Inhibition of oligosaccharide processing and membrane morphogenesis in retinal rod photoreceptor cells

(castanospermine/glycoprotein/membrane/rhodopsin/retina)

STEVEN J. FLIESLER^{*†}, MARY E. RAYBORN[‡], AND JOE G. HOLLYFIELD[‡]

^{*}Departments of Ophthalmology and Biochemistry, University of Miami School of Medicine, Miami, FL 33101; and [‡]Cullen Eye Institute, Baylor College of Medicine, Houston, TX 77030

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ABSTRACT Castanospermine (Cas), an inhibitor of α -glucosidase I, blocks "trimming" of the N-linked oligosaccharide $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$, thus preventing normal glycoprotein maturation. With use of a dual-label protocol, *Xenopus* retinas incubated in the presence of Cas exhibited at least a 2.3-fold increase in the incorporation of [^3H]mannose into total retina Cl_3CCOOH -precipitable material, whereas incorporation of [^{14}C]leucine was not significantly affected, relative to controls. Analysis of NaDodSO₄/PAGE fluorograms of solubilized retinas and rod outer segment (ROS) membranes indicated a relatively selective effect of Cas on opsin (the rod visual pigment apoglycoprotein). The apparent molecular mass of opsin was increased by ≈ 2500 in the presence of Cas; the incorporation of [^3H]mannose into opsin was enhanced about 2.3-fold without a significant effect on [^{14}C]leucine incorporation, relative to controls. Electron microscopic autoradiography of retinas incubated for 4 hr with [^3H]mannose showed that the number of newly formed ROS discs in Cas-treated retinas was not significantly different from controls, but the silver grain density over those discs was about 2.6-fold greater than in controls. The morphology of the newly formed discs was comparable under both conditions. Thus, opsin bearing abnormally large oligosaccharides can be accommodated in the process of disc morphogenesis. These results suggest that the structural requirements for opsin's oligosaccharides, with regard to their potential role as determinants of disc morphogenesis, are not stringent. Furthermore, post-translational processing of N-linked oligosaccharides is not essential for the normal intracellular routing and cell surface expression of membrane glycoproteins.

Most eukaryotic plasma membrane integral proteins are glycoproteins, often containing N-linked oligosaccharide chains. Although glycosylation may subserve particular biological functions of the cell (1-3), the exact relationship of oligosaccharide structure to the function of glycoproteins remains largely obscure. One approach to elucidating such structure-function relationships is to expose cells to compounds that selectively inhibit discrete steps of the multienzyme pathway responsible for the initial synthesis and subsequent processing of N-linked glycans (4, 5), examining the consequences of such treatment on a particular glycoprotein's oligosaccharide structure and some specific cellular process in which it is involved. Using this approach, we have been examining the putative role played by N-linked glycans, particularly the oligosaccharide chains of opsin (the apoglycoprotein moiety of the retinal rod visual pigment, rhodopsin), on the process of rod outer segment (ROS) disc morphogenesis.

The rod photoreceptor is a highly compartmentalized and polarized neuron (see Fig. 1). The ROS consists of several

hundred flattened double-membrane saccules (discs) arranged in an orderly stack, enveloped by the plasma membrane of the cell. The discs are composed of approximately equal amounts by weight of proteins (primarily opsin) and lipids (primarily glycerophospholipids) (7, 8). Normally, ROS length is maintained relatively constant by two dynamic processes: "new" discs are added at the ROS base and "old" discs are lost from the distal ROS tip (9, 10). New discs are derived from the plasma membrane (11, 12): as newly synthesized membrane constituents are added to the plasma membrane near the ROS base, the membrane buckles and expands outward from the ciliary stalk to form an "open disc" (see Fig. 1 *Inset*). Adjacent open discs fuse circumferentially along their outer margins and detach from the plasma membrane, forming a "closed disc," which is concomitantly added to the growing stack of discs sequestered within the ROS.

Opsin is a transmembrane constituent of the discs and the ROS plasma membrane (13, 14) (see Fig. 1 *Inset*). In the plasma membrane (and open discs), opsin's amino terminus is exposed to the extracellular milieu; in the closed discs, the amino terminus faces the intradiscal lumen. The amino terminal region contains two "hybrid"-type, N-linked oligosaccharides having the formula $\text{GlcNAcMan}_3\text{GlcNAc}_2$ (15-19). Incubation of retinas with tunicamycin, a selective inhibitor of dolichyl phosphate-dependent oligosaccharide biosynthesis (4, 5), blocks opsin glycosylation (20-22) and concomitantly results in aberrant disc morphogenesis (22). Rather than forming disc-like evaginations of the ROS plasmalemma, tunicamycin-treated rod cells accumulate tubulovesicular material in the compartment between the rod inner segment (RIS) and ROS (the "intersegmental space"). Evidence obtained by light and electron microscopic autoradiography (22), scanning electron microscopy (23), and quantitative freeze-fracture microscopy (24) strongly suggests that this intersegmental material represents nascent disc membranes that contain nonglycosylated opsin but apparently lack some essential architectural feature necessary for normal disc structure. Furthermore, when tunicamycin is injected intravitreally in frogs, a retinal degeneration ensues that is characterized by cessation of ROS membrane renewal, progressive shortening of ROS and eventual photoreceptor cell death (25, 26). Similar results have been obtained with other animals having either rod-dominant (27) or cone-dominant retinas (28). These findings suggest that N-linked oligosaccharides (e.g., those of opsin) may be important determinants in the process of disc morphogenesis and in the maintenance of normal photoreceptor outer-segment structure (6).

Abbreviations: Cas, castanospermine; RIS, rod inner segment; ROS, rod outer segment.

[†]To whom reprint requests should be addressed at: Bascom Palmer Eye Institute, P.O. Box 016880, Miami, FL 33101.

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FIG. 1. Schematic diagram depicting the major structural features and cellular compartments of a retinal rod photoreceptor cell. (Inset) Magnified view of the basal region of the ROS (the compartment of disc morphogenesis), illustrating the distribution of rhodopsin, with its two N-linked oligosaccharide chains, in the ROS plasma membrane and discs (modified from ref. 6).

N-glycosylation is not required for transport of opsin from its site of synthesis in the RIS to the site of disc assembly near the base of the ROS (21, 22). However, the biological significance of opsin's truncated oligosaccharides remains enigmatic. Why are they so extensively "processed" and simple, relative to the N-linked oligosaccharides of other glycoproteins, and what role do they play in the physiology of the photoreceptor cell? We wished to examine whether or not opsin would undergo normal intracellular transport and disc membrane assembly if its oligosaccharide chains were markedly altered. Our approach was to inhibit processing of the common N-linked precursor oligosaccharide $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$, the first protein-bound oligosaccharide generated via the "lipid intermediate pathway" (29, 30), resulting in formation of hyperglycosylated opsin molecules. This was achieved by incubating *Xenopus* retinas in the presence of castanospermine (Cas), an inhibitor of α -glucosidase I that prevents the conversion of $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ to nonglycosylated, "high mannose"-type structures (5, 31–33). Herein, we report that retinas incubated in the presence of Cas synthesize hyperglycosylated opsin, which undergoes normal intracellular transport and is utilized for the biogenesis of ROS membranes having normal disc morphology. A prelim-

inary account of this study has been published elsewhere (34).

MATERIALS AND METHODS

Animals and Reagents. Juvenile *Xenopus laevis* toads (Nasco, Fort Atkinson, WI) were maintained at 21°C under cyclic lighting (12 hr dark/12 hr light) for at least 2 wk prior to use. Retinas from dark-adapted animals were dissected free of pigment epithelium under dim red light, ≈ 1 hr prior to the time of normal light onset. Incubation medium was prepared from a Ringer's bicarbonate/pyruvate solution (21) containing 2 mg of Casamino acids (Sigma) per liter, was aerated with 95% O_2 /5% CO_2 on ice for 30 min prior to use, and was supplemented with 5% (vol/vol) amphibian tissue culture medium (GIBCO). Cas (gift of Alan Elbein) was dissolved in dimethyl sulfoxide (Burdick and Jackson, Muskegon, MI) to obtain a stock solution concentration of 100 mg/ml. D-[2- ^3H]Mannose (20 Ci/mmol; 1 Ci = 37 GBq) and L-[U- ^{14}C]leucine (354 mCi/mmol) were obtained from ICN and Amersham, respectively. Protein standards and reagents for NaDodSO₄/PAGE were obtained from Bio-Rad.

Incubation of Retinas. Retinas were incubated in 50-ml flasks (four groups of eight retinas per 5 ml of medium per flask) at 22°C in darkness under 95% O₂/5% CO₂ for 1 hr without or with addition of Cas in dimethyl sulfoxide (final concentrations, respectively, 0.1 mg/ml and 0.1%). For biochemistry, retinas (four experimental and four control) were transferred to 25-ml flasks containing fresh medium (3 ml, with or without Cas/dimethyl sulfoxide) and [³H]mannose (0.23 mCi/ml)/[¹⁴C]leucine (0.01 mCi/ml). For microscopy/autoradiography, retinas (four experimental and four control) were transferred to fresh medium (as above) containing [³H]mannose (0.20 mCi/ml) as the radiolabeled substrate. Incubations were continued an additional 4 hr in the presence or absence of Cas under normal room lighting. Retinas were then serially transferred through four 10-ml rinses of fresh incubation medium on ice prior to further processing.

Biochemical Analyses. Procedures used for Cl₃CCOOH precipitation of retinal proteins, determination of protein content and specific radioactivity of Cl₃CCOOH-precipitable material, solubilization of retinas and ROS membranes, and NaDodSO₄/PAGE, and fluorography were as described (21, 22). The NaDodSO₄/PAGE system used a 10% T/2.7% C separating gel and a 4% T/2.7% C stacking gel (T and C are polyacrylamide and crosslinker percentages of total monomer). In two experiments, ROS membranes were prepared from retinas incubated with [³H]mannose/[¹⁴C]leucine in the presence or absence of Cas, and the ROS proteins were analyzed in parallel with the whole retina samples (21, 22). In one experiment, the band corresponding to opsin in the NaDodSO₄/PAGE gel (for each of the two incubation conditions) was excised and solubilized in NCS Tissue Solubilizer (Amersham), and the radioactivity was measured by liquid scintillation counting.

Microscopy and Autoradiography. Procedures utilized for processing and analysis of retinas by electron microscopy and autoradiography were as described (22, 35, 36). Only well-oriented rods in longitudinal sections were used for quantitative analysis, surveying multiple sections from at least three retinas per incubation condition.

RESULTS

In the first dual-label experiment, we wished to ascertain whether or not Cas (at 0.1 mg/ml) would block N-linked oligosaccharide processing without appreciably inhibiting more general aspects of retina metabolism (e.g., protein synthesis) under our *in vitro* conditions. This was assessed indirectly, taking advantage of the fact that the precursor oligosaccharide Glc₃Man₉GlcNAc₂ contains nine mannose residues, while the fully processed oligosaccharides of opsin [the predominant glycoprotein in *Xenopus* retinas (22)] would be expected to contain between three and five mannose residues per chain (17–19). Incubation of retinas in the presence of Cas caused a significant (*ca.* 2.3-fold) increase in the incorporation of [³H]mannose into total Cl₃CCOOH-precipitable material, whereas [¹⁴C]leucine incorporation was not appreciably affected by Cas, relative to controls (Table 1).

In a second experiment, we analyzed the incorporation of [³H]mannose and [¹⁴C]leucine into proteins of whole retinas and purified ROS membranes by NaDodSO₄/PAGE and fluorography. The results are shown in Fig. 2. In the control retina sample, the major radiolabeled component exhibited an intense, broad band corresponding to an apparent *M_r* of ≈36,000, consistent with the electrophoretic behavior of opsin on this type of PAGE system (21, 22). Another relatively intense, broad band was observed with an apparent *M_r* of ≈66,000, consistent with the electrophoretic behavior of opsin dimers (21, 22). The corresponding components in

Table 1. Effect of Cas on incorporation of [³H]mannose and [¹⁴C]leucine into retina Cl₃CCOOH-precipitable material

	Specific activity, dpm/μg of protein		³ H/ ¹⁴ C
	[³ H]Man	[¹⁴ C]Leu	
Control	326 ± 32	682 ± 96	0.48
Cas	758 ± 127	609 ± 38	1.25
Ratio	2.33	0.89	2.60
	(<i>P</i> < 0.001)	(NS)	

Values are expressed as means ± SD; *n* = 3. NS, not significant.

the Cas-treated retina sample were similar in intensity, but their migration was slightly retarded, consistent with an increase in apparent *M_r* by ≈2500 ± 500. The intensities and relative mobilities of the other radiolabeled components showed no apparent differences as a function of the presence of Cas. The fluorogram of the control ROS sample exhibited one major radiolabeled component, a broad, partially resolved doublet corresponding to an apparent *M_r* of ≈36,000. The fluorogram of the corresponding Cas-treated ROS sample was virtually identical, except that the major band was retarded in migration, corresponding to an increase of *M_r* of ≈2500 ± 500. The ROS membrane samples were run on a companion NaDodSO₄/PAGE gel, the band corresponding to opsin in each lane was excised from the gel and solubilized, and the radioactivity was measured. The control sample contained 4100 dpm of ³H and 12,100 dpm of ¹⁴C; the Cas-treated sample contained 9400 dpm of ³H and 13,200 dpm of ¹⁴C. Thus, the incorporation of [³H]mannose into opsin in Cas-treated retinas was about 2.3-fold greater than in controls, whereas the incorporation of [¹⁴C]leucine was nearly identical in each.

Retinas which had been incubated with [³H]mannose in the

FIG. 2. NaDodSO₄/PAGE fluorogram (2-day exposure) of proteins from retinas (A) and ROS membranes (B) after incubation of *Xenopus* retinas for 4 hr with [³H]mannose (0.23 mCi/ml)/[¹⁴C]leucine (0.01 mCi/ml) in the absence (–) and presence (+) of Cas (0.1 mg/ml). Under the conditions used, only the ¹⁴C-labeled components are visualized. Note the electrophoretic mobility of opsin in controls (arrow, *M_r* ≈ 36,000) and its apparent shift to increased *M_r* (open arrowhead) after Cas treatment. All other radiolabeled proteins (except for the opsin dimer, *M_r* ≈ 66,000) appear to be unaffected by Cas, with regard to electrophoretic mobility and densitometric intensity.

presence or absence of Cas were analyzed further by electron microscopic autoradiography. The ROS, apical RIS, and nascent disc membranes in Cas-treated retinas were similar in ultrastructure to the corresponding rod cell components of control retinas (Fig. 3). Notably, rod cells from retinas incubated under both conditions exhibited moderate dilation of the intersegmental space (in comparison with freshly fixed, unincubated retinas) as well as a discrete population of undulating discs just beneath the basal ROS surface, clearly separate from the overlying, well-aligned stack of discs sequestered within the ROS. Furthermore, only this distinct population of basal discs was labeled with silver grains because of incorporation of newly synthesized glycoproteins containing [^3H]mannose. These features identify those discs that were newly synthesized within the incubation period and facilitate their quantitative analysis. The number of newly formed discs in Cas-treated retinas was not significantly different from that of controls (Table 2). However, the silver grain density over the newly formed discs of rods in Cas-

FIG. 3. Electron microscopic autoradiograms of the junctional region between a RIS and ROS from retinas incubated for 4 hr with [^3H]mannose (0.2 mCi/ml) in the absence (A) or presence (B) of Cas (0.1 mg/ml). Note that the newly assembled, basal-most discs (separated from the overlying ROS membranes) exhibit incorporation of radioactivity, represented by the black silver grains in the autoradiograms (discs of the Cas-treated sample appear to be more heavily labeled than do those of the control sample), and that the morphology of the discs is similar under both incubation conditions. (Bar = 1 μm .)

Table 2. Effect of Cas on assembly of ROS disc membranes

	New discs, no.	Grains per μm^2
Control	9.3 $\pm$ 2.6 (19)	3.38 $\pm$ 1.48 (9)
Cas	8.4 $\pm$ 3.0 (22)	8.63 $\pm$ 4.26 (13)
% of control	90.3 (NS)	255 ($P < 0.005$)

Values are expressed as means $\pm$ SD, with the number of independent measurements given in parentheses. New discs were defined as those that were distinctly separated from the bulk of overlying membranes at the base of the ROS and exhibited silver grains due to incorporation of [^3H]mannose.

treated retinas was nearly 2.6-fold greater than that of rods in control retinas.

DISCUSSION

In comparing the structure $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ with the oligosaccharides of bovine opsin (19), the expected ΔM_r is approximately 1160. Thus, since opsin has two N-linked oligosaccharides per polypeptide chain, the expected ΔM_r between normal opsin and opsin from Cas-treated retinas is ≈ 2320 , assuming that the oligosaccharides of *Xenopus* and bovine opsin are identical in composition and structure. This is in good agreement with the observed results. It should be noted that the Cas-treated sample exhibited no labeled component corresponding to M_r 36,000, suggesting that the inhibition of oligosaccharide processing was relatively efficient under the conditions used. However, the broadness and obvious heterogeneity of the opsin band in the Cas-treated samples suggests that some limited processing of the oligosaccharides did occur, resulting in multiplicity of opsin species. From the published mole ratio of the three oligosaccharide structures found in bovine opsin (19), one can calculate an average value of 3.5 mannose residues per N-linked oligosaccharide chain. If one assumes that the oligosaccharide composition of *Xenopus* and bovine opsin are identical and that the oligosaccharides of opsin in the Cas-treated sample contain nine mannose residues per chain, the theoretically expected enhancement of [^3H]mannose incorporation into opsin in the presence of Cas would be approximately 2.6-fold. Considering that the oligosaccharide composition and structures of *Xenopus* and bovine opsin may not be identical (see the discussion of ref. 22) and that some limited processing of opsin's oligosaccharide chains is evident even in the presence of Cas, the agreement between the theoretical and observed values for enhancement of [^3H]mannose incorporation is remarkable. We have obtained similar results with *Rana pipiens* retinas *in vitro* (6). It should be appreciated that the enhancement of [^3H]mannose incorporation in response to Cas cannot be ascribed solely to an increase in the specific activities of the biogenically relevant substrate pools of mannose because the apparent molecular mass of opsin increased by an amount consistent with hyperglycosylation of opsin.

Studies by Elbein and coworkers (31–33) using other cell types have demonstrated that some removal of mannose residues can occur in the presence of Cas without removal of glucose residues, resulting in structures of the type $\text{Glc}_3\text{Man}_{5-7}\text{GlcNAc}_2$. Notably, oligosaccharide structures of the type $\text{Man}_{5-9}\text{GlcNAc}_2$ were not found. Although we have no direct evidence to indicate the exact number of glucose and mannose residues in the abnormally large oligosaccharides attached to opsin, the increase in [^3H]mannose incorporation and concomitant increase in the molecular mass of opsin in the presence of Cas, in conjunction with the known effects of Cas on glycoprotein processing in other systems (31–33), are consistent with a structure containing between five and nine additional sugars per chain.

Previous studies (6, 22–25) have suggested that the presence of oligosaccharides on opsin may be essential for the normal morphogenesis and renewal of ROS discs. In the present study, *Xenopus* retinas were incubated in the presence of Cas under conditions that caused hyperglycosylation of opsin. Our data suggest that the mechanism of ROS disc morphogenesis can accommodate opsin molecules having oligosaccharide structures different from those normally present. The apparent lack of stringency in specification of opsin oligosaccharide structure with regard to disc morphogenesis also may be inferred *a priori* from the fact that these oligosaccharides normally exhibit microheterogeneity (17–19).

In addition to the biochemical data (see Table 1 and *Results*), the fact that newly synthesized, hyperglycosylated opsin was localized to nascent discs and that these discs were quantitatively similar to those of control retinas indicates that Cas did not affect either the rate of opsin synthesis or the rate of intracellular transport of opsin to the locus of disc morphogenesis. Thus, the stepwise enzymatic processing of N-linked glycans normally associated with the compartmentalized intracellular transport of such molecules from the endoplasmic reticulum through the Golgi apparatus to the plasma membrane (29, 30) is not essential for such transport to occur. A similar conclusion has been reached by Burke *et al.* (37), who showed (using two different inhibitors of oligosaccharide processing) that vesicular stomatitis virus G protein, influenza virus hemagglutinin, and human class I histocompatibility antigens containing unprocessed or partially unprocessed N-linked oligosaccharides can be expressed at the surface of host cells in culture.

We have proposed (6) that the oligosaccharides of opsin may be involved in some significant manner in the mechanism of disc morphogenesis. Specifically, we hypothesize that opsin's oligosaccharides may serve either as ligands or as allosteric modulators in the course of protein–carbohydrate or protein–protein interactions that promote and stabilize the close apposition of membrane surfaces during disc formation. Accommodation of structures as divergent as those normally present on opsin compared with those generated in the presence of Cas may signify that some specific chemical or geometrical feature common to both types of oligosaccharide structures is recognized and utilized during disc morphogenesis. In most cells, N-linked oligosaccharides, once trimmed to the form GlcNAcMan₃GlcNAc₂ are often modified in the Golgi apparatus by addition of “peripheral sugars” (29, 30). Apparently this is not the case for opsin's oligosaccharides, which normally retain their relatively stunted form and simple composition. However, under certain conditions, opsin can serve as a substrate for galactosyl and fucosyl transferases (13, 38–40). Perhaps such post-translational modifications of opsin's carbohydrate moieties are not compatible with the mechanism of disc morphogenesis? This question remains to be answered.

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