Glycoproteins were metabolically labeled with \(^{3}{H}\)-fucose in cultured RPE cells from RCS \(r^{dy-p^{+}}\) and Long Evans rats. \(^{3}{H}\)-labeled glycoproteins associated with a plasma membrane-enriched subcellular fraction were separated by two-dimensional gel electrophoresis. Relative incorporation of \(^{3}{H}\)-fucose into high molecular weight cell surface glycoproteins (Mr of 128,000–183,000) was measured by quantitative autoradiography and densitometry. The results of these experiments show that \(^{3}{H}\)-fucose incorporation into four glycoproteins (Mr of 183,000, 175,000, 164,000 and 156,000) was reduced by 30–50% in the dystrophic RPE as compared to the normal cells. This reduction was not due to an absence of the protein core of glycoproteins on the dystrophic RPE cell surface; when RPE cells were labeled with \(^{3}{H}\)-leucine prior to analysis, no reduction in label was found in the dystrophic RPE as compared to normal. Therefore, the results of this study suggest that the RCS rat RPE processes the oligosaccharide portion of some cell surface glycoproteins differently than normal rat RPE.

In the Royal College of Surgeons (RCS \(r^{dy-p^{+}}\)) dystrophic rat, retinal degeneration results because the retinal pigment epithelial (RPE) cells fail to phagocytize shed photoreceptor outer segment (OS) debris.\(^{1,2}\) The RPE cell is specifically affected by the \(r^{dy}\) mutation,\(^{3}\) and RPE cells from the RCS rat continue to express the \(r^{dy}\) phenotype in cell culture.\(^{4}\) Use of pure cultures of RPE cells for experimentation has yielded important information about the phagocytic process in both normal and dystrophic rats.

Cultured RPE cells from dystrophic rats bind OS fragments at the cell surface in normal amounts, but fail to ingest the OS, once bound.\(^{5}\) One hypothesis to explain this observation is that the defect in the dystrophic rat RPE involves a cell surface molecule that is responsible for signalling the RPE cell to begin ingestion of bound OS.\(^{5}\) There is some evidence that a specific cell surface receptor (possibly a protein) is responsible for the binding and phagocytosis of OS in normal rat RPE. Treatment of cultured rat RPE cell surfaces with proteases results in reduced ingestion of bound OS.\(^{6}\) Furthermore, the binding of OS to normal rat RPE displays classic characteristics of receptor mediated binding, such as saturation kinetics and temperature dependency.\(^{7}\)

The objective of the present ongoing work has been to characterize and compare the cell surface proteins in cultured normal and dystrophic RPE in order to determine if there are any alterations in these molecules in the dystrophic cells. In a previous study,\(^{8}\) proteins of normal and dystrophic rat RPE cell plasma membrane were mapped by two-dimensional gel electrophoresis. A small group of cell surface glycoproteins was identified due to the fact that they could be labeled metabolically with \(^{3}{H}\)-fucose and \(^{3}{H}\)-glucosamine, and labeled at the external cell surface with \(^{125}\)I by lactoperoxidase-catalyzed iodination. When the patterns of \(^{3}{H}\)-fucose-labeled cell surface glycoproteins present in dystrophic and normal RPE were visually compared, it appeared that there was less \(^{3}{H}\)-fucose incorporated into two glycoproteins with Mr of 183,000 and 175,000, in the dystrophic RPE. The current study examines this observation more closely by using quantitative methods to measure the relative incorporation of \(^{3}{H}\)-precursors into the dystrophic and normal RPE cell surface glycoproteins.

### Materials and Methods

#### Animals

Long Evans rats were originally obtained from Simonsen Labs, Inc. (Gilroy, CA), while RCS \(r^{dy-p^{+}}\) breeding pairs were obtained from Dr. M. LaVail, University of California (San Francisco, CA). The
method of animal sacrifice used was in compliance with the ARVO Resolution on the Use of Animals in Research.

Preparation of $^3$H-Labeled RPE Cell Membranes

Primary cultures of RPE cells were established from 10-day-old normal (Long Evans) and dystrophic (RCS) rats. Parallel cultures of normal and dystrophic RPE cells were initiated at the same time. The RPE cells were cultured for 6 days in the presence of 5 $\mu$Ci/ml of L-(5,6-$^3$H)-fucose (48 Ci/m mole, New England Nuclear, Boston, MA) or 10 $\mu$Ci/ml of L-(4,5-$^3$H(N))-leucine (60 Ci/m mole, New England Nuclear). Growth medium containing diluted isotopes was always prepared in a single batch for both cell types. Fresh medium with fresh isotope was added at day 3. Plasma membrane enriched fractions (Peak II) were prepared from the cultured cells as described previously.

Two-Dimensional Gel Electrophoresis

$^3$H-labeled membrane bound proteins were separated by two-dimensional gel electrophoresis by a published method. Gels to be compared were loaded with equal dpms, as detailed in a previous publication.

Quantitative Autoradiographic Analysis

Two-dimensional gels were fixed, soaked in Auto-fluor (National Diagnostics, Mannville, NJ), dried and exposed to preflashed XOMAT-AR (Kodak, Rochester, NY) film at $-70^\circ$ for 1-2 weeks. Autoradiograms were scanned using an LKB Ultrascan XL laser densitometer, set to scan two-dimensional images. Absorbance volumes of each spot were calculated using the Gelscan 2D software. Spot 12 was chosen as an internal point of reference within each autoradiogram, due to its consistency with regard to shape and density. The absorbance volume of each spot in dystrophic samples was compared to that of the corresponding spot in normal samples as follows:

1. Adjust the absorbance volume of dystrophic (RCS) spot 12, making it equal to the absorbance volume of normal (Long Evans) spot 12.
2. Normalize (N) the absorbance volumes of spots 5, 7, 8, 9, 11 and 13 in the dystrophic sample by multiplying by the same factor that was used to adjust spot 12.
3. Calculate the absorbance volume ratios by dividing the adjusted or normalized dystrophic absorbance volume by the corresponding control value.

Results

Quantitative Measurement of $^3$H-Fucose Incorporation

Figure 1 shows a schematic of Peak II membrane glycoproteins that were metabolically labeled with $^3$H-fucose and/or $^3$H-glucosamine, and surface-labeled with lactoperoxidase and 125I. The numbering system is the same as in a previous publication. Densitometry studies were conducted on a cluster of spots in the area of the gel that falls between the 115,000 and 200,000 $M_r$ markers, since the data in Figure 1 suggest that these are cell surface glycoproteins. This analysis specifically focused on spots 5, 7, 8, 9, 11, 12 and 13, which range in $M_r$ from 128,000 to 183,000 (Table 1), and are always labeled with $^3$H-fucose.

Table 1. Estimated molecular weights of RPE cell surface glycoproteins

<table>
<thead>
<tr>
<th>Spot</th>
<th>$M_r$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>183,000</td>
</tr>
<tr>
<td>7</td>
<td>164,000</td>
</tr>
<tr>
<td>8</td>
<td>175,000</td>
</tr>
<tr>
<td>9</td>
<td>156,000</td>
</tr>
<tr>
<td>11</td>
<td>152,000</td>
</tr>
<tr>
<td>12</td>
<td>136,000</td>
</tr>
<tr>
<td>13</td>
<td>128,000</td>
</tr>
</tbody>
</table>
Fig. 2. Two-dimensional gel separation of Peak II membrane proteins from \(^3\)H-fucose-labeled RPE cells. Autoradiograms show \(^3\)H-labeled proteins from (A) Long Evans and (B) dystrophic (RCS rd^p+) rats. The area of each autoradiogram that was scanned for quantitative densitometry is enclosed in the rectangular box.

Figure 2 shows typical patterns of \(^3\)H-fucose-labeled spots obtained for normal (A) and dystrophic (B) RPE membranes. The upper region of the pattern, enclosed by the box, was quantitatively scanned with the Ultrosan XL. Absorbance data were then analyzed, using the Gelscan software. Figure 3 shows a reconstruction of the images produced by Gelscan from the scanned areas of the autoradiograms shown in Figure 2. The shading indicates increasing levels of absorbance, with darkest grey indicating areas of greatest image density, and lightest grey being areas of least absorbance.

In most cases it was necessary to use several settings for the lowest absorbance (lightest grey) cutoff, in order to obtain the best images for each spot. In general, the image for each spot was maximized prior to integration of absorbance volume. Using Gelscan, the absorbance volumes of spots 5, 7, 8, 9, 11, 12 and 13 for normal and dystrophic RPE membranes were calculated. All of the values from the dystrophic sample were normalized so that the absorbance volume for spot 12 equaled that of the control spot 12. For each spot, Table 2 shows the ratio of the normalized absorbance volume of a particular fucose-containing spot in the dystrophic sample, to that of the same spot in the normal sample. Spots 5, 7, 8 and 9 display a 30–50% reduction in fucose incorporation in the dystrophic membranes.

By contrast, in control experiments, in which two sets of normal RPE tissue were processed side by side (as if one were dystrophic), the absorbance volume ratios are much closer to 1 for each of the spots (Table 2).
Quantitative Measurement of $^3$H-Leucine Incorporation

The observed decrease in $^3$H incorporation into spots 5, 7, 8 and 9 as a result of labeling with $^3$H-fucose could be due to a decrease in the amounts of these glycoproteins on the dystrophic RPE cell surface. To test this possibility, RPE cells were labeled with $^3$H-leucine prior to two-dimensional gel analysis of the cell surface proteins. Figure 4 shows the pattern of $^3$H-leucine labeled proteins in normal (A) and dystrophic (B) membranes. As expected, the pattern is more complex than that resulting from $^3$H-fucose labeling. Once again, the areas scanned are enclosed in boxes. Figure 5 shows the Gelscan reconstruction of spots 5, 7, 8, 9, 11, 12 and 13. (Other spots are omitted for clarity.)

Absorbance volume ratios for leucine incorporation into normal and dystrophic membranes are shown in Table 3. The results suggest that there is no difference in the relative incorporation of $^3$H-leucine into spots representing glycoproteins 5, 7, 8 and 9 in the dystrophic as compared to normal RPE. Thus, the observed reduction in $^3$H-fucose incorporation is not due to a reduced amount of these glycoproteins on the surface of the dystrophic RPE cell. The apparent increase in $^3$H-leucine labeling of spot 11 in the dystrophic RPE was reproducible, although its meaning is not clear at this time.

Table 2. Relative incorporation of $^3$H-fucose into RPE cell surface glycoproteins

<table>
<thead>
<tr>
<th>Spot #</th>
<th>[dyst. abs. vol. (N)]*</th>
<th>[normal abs. vol]</th>
<th>[normal 1 abs. vol (N)]</th>
<th>[normal 2 abs. vol]</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.56† (0.02)‡</td>
<td>0.99§ (0.01)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0.62 (0.17)</td>
<td>0.99 (0.12)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0.49 (0.02)</td>
<td>0.89 (0.04)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>0.74 (0.13)</td>
<td>0.97 (0.03)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>0.82 (0.15)</td>
<td>0.93 (0.08)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>1.00 (-)</td>
<td>1.00 (-)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>0.81 (0.09)</td>
<td>1.02 (0.01)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Calculation of absorbance volume ratios is explained in Materials and Methods.
† Average of values obtained from four separate labeling experiments.
‡ Average deviation from the mean.
§ Average of values obtained from two separate labeling experiments.

Comparison of the relative incorporation of $^3$H-fucose into spots representing glycoproteins 5, 7, 8 and 9 in normal and dystrophic RPE membrane samples shows a 30–50% reduction in the dystrophic membranes. Because this reduction is not an "all-or-none" phenomenon, a number of precautions were taken to ascertain that this was a reproducible difference. The measurement was repeated four times, over the course of 6 months, using primary cultures of RPE prepared from offspring of different mating pairs. Preflashed X-ray film (to a background fog of 0.15 absorbance units) was used to develop autoradiograms, thus ensuring a linear relationship between the number of $^3$-emissions and silver grain density, even for less radioactive spots. For each two-dimensional gel, scanning measurements were repeated on a number of autoradiograms of varying exposure underglycosylated in the dystrophic RPE as compared to the controls. This observation was made by visual inspection of autoradiograms. In the current work, this initial observation has been confirmed by quantitative densitometry.

Discussion

In a previous study, $^3$H-fucose-labeled cell surface glycoproteins from normal and dystrophic (RCS) rat RPE were analyzed by two-dimensional gel electrophoresis and autoradiography. The results of that analysis suggested that two high molecular weight glycoproteins (M, 183,000 and 175,000) were underglycosylated in the dystrophic RPE as compared to the controls. This observation was made by visual inspection of autoradiograms. In the current work, this initial observation has been confirmed by quantitative densitometry.
density. This was to ensure that selection of spot 12 as a point of internal reference was not creating an artifactual phenomenon. If spot 12 were exposed beyond the saturation limits of the X-ray film, then its use as a normalization standard could lead to erroneous conclusions. However, measurements made with both "light" and "dark" exposures of autoradiograms produced the same results. Furthermore, the raw values for absorbance volume of spot 12 in the control and dystrophic samples were usually within 5-10% of each other, thus making the normalization factor very small.

$^3$H-Fucose incorporation into glycoproteins 5, 7, 8 and 9 could be decreased if less of these glycoproteins were present on the cell surface in the dystrophic RPE, or if these glycoproteins actually contain less fucose in the dystrophic RPE membranes. In order to address the first possibility, RPE cells were labeled with $^3$H-leucine prior to analysis. The relative incorporation of $^3$H-leucine into spots 5, 7, 8 and 9 was the same for normal and dystrophic RPE. Thus, the observed reduction in $^3$H-fucose incorporation into these glycoproteins is not due to a reduced quantity of the glycoprotein in the plasma membrane.

The results of this study suggest that the dystrophic rat RPE processes cell surface glycoproteins differently than normal RPE. The observed reduction in $^3$H-fucose incorporation into glycoproteins 5, 7, 8 and 9 could be due to:

1. A mutation that has caused an alteration of the amino acid sequence of the glycoproteins at an oligosaccharide acceptor site, resulting in complete loss of the acceptor site.

2. A mutation causing alteration of amino acid residues adjacent to the oligosaccharide acceptor site. This type of change has been shown to influence the final processing of oligosaccharides in some glycoproteins, resulting in microheterogeneity of the oligosaccharide structure. Explanations 1 or 2 are likely only if glycoproteins 5, 7, 8 and 9 are related molecules, sharing the same amino acid sequence.

3. A mutation that has resulted in reduced activity of a specific glycosylating enzyme. Such an enzyme could be active either in early or late stages of oligosaccharide processing. Alteration of either an early or late processing event could reduce fucose incorporation, by eliminating a sugar residue to which fucose is added, or by affecting the addition of fucose itself by a specific fucosyltransferase.

4. Mutations that affect synthesis of substrate molecules (eg, GDP-fucose) or alter transport of glycoproteins through various cellular compartments during processing. In cases 3 or 4, other cell surface glycoproteins besides 5-9 are likely to be affected.

An experiment was conducted to determine if the asparagine-linked oligosaccharides of glycoproteins 5, 7, 8 and 9 in the RCS RPE, if less processed, are of the high mannose precursor type. Glycoproteins in the molecular weight range of 116,000-180,000 Mₗ from dystrophic and normal membranes were digested with endoglycosidase H, and found to be equally resistant to the enzyme (results not shown). This suggests that differences in processing of RCS RPE glycoproteins occur at a later stage of processing.

### Table 3. Relative incorporation of $^3$H-leucine into RPE cell surface glycoproteins

<table>
<thead>
<tr>
<th>Spot #</th>
<th>$^{[\text{dist. abs. vol (N)}]}$/ $^{[\text{normal abs. vol}]}$</th>
<th>$^{[d]}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.99 (0.15)†</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>1.04 (0.17)‡</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0.82 (0.09)</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>1.00 (0.18)</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>1.38 (0.27)</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>1.00 (―)</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>1.00 (0.13)</td>
<td></td>
</tr>
</tbody>
</table>

* Calculation of absorbance volume ratios is explained in Materials and Methods.

† Average of values obtained from seven separate labeling experiments.

‡ Average deviation from the mean.
Such differences could effect both N- and O-linked carbohydrates, as they may share later processing pathways. There have been reports of differences in lectin binding to intact plasma membrane, and to plasma membrane glycoproteins from RPE of normal and dystrophic rats. McLaughlin et al18 quantitated the amount of ferritin-conjugated lens culinaris haemagglutinin (LcH-fe) that bound to normal and dystrophic RPE microvilli in freshly isolated tissue. (LcH preferentially binds to core glycopeptide structures containing both mannose and fucose). They found that the dystrophic RPE bound more LcH (approximately 12.7x more) than the normal tissue. These results imply that there is less final processing of glycoproteins on the surface of the dystrophic RPE, thus making mannose- and fucose-containing core oligosaccharide structures of some glycoproteins more accessible to LcH binding in dystrophic RPE. Cooper et al19 reported a 175,000 M r wheat germ agglutinin (WGA, specific for terminal GlcNAc or sialic residues) binding protein in apical microvilli preparations from Long Evans RPE. This same glycoprotein would not bind WGA in similar preparations from RCS tissue. These observations support the hypothesis that dystrophic rat RPE cells process glycoproteins differently than normal RPE cells. They also lend validity to the present observations, made in primary cultures of rat RPE, that is, that such differences are not merely an artifact of cell culture. There are a number of examples in which faulty glycosylation has been found to be responsible for malfunctioning of a cell surface receptor.20-22 Whether the alteration of cell surface glycoproteins in the dystrophic rat RPE is related to its impaired phagocytic capabilities remains to be determined.

Key words: inherited retinal dystrophy, RCS rat, retinal pigment epithelium, cell surface glycoproteins, fucose

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