane reaction, but other cells also showed a kind of spot-like pattern, possibly representing aggregated protein (Figure 5B). These spots were located in the plasma membrane because analysis of intact Sf9 cells showed the same pattern with CERNJS858, and subsequent fixation and incubation with 1D4 or R2-12N revealed no additional large intracellular storage sites of opsin (results not shown).

DISCUSSION

Apparently the biosynthesis, membrane translocation and glycosylation of opsin can be severely affected by point mutations. Only palmitoylation seems to occur fairly normally in most mutants described here. Hence the palmitoylation sites are apparently recognized and modified, whether located on the intracellular or the intraluminal side of the endoplasmic reticulum. Indeed, extracellular thiopalmitoylation sites have been described in plasma membrane proteins [28], suggesting that thiopalmitoyl modification of Cys residues can occur inside the lumen of the endoplasmic reticulum. However, replacement of Asp-83 with Asn or Trp-265 with Phe decreased the extent of palmitoylation. This is currently unexplained.

It should be noted that our observations stem from heterologous expression in insect cell lines that perform all essential eukaryotic protein processing but still differ in some aspects from the mammalian environment, from which these proteins originate. Nevertheless we do not consider this to have a major influence. Indeed, wild-type opsin is fully processed in these insect cells up to quite high expression rates (10–15 pmol/day per 10^6 cells, which is comparable to a photoreceptor cell). Definite proof that the effects of point mutations that we observed also operate in the photoreceptor cell will have to come from studies with transgenic mice.

From the overall results we can distinguish four types of mutant, types A to D.

Type A: amino acid substitutions that do not significantly affect opsin biosynthesis and folding of opsin

In this category are D83N, A299C-V300A-P303G and P291A. The presence of a Pro residue in a transmembrane domain is usually considered to be of structural importance [29,30]. Although Pro-291 is not generally conserved in the G-protein-coupled receptor family, it is fully conserved in the vertebrate visual pigments. Nevertheless the P291A mutation shows few structural consequences. This also agrees with our observation that this mutant shows normal regeneration capacities and spectral properties [22].

Pro-303 is fully conserved in the G-protein-coupled receptor family, but its replacement with Gly does not affect the biosynthesis of opsin. Spectral properties of rhodopsin are not affected either [22]. However, transducin activation needs to be examined because replacement of this residue in the m3 receptor with Ala increased the affinity for agonists but severely impaired stimulation of PtdIns hydrolysis. It might therefore represent a key residue involved in mediating agonist-induced conformational changes required for G-protein activation [31]. In contrast, substitution of the corresponding Pro by Ser in the β_2 -adrenergic receptor seemed to affect post-translational processing (non-glycosylated and partly glycosylated forms) [32]. It is important to note that this mutant contains two additional mutations (A299C and V300A), which represent unconserved residues and are adjacent to residue Y301, which will be discussed below.

The relatively innocuous effect of these Pro mutations supports other evidence indicating that the replacement of single Pro

residues in transmembrane domains by Gly or Ala are generally structurally well tolerated [33].

Although Asp-83 is highly conserved among the entire family of G-protein-coupled receptors, replacement with Asn influences neither opsin biosynthesis or folding, nor has an effect been observed on regeneration or transducin activation [20,34–37]. In retrospect this is not unexpected because the D83N mutation has meanwhile been detected in several rod visual pigments [38–41] and hence is unlikely to perturb the fine tuning of the protein sequence for correct biosynthetic processing. This seems to be in contrast with adrenergic receptors, where replacement with Ala/Arg affected the affinity for agonists and activation of the G-protein [42,43].

Type B: amino acid substitutions that result in the production of a truncated protein

To this category belong R69H and Y301F. Interestingly, the resulting incomplete protein was transferred to the plasma membrane and the N-terminus was correctly located extracellularly. Hence, as expected, the first translocation-start signal was properly recognized. However, the cause of the eventual truncation was probably different. The Y301F mutant produces a protein of approx. 30 kDa that lacks the C-terminus. Hence translation and translocation probably proceed normally until the point mutation is reached. Then either translocation and/or translation are aborted. Correct translation and translocation of the intact protein followed by removal of the C-terminal part, due to increased susceptibility of the mutant to proteolysis, is less likely because (1) no trace of the intact protein is detectable and (2) the point mutation resides within transmembrane domain VII and is not expected to strongly change proteolytic sensitivity outside that domain. Because Tyr-301 is highly conserved in the G-protein-coupled receptor family, we propose that it is essential for proper translocation and folding of the protein. Our observation that mutations at positions 299, 300 and 303 are quite 'harmless' in this respect (see above) again shows how delicately tuned these functions are.

The situation for R69H is quite different. This mutant mainly produces an approx. 22 kDa truncated protein, which is glycosylated and still contains the e2 loop. It is currently unclear why translation and/or translocation are aborted shortly after the e2 loop by a mutation in the i1 loop. Although the complete protein is detectable in very small amounts, this probably indicates that a minor part of the translational events proceed to the intact protein. The alternative, complete translation to the intact protein followed by intracellular proteolysis is highly unlikely in our opinion because the required cleavage near the C-terminus and/or in the i3 loop would result in products of approx. 33 kDa or approx. 26 and 13 kDa respectively [44]. Fragments smaller than 20 kDa were never observed. In addition, we did not observe larger amounts of intact protein at earlier time points (1 or 2 dpi).

Type C: amino acid substitutions that affect membrane translocation and glycosylation

Substitutions at the positions Glu-113, Glu-122, Glu-134, Arg-135 and Lys-248 belong to this category. We had already suggested that the charged pair Glu-134 and Arg-135 could be part of a stop-transfer site [18]. The mutations in transmembrane domain (TM) III (positions 113, 122, 134 and 135) pertain to highly polar residues localized within the membrane or at the membrane interface. Protein transfer signals consist of a combination of polar and hydrophobic residues and probably require a specific three-dimensional arrangement, clear insight in which

is still lacking [45]. We propose that these four positions are important elements for a transfer signal, which in wild-type opsin functions as a transfer stop, thereby positioning TM III in the membrane. When point mutations decrease the affinity of the signal site for the translocation machinery, there is a chance that it is not recognized as such and the signal 'slips' through. In that case TM III is fully translocated and translocation stops only at the next signal in TM IV, which in wild-type opsin functions as a start-transfer signal (transfer signals can probably function as both stop- and start-transfer sites, depending on the current activity of the translocase [45,46]). Hence, if a slip in the TM signal occurs, TM III and loop i2 are translocated and the rest of the topography of opsin reverses, the C-terminus finally being located extracellularly. This model would explain the partial accessibility of these mutants to 1D4 and thermolysin in intact cells. Interestingly, the chance of such a slip's occurring clearly depends on the type of substitution (E134R-R135E > E134R > E134D; E113Y > E113D > E113Q). This suggests that in wild-type opsin the signal is very delicately tuned.

A similar model can be put forward for the K248L mutant. Lys-248 is located in the interface region of TM VI. If replacement of the polar Lys-248 with the apolar Leu interferes with the proper functioning of the start-transfer signal in TM VI, this signal might 'slip through'. TM VI and loop e3 will not be translocated and the signal in TM VII will induce transfer start, again with extracellular location of the C-terminal as a result.

Hence our results can be explained by assuming that the type C mutations interfere with the function of a transfer signal, resulting in a significant 'slip' of the signal. It should be further noted that in all positions in this category (E113C, E122L, E134D, K248L; Table 3) the non-glycosylated forms are less susceptible to proteolysis in intact cells than the glycosylated forms but are rapidly proteolysed in lysed cells. Our interpretation is that these non-glycosylated species have not been targeted to the plasma membrane but, probably because of folding problems, became stuck in traffic, possibly in the endoplasmic reticulum. We are currently trying to locate these intracellular species more precisely by confocal microscopy.

Although the effect of mutations on folding and targeting has not been reported in much detail in the literature, scattered results generally support our model. First, the extracellular domain is very sensitive to point mutations and deletions. These usually lead to incorrect folding and loss of glycosylation [4,47–52], indicating that the structural organization of this domain is easily perturbed and that glycosylation is sensitive to the structure of this entire domain. Furthermore the mutation R135L/W is found in patients with autosomal dominant retinitis pigmentosa [50,53] and seems to be associated with glycosylation and targeting [50]. Mutation of Glu-113 to Lys also resulted in misfolding and impaired glycosylation [16,54]. This is also described for the mutant E122K [16].

It should be emphasized that, when correctly folded and glycosylated, these mutant opsins do generate a corresponding photopigment [22]. An interesting phenomenon, however, is that all of these mutant pigments are also affected with respect to signal transduction. The effects vary from a complete loss of transducin activation (R135Q/W/L) [16,17,50,53] to a decrease (E122Q/D/A, E134D, K248L) [16,17,35,55]. Several mutants in these positions show constitutive activity (E113Q [54,56]; E134A [36]). In most other G-protein-coupled receptors an Asp residue at the position corresponding to Glu-134 is fully conserved and seems to be required for efficient signal transduction [31,42]. Residue Lys-248 also seems to be of similarly general importance [57,58]. Glu-113 is considered to be the Schiff base counterion in visual pigments [16,34,37] and a ligand counterion in several G-

protein-coupled receptors [31,42]. Replacement of this residue with one lacking a negative charge leads to constitutive activity in visual pigments, suggesting that it helps to constrain opsin into an inactive conformation via a charge-pair interaction [54,56].

Type D: amino acid substitutions that produce an intact protein without detectable non-glycosylated product but lead to heterogeneous distribution in the plasma membrane and decreased regeneration

The mutations E113Q/D, W265F and Y268S belong to this category. Immunohistochemical analysis of these mutants produces a patchy distribution in the plasma membrane. They have a strongly decreased regeneration capacity [16,22,34,35,37] and the respective residues reportedly all interact with the retinal chromophore in bovine rhodopsin [35]. The structural effects of the E113Q mutation, which among others result in constitutive activity [54,56], might lead to lower stability, an enhanced tendency to aggregate and a decrease in regeneration capacity. Mutation of Trp-265 might also induce structural changes, as evident from lower regeneration capacity, a blue shift of 20–30 nm and a decrease in transducin activation [35]. In the m3 receptor, decreased affinities for both agonists and antagonists are observed on mutation of this residue. Hence this residue probably contributes to stabilizing the ligand–receptor complex [31].

Evidence is also available for structural perturbation in Tyr-268 mutants. The mutation Y268F in rhodopsin decreases transducin activation [35] and in the m3 receptor leads to decreased ligand-binding affinities [31].

Conclusions

In conclusion, we can state that single substitution of amino acids in the opsin sequence generally affects a combination of structural and/or functional properties. This again emphasizes that structure and function of this G-protein-coupled receptor are intimately related and delicately tuned. In addition, in view of the delicate tuning of the opsin sequence towards correct translocation and folding it can be expected that these functions are easily perturbed by single mutations. Hence we do not consider it remarkable that a large number of point mutations in rhodopsin are connected to autosomal dominant retinitis pigmentosa.

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