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Transient Hyperglycosylation of Rhodopsin with Galactose

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Rhodopsin’s oligosaccharide chains contain predominantly two types of sugar residues: mannose and N-acetylglucosamine. In the present work, bovine and rat rhodopsin were analysed biochemically for the presence of a third sugar, galactose. Treatment of bovine rod outer segments (ROS) with galactose oxidase followed by reduction with tritium-labeled sodium borohydride revealed the presence of existing molecules of galactose on rhodopsin. Rats injected intravitreally with [3H]galactose and [14C]leucine and maintained in darkness were killed 1 hr, 6 hr, 1, 3 or 5 days following the injection. Retinas were collected for subcellular fractionation and rhodopsin from each of the fractions was purified by ConA sepharose chromatography and SDS-PAGE. During the first 6 hr, galactose selectively labeled rhodopsin in the Golgi-enriched fraction resulting in increased [3H]/[14C] ratios in both Golgi and ROS. The data suggested that trimming was occurring at the transition from Golgi to ROS. Furthermore, a decrease in isotope ratio in the ROS between 6 hr and 1 day suggested further trimming of rhodopsin after membrane assembly in the ROS. Additional in vivo experiments demonstrated existing molecules of galactose on rhodopsin’s oligosaccharide chain using lectin affinity chromatography. Rats injected intravitreally with [3H]methionine were dark-adapted for 2 hr. Following subcellular fractionation of retinas, ConA purified rhodopsin from ROS was applied to one of two additional lectin columns: Ricinus communis agglutinin (RCA) or Griffonia simplicifolia I (GSA). Eight to nine per cent of the labeled rhodopsin was bound to and eluted from RCA, whereas none bound to GSA, indicating the presence of a β-galactoside. The RCA agaroase eluted puruless ω-electrophoresed with a rhodopsin standard and was light sensitive. Galactose was shown to be the terminal sugar on this subset of rhodopsin and was not capped by neuraminic acid. Binding of rhodopsin’s oligosaccharide to RCA was abolished by pre-treatment with β-galactosidase. Decreased binding of rhodopsin to RCA was observed following intravitreal injection of castanospermine but not swainsonine. Of those two inhibitors of glycoprotein trimming, only castanospermine would be expected to prevent the addition of galactose to the oligosaccharide. The association of galactose with rat rhodopsin appeared to be a transient one. At 2 hr, 8-9% of rhodopsin contained galactose, at 6 hr only 2-2% had galactose and by 24 hr less than 1% did. The galactose was trimmed from rhodopsin’s oligosaccharide presumably after its role was complete. Separation of rhodopsin of the plasma membranes from rhodopsin of discs indicated that 75% of the galactose-containing rhodopsin was in the plasma membrane and only 25% was in the discs. These findings suggested a possible role for galactose in new disc formation with subsequent removal after the discs are sealed.

Key words: rhodopsin; rat; retina; glycosylation; galactose; lectin affinity chromatography; RCA; ricin.

1. Introduction

Rhodopsin contains two oligosaccharide chains linked to Asn, and Asn, (Hargrave, 1977). For the most part, these chains contain only two types of sugar residues: mannose and N-acetylglucosamine (Heller, 1968; Shichi et al., 1969; Heller and Lawrence, 1970; Plantner and Kean, 1976). The synthesis of asparagine-linked glycoproteins typically involves the rough endoplasmic reticulum for the initiation of oligosaccharide synthesis and both the rough endoplasmic reticulum and Golgi complex for modification of the oligosaccharide chain (Kornfeld and Kornfeld, 1985). The oligosaccharide chain of rhodopsin is reminiscent of the ‘core’ oligosaccharide found in more complex glycoproteins prior to the addition of a terminal trisaccharide consisting of N-acetylglucosamine, galactose and neuraminic acid. Rhodopsin actually contains the first sugar of the terminal trisaccharide as demonstrated by glucosamine incorporation studies (O’Brien and Muellenberg, 1974; Bok, Hall and O’Brien, 1977). The remaining sugars, galactose and neuraminic acid have not usually been detected in the rhodopsin oligosaccharide chain. However, O’Brien (1976) demonstrated the direct transfer of galactose in vitro to bovine rhodopsin and showed chromatographically that the radiolabeled-galactose was incorporated as galactose since the radioactivity coincided with a galactose standard and was not converted to some other sugar (such as mannose). More recently, galactose was successfully incorporated into rat rhodopsin in vitro following incubation of whole retinas with [3H]galactose (St Jules, Smith and O’Brien, 1990). Fukuda, Papermaster and Hargrave (1979) analysed bovine rhodopsin’s...
amino terminal glycopeptide using gas chromatography and mass spectrometry and reported that in addition to the major oligosaccharide structure which contained three N-acetylglucosamine residues and three mannose residues, a minor variation of rhodopsin contained 0.1 mol galactose per three N-acetylglucosamine residues and three mannose residues. These data suggested that up to 10% of rhodopsin contained galactose.

Morphologic studies of carbohydrate-containing molecules in photoreceptor cells have invoked a different methodology utilizing lectins for detecting the presence of specific sugars. Retina communis agglutinin (RCA) is a lectin that specifically recognizes terminal galactose moieties associated with either glucosamine or N-acetylglucosamine in a β1–4 linkage (Baenziger and Fiete, 1979). Transmission electron microscopic studies of frog, bovine, monkey and rat retinas have shown binding of RCA to rod outer segments and in some cases rod inner segments (Nir and Hall, 1979; Hicks and Molday, 1985; Koide et al., 1986; Hicks and Barnstable, 1986). Likewise, similar results were obtained in fluorescent microscopic studies detecting RCA binding in frog, monkey, mouse, human, rabbit, and fish retinas (Bridges and Fong, 1979; Bridges, 1981; Uchera et al., 1983; Blanks and Johnson, 1984). Of great interest was the work by Hicks and Molday (1985) who published photomicroscopic evidence of post-embedding, direct labeling by RCA in the basal area of the ROS.

In the present study, we used biochemical techniques to characterize the subset of rhodopsin which appears to contain galactose. We present evidence to confirm the incorporation of galactose into rhodopsin in vivo experiments. Additionally, we demonstrate the presence of galactose in bovine rhodopsin by radiolabeling existing moieties. Finally, using lectin affinity chromatography, an analytical procedure which is rapid, reliable and highly sensitive (Cummings, Merkle and Stults, 1989), we are able to separate the galactose-containing species of rhodopsin and study its transient association with the oligosaccharide chain.

2. Materials and Methods

Animals

For most experiments, Sprague-Dawley rats, 125–150 g (Taconic Farms, Germantown, NY) were used. One set of experiments utilized bovine retinas dissected from bovine eyes obtained fresh from a slaughterhouse.

Isotopic Labeling

For studies of in vivo labeling of rhodopsin with galactose, rats were anesthetized with ether and injected intravitreally with 2 μL per eye of a saline solution containing 2 μCi [14C]leucine and 87.5 μCi [3H]galactose (44.1 Ci mmol⁻¹). In other in vivo experiments, eyes were injected with 20 μCi [3H]leucine (60 Ci mmol⁻¹) or 3 μCi [35S]methionine (1105 Ci mmol⁻¹) or 3 μCi [3H]methionine and 20 μCi [3H]glucosamine (44.8 Ci mmol⁻¹). The rats were maintained in darkness and at various intervals were killed with CO₂. The retinas were removed by propoting the eye using a curved forceps, the prongs of which were enmeshed in polyethylene tubing. The cornea was slit with a scalpel blade causing the lens and vitreous humor to be extruded. The retina was lifted free of any other ocular material simply by squeezing and lifting the forceps upwards. The sclera remained attached to the optic nerve. This procedure takes only 10–15 s and the retinas are removed intact and ready for biochemical workup. No elaborate dissection is required.

[3H]Galactose, [3H]glucosamine, [3H]leucine and [3H]methionine were purchased from New England Nuclear; [35S]methionine was purchased from American Radiolabeled Chemicals, Inc.

Subcellular Fractionation and Purification of Rhodopsin

Isolated retinas were fractionated in dim red light by a method described previously (St Jules, Smith and O'Brien, 1990). Four to six retinas were suspended in 2 vol 40% sucrose containing 65 mM NaCl, 2 mM MgCl₂, and 5 mM Tris acetate pH 7.4. Rod outer segments were broken off with 15 3-sec bursts of a vortex mixer. After a 15-min centrifugation at 27,000 g the floating ROS were removed with the supernatant and the retinal debris was treated again as above. The pooled crude ROS suspension was diluted with 3 volumes of buffered 10% sucrose, or in some cases 0.9% NaCl, and were sedimented by centrifugation at 27,000 g for 15 min. Both the ROS and retinal debris were separately homogenized in 2 ml 20% sucrose made up in 65 mM NaCl, 2 mM MgCl₂, and 5 mM Tris acetate pH 7.4 using seven strokes of a Dounce tissue grinder (Wheaton 7 ml B pestle with 0.0025–0.0035-inch clearance). These homogenates were layered over separate linear 50–25% sucrose gradients made up in the same buffer. After centrifugation for 2 hr in a Spincro SW 41 rotor at 35,000 rpm, bands were removed with a Pasteur pipet, diluted with 10% sucrose and sedimented by centrifugation for 15 min at 27,000 g. Two bands of unsealed and sealed ROS were near the top of the ROS gradient (Godchaux and Zimmerman, 1979). Below these was a zone of fine particles, enriched in Golgi. In the retinal debris gradient was a trace of the Golgi-enriched fraction and a prominent zone of coarse particles, including the rough endoplasmic reticulum, nuclei and synaptosomes. These fractions are described in more detail in St Jules and O'Brien (1986).

With the bovine retinas, discontinuous gradients were prepared by overlaying the bovine ROS sus-
pension with the following sucrose solutions: 2 ml of 
31.15, 5 ml of 31.13 and 2 ml of 31.11. The sucrose 
solutions were made up in 1 mM Tris acetate, pH 7.4,
containing 0.1 mM MgCl₂. After centrifugation for 
45 min at 35 000 rpm in a Spinco SW 40Ti rotor, the 
ROS were removed from the interface at 31.11/31.13 
with a Pasteur pipette, diluted with 10% sucrose and 
sedimented by centrifugation for 15 min at 27 000 g.

Purification of Rhodopsin

Rhodopsin-containing membranes from four to six 
rat retinas were extracted in dim red light for 1 hr at 
0°C with 2 ml 1% Emulphogene BC 720 (General 
Aniline and Film) in 50 mM Tris acetate buffer, pH 6.9,
containing 1 mM MgCl₂ and 1 mM CaCl₂. The extract 
was clarified by centrifugation at 27 000 g for 15 min 
and applied to a 0.8 ml column of ConA sepharose 
(Pharmacia). After washing with 25 ml 0.1% Emul-
phogene in the same buffer, the rhodopsin was eluted 
with eight applications of 200 μl aliquots of 0.5 M α-
methylmannoside (Sigma) also made up in the same 
buffer with 0.1% Emulphogene. Twenty microliters of 
each 200-μl fraction were sampled for radioactivity. 
Peak fractions were pooled and aliquots were adjusted 
to 2% sodium dodecyl sulfate (SDS) and 5 mM EDTA. 
Samples were incubated at room temperature (25°C) 
for 1 hr in dim red light. After the addition of use lalf 
volume of 50% glycerol containing 0.002% Bromo-
phenol blue (Biorad) the samples were applied to 
10% polyacrylamide disc gels with 3% stacking gels 
for electrophoresis by the method of Laemmli (1970). 
Gels were stained with Coomassie brilliant blue 
(Biorad) and cut into 1.2 mm slices with a Hoefer gel 
slicer and the slices containing the stained opsin were 
noted. Slices were digested overnight at 50°C in NCS 
protein solubilizer (Amersham) with 2.1% water. 
Slices were counted (two per vial) in Econofluor-2 
(Biorad) and cut into 1.2 mm slices with a Hoefer gel 
slicer. Those experiments utilizing two isotopes (i.e. [3H]galactose and [14C]leucine) permitted determination of the ratio of these isotopes in the opsin 
band of the gels.

Labeling of Bovine ROS with Galactose Oxidase and 
Sodium Borohydride

To radiolabel existing galactose moieties on rho-
dopsin, bovine ROS purified by a discontinuous sucrose 
density gradient procedure, were subjected to en-
zymatic treatment with galactose oxidase followed by 
a reduction with tritium labeled sodium borohydride. 
In these experiments no external source of radioactive 
galactose was provided.

ROS from two bovine retinas were washed by 
centrifugation with PBS for 10 min at 27 000 g. They 
were suspended in 1 ml PBS, an additional 1 ml of PBS 
containing 200 U of galactose oxidase (Worthington) 
was added to one ROS sample and 1 ml PBS without 
the enzyme was added to a second ROS sample as a 
control. Five microliters 0.1 M PMSF in ethanol were 
added to each sample. ROS were incubated at room 
temperature with occasional swirling for 30 min in 
the dark. ROS were centrifuged at 27 000 g for 10 min, 
then resuspended in PBS and centrifuged for the same 
length of time and speed. ROS were suspended in PBS. 
To each sample was added 2.5 ml of tritium labeled 
sodium borohydride (New England Nuclear) in 0.01 N 
NaOH made up in 1 ml PBS. After 30 min at room 
temperature, additional PBS was added and ROS were 
centrifuged at 27 000 g for 10 min. Subsequently, 
ROS were washed twice by centrifugation in PBS. In a 
repeat of this procedure, one sample received pre-
treatment with 1 U neuraminidase from Clostridium 
perfringens (Sigma) at pH 5.3 for 1 hr at 37°C and the 
subsequent galactose oxidase incubation time was 
doubled. The ROS were solubilized and rhodopsin was 
analysed by SDS–PAGE as described above.

Lectin Affinity Chromatography

To further characterize the subset of rhodopsin 
which contains galactose (without introducing an 
external source of galactose) lectin affinity chromatog-
raphy was used. In this method, ConA purified 
rhodopsin or in some cases the clarified extract of ROS 
from six rat retinas labeled with [3H]methionine was 
applied to columns of either Ricinus communis agglu-
tinin I (RCA) or Griffonia simplicifolia I (GSA), specific 
for β-linked or α-linked galactose residues, respect-
ively. RCA agarose was purchased from U.S. Bio-
chemical Corp., Cleveland, OH and GSA agarose was 
purchased from Biocarb Chemicals, Lund, Sweden. 
Columns were washed with 0.1% Emulphogene in 
50 mM Tris acetate buffer, pH 6.9, containing 1 mM 
MgCl₂ and 1 mM CaCl₂. Samples were eluted from RCA 
agarose or GSA agarose columns with 200 μl aliquots 
of either 0.5 M β-methyl-d-galactopyranoside or α-
methyl-d-galactopyranoside (U.S. Biochemical Corp.), 
respectively. Aliquots (20 μl) were analysed for radio-
activity using a Beckman LS 2800 scintillation coun-
ter. Aliquots of the peak fractions were applied to 
SDS–polyacrylamide gels and analysed for radio-
activity of opsin as described above.

Neuraminidase Treatment

To determine if the galactose of rhodopsin was 
capped with a neuraminic acid, rat ROS labeled with 
[3H]methionine were incubated with 1 U neuraminidi-
dase from Vibrio cholerae (Boehringer-Mannheim) at 
37°C for 2 hr. A second ROS sample was incubated 
with no enzyme. The incubated suspension was 
centrifuged for 15 min at 27 000 g. The ROS were 
extracted for 1 hr and the clarified extracts were
of the rhodopsin band of the gels of the RCA eluate as percentage of ConA purified rhodopsin which contained galactose was determined from the radioactivity of the fraction by ConA sepharose chromatography as described above. The amount of radioactivity eluted from RCA after galactosidase treatment was compared to that eluted from RCA without the enzyme treatment.

In vivo Treatment with Castanospermine or Swainsonine

To determine the consequences of the interruption of glycoprotein processing on the galactosylation of rhodopsin, two inhibitors of the glycoprotein processing pathway, castanospermine and swainsonine (Boehringer-Mannheim) were used in in vivo experiments. The concentration of the stock solution of castanospermine was 100 mg ml⁻¹. It was prepared by dissolving 5 mg of the inhibitor in 50 μl dimethyl sulfoxide (DMSO, Sigma). The concentration of the stock solution of swainsonine was 1 mg ml⁻¹. It was prepared by dissolving 500 μg of the inhibitor in 500 μl water. Rats were injected intravitreally with [³⁵S]methionine as a control or [¹⁴C]glucosamine was incubated at 37°C for 2 hr at 4°C. After centrifugation at 27,000 g for 15 min, the pellet was suspended in 1 ml of homogenizing buffer to which ricin agarose (U.S. Biochemical Corp.) was added. This mixture was incubated for 2 hr at 4°C. Ricin agarose was used rather than ricin–gold–dextran because it is sufficiently dense to pellet through the sucrose gradient.

B-Galactosidase Treatment of Rhodopsin's Oligosaccharide

ConA purified rhodopsin labeled with [¹⁴C]methionine and [³⁵S]glucosamine was incubated at 37°C for 2 hr with 20 U of N-glycanase (Genzyme, Boston, MA) in 0.15% SDS, 10 mM β-mercaptoethanol, 100 μM PMSF, 15 mM EDTA, 1-25% NP-40, pH 7.8. At the end of the incubation the pH of the sample was adjustd to 6.0 by the addition of acetic acid. The solution was buffered with MES (Sigma). β-Galactosidase (200 mU) from Diplococcus pneumoniae (Boehringer-Mannheim) was added to half of the sample. Both the enzyme treated sample and the control were incubated at 37°C for 24 hr. The samples were applied to separate RCA agarose columns and subsequently eluted with 200-μl aliquots of β-methyl-N-galactopyranoside as described above. The amount of radioactivity eluted from RCA after β-galactosidase treatment was compared to that eluted from RCA without the enzyme treatment.

Light-sensitivity Experiments

ROS labeled with [³⁵S]methionine and purified on a linear sucrose gradient were extracted with 10% Emulphogene as described above. Prior to purification of rhodopsin over ConA sepharose, half of the clarified extract was exposed to light for 10 min at 4°C. Each sample was then applied to a ConA sepharose column and chromatographed as described above. The eluted samples were each applied to separate RCA agarose columns and eluted according to the previously described procedures. Selected eluted fractions were applied to SDS polyacrylamide gels along with a [¹⁴C]rhodopsin standard for determination of the amount of radioactivity present in opsin under the conditions of light exposure and darkness.

Plasma Membrane Preparation

In order to determine whether the galactose-containing rhodopsin was present in photoreceptor discs or plasma membrane a modification of the method of Molday and Molday (1987) was used. [³⁵S]methionine-labeled ROS from 18 rats were prepared using the linear sucrose gradient procedure described previously. ROS were suspended in 1 ml homogenizing buffer consisting of 20 mM Tris acetate, pH 7.4, 2 mM MgCl₂, 20% sucrose. Neuraminidase (0.1 U) from Anthrobacter ureafaciens (Boehringer-Mannheim) was added to the ROS suspension and incubated for 2 hr at 4°C. After centrifugation at 27,000 g for 15 min, the pellete was suspended in 1 ml of homogenizing buffer to which ricin agarose (U.S. Biochemical Corp.) was added. This mixture was incubated for 2 hr at 4°C. Ricin agarose was used rather than ricin–gold–dextran because it is sufficiently dense to pellet through the sucrose gradient.

S. B. SMITH ET AL
in 65 mM NaCl, 2 mM MgCl₂, and 5 mM Tris acetate pH 7.4. After centrifugation for 3 hr in a Spinco SW 41 rotor at 35 000 rpm, the band of discs was drawn off using a Pasteur pipet and the plasma membranes at the bottom of the tube were also removed. These two fractions were separately centrifuged at 27 000 g for 15 min followed by the addition of 1% Emulphogene to each pellet to extract rhodopsin. Extracts were applied to RCA agarose columns, washed with 0.1% Emulphogene, and eluted according to the previously described procedures. Selected eluates were applied to SDS-polyacrylamide gels and the amount of radioactivity present in rhodopsin was determined for the discs and plasma membrane.

3. Results

**Labeling of Bovine ROS with Galactose Oxidase and Sodium Borohydride**

In the experiments using bovine retinas, purified ROS were treated with galactose oxidase to oxidize the sixth carbon hydroxyl group of galactose to a carbonyl group. This treatment was followed by exposure to tritium labeled sodium borohydride which chemically reduced the carbonyl group of galactose back to a hydroxyl with the introduction of a tritium atom (Gahmberg and Hakomori, 1973; Carubelli and Wen, 1990).

Figure 1 shows that without galactose oxidase treatment there was tritium incorporation into rhodopsin. There was a twofold increase in label following the galactose oxidase treatment. The galactose oxidase-dependent label tended to be in a species that migrated slower than rhodopsin as is indicated in the figure by the dashed line representing the difference between the two treatment groups.

Table I illustrates another experiment in which the galactose oxidase incubation time was doubled and the amount of radioactivity in the galactose oxidase treated ROS was 6.8-fold greater (over 70 000 dpm) than the samples not treated with the enzyme. Pretreatment with neuraminidase did not increase the labeling. The non-specific labeling of opsin in the samples not treated with the enzyme was due perhaps to reductions of the retinyl-lysine Schiff's base linkage. These experiments demonstrate the presence of galactose in the oligosaccharide chains of native rhodopsin in ROS plasma membranes and basal folds. Since the enzyme does not have access to the carbohydrate chains within the sealed discs, it is not known whether there are any galactose residues in the disc membrane rhodopsin.

**In Vivo Labeling of Rat Rhodopsin with Galactose**

Rats injected intravitreally with [³H]galactose and [¹⁴C]leucine were maintained in darkness. At various times (1 hr, 6 hr, 1, 3 or 5 days) following the injection, four retinas were collected for subcellular fractionation. Rhodopsin from each of the fractions was purified by ConA sepharose chromatography and SDS-PAGE. Table II provides the ratios of [³H] derived from galactose to [¹⁴C]leucine in rhodopsin from the subcellular fractions enriched in either rough endoplasmic reticulum, Golgi or ROS. The labeling pattern was complex. Galactose was probably converted to glucose and mannose residues of the core.

**Table I**

Reduction of bovine ROS with tritium-labeled sodium borohydride

| Pre-treatment | Galactose oxidase | dpm in opsin |
|---------------|------------------|--------------|
|               | −                | 9006         |
|               | −                | 12 287       |
| +             | +                | 74 809       |
| +             | +                | 70 674       |

Bovine ROS were pre-incubated for 1 hr at 37°C with neuraminidase added where indicated. Subsequently, the ROS were incubated at room temperature for 1 hr with galactose oxidase added where indicated. Finally, all ROS samples were reduced with [³H]sodium borohydride, solubilized and an aliquot representing the ROS from 0.05 retina analyzed by SDS-PAGE. The gels were sliced and counted and the opsin zone located by comparison with a standard on a parallel gel.
TABLE II

Ratio of \([^{1}H]\) derived from galactose to \([^{14}C]\)leucine in rat rhodopsin from major cell fractions

| Post-injection time | Sub-cellular fraction | Rough ER | Golgi | ROS |
|---------------------|----------------------|----------|-------|-----|
| 1 hr                |                      | 4.8      | 8.3   | 2.5 |
| 6 hr                |                      | 3.6      | 4.9   | 2.9 |
| 1 day               |                      | 4.2      | 2.6   | 0.9 |
| 3 day               |                      | 5.6      | 2.7   | 0.8 |
| 5 day               |                      | 3.4      | 2.5   | 1.2 |

Rats were injected intravitreally with \([^{1}H]\)galactose and \([^{35}S]\)-methionine. At the indicated times four retinas were taken for subcellular fractionation. Rhodopsin, extracted from each fraction, was purified on ConA sepharose and analysed by SDS-PAGE. The dual radioactive opsin peaks all migrated slower than the Coomassie blue stained mature opsin except in the ROS samples at 1, 3 and 5 days where they all coincided.

![Graph](image)

During the first 6 hr, however, galactose selectively labeled rhodopsin in the Golgi-enriched fraction resulting in increased \([^{1}H]/[^{14}C]\) ratios in both Golgi and ROS. By day 1 the galactose pool was exhausted and carbohydrate labeling that occurred in the rough endoplasmic reticulum-enriched fraction was probably due to glycogen turnover. The decreasing isotope ratios at each time point suggest that trimming was occurring at the transition from Golgi to ROS. Furthermore, the decrease in isotope ratio in the ROS between 6 hr and 1 day suggests further trimming of rhodopsin after membrane assembly in the ROS. Some of this lost carbohydrate may have been galactose, particularly in the ROS.

**Studies to Determine the Nature of the Linkage of Galactose to Rhodopsin**

Rats injected intravitreally with \([^{35}S]\)methionine were maintained in darkness for 2 hr at which time 12 retinas were collected for subcellular fractionation and rhodopsin purification by ConA sepharose chromatography. The ConA-purified rhodopsin was then applied to one of two lectin columns: RCA or GSA. RCA specifically recognizes \(\beta\)-linked galactose moieties (Baenziger and Fiete, 1979) and GSA is specific for \(\alpha\)-linked galactose residues (Blake and Goldstein, 1980). At 2 hr, 8–9.8% of the labeled rhodopsin bound to and was eluted from the RCA column, whereas none bound to GSA.

![Graph](image)

During the first 6 hr, however, galactose selectively labeled rhodopsin in the Golgi-enriched fraction resulting in increased \([^{1}H]/[^{14}C]\) ratios in both Golgi and ROS. By day 1 the galactose pool was exhausted and carbohydrate labeling that occurred in the rough endoplasmic reticulum-enriched fraction was probably due to glycogen turnover. The decreasing isotope ratios at each time point suggest that trimming was occurring at the transition from Golgi to ROS. Furthermore, the decrease in isotope ratio in the ROS between 6 hr and 1 day suggests further trimming of rhodopsin after membrane assembly in the ROS. Some of this lost carbohydrate may have been galactose, particularly in the ROS.

**Experiments to Determine Whether the Galactose of Rhodopsin is Capped by Neuraminic Acid**

Rats injected intravitreally with \([^{35}S]\)methionine were maintained in darkness for 2 and 4 hr at which time 12 retinas were collected for subcellular fractionation. ROS were incubated with and without neuraminidase. The amount of rhodopsin bound to and eluted from the RCA agarose columns was not increased by treatment with neuraminidase. The total dpm in the opsin region of SDS–polyacylamide gels for the two conditions and time points are shown in
Table III

Effect of neuraminidase treatment of ROS on binding of rhodopsin to RCA agarose

| Post-injection time (hr) | Neuraminidase | dpm in opsin |
|-------------------------|---------------|--------------|
| 2                       | +             | 1137         |
| 2                       | -             | 1132         |
| 4                       | +             | 721          |
| 4                       | -             | 729          |

Two groups of six rats were injected intravitreally with \[^{35}S\]-methionine and killed at the indicated times. ROS were prepared and half of each sample incubated with neuraminidase. After extraction, the rhodopsin samples were purified on ConA sepharose and subsequently chromatographed on Ricin agarose.

Table III. These data suggest that although complex asparagine-linked oligosaccharides often have a terminal trisaccharide containing N-acetylglucosamine linked to galactose and capped with neuraminic acid (Kornfeld and Kornfeld, 1985), rhodopsin lacks neuraminic acid.

Demonstration that Binding of the Rhodopsin Oligosaccharide to RCA can be Decreased with β-galactosidase Treatment

Rats were injected intravitreally with \[^{35}S\]methionine and \[^{3}H\]glucosamine to provide markers for the polypeptide and the oligosaccharide, respectively. They were maintained in darkness for 2 hr at which time 24 retinas were collected for subcellular fractionation and rhodopsin purification by ConA sepharose chromatography. The ConA-purified rhodopsin was treated initially with N-glycanase to hydrolyze the asparagine-linked oligosaccharides from rhodopsin. This treatment was followed by incubation of half of the sample with β-galactosidase which specifically hydrolyses terminal galactose residues that are β1-4 linked to N-acetylglucosamine. As shown in Fig. 4, the amount of radioactivity (dpm \[^{3}H\]) eluted from RCA agarose after β-galactosidase treatment was significantly less (0.6%) than that of the sample eluted from RCA agarose without the enzyme treatment (3.5%).

Table IV

Effect of swainsonine or castanospermine on the fraction of ConA purified rhodopsin that binds to RCA

| Inhibitor          | Control | Swainsonine | Castanospermine |
|--------------------|---------|-------------|-----------------|
| Percentage of ConA purified rhodopsin from ROS that binds RCA* | 3.30% ± 0.12 | 5.77% ± 2.0 | 0.64% ± 0.3 |
| Percentage of ConA purified rhodopsin from Golgi that binds RCA | 4.06% ± 1.80 | 4.26% ± 1.1 | 1.08% ± 0.3 |

Rhodopsin was labeled for 1 hr following simultaneous intravitreal injection of \[^{35}S\]methionine and swainsonine or castanospermine, or neither in three groups of six rats.

* Data are expressed as the mean percentages of three experiments ± s.d. One-way analysis of variance for the ROS and Golgi samples indicated that there was a significant difference among the three groups (ROS: F = 11.7, P = 0.01; Golgi: F = 21.5, P = 0.003). Tukey’s paired comparisons test confirmed that the percentages obtained for the castanospermine treated group differed significantly from the control or swainsonine treated group, but these latter two groups did not differ significantly (P < 0.05).
Demonstration that Binding of Rhodopsin to RCA is Abolished by Treatment with Castanospermine But Not Swainsonine

Rats in groups of six injected intravitreally with \(^{[35}S\)methionine only or \(^{[35}S\)methionine and either castanospermine or swainsonine were dark adapted for 1 hr at which time retinas were collected for subcellular fractionation. Rhodopsin was purified from the ROS- and Golgi-enriched fractions by ConA sepharose chromatography and the eluates were applied to RCA agarose columns. Both the RCA agarose eluates and a fraction of the ConA Sepharose eluates were applied to SDS-PAGE gels. The data in Table IV, representing three replicates of this experiment, show that the mean percentage of ConA purified rhodopsin from ROS which bound to RCA was greatly reduced in the castanospermine exposed group. A similar effect was seen in the Golgi-enriched fraction. The statistical analysis of these data indicated that the percentages obtained in the castanospermine treated ROS and Golgi samples were significantly different from the control or swainsonine treated group. These data demonstrate that castanospermine, which inhibits glucosidase I, disrupted the binding of rhodopsin to RCA, whereas swainsonine, which inhibits Golgi mannosidase II, did not. Since RCA is specific for \(\beta\)-galactose residues, it appears likely that in the absence of castanospermine, galactose was never linked to the oligosaccharide chain of rhodopsin.

Light-sensitivity Experiments

Rats injected intravitreally with \(^{[35}S\)methionine were maintained in darkness for 2 hr at which time 12 retinas were collected for subcellular fractionation. Half of a sample of detergent extracted purified ROS labeled in vivo with \(^{[35}S\)methionine was exposed to light while the remainder of the sample was kept dark. Each sample was chromatographed on ConA sepharose and eluted with \(\alpha\)-methyl mannoside. The ConA sepharose column eluates were applied to RCA agarose which was washed with buffer and then eluted with \(\beta\)-methyl-D-galactopyranoside beginning at fraction 1. As would be expected, the dark sample bound to and was eluted from ConA sepharose. 7.0% of that sample was bound to and eluted from RCA. In contrast, considerably less radioactivity was eluted when the light-exposed sample was chromatographed on ConA. What little was eluted did not bind to RCA. (\(\bullet\)) Dark; (\(\bigcirc\)) light.

 chargide using this highly specific enzyme dramatically reduces the binding of the oligosaccharide to RCA. These data provide further support for the presence of galactose on the oligosaccharide of rhodopsin.

Determination of which Photoreceptor Cellular Compartments have Galactose-containing Rhodopsin

N-linked oligosaccharides are typically assembled in the endoplasmic reticulum on dolichylphosphate and are then transported by means of vesicles to the Golgi membrane (Kornfeld and Kornfeld, 1985). In the trans Golgi, galactose may be added to a terminal N-
TRANSIENT GALACTOSYLATION OF RHODOPSIN

Fig. 6. SDS-polyacrylamide gels of the material eluted from RCA agarose [described in Fig. 5(B)]. The dark sample coincided with a rhodopsin standard labeled in vivo with [3H]leucine, whereas there was virtually no radioactivity detected on the gel of the light-exposed sample. As before (Fig. 3) the radioactive opsins migrated slightly slower than the Coomassie blue-stained mature opsin. (○—○) Light; (●—●) dark; (△—△) rhodopsin standard.

Fig. 7. Elution profiles from RCA agarose columns of bands separated from a linear 50-25% sucrose gradient over which had been layered a crude ROS suspension. The ROS suspension was prepared from retinas of six rats which had been intravitreally injected with [35S]methionine and dark adapted for 2 hr. This procedure typically yields four bands on the gradient: a faint uppermost band 1.5-1.8 cm from the top of the gradient, two bands of unsealed and sealed ROS (1 and 2, respectively) and a zone of fine particles, enriched in Golgi. Pellets of each of these bands were detergent extracted and applied to separate RCA columns. As shown in Fig. 7, the Golgi-enriched fraction and both ROS fractions (ROS 1 and ROS 2) bound to and were eluted from RCA. The uppermost band did not demonstrate RCA binding. SDS-PAGE of these fractions showed that the Golgi and ROS fractions co-electrophoresed with a rhodopsin standard. Although the Golgi-enriched fraction is not entirely free of ROS, the data suggest that the galactose residue of rhodopsin is present on the oligosaccharide in the Golgi as it is in other glycoproteins containing N-linked oligosaccharides.

acetylglucosamine of the oligosaccharide chain. In an effort to determine if the Golgi apparatus, as well as the ROS, of rod photoreceptor cells contained galactose, subcellular fractionation of ROS was performed. Rats were injected intravitreally with [35S]methionine and were maintained in darkness for 2 hr at which time 12 retinas were collected and crude ROS preparations were made using the vortexing procedure described. Linear 50-25% sucrose gradients of crude ROS suspensions which have been centrifuged for 2 hr typically have four bands. A very faint uppermost band is approximately 1.5-1.8 cm from the top of the gradient. Two bands of unsealed and sealed ROS are below the 1.5 cm band (Godchaux and Zimmerman, 1979). Below these is a zone of fine particles, enriched in Golgi. Pellets of each of these bands were detergent extracted and applied to separate RCA columns. As shown in Fig. 7, the Golgi-enriched fraction and both ROS fractions (ROS 1 and ROS 2) bound to and were eluted from RCA. The uppermost band did not demonstrate RCA binding. SDS-PAGE of these fractions showed that the Golgi and ROS fractions co-electrophoresed with a rhodopsin standard. Although the Golgi-enriched fraction is not entirely free of ROS, the data suggest that the galactose residue of rhodopsin is present on the oligosaccharide in the Golgi as it is in other glycoproteins containing N-linked oligosaccharides.

Time-course of the Trimming of Galactose from Rhodopsin

The results of the in vivo experiments in which [3H]galactose was used to label rhodopsin over several days (described above) suggested that galactose might be trimmed from the oligosaccharide of rhodopsin in the transition from Golgi to ROS as well as in ROS over time. To test this, a time course experiment was conducted. Rats were injected with [35S]methionine and [3H]galactose and groups of 6 animals were killed 2, 6 and 24 hr following injection. Retinas were collected for subcellular fractionation, ROS were extracted and rhodopsin was purified by ConA sepharose chromatography. A small fraction of the ConA eluate was applied to SDS-polyacrylamide gels and the remainder was applied to RCA. Table V provides the data obtained from gel electrophoresis of the ConA- and RCA-eluted samples. Electrophoresis of the RCA agarose eluates revealed that the percentage of the ConA-purified rhodopsin that bound to RCA decreased over time. At 2 hr, 8.69% of the rhodopsin bound to RCA, after 6 hr 2.17% was bound and by 24 hr only 0.89% was bound. Electrophoresis of the ConA-purified rhodopsin showed that the ratio of the isotopes (that is the ratio of [3H]galactose to [35S]-methionine) decreased over time from 0.40 at 2 hr to 0.15 by 24 hr. Clearly, trimming of galactose from rhodopsin had occurred suggesting that galactose may be an early component of rhodopsin, but as the molecule progresses through the ROS the galactose appears to be lost. In contrast, the ratio of the isotopes ([3H]galactose to [35S]-methionine) in the rhodopsin that bound to RCA was higher than in the total pool eluted from ConA and did not change significantly over time. Thus, the ratio changes were due to removal of galactose residues.
Table V

The percentage of ConA-purified rhodopsin that binds to RCA and the ratio of [3H] to [35S] in gel electrophoresis of the purified rhodopsin

| Hours post-injection | Percentage of ConA purified [35S] rhodopsin that binds RCA | Isotope ratio in ConA purified rhodopsin |
|----------------------|------------------------------------------------------------|----------------------------------------|
| 2                    | 8.69%                                                      | 0.40                                   |
| 6                    | 2.17%                                                      | 0.29                                   |
| 24                   | 0.89%                                                      | 0.15                                   |

| Isotope ratio in ConA purified rhodopsin that binds to RCA |
|-----------------------------------------------------------|
| 0.77                                                      |
| 0.71                                                      |
| 0.93                                                      |

Three groups of six rats were injected intravitreally with [3H]galactose and [35S]methionine. Retinas were collected at the indicated times. ROS were prepared and rhodopsin was purified on ConA sepharose. Aliquots were analysed by SDS-PAGE and the rest chromatographed on ricin agarose. Ricin eluates were also analysed by SDS-PAGE.

Plasma Membrane Preparation

It was of interest to determine whether the galactose-containing rhodopsin was present in photoreceptor discs or plasma membrane. A modification of the method of Molday and Molday (1987) was used in which nine rats were injected intravitreally with [35S]methionine and were maintained in darkness for 2 hr, at which time ROS were prepared using the linear sucrose gradient procedure described previously. After treatment with neuraminidase ROS were incubated with ricin-agarose. Subsequent to lysis and trypsin treatment, the ROS membranes were layered on linear 30–25% sucrose gradients underlaid with 60% sucrose. Following centrifugation, discs and the plasma membranes were removed. Detergent extracts of these two components were applied to RCA agarose columns. Elution patterns from RCA revealed significant binding of plasma membrane extracts and a small amount of binding of disc extracts. SDS-polyacrylamide gels of the eluates showed that the total amount of radioactivity present in rhodopsin of the plasma membrane (1149 dpm) was 3-6 times greater than the total amount of radioactivity present in disks (318 dpm). From these experiments it appears that at 2 hr post-injection, 74.5% of the labeled galactose-containing rhodopsin is in the plasma membrane and 25.4% is in discs.

4. Discussion

Although rhodopsin’s oligosaccharide chains have been thought to contain mainly two types of sugar residues [mannose and N-acetylglucosamine (Heller, 1968; Shichi et al., 1969; Heller and Lawrence, 1970; Plantner and Kean, 1976)], there is evidence that a small fraction of rhodopsin also may contain galactose. Fukuda et al. (1979) found that 10% of the oligosaccharides in bovine rhodopsin contained galactose. Furthermore, O’Brien (1976) showed that bovine ROS preparations support the transfer of galactose from UDP-galactose to rhodopsin. St. Jules et al. (1990) demonstrated the in vitro incorporation of galactose into rhodopsin and its subsequent removal after rhodopsin had reached the outer segment. These findings suggested that galactose may in fact be associated with rhodopsin of the ROS, if only for a short period of time. Perhaps the transient nature of its association explains its rather elusive detection by some biochemical techniques. In the current experiments, efforts were focused on demonstrating the presence of galactose by methods which could detect existing molecules, rather than solely by methods to show incorporation of exogenous galactose.

Galactose molecules were shown to exist on bovine rhodopsin using the galactose oxidase–sodium borohydride treatment. The results clearly demonstrated that there were galactose molecules present on rhodopsin which were altered by the highly specific galactose oxidase treatment and were then reduced by the sodium borohydride with the introduction of radiolabel. The bovine model does not permit in vivo studies of oligosaccharide synthesis, however, so it became necessary to investigate the incorporation of galactose in the rat model. The studies in which galactose was injected intravitreally in rats and monitored over several days demonstrated a selective labeling during the first 6 hr in the Golgi-enriched and the ROS fractions. Thus, even though conversion of galactose to other sugars commonly found on rhodopsin’s oligosaccharide chain probably occurred, the initial pulse of galactose was apparently incorporated as galactose in the Golgi where galactosyl transferase is known to be localized. The results strongly suggested that the galactose had been added to rhodopsin and then trimmed over time.

The need to demonstrate the presence of galactose without directly incorporating it into rhodopsin lead to the use of lectin affinity chromatography. This powerful technique allows for the separation of glycoproteins based on the affinity for certain sugar moieties (Cummings et al., 1989). ConA, which has an affinity for mannose residues, has long been used in the purification of rhodopsin. In the present work, a portion of the ConA-purified rhodopsin was shown to bind to the lectin RCA which specifically recognizes terminal galactose moieties associated with either glucosamine or N-acetylgalactosamine in a β1–4 linkage (Benzinger and Fiete, 1979), whereas no rhodopsin bound to GSA which has an affinity for α-linked galactose (Blake and Goldstein, 1980). Methionine was injected intravitreally into rats in order to label the polypeptide chain of rhodopsin. That the
TRANSIENT GALACTOSYLATION OF RHODOPSIN

In addition, the binding of the rhodopsin oligo-
radiolabeled protein which bound to RCA was indeed
rhodopsin was verified by light sensitivity experiments.
In the present work, it was of interest to
determine if galactose was associated with rhodopsin
purified from the Golgi-enriched fraction as well as
ROS. It was determined by subcellular fractionation
and subsequent RCA chromatography that the rho-
dopsin of the Golgi-enriched fraction did indeed
contain galactose. Furthermore, it was shown that the
addition of galactose to N-acetylglucosamine could
be inhibited by in vivo exposure to castanospermine
which specifically inhibits Glucosidase I. Glucosidase I
is a rough endoplasmic reticulum enzyme that cleaves
the terminal glucose from the GlcManGlcNAc (Repp
et al., 1985). With this early step in the processing of
the oligosaccharide disrupted, the subsequent steps in
the processing pathway could not continue. The
addition of galactose to N-acetylglucosamine was
prevented because the oligosaccharide chain was
never processed to the point where galactose is
typically added to N-acetylglucosamine. By way of
contrast, the addition of galactose to N acetyl
glucosamine was not affected by swainsonine which
inhibits the Golgi mannosidase II. This enzyme
typically cleaves two mannose residues from one of
the branches of the oligosaccharide chain (Elbein, 1983).
The processing of the other branch of the oligo-
saccharide chain can continue normally. It is to this
other chain, which contains a mannose and an N-
acetylglucosamine, that galactose is typically added.
However, it should be noted that a negative result
with swainsonine is of limited significance since there
is no assurance that it reached and inhibited the target
enzyme. To the best of our knowledge, these experi-
ments provide the first evidence in a mammalian
system that glycoprotein processing can be modified in
rhodopsin following in vivo exposure to the appro-
priate inhibitory agent. Chambers et al. (1986)
reported that intraocular injection of frogs with up to
250 mg of castanospermine and swainsonine resulted
in neither a decrease in rhodopsin content nor a
change in the length of photoreceptor outer segments.
Fliesler, Rayborn and Hollyfield (1986a) demonstrated
that in vitro exposure of Xenopus retinas to castano-
spermine resulted in the hyperglycosylation of opsin.
The opsin underwent normal intracellular transport
and was utilized for the biogenesis of ROS membranes
having normal disc morphology.

The 1 hr to 5 day in vivo galactose labeling studies
of rhodopsin purified from rough endoplasmic reticu-
lum- Golgi-enriched fractions and ROS, suggested
that the amount of galactose-derived label associated
with rhodopsin was not constant. It appeared from
this work that trimming had occurred in the ROS over
the course of several hours and also had occurred in
transit from one subcellular compartment to another,
namely, the Golgi-enriched fraction to ROS. We chose
to investigate the trimming phenomenon which was
apparently occurring in the ROS using lectin affinity
chromatography. Two hours after the injection,
approximately 8-9% of the labeled rhodopsin bound
to RCA suggesting that about 8-9% of newly
synthesized rhodopsin contains a β-galactose residue.
The percentage of rhodopsin which contained
galactose decreased by more than half within 6 hr and
by 24 hr of the injection less than 1% of rhodopsin
contained galactose. One might surmise that if the
galactose concentration is greatest within 2 hr of
injection, perhaps it constitutes a transient component
of rhodopsin's oligosaccharide chain. One possibility is
that it is associated with the rhodopsin of the plasma
membrane and basal folds of the ROS and is removed
as discs are scaled and move apically. The work of
Hicks and Molday (1985) emphasized the presence at
the basal area of the outer segments of ricin-binding
compounds.

The possible function of a galactose on rhodopsin at
this one area is open to speculation. It has been shown
that the oligosaccharide of rhodopsin or some other
ROS protein such as peripherin is essential for the
process of disc morphogenesis by Fliesler and Basinger
(1985) and by Fliesler, Rayborn and Hollyfield (1985)
who successfully disrupted disc formation with tunic
amycin, an inhibitor of oligosaccharide synthesis.
Mechanisms have been proposed (Fliesler, Rayborn
and Hollyfield, 1986b; Fliesler, 1988) showing how
the oligosaccharide of rhodopsin could play an
important role in disc formation. As new discs are
formed, presumably the opposite faces of a newly
forming disc must be aligned and brought into close
apposition. It might be that the oligosaccharide plays
some role in the process in much the same way that a
lectin binds a ligand or a hydrolase interacts with an
oligosaccharide substrate. If there were an enzyme-
substrate interaction, perhaps a carbohydrate residue
such as galactose on one surface might be cleaved by
a hydrolase such as a galactosidase on the opposing
surface. During this process the two membrane
surfaces would come into close apposition with the
exclusion of extracellular matrix allowing fusion of the
new disc. This hypothesis led to the experiment in
which ROS plasma membranes were separated from
36-2
discs for the purpose of determining in which compartment the galactose-containing rhodopsin was most prevalent. The experiments showed that the greatest preponderance of the galactose-containing rhodopsin was in the plasma membrane component. This finding lends support to the hypothesis that a transiently present galactose is important in new disc formation in bovine and rat retinas because the galactose appears to be associated with the plasma membrane until the discs are finally fused. This proposed mechanism would not specifically require galactose as the substrate, only that the components of the oligosaccharide, however they may vary among species, be the substrates of the appropriate hydrolases.

In the summary, the present work has shown through biochemical techniques that a subset of rhodopsin contains galactose. In fact, shortly after it is synthesized, approximately 8–9% of the rhodopsin in the ROS contains this sugar residue. The galactose appears to be added in the Golgi complex in the same way that a terminal trisaccharide is assembled on a complex oligosaccharide chain. The galactose is not capped, however, with neuraminic acid. We presented evidence to confirm the incorporation of galactose into rhodopsin by radiolabeling existing moieties. Finally, using lectin affinity chromatography, we were able to separate the galactose-containing species of rhodopsin and study its transient association with the oligosaccharide chain. We determined that galactose is trimmed from rhodopsin over the course of about 24 hr, presumably after it has served its function which may be related to new disc formation.

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