SPECIFIC GLYCOPROTEIN CHANGES DURING DEVELOPMENT OF THE CHICK NEURAL RETINA

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ABSTRACT

After separation of whole proteins of chick neural retina by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS), a number of glycoproteins can be detected by staining the gels with ^125I-labeled wheat germ agglutinin (WGA) and other lectins. The glycoprotein patterns show both quantitative and qualitative changes between days 7 and 13 of development. Some of these glycoproteins can be separated by chromatography on columns of insolubilized lectins. These observations suggest that purification of some of these glycoproteins identified by staining with radioactive lectins would yield retinal antigens which may be specific for developmental stage and cell type.

KEY WORDS chick retina • glycoproteins • lectins • affinity chromatography • differentiation • autoradiography

Changes in cell surface proteins are presumed to be of importance during development, both for cell-cell interactions and for other interactions of the cell with its environment. Developmentally regulated changes in cell surface glycoproteins (11) as well as carbohydrate-binding proteins that interact with them to mediate cell adhesion (5, 9, 21) have been documented in cellular slime molds. Similar changes in more complex eukaryotic systems have been difficult to demonstrate. Glycoproteins are generally located on the cell surface, and a demonstration of developmentally regulated changes in glycoproteins would be of considerable interest. It has been suggested that glycoproteins are important components of cell recognition systems including synaptogenesis (2). Previous attempts to study these changes include the histological demonstration of changes in cell surface glycosyl transferases, changes in lectin agglutinability of dissociated cells, as well as lectin-staining patterns of intact cells or cell fractions (for review see references 3, 12, 14, and 20). In none of these cases do the techniques allow a determination of the type of molecules involved and whether the observed changes are quantitative or qualitative.

Recently, several investigators have independently demonstrated that, after separation of proteins on polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS), glycoproteins can be specifically localized by the use of fluorescent or radioactive lectins (4, 22–25). This method allows the selective visualization of a subclass of proteins whose carbohydrate moiety can bind to certain lectins. This technique has been used to demonstrate developmental changes in glycoproteins in D. discoideum (11) and erythroid cells (16). In this communication, we show that specific glycoprotein changes occur during development of neural retina from chick embryo. These changes can be visualized with wheat germ agglutinin (WGA), a lectin that reacts with N-acetylgalactosamine, and ricin or RCAI (1) lectins that react with terminal galactose residues. The potential impli-
Preparation of Retina
egg incubator. Cell lysates were prepared from chick embryo neural retina at different stages of development. The tissue was placed directly into a 1/10 dilution of the forced through a syringe four to five times, using 21 and 25 gauge needles. The SDS should rapidly inactivate hydrolytic enzymes that may alter the retinal glycoproteins. Protein concentrations were determined by the method of Lowry et al. (18) using bovine serum albumin as a standard.

Lectins

Purified WGA was obtained from Sigma Chemical Co. (St. Louis, Mo.). Ricin and RCA₁₅₀ were purified from castor bean (1) on an ovomucoid-Sepharose affinity column prepared by the cyanogen bromide method (6). The lectins, 0.5-0.8 mg/ml, were iodinated by the chloramine T method (10). Iodinated lectins were repurified by affinity chromatography on ovomucoid-Sepharose. WGA was eluted with 0.1 M acetic acid, whereas ricin and RCA₁₅₀ were eluted with 0.1 M lactose. Eluted fractions containing the peak radioactivity were pooled and dialyzed for 48 h against phosphate-buffered saline (PBS) (0.14 M NaCl, 10 mM Na phosphate, pH 7.2). Radioactivity was measured in a Beckman gamma counter Beckman Instruments, Inc. (Fullerton, Calif.). The lectins had specific activities between 10 × 10⁷ and 25 × 10⁸ cpm/mg protein. Iodinated lectins were stored at 4°C for up to a month. Longer storage resulted in lectins with altered binding properties.

Triton X-100 Extracts and Affinity Chromatography on RCA₁₅₀-Sepharose Columns

Neural retinas were dissected from 7-, 8-, 10-, and 13-day-old chick embryos and solubilized in 10 mM K phosphate, pH 8.0, containing 0.5% Triton X-100 and 20 U/ml Trasylol (Sigma Chemical Co.). After stirring for 20-30 min at 4°C, the extract was centrifuged at 27,000 g for 20 min. The supernate was removed and stored frozen at -20°C and used for the electrophoretic separation of the retinal proteins.

The Triton X-100 extracts (10 mg of protein) were chromatographed on RCA₁₅₀-Sepharose affinity columns (0.8 × 3 cm) equilibrated in the same buffer. Glycoproteins bound to the column were specifically eluted with column buffer containing 0.1 M lactose (17). Eluted fractions were dialyzed against 10 mM K phosphate, pH 8.0, to remove lactose. Protein concentrations were determined by a modified Lowry procedure in the presence of SDS (7).

Polyacrylamide Gel Electrophoresis

Samples of retina cell extracts were separated by polyacrylamide gel electrophoresis in the presence of SDS on slab gels (1.5-mm thickness, 13-cm length of the running gel) containing 7.5% acrylamide (wt/vol) by the method of Laemmli (13). After electrophoresis, the gel was fixed and “stained” with lectin by a modification of previously described methods (4, 24). SDS was removed by placing the gel in 100 ml of 50% (vol/vol) methanol for 30 min; fresh 50% methanol containing 0.05% glutaraldehyde (Sigma Chemical Co.) was added and left at 25°C for 1.5 h. The fixing solution was removed and the gel placed in 150-200 ml of a solution containing 0.1 M NaCl, 33 mM Na phosphate, pH 8.0. The cross-linked proteins were reduced by addition of the solid NaBH₄ (Sigma Chemical Co.) to a final concentration of 20 μg/ml. After 1 h, the solution was decanted and replaced with fresh buffer containing NaBH₄ and left at 25°C overnight. The gel slices were then soaked in two to three washes of 150-200 ml of PBS. The wash was changed every 3-4 h.

Staining Gels with Lectins

The gel slices were placed in a flat plastic box containing 100 ml of PBS, 0.05% NaN₃ and 1 mg/ml of bovine serum albumin (Sigma Chemical Co.) as a protein carrier, and the labeled lectin. The incubation mixture was shaken gently at room temperature for 2 days on a gyratory water-bath shaker (New Brunswick Scientific Co., Inc., Edison, N. J.). After lectin incubation, the solution was decanted and the gel slices were washed for 3 days in 100 ml of PBS/azide solution. The wash solution was changed every 3-4 h during the day and then left shaking overnight. Monosaccharide hapten (0.1 M) was added to control gels only during the overnight wash. Gels were dried on Whatman no. 54 filter paper with a Hoefer gel drier (Hoefer Scientific Instruments, San Francisco Calif.). Labeled bands were detected by autoradiography at -60°C on RP Royal X-OMAT x-ray film (Kodak) with the aid of a Cronex Lighting Plus (calcium tungstate phosphor) intensifying screen (DuPont Instruments, Wilmington, Del.) (15). Gels were stained for protein with Coomassie Blue R250 (Miles Laboratories Inc. Miles Research Products, Elkhart, Ind.). As a reference, an extract from Swiss 3T3 cells was included in all electrophoresis runs.

RESULTS

The glycoproteins from whole neural retina which bind to [¹²⁵I]WGA under our electropho-
resin and staining conditions are shown in Fig. 1, for neural retina from days 7 to 13 of development. It should be clear that the observed bands represent the minimal number of glycoproteins that can bind WGA. Not only may each visible band consist of more than one glycoprotein, but minor glycoproteins or glycoproteins to which the lectin can only bind weakly may be missed by these techniques. The observed pattern has been seen in at least three separate dissections, and at least 10 separate gels. None of the bands are visible if the gel is incubated with [125I]WGA in the presence of 0.1 M GlcNAc (data not shown).

Both qualitative and quantitative changes in glycoproteins are visible in Fig. 1 and are indicated by the arrows and letters on the right margin of the gel.

The major changes are bands c, g, and i, which are present in retinas from days 7, 8, and 9, and are no longer detectable in older neural retinas. In contrast, bands designated as e, f, h, k, and possibly l, and m (as revealed in longer exposure of the gels to film) appear only later in development. Quantitative changes in other bands are also seen.

The gels also show some staining at the dye front which could be due to glycolipids or glycoprotein of low molecular weight. No attempt has been made so far to examine this material in detail.

In contrast to the changes observed with [125I]WGA, staining of the same extracts with Coomassie Blue reveals that the major protein bands detected at this low level of resolution remain invariant during the whole developmental period that we have examined (see Fig. 4 below).

Fig. 2 shows that at least one of the major developmental changes observed with WGA can also be observed with [125I]ricin. Protein(s) in the position of band i appear at days 7 and 8.
and are absent after day 10. Similarly, the appearance of proteins with the mobility of bands k and n are also seen at later stages of development. Higher molecular weight bands which stain with WGA appear not to be stained by ricin. None of the major glycoprotein bands are observed when ricin is incubated with the acrylamide gel in the presence of 0.1 M lactose (data not shown).

Ricin and RCA4 share some of the same carbohydrate specificity; however, when we attempted to use [3H]RCA4 to locate glycoproteins on gels, we found that this lectin stained a variety of proteins, but this staining appeared to be nonspecific because the lectin could not be displaced with 0.1 M lactose. Thus, under our assay conditions, RCA4 appears to bind to several glutaraldehyde-fixed proteins in a manner unrelated to its carbohydrate specificity. Affinity columns prepared with RCA4, however, have proven useful in preliminary separation of retinal glycoproteins.

Triton X-100 extracts were prepared from whole neural retina. When fractionated by acrylamide gel electrophoresis in SDS and stained with [3H]WGA, these extracts showed the same glycoprotein patterns as SDS extracts (data not shown). Such extracts of whole neural retina were adsorbed on columns of RCA4 linked to Sepharose and the bound glycoproteins were eluted with lactose. Fig. 3A shows the electrophoretic pattern of the lactose-eluted material obtained by affinity chromatography, stained with [3H]WGA for days 7, 8, 10, and 13 of development, compared to an SDS extract of whole neural retina from the same days (Fig. 3B). The same changes are noted as in Fig. 1, particularly the absence of band b on days 7, 8, and 10, the absence of bands g and i on days 10 and 13. The glycoproteins have been purified about 15-fold by this procedure, and there appears to be some differential enrichment of some of the bands. It should be clear that, when a band is not present in our gels, this simply means that at the present level of resolution it is not detected. Thus, for example, very small quantities of band g could be present in day-13 retina and would not be detected. Longer exposure of the gel to photographic film would simply obscure this area due to the presence of band f. Only after isolation and characterization of these glycoproteins will we be able to clarify this potential ambiguity.

**Figure 3** Separation of glycoproteins by affinity chromatography on RCA4. Triton X-100 extracts from neural retina from embryos of 7, 8, 10, and 13 days of age were adsorbed to RCA4-Sepharose as indicated in the text, and the bound glycoproteins were eluted with 0.1 M lactose. Samples of 5 μg each of protein were fractionated by gel electrophoresis and stained with [3H]WGA (part A). (For comparison part B shows the fractionation of SDS extracts of whole neural retina of the same age embryos [80 μg protein/lane]). The dried gel was exposed to film for 5 h. Straining was with 22 μg of lectin (6 × 10^5 cpm).

Fig. 4 illustrates a Coomassie Blue-stained gel of neural retina cell SDS extracts and Triton X-100 extracts specifically eluted from the RCA4 column. It is noteworthy that none of the bands that stain with [3H]WGA are visible, either because they are present at too low concentrations or because they, like other glycoproteins (8), fail to stain with Coomassie Blue. However, the glycoprotein fraction on day 13 appears to contain two proteins labeled “1” and “3” which are undetectable in the glycoprotein fraction obtained from day-7 and day-8 neural retina. These proteins presumably do not bind to WGA but will bind to RCA4. In contrast, bands “2” and “4” are present at all stages of development.

**DISCUSSION**

The results presented in this paper show that there are major changes in the detectable gly-
FIGURE 4 Separation of proteins by affinity chromatography. The lanes contain either 80 μg of neural retina extract from the day indicated (labeled SDS 7 for extract of 7-day-old neural retina, etc.) or glycoproteins obtained by chromatography on RCA₁₁ affinity column of Triton X-100 extracts of neural retina of the day indicated (see Fig. 3 and text); the gel was stained with Coomassie Blue. 3T3 is a reference extract from 3T3 cells; the numbers on the right margin indicate bands discussed in the text.

copolyprotein pattern of chick neural retina during development. The presence of glycoproteins which are unique to certain developmental stages could arise either because these proteins are unique to certain cell types present in the neural retina, or because they represent changes in the glycoprotein pattern of most of the cells in the neural retina. These possibilities can potentially be distinguished by the purification of these proteins and preparation of suitable antibodies. If a unique population of cells can be labeled by such antibodies, this would provide a method for the separation of specific neuronal cell populations from the neural retina. The purification of small quantities of proteins is difficult but will be aided in this case by the possibility of using different lectin affinity columns for the separation.

The combination of polyacrylamide gel electrophoresis and staining with ¹²⁵I-labeled lectins is clearly a powerful tool for the study of the development of a specific group of proteins. Not all lectins will, however, detect significant developmental differences; for example, we could not detect any major differences in the staining pattern of glycoproteins with ¹²⁵I-labeled succinylated concanavalin A between days 7 and 13 of development in chick neural retina. Exploration of developmental differences with a number of different lectins may prove extremely valuable.

The precise cellular location of the glycoproteins stained by our methods are not known, although we presume that at least some of them are present on the cell surface. Previously, Merrell et al. (19) reported temporal changes in cell adhesion in neural retina between days 7 and 9 of development. It is interesting, although possibly a coincidence, that major changes in glycoprotein pattern as revealed by WGA and ricin staining also occur during this period of development. It is important to caution again that the observed changes are minimal numbers, and that more sophisticated separation methods may reveal even more complex patterns of glycoprotein development. The possible importance of glycoproteins in phenomena such as cell adhesion have been emphasized by a number of laboratories. The sensitive detection methods applied in this study should allow the correlation of observed functional changes with structural alteration in the glycoprotein composition of the cell surface.

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