Rod Outer Segment-Associated N-Acetylgalactosaminylphosphotransferase

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Purpose. To determine the exact location of a cell surface glycosyltransferase (N-acetylgalactosaminylphosphotransferase, GalNAcPTase) immunochemically identified in mammalian rod outer segments (ROS), to determine whether anti-GalNAcPTase antibody recognizes retinal molecules that possess transferase activity and to characterize ROS transferase enzyme activity and acceptors. The GalNAcPTase is known to be associated with the adhesion molecule N-cadherin in embryonic avian retinas and with E-cadherin in mammalian pancreatic islet cells.

Methods. Purified, fixed ROS were reacted with anti-chick GalNAcPTase antibody followed by secondary antibody conjugated to colloidal gold and were examined by electron microscopy. Fractions of retinal and ROS proteins enriched in the transferase were obtained through batch adsorption on Sepharose, separated by gel electrophoresis, transferred to nitrocellulose, and either reacted with anti-GalNAcPTase antibody or assayed for transferase activity. Interphotoreceptor matrix (IPM) was examined for the presence of immunoreactive GalNAcPTase by gel electrophoresis and immunoblot. The kinetics and endogenous acceptors of the cow ROS transferase were characterized.

Results. ROS are specifically labeled by anti-GalNAcPTase antibody at the cell surface. The immunogold label was associated with the cell surface and with flocculent material adherent to the cell surface. In addition, soluble and particulate fractions of the IPM showed GalNAcPTase-like immunoreactivity. The transferase appears as single immunoreactive band at or near 220 kd. Transferase enzyme activity was present at this position on Western transfers of retinal and ROS proteins. In whole ROS, transferase activity was directed toward endogenous acceptors of very high molecular mass.

Conclusions. The GalNAcPTase is localized on ROS in association with the cell surface and with components of the IPM. The molecule recognized by the anti-GalNAcPTase antibody possesses transferase activity toward itself and a few other proteins, but mostly toward very large molecules that may be IPM proteoglycans. It is not yet known whether the enzyme of the adult retina specifically transfers sugar or sugar-phosphate groups to its acceptors. It is proposed that the ROS GalNAcPTase is involved in the modulation of adhesive phenomena between or within photoreceptors or between photoreceptors and the interphotoreceptor matrix. Investig Ophthalmol Vis Sci. 1995;36:163-173.
linked oligosaccharide of the acceptor. It is not known whether this catalytic activity is directly involved in the ability of the GalNAcPTase to modulate N-cadherin-mediated adhesion. Similar intracellular phosphodiester-linked sugars are added to lysosomal enzymes. This is followed by removal of the terminal sugar, exposing mannose-6-phosphate, which is necessary for targeting to lysosomes.9

The GalNAcPTase is widespread at the cell surface in the developing retina but becomes concentrated over the photoreceptor layer at hatching.10 GalNAcPTase-like immunoreactivity is also present in the retinas of adult cows, rats, frogs (Xenopus laevis), and non-human primates. Primate cones are particularly immunoreactive.11 Results of immunoblot studies showed that GalNAcPTase immunoreactivity was associated with purified cow rod outer segments (ROS) and with soluble interphotoreceptor matrix (IPM). Similar work with the frog revealed GalNAcPTase immunoreactivity only in the soluble IPM.12 These results complement the recent finding that the GalNAcPTase of the embryonic chick retina is anchored to the cell surface by a glycosylphosphatidylinositol linkage.13 Appearances of the GalNAcPTase in soluble fractions may be explained by the ready cleavage of such an anchor.

In adult retinas, GalNAcPTase of the photoreceptors or IPM may modulate adhesive interactions between components of the IPM, photoreceptors, apical retinal pigment epithelium, or Müller cells. Such interactions could involve analogs of the N-cadherin-based adhesion system of the embryonic retina. The present work was undertaken to clarify further the location of the GalNAcPTase associated with ROS and to determine whether the immunoreactive molecule of the outer segments has transferase enzyme activity. It is shown that immunoreactive GalNAcPTase is associated with the surface of purified bovine ROS and with IPM, that the anti-GalNAcPTase antibody recognizes an enzymatically active molecule of the cow retina and ROS, and that outer segments have transferase activity.

MATERIALS AND METHODS

Preparation of Bovine ROS

Rod outer segments were purified from fresh or frozen, dark-adapted cow retinas using established methods.13 Unless otherwise noted, all procedures were performed in darkness or under dim red illumination (Kodak 2A filter; Eastman Kodak, Rochester, NY). Frozen retinas were obtained from G.A. Hormel & Co. (Austin MN), and fresh cow eyes were obtained from Randolph Packing Co. (Asheboro NC) or from A. L. Beck & Sons (Winston-Salem, NC). Retinas were gently homogenized in 20 mM Tris acetate buffer, pH 7.2, containing 2 mM MgCl₂, 1 mM CaCl₂, 10 mM glucose, 5 mM taurine, 5% sucrose, and the protease inhibitor phenylmethylsulfonylfluoride at 1 mM (buffer: 30% SucTrAc; 50 retinas/40 ml buffer). Calcium was included in the buffer to inhibit proteolysis of the transferase.14 Glucose was included as an energy source, and taurine was included to improve the preservation of ROS structure.15 All reagents were from Sigma Chemical (St. Louis, MO). The homogenates were centrifuged at 1900g for 5 minutes (4000 rpm, Sorvall SS-34 rotor). The combined supernatants were slowly diluted with two volumes of Tris acetate buffer without sucrose and centrifuged at 3000g for 5 minutes (5000 rpm, SS-34 rotor). The ROS-containing pellets were combined in 20% SucTrAc, layered on 25% to 60% sucrose gradients, and centrifuged at 100,000g for 45 minutes (25,000 rpm, Beckman SW-28 rotor). Banded ROS were collected from the gradients, diluted with six volumes of 20% SucTrAc, and centrifuged at 7650g for 10 minutes (8,000 rpm, SS-34 rotor). The final pellets were combined in a small volume of 20% SucTrAc and either frozen for later use or fixed for immunocytochemistry, as described in the next section. Protein concentrations of aliquots of the ROS were measured by the Lowry method.16

Electron Microscopic Immunocytochemistry

Numerous attempts were made to immunolocalize GalNAcPTase in fixed, embedded retinas. These were uniformly unsuccessful and led to the choice of a preembedding method, modeled on that described for ROS.17 Freshly purified cow ROS in 20% SucTrAc were pelleted and resuspended in fixative consisting of 1% glutaraldehyde/1% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. ROS were fixed at 4°C for 5 hours, washed in phosphate buffer, and stored for up to 16 months at 4°C in the buffer, with 0.1% azide added to retard microbial contamination. The fixed ROS were rinsed extensively with phosphate-buffered saline (PBS: 50 mM phosphate, 150 mM NaCl, pH 7.4), and allowed to settle overnight (at 4°C) on glass coverslips coated with poly-L-lysine. The coverslips were rinsed for 20 minutes with tris-buffered saline (TBS: 25 mM Tris, 150 mM NaCl, pH 7.4), and blocked for 2 hours with 0.1% nonfat dry milk/3% normal goat serum (NGS) in TBS. Coverslips were exposed overnight at 4°C to anti-chick GalNAcPTase, a mouse monoclonal IgM antibody (mAb) designated mAb 7A2 (84 µg/ml)18 or to control mouse IgM (100 µg/ml, Sigma), both diluted in PBS/NGS/0.1% Tween-20. After being rinsed in PBS, coverslips were exposed to 20-nm colloidal gold-conjugated goat anti-mouse IgM (E-Y Labs, San Mateo, CA) for 90 minutes.

The coverslips were immersed for 30 minutes in 4% paraformaldehyde/2.5% glutaraldehyde in 0.1 M
Adsorption of Retinal GalNAcPTase on Sepharose bead-retinal homogenate suspension was shaken on ice for 1 hour and then centrifuged at 25,000g for 10 minutes (14,500 rpm, SS-34 rotor). The supernatant was removed, and the beads were washed three to five times by resuspension and recentrifugation in three volumes of TBSTX. Sepharose-associated proteins were solubilized by addition of SDS-DTT electrophoresis sample buffer to the final bead pellet. (Sample buffer composition: 75 mM Tris, 15 mM dithiothreitol, 15% sucrose, 4% SDS, 0.04% bromophenol blue, pH 8.9). The Sepharose beads and SDS-DTT were stored at 4°C. Before protein determination, aliquots of the SDS-DTT-solubilized bead preparation and of SDS-DTT solubilized crude retinal homogenate were diluted fourfold with deionized water, followed by the addition of 50% trichloroacetic acid (TCA) to achieve a final concentration of 10% TCA. The protein content of the TCA-precipitates was determined using a modification of the Lowry protein assay.19 Where protein content was not measured, loads on gels are expressed in terms of the number of retinas represented.

Fresh, light-adapted cow retinas were homogenized in ice-cold TBS (10 to 24 retinas, at 1 retina per milliliter) containing 1% Triton X-100 (TBSTX), 0.2 mg/ml DNase (5-Prime 3-Prime, Boulder, CO), and protease inhibitors as described for the IPM preparation. To remove SDS, gels were stained with Coomassie brilliant blue, followed by silver staining (Bio Rad). Other gels were prepared as follows for immunoblotting and transferase assay. To remove SDS, gels were rinsed three times (10 minutes each) in 25 mM Tris buffer (pH 7.2) containing 25% isopropyl alcohol, followed by three 10-minute rinses in Tris buffer alone. After equilibration in blot buffer, proteins were transferred to nitrocellulose using standard methods.22 Before immunoblotting selected lanes of the Western transfers, the nitrocellulose was blocked with TBS (pH 7.4) containing 5%...
nonfat milk, 0.05% Tween-20, and 1% normal goat serum. Transfers were probed with anti-chick GalNAc-PTase mAb 7A2 (5-10 μg/ml). Afterward, blots were rinsed with TBS and exposed to goat anti-mouse IgM conjugated to alkaline phosphatase (1:2000; Organon Teknika, Durham NC). Alkaline phosphatase color development was performed with the bromochloroindolyl phosphate/nitro-blue tetrazolium system (BCIP/NBT). SDS-PAGE and immunoblot and transferase assay of IPM components were performed in the same manner.

**Transferase Assay of Proteins on Nitrocellulose Blots**

For enzyme assays, lanes of the nitrocellulose blots of retinal, ROS, or IPM proteins, each approximately 1.5-cm wide, were excised and cut into 3-mm sections containing proteins of a small range of molecular masses. A control strip of nitrocellulose was obtained from an area of the blot that had been exposed to a region of the gel below the dye front during transfer. The nitrocellulose strips were placed in 75 μl of assay buffer (25 mM Hepes, 150 mM NaCl, 1% Triton X-100, 2 mM MnCl₂, 1 mM AMP, pH 7.2), and kept on ice for 0.5 hour. The transferase reaction was initiated by the addition of 50 μl of assay buffer containing 1.5 μl of the radioactive sugar donor UDP-[H-3]-GalNAc (uridine diphosphate N-acetyl-D-galactosamine, [galactosamine-1-]; New England Nuclear, Boston, MA). Specific activity of the donor was 8.3 Ci/mmol, with a final donor concentration 0.14 μM. The tubes were transferred to a 37°C shaking water bath for 1 hour, after which the reaction medium was transferred to a tube containing 100 μl ice-cold assay buffer containing 1 mg bovine serum albumin as carrier protein. The tubes with the nitrocellulose strips were rinsed with 200 μl deionized water added to the iced reaction mixture. Proteins and glycoconjugates in the reaction mixture were precipitated by the addition of 500 μl ice-cold phosphotungstic acid:trichloroacetic acid mixture. Proteins and glycoconjugates in the reaction mixture were precipitated by the addition of 500 μl ice-cold phosphotungstic acid:trichloroacetic acid (0.5%;6% at final dilution). Precipitates were collected on 2.4-cm glass fiber filters (Whatman GF/F) and washed under vacuum with PTA:TCA and ethanol. The filters were placed in scintillation vials with 200 μl scintillation cocktail (ReadySafe, Beckman, Fullerton, CA), and counted 24 to 48 hours later in a liquid scintillation spectrometer. Data were expressed as the dpm per section, minus the value (240 to 250 dpm) obtained from the control strip of nitrocellulose.

**Transferase Assay of Rod Outer Segments**

Purified whole ROS were resuspended in assay buffer consisting of 20% sucrose in 25 mM Hepes, 150 mM NaCl, 1 mM CaCl₂, 3mM AMP, 0.5% to 1% Triton X-100, pH 7.2. Assays included 100 μg ROS protein and 2 to 4 mM MnCl₂, in a final reaction volume of 50 μl. Except in studies of activity as a function of donor concentration, unlabeled UDP-GalNAc was present at 8 μM with radiolabeled UDP-GalNAc at 0.5 μM and a specific activity of 470 mCi/mmol (total donor concentration: 8.5 μM). Experimental additives included the exogenous acceptor asialo-agalactofetuin (20 μg/tube) or the nucleotides UMP and UDP, both at 250 μM. Four replicate tubes were included in each of the various treatment conditions. Tubes were incubated on ice or at 37°C, in the light, for 3 hours. Assays were terminated by precipitation with ice-cold PTA:TCA, and the precipitates were collected on filters and analyzed as described above. Filter blanks of 20 to 30 dpm were subtracted from all experimental values. In calculating the dependence of transferase activity on donor concentration, the radioactivity of an aliquot of radiolabeled donor nucleotide sugar was measured and used to determine the dpm/mol of donor. Dpm values from assays were then converted to moles (fmol) sugar transferred to precipitable material.

**Acceptor Identification**

Thawed ROS were resuspended at 1 to 2 mg protein in 300 μl assay buffer containing UDP-[H-3]-GalNAc (specific activity 8.3 Ci/mol, final concentration 2 μM). The reaction mixture was incubated at 37°C for 3 hours. Ice-cold ethanol was added to precipitate macromolecules, and the mixture was kept at −20°C for at least 8 hours. The precipitate was collected by centrifugation and dissolved in SDS-DTT sample buffer. Proteins in the sample were separated by SDS-PAGE on a 5% to 10% gradient gel. The gel lane containing the sample was cut into 1-mm horizontal strips dissolved by overnight incubation in 30% H₂O₂ at 60°C. Scintillation cocktail was added, and dpm per gel slice was determined.

**RESULTS**

**EM Immunolabeling of Rod Outer Segments**

Results of electron microscopic immunolabeling of the GalNAcPTase on ROS are shown in Figure 1. Panels A and B are controls which show no binding of immunogold reagent to ROS after exposure to non-specific mouse IgM. This was true of intact ROS (Fig. 1A) as well as of disks exposed in broken ROS (Fig. 1B). Exposure of ROS to mAb 7A2 resulted in focal immunogold labeling of ROS surfaces and of flocculent material associated with the ROS (Figs. 1C, 1D, 1E). ROS that were broken open during treatment showed immunolabel of whorls of membrane, as well as of areas near the edges of disks (Fig. 1F). Exposed disk surfaces were not appreciably immunolabeled.
**Figure 1.** Preembedding electron microscopic immunocytochemistry of isolated cow rod outer segments. Panels A and B are control preparations exposed to nonspecific mouse IgM. Panels C to F are examples of anti-GalNAcPTase-immunogold labeling of ROS surfaces (C), associated flocculent material (D, E), and partially dissociated disc and plasma membranes (F). All scale bars = 250 nm.

**Transferase Immunoreactivity of Sepharose-Binding Retinal Proteins**

In the course of immunoprecipitation studies using agarose- and Sepharose-conjugated secondary antibodies, it was discovered that a number of ROS and retinal proteins, including those with GalNAcPTase-like immunoreactivity, bind to control bead preparations. The agarose-binding property of a GalNAc transferase was originally described for the blood serum enzyme. We used this rediscovered property to obtain a transferase-enriched fraction of retinal proteins for use in studies relating GalNAcPTase immunoreactivity with transferase enzyme activity. Our goal was to enrich for the retinal GalNAcPTase, making detection and assay easier, and not to purify the enzyme.

The silver-stained proteins present in crude retinal homogenates (25,000 g supernatant) and in material adsorbed to Sepharose 4B and solubilized by SDS-DTT buffer is shown in Figure 2, lanes 1 and 2. Both preparations were loaded at 7.5 μg/lane. It is clear from this figure that although many retinal proteins are included in the Sepharose-bound material, there is a relative increase in the proportion of high molecular mass proteins in the Sepharose-bound material (this difference was not as pronounced after staining only with Coomassie blue). Some of the bands among the Sepharose-bound proteins were expected to represent transferase molecules, whereas others may be proteins associated with the transferases in detergent extracts.

**Figure 2.** Analysis of Sepharose-4B adsorbed retinal proteins. Crude cow retinal homogenate was centrifuged, and the supernatant was incubated with Sepharose 4B beads. Sepharose-bound components were solubilized in SDS-DTT sample buffer and electrophoresed on 7.5% acrylamide gels and transferred to nitrocellulose. Protein load in each lane was 7.5 μg. Silver-stained gels show proteins of the retinal homogenate (lane 1) and of retinal proteins bound to Sepharose 4B (lane 2). GalNAcPTase immunoblot of retinal homogenate (lanes 3 and 4) and of retinal proteins bound to Sepharose 4B (lanes 5 and 6). Lanes 3 and 5 are controls exposed only to secondary antibody. Lanes 4 and 6 were exposed to anti-GalNAcPTase MAb 7A2, followed by goat anti-mouse IgM conjugated to alkaline phosphatase. Color development was with NBT + BCIP. Immunoreactive GalNAcPTase (arrow) in the region of 220 kd appears only in the sample derived from the Sepharose adsorption procedure.
or proteins that bind independently to the Sepharose. A comparison of GalNAcPTase immunoreactivity between samples of retinal homogenate and of solubilized Sepharose-bound material, each loaded at 7.5 μg/lane, is shown in Figure 2, lanes 3 to 6. No immunoreactivity is seen among the proteins of the crude retinal homogenate at this protein load, whereas a distinct immunoreactive band is seen at a position corresponding to 220 to 250 kd among retinal proteins bound and subsequently solubilized from the Sepharose beads. These results demonstrate that the Sepharose adsorption technique produces a protein sample enriched in immunoreactive GalNAcPTase.

Assay of Transferase Enzyme Activity on Western Blots

The profile of GalNAc transferase activity of a lane from a nitrocellulose transfer of Sepharose-binding retinal proteins is shown in Figure 3, top panel. A distinct, isolated peak of transferase activity was evident in the second section from the top of the transfer (3 mm to 6 mm). Superimposed on the bar graph is a photograph of an immunostained companion lane (identically loaded) from the nitrocellulose transfer, showing that the position of GalNAcPTase immunoreactivity corresponds to the position of enzyme activity. In addition, a broad region of transferase activity was seen for components with masses in the range 30 to 150 kd.

A plot of transferase activity on a Western transfer of Sepharose 4B-binding ROS proteins is shown in the lower panel of Figure 3. Transferase activity had a distribution similar to that seen for Sepharose-binding proteins from the intact retina. The position of transferase activity near 200 kd in the ROS preparation corresponds to the migration position of immunoreactive transferase of the Sepharose-binding retinal proteins, as well as to that of the ROS transferase seen in our earlier work.11 This position also corresponds to that of the original embryonic chick transferase.10 Transferase activity at lower masses may represent activity among nonimmunoreactive breakdown products of the GalNAcPTase.

Immunoechemical Characterization of GalNAcPTase of the IPM

The presence of GalNAcPTase immunoreactivity over flocculent material associated with the ROS surface suggested that GalNAcPTase in the IPM is associated with particulate material, as well as with soluble components as shown previously.11 To confirm this, we examined GalNAcPTase immunoreactivity in soluble and particulate fractions of IPM. Western blots of immunoreactive IPM components are shown in Figure 4. GalNAcPTase immunoreactivity was evident in the region near 220 kd both in the soluble fraction and in a particulate fraction obtained after centrifugation at 170,000g (Fig. 4). Protein derived from 0.09 retina is represented in each gel lane. Therefore, per retina, it appears that somewhat more immunoreactive GalNAcPTase is in the soluble fraction than in the particulate material. The immunoreactive species in the pellet was not solubilized by prior treatment of the sample.
FIGURE 4. Transferase immunoreactivity of IPM components. Supernatants and pellets from 170,000g centrifugation of IPM were analyzed by SDS-PAGE, followed by immunoblot with anti-GalNAcPTase mAb 7A2. (lanes 1 and 2) Supernatant. (lanes 3 and 4) Pellet. (lanes 5 and 6) Supernatant from sample treated with Triton X-100 before high-speed centrifugation. (lanes 7 and 8) Pellet from Triton-treated sample. (lanes 1, 3, 5, 7) No primary antibody. (lanes 2, 4, 6, 8) Exposed to anti-transferase. Soluble and particulate components of the IPM show transferase immunoreactivity in the region near 220 kd. Each lane represents protein derived from 0.09 retina.

with Triton X-100 (Fig. 4, lanes 6 and 8). The small differences in the migration positions of the immunoreactive material (Fig. 4) were not due to variations between gels or blots, because the immunoblot was prepared from a single gel. Similar variations in migration position have been observed for the embryonic chick GalNAcPTase (Balsamo and Lilien, unpublished observations, 1990). This variation may reflect differences in chemical composition between variants of GalNAcPTase associated with the soluble and particulate IPM fractions. Immunoreactive 220-kd GalNAcP-Tase was also present on Western transfers of Sepharose-adsorbed IPM proteins (data not shown). Repeated attempts to measure enzyme activity on transfers of Sepharose-adsorbed and of unfractionated IPM (0.05 to 0.5 retinas represented per assay) were not successful.

Characterization of ROS Transferase Activity and Acceptors

In preparations of purified whole cow ROS, transferase activity showed typical enzyme characteristics, with activity dependent on donor concentration (Fig. 5). The reaction was also temperature-dependent: Incubation of ROS homogenates with assay components at 0°C significantly decreased transferase activity relative to that seen at 37°C (Fig. 6). Inclusion of an exogenous acceptor molecule, asialo-agalactofetuin, in the assay medium had no effect on ROS transferase activity, suggesting availability of or preference for endogenous acceptors in these biochemically complex mixtures of ROS-associated components (Fig. 6). Finally, inclusion of UDP and UMP in the assay medium reduced enzyme activity, as was previously shown for the embryonic chick transferase23 and as would be expected for an end-product inhibition mechanism specific for a GalNAc or GalNAcP transferase (Fig. 6).

Using SDS-PAGE–gel fluorography of radiolabeled transferase acceptors present in whole ROS, it was evident that the great majority of the radiolabel was incorporated into very large molecules that remained in the stacking gel or migrated only a short distance into the resolving gel (Fig. 7). Glycoproteins with masses of approximately 70 kd and 180 kd were also labeled, but not to the same extent as the high molecular mass components.

DISCUSSION

Results of our previous work indicated that immunoreactive GalNAcPTase is associated with cow ROS and soluble IPM.13 The present study supports and refines those results. Electron microscopic immunocytochemistry reveals immunolabeling of ROS extracellular surfaces, as well as of flocculent material adherent to the...
FIGURE 6. Effects of temperature, acceptors, and inhibitors on ROS GalNAc transferase activity. The transferase assay was conducted with various additives at 0°C (black bars) or at 37°C (gray bars): Buf = Buffer components only; Ace = Buffer + exogenous acceptor, asialo-agalactofetuin (ASAGF, 20 μg/tube); ROS: buffer + rod outer segments; ROS + Ace: ROS + ASAGF; ROS + Nuc: ROS + Nucleotides, UDP and UMP (250 μM each); ROS + Ace + Nuc: ROS + ASAGF + UDP and UMP. Error bars = 1 SD. Data based on four samples per condition. Specific activity of UDP-[H-3]-GalNAc = 470 mCi/mmol. The reaction was inhibited by cold and by UDP + UMP. Inclusion of the exogenous acceptor (ASAGF) did not increase the recovery of glycosylated products.

ROS (Fig. 1, panels C, D, and E). Where ROS had been damaged during preparation, exposed disc faces did not show significant immunolabeling. The density of immunogold along the ROS surface was much lower than that seen for integral proteins of the ROS plasma membrane, such as the Na+/Ca2+ exchanger.

We also found GalNAcPTase immunoreactivity in the IPM, in both soluble and particulate fractions. In earlier work, it was shown that cow soluble IPM has GalNAcPTase-like immunoreactivity near 220 kd, but mostly at 180 kd.11 In the present work, the IPM immunoreactivity at 220 kd corresponds to the position of retinal GalNAcPTase immunoreactivity, to the position of ROS immunoreactivity (shown in the earlier work11), and to the position of the original chick transferase.10 The consistent preservation of immunoreactivity of the 220-kd GalNAcPTase in the present work may have been due to inclusion of a spectrum of protease inhibitors in the isolation buffer instead of the single, short-lived inhibitor, phenylmethyl-sulfonyl-fluoride, used in our previous study.

The GalNAcPTase in the cow IPM may be secreted directly into the IPM as a soluble molecule or it may be released from the cell surface. GalNAcPTase in the chick embryonic retina is glycosylphosphatidylinositol-linked and can be recovered in membrane and soluble fractions.17 Based on the association of the GalNAcPTase with plasma membranes in the chick, we favor the interpretation that the cow GalNAcPTase is released into the IPM from photoreceptor plasma membranes, most likely from the ROS plasma membrane. Appearance of the GalNAcPTase in insoluble IPM material may be due to its subsequent adsorption to particulate components. Evidence to test these ideas will come through characterization of the location of the cow GalNAcPTase in intact tissue and through analysis of glycosylphosphatidylinositol-linkage of the cow GalNAcPTase.

Adsorption of retinal proteins on Sepharose 4B, followed by solubilization with SDS-DTT buffer, led to retrieval of at least 20 proteins, as seen in stained gels. Among the proteins of the Sepharose-binding fraction, anti-GalNAcPTase recognized one band of about 220 kd. This mass is close to that determined for the cell surface GalNAcPTase present in other retinas, in cow ROS, and in nonneural tissues.3,10,11,18 On Western transfers of the Sepharose-binding fraction from whole retinas and from ROS, a number of areas showed transferase activity. In particular, the region near 220 kd showed a distinct, isolated peak of enzyme activity. This activity correlates well with the position of the immunoreactive transferase. Some of the transferase activity among lower molecular mass components may represent glycosylation by proteolytically released active fragments of the 220-kd transferase, or, in the case of the retinal preparation, may be due to...
the presence of Golgi-derived transferases, the masses of which lie in the 38- to 60-kd range.26-28 In initial attempts to measure transferase activity of blotted proteins, the exogenous acceptor asialo-agalactofetuin was added to the reaction medium. However, the highest transferase activities of retinal proteins were obtained when no exogenous acceptor was included in the assay. Thus, the radiolabeled products measured in the assays may represent auto-glycosylated transferases and/or glycosylated acceptors eluted from the same strip of nitrocellulose that contained the transferase. In addition, because the glycosylation by GalNACPTase involves transfer of GalNAc-phosphate to acceptors,7 the reaction measured for the GalNACPTase may represent a form of autophosphorylation. In previous studies of the acceptors of the embryonic chick retinal GalNACPTase, it was shown that a GalNACPTase acceptor of molecular weight near 220 kd is present in a macromolecular complex containing GalNACPTase, N-cadherin, and other components.7,8,10 This 220-kd acceptor may represent the same transferase acceptor in the cow as in the embryonic chick.

In contrast to the situation with retinal proteins, transferase activity near the 220-kd position on blots of Sepharose-binding ROS proteins is enhanced by an exogenous acceptor (asialo-agalactofetuin). This may be due to differences in the state of glycosylation of acceptors, including the transferase itself, or to differences in the availability of a comigrating acceptor. Though it was not confirmed that the exogenous acceptor was glycosylated, a difference in acceptor requirement may reflect differences in the state of glycosylation (or phosphorylation) of the GalNACPTase of ROS versus retina. A form of GalNACPTase present in the retina, but not in ROS, may retain an acceptor site, whereas that associated specifically with ROS may not. Alternatively, the difference may have been due to the absence of a potential acceptor molecule of about 200 kd among the Sepharose-binding ROS proteins on the nitrocellulose blot. The lack of transferase activity at the position of immunoreactive GalNACPTase of the IPM may have been due either to low recovery of the IPM enzyme, to loss of enzyme activity during the collection of the IPM, or to a change in enzyme activity upon appearance of the transferase in the soluble IPM. Further studies will be necessary to examine these possibilities.

The Sepharose adsorption procedure was developed specifically to obtain transferase-enriched preparations for correlation of immunoreactivity and enzyme activity on blots. The mode of interaction of GalNACPTase with Sepharose, a polymer of galactose, was not investigated, nor was the binding capacity of the Sepharose. The variation in activity per retina represented on the nitrocellulose blots may reflect differences in adsorption to Sepharose of the GalNACPTase from whole retinas as opposed to the enzyme specifically associated with ROS (or with the IPM). It should also be recognized that the activity measured on the blots represents only the portion that remains after subjecting the enzyme to denaturing conditions (detergent, reducing agent, heat).

To examine some of the properties of the ROS GalNACPTase of the adult cow retina, we studied transferase activity toward endogenous acceptors in preparations of purified whole ROS. Under the conditions used, the transfer reaction proceeded slowly. Even when donor concentration was raised by the addition of unlabeled UDP-GalNAc, the amount of sugar transferred per 100-µg aliquot of ROS protein remained in the fmol range (Fig. 5). This can be interpreted as indicating that the reaction is limited by either the amount of enzyme or acceptor. GalNAc transfer in the ROS system is inhibited by expected products of the transferase reaction (UDP and UMP; Figure 6).22 Because the addition of a known acceptor for the chick GalNACPTase, asialo-agalactofetuin, did not cause an appreciable increase in enzyme activity (Fig. 6), endogenous acceptors seemed to be preferred. Analysis of these acceptors by SDS-PAGE showed that most of the GalNAc transferred in the ROS preparation is associated with high-molecular mass components that remain in the stacking gel or that migrate only a short distance into a 5% to 10% gel (Fig. 7). These high-molecular mass glycosylated acceptors may be proteoglycans or aggregates of other heavily glycosylated proteins. This is consistent with the ultrastructural immunolocalization of the GalNACPTase to the ROS surface, where it appears to be associated with flocculent material derived from the IPM. The numerous exposed oligosaccharide chains of the IPM proteoglycans (e.g., chondroitin sulfate) may provide acceptor sites for the transferase.

Among the lower mass acceptors in whole ROS, significant glycosylation of proteins occurred at positions corresponding to 65 kd and from 180 kd up to the stacking gel. The peaks near 65 and 180 kd may represent other acceptors, degraded forms of self-glycosylated 220-kd transferase, or products of glycosylation by other ROS-associated transferases, if such exist. In the embryonic chick retina, N-cadherin, with a mass of 130 kd, serves as an acceptor for the GalNACPT transfer reaction.8 In ROS, no component near 130 kd was significantly glycosylated, indicating that a cadherin-like molecule was either not present or was not glycosylable.

These results demonstrate that a ROS-associated transferase shows activity toward acceptors that have characteristics of cell surface or IPM components, some of which could be the transferase itself. We have not yet shown that the retinal or ROS GalNACPTase

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transfers GalNAc-phosphate to its acceptors, as does the chick transferase. However, the close correlation of a GalNAcP Tase-immunoreactive 220-kd protein with transferase activity supports the proposal that the cow molecule is also a GalNAcTase transferase.

The present work on ROS GalNAcPTase has implications for adhesion systems that involve N-cadherin. In the embryonic chick retina, GalNAcPTase is associated at the cell surface with the adhesion molecule N-cadherin. In the developed chicken retina, N-cadherin is present at the outer limiting membrane, but immunohistochemical evidence shows that the GalNAcPTase is present on outer segments and in the outer plexiform layer at this stage. This apparent separation of the GalNAcPTase and N-cadherin may reflect a changing role for the transferase during maturation of the retina. In the developed retina, the ROS GalNAcPTase may be part of an adhesion system analogous to that involving N-cadherin but that includes a ROS-specific adhesion molecule. An attractive hypothesis is that the ROS GalNAcPTase is involved in modulating adhesive interactions between ROS membrane systems (e.g., between adjacent forming discs) or between ROS and the IPM. Tests of this hypothesis will require studies of GalNAcPTase distribution and function in ROS membrane systems in the intact retina.

Key Words
adhesion, cow, glycosyltransferase, interphotoreceptor matrix (IPM), N-acetylgalactosaminylphosphotransferase, N-cadherin, photoreceptor, rod outer segment (ROS)

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References


