versus transparent sections of the same lens. The results of our analyses using polyclonal antiserum to the beta crystallin fraction and to synthetic peptides from the N- and C-terminus of the beta crystallin molecule suggest that changes in some, but not all, opacification processes involve the beta crystallin class of polypeptides.

When direct comparison is made of antisera binding to proteins from the anterior sections of lenses 5668 and 5669, anti-beta crystallin class of molecules. With the development of antigen probes to more synthetic peptides from clear versus opaque sections, the increase in binding of anti-beta crystallin to opaque sections may result from greater accessibility of the N- and C-terminal sections. Such a change would imply structural changes of this molecule during the opacification process. The decrease in binding of anti-beta crystallin to opaque sections may result from insolvulization of a class of beta crystallins other than beta crystallin during the opacification process. Such a change would also result in a relative increase in anti-beta crystallin sera binding to samples from the opaque sections. Whatever the molecular mechanisms responsible for the changes seen in the anterior sections of lenses 5668 and 5669, it is clear from these studies that not all opacification processes involve this change in the beta crystallin class of molecules. With the development of polyclonal antisera to more synthetic peptides of lens crystallin, it should be possible to investigate possible changes in defined epitopes of the alpha and gamma crystallins from microdissected sections of the same human lens.

A 31,000-Dalton Protein Released by Cultured Human Retinal Pigment Epithelium

Ross D. Edwards, Junelle T. Brandt, and Gordon S. Hardenbergh

Confluent primary cultures of human retinal pigment epithelium (RPE) were incubated for 3–14 hr with 35S-methionine, 3H-leucine, or 2-3H-mannose, and the released proteins were analyzed by SDS-polyacrylamide gel electrophoresis. A 31,000-dalton protein constituted 10–70% of the released radioactive proteins. The peak was degraded by pronase and its synthesis was inhibited by cycloheximide. The incorporation of 2-3H-mannose into this protein, and its inhibition by tunicamycin, showed that the protein is glycosylated. Monensin (10−5 M) also inhibited the release of the 31,000-dalton glycoprotein. The released 31,000-dalton glycoprotein from human RPE comigrated on gels with a protein present in extracts of human interphotoreceptor matrix, raising the possibility that the released protein corresponds to a component of the matrix. Invest Ophthalmol Vis Sci 28:1213–1218, 1987

The retinal pigment epithelium (RPE), as one of the tissues bordering the interphotoreceptor space, is a potential source of components of the interphotoreceptor matrix (IPM). Cultured human RPE synthesizes and releases hyaluronic acid, chondroitin sulfates, and dermatan sulfates, some of which resemble the glycosaminoglycans in extracts of IPM from human eyes. Extracts of IPM also contain proteins. The most prominent of these is interstitial retinol binding protein (IRBP), which appears to be syn-

Key words: microdissected sections, peptide antisera, cataracts

From the *Division of Biology, Ackert Hall, Kansas State University, Manhattan, Kansas, and the †Howe Laboratory of Ophthalmology, Massachusetts Eye and Ear Infirmary, Harvard Medical School, Boston, Massachusetts. Supported by grants from the National Eye Institute to L.T., J.W., and L.C. and to the Cooperative Cataract Research Group. Submitted for publication: November 5, 1986. Reprint requests: Larry Takemoto, Division of Biology, Ackert Hall, Kansas State University, Manhattan, KS 66506.

References
Chemicals, Milwaukee, WI), tunicamycin (B2 homo-

Nuclear, Boston, MA). Cultures were incubated for

the appropriate radioactive precursor (New England

preincubation except that it contained 250 juCi/ml of

Radiopaque (for incubations with 2-3H-mannose). After

cose and 3 JUM leucine (for incubations with 3H-leu-

medium of the same composition as used for the

cell-associated proteins:

ning. Cycloheximide (100 ng/ml, Immuno Nuclear Corp., Still-

water, MN), or monensin (10^-5 M, Calbiochem-

Behring, La Jolla, CA), when used, were present during

both the preincubations and the incubations with

radioactive precursors. Following the incubations

with radioactive precursors, the medium was re-

moved and centrifuged at 165 g for 10 min at 4°C. The

supernatants were removed, combined with the

protease inhibitors pepstatin A (5 ju/ml final con-

centration), phenylmethylsulfonylfluoride (PMSF, 0.1 mM), EDTA (1 mM, to inhibit metalloproteases),

and iodoacetate acid (1 mM, to inhibit proteases that

require sulfhydryl groups), and centrifuged again for

1 hr at 170,000 g. The supernatants were dialyzed for

24 hr against protease inhibitors and 0.05 M (NH4)2CO3, freeze-dried, and dissolved in SDS-mer-
captoethanol sample buffer. Rinsed cells were re-

moved from the culture dish with a rubber scraper,

resuspended in 0.6 mL of 0.05 M Tris-HCl with pro-

tease inhibitors, and sonicated briefly; aliquots were

centrifuged at 170,000 g for 1 hr in order to prepare a

membrane pellet and an aqueous supernatant. Ali-

quots of medium, sonicated cells, or centrifuged cell

fractions were analyzed for protein and trichloroac-

eic acid (TCA)-precipitable radioactivity. Other ali-

quots were dissolved in SDS sample buffer, boiled for

2 min, and analyzed by electrophoresis at room

temperature on 7.5–17.5% linear gradient SDS poly-
crylamide slab gels. Protein standards (Biorad, Rich-

mond, CA) were included on separate lanes of
different gels. Gels were fixed, stained with Coomassie

blue or silver nitrate (Biorad) and sliced (1.25

± 0.15 mm). Slices were incubated with Protosol

Radiopaque (Enhance, New England Nuclear), dried, and ex-

posed to Kodak (Rochester, NY) AR X-ray film at

–80°C.

Results. Cultured normal human RPE incubated

with 35S-methionine or 3H-leucine synthesized and

released radioactive proteins. The most prominent of

these migrated at a position equivalent to a molecular

weight of 31,000 ± 1,000 daltons (31 kd) and com-

prised 10–70% of the total released proteins (Fig. 1A).

Other proteins which were present in most profiles of

released proteins were of approximate molecular

weights 40, 46, 59, 72, 92, 120, 140, 167, and 240 kd

(Fig. 1A). One or two proteins smaller than 31 kd

were also sometimes seen (see Fig. 4). Cycloheximide

(100 ju/ml) inhibited by over 95% the synthesis and

release of these proteins. Analysis by electrophoresis

showed that cycloheximide reduced the amounts of

the 31-kd peak as well as the other radioactive re-
Radioactive proteins released by cultured normal human RPE. RPE cultured from autopsy eyes of a 19-yr-old male were incubated with 250 μCi/ml [35S]-methionine for 6.4 hr and analyzed by SDS polyacrylamide gel electrophoresis and fluorography as described in Methods. The scale indicates the position of standard proteins analyzed in an adjacent lane (myosin, 200 kd; ovalbumin, 45 kd; β-galactosidase, 116 kd; phosphorylase b, 93 kd; bovine serum albumin, 66 kd; ovalbumin, 45 kd; and carbonic anhydrase, 30 kd). The principal released radioactive proteins are indicated by arrows and approximate molecular weights. (B) Released and cellular proteins of cultured normal human RPE. A culture from a 68-yr-old female was incubated for 9 hr with 250 μCi/ml [3H]-leucine. The proteins released into the medium (Med) were compared with the proteins of the sonicated cells (Cells) and of the supernatant (Sup) and pellet (Pel) prepared by centrifugation of the sonicated cells as described in Methods. A radioactive peak with an electrophoretic mobility of about 31 kd was present in the pellets obtained by sonicating and centrifuging cultured human RPE cells (Fig. 1B). The RPE cell supernatant also contained a small amount of radioactive 31-kd protein; the amounts and proportions of 31-kd protein in the cell supernatant differed between RPE cells from different individuals, but was usually lower than in the cell pellet from the same individual. Each fraction (released, cell supernatant, and cell pellet) contained some proteins which were enriched relative to the other fractions (Fig. 1B).

2-[3H]-mannose was incorporated into the 31-kd peak and other released proteins (Fig. 2A). Tunicamycin inhibited the incorporation of [3H]-mannose into total released proteins and total cell-associated proteins to 7% and 12%, respectively, of control values. Tunicamycin also inhibited the incorporation of [3H]-leucine into released and cell-associated proteins (26% and 64%, respectively, of controls), but to a lesser extent than the inhibition of [3H]-mannose incorporation. Amounts of the [3H]-leucine-labeled 31-kd protein and other higher molecular weight released proteins were reduced in the presence of tunicamycin, and a protein with an estimated molecular weight of about 24 kd, not observed under control conditions, was the principal released protein (Fig. 2B).

In the presence of monensin (10^-5 M) the release of the 31-kd protein was inhibited over 90% (Fig. 3). The amount of total [3H]-leucine-labeled proteins released into the medium was 20-40% of controls, while incorporation into total cell protein was over 80% of controls.

Coelectrophoresis of released radioactive proteins and IPM extracts was carried out with released proteins from RPE cultures of five individuals and with IPM extracts from two individuals. The 31-kd protein from each donor migrated precisely with a silver-stained 31-kd protein of IPM extracts, an example of which is shown in Figure 4.

Discussion. A 31-kd protein was the principal radioactive protein released into the medium by cultured human RPE cells incubated with radioactive precursors. Degradation of the radioactivity by protease and inhibition of precursor incorporation by
contamination with whole cells or with the soluble components of cells. Furthermore, monensin, a sodium ionophore that inhibits the passage of some secreted proteins through the Golgi body, significantly inhibited the amount of released 31-kd protein but only slightly inhibited total protein synthesis; this finding is consistent with the idea that release of the 31-kd protein was by a cellular secretory pathway.

Cultured human iris pigment epithelium, lens epi-

cycloheximide confirmed the protein nature of the material and that the radioactive peak was synthesized de novo by the cells. Since 2-3H-mannose was incorporated into the 31-kd protein, and since the amount of released 3H-mannose-labeled 31-kd protein was reduced to less than 10% of controls by tunicamycin, an inhibitor of asparagine-linked protein glycosylation11 (Fig. 2A), the 31-kd protein appears to be glycosylated. Additional support for this conclusion is suggested by the observation that, in the presence of tunicamycin, the amount of released 3H-leucine-labeled 31-kd protein was significantly reduced and a 3H-leucine-labeled protein of approximately 24 kd became the most prominent protein; the latter may be the unglycosylated form of the 31-kd protein (Fig. 2B). Proportions of 31-kd protein in the proteins released by cultured RPE cells were significantly greater than the proportions of 31-kd protein in the proteins of RPE whole cells or in the RPE cell soluble fraction (Fig. 1B), which indicates that the radioactive proteins in the culture medium were not due to contamination with whole cells or with the soluble components of cells. Furthermore, monensin, a sodium ionophore that inhibits the passage of some secreted proteins through the Golgi body, significantly inhibited the amount of released 31-kd protein but only slightly inhibited total protein synthesis; this finding is consistent with the idea that release of the 31-kd protein was by a cellular secretory pathway.

Cultured human iris pigment epithelium, lens epi-
Fig. 4. Coelectrophoresis of released radioactive proteins and extracts of human interphotoreceptor matrix (IPM). RPE cultured from an 87-yr-old female was incubated with 250 μCi/ml 35S-methionine, and a culture from an 18-yr-old male was incubated with 250 μCi/ml 2-3H-mannose, for 3 hr. To an aliquot of the released proteins of each culture was added an aliquot of IPM extract (25 μg protein) prepared from the eyes of the same 87-yr-old female within 4 hr after death. Each mixture was analyzed by electrophoresis on separate lanes of the same slab gel. The gel was silver-stained and photographed. Silver-stained IPM proteins are shown in the strip below the graph. Each gel lane was sliced so that a silver-stained 31-kd protein of IPM (arrowhead) was centered in an identified slice, and radioactivity was analyzed as described in Methods. The major radioactive peak of each sample coincided with the IPM protein indicated by the arrowhead.

The retinal pigment epithelium, choroidal melanocytes, and rat RPE also release proteins similar in size to the 31-kd protein (R.B. Edwards, unpublished results). Analysis by gel electrophoresis is not sufficient to determine if these proteins are the same as the 31-kd protein released by cultured human RPE, but these observations raise the possibility that the 31-kd protein may not be unique to the RPE. Not all cultured tissues release a 31-kd protein, since it is not released by cultured human fibroblasts, undifferentiated rat neural retinal cells, nor differentiated rat heart muscle cells (R.B. Edwards, unpublished results).

Extracts of human IPM contained a protein which comigrated on polyacrylamide gels with the 31-kd protein released by cultured human RPE (Fig. 4), and a similar protein is present in extracts of bovine IPM. These observations raise the possibility that the 31-kd protein is a component of IPM synthesized and released by the RPE. Further support for this idea will require localization of the 31-kd protein to the IPM in situ and determination that the IPM and RPE proteins share properties additional to a similar electrophoretic mobility. These results provide a basis for further characterization of the 31-kd and other proteins released by cultured RPE and provide a framework for similar studies with RPE cultured from eyes with retinitis pigmentosa and other hereditary retinal degenerations.

Key words: retinal pigment epithelium, human, glycoproteins, interphotoreceptor matrix, protein secretion, cell culture

Acknowledgments. The authors thank the New England Eye Bank, Boston, Massachusetts, and the National Disease Research Interchange, Philadelphia, Pennsylvania, for providing normal autopsy eyes.

From the Berman-Gund Laboratory for the Study of Retinal Degenerations, Department of Ophthalmology, Harvard Medical School, Massachusetts Eye and Ear Infirmary, Boston, Massachusetts. Supported by National Eye Institute Grant EY-02028, NEI Specialized Research Center Grant EY-02014 and in part by the Retinitis Pigmentosa Foundation Fighting Blindness, Baltimore, Maryland. G.S.H. was the recipient of a Student Fellowship provided by the Albert G. and Herman B. Mosler Fund of Fight for Sight, Inc., New York, New York. Submitted for publication: February 12, 1986. Reprint requests: Ross B. Edwards, PhD, Berman-Gund Laboratory, 243 Charles Street, Boston, MA 02114.

References


2. Edwards RB: Glycosaminoglycan synthesis by cultured human retinal pigmented epithelium from normal postmor-
Inhibition of β-Crystallin Cross-Linking in the Ca\[^{2+}\]-Treated Lens

L. Lorand, S. M. Conrad, and P. T. Velasco

β-Crystallin dimers of approximately 55,000 weight are among the early products of protein cross-linking in Ca\[^{2+}\]-treated rabbit lens, caused by the activation of intrinsic transglutaminase; formation of the cross-linked species can be blocked by 75 mM histamine (Lorand et al, Biochemistry, 24:1525–1531, 1985). As an extension of this work, we initiated a search for more specific inhibitors of cross-linking in this system. Of the compounds tested so far, hydroxylamine, methoxyamine, bisaminoxyp propane and aminoacetoneitrile were particularly effective, inhibiting the generation of the cross-linked dimer in the 5 to 10 mM concentration range during a 4 hr treatment of the lens with Ca\(^{2+}\)-ions. Invest Ophthalmol Vis Sci 28:1218–1222, 1987

Post-translational protein modifications, in general, might play important roles in the process of lens aging and cataract formation. They could change the solubilities of crystallins, disturb their normal aggregation characteristics and alter the interactions of cytoplasmic proteins with membrane components. The isolation of N\(^{-}(\gamma\text{-glutamyl})\)lysine peptides from polymers typically present only in cataractous lens\(^1\) called attention to the biochemical reactions catalyzed by transglutaminase.\(^2\)

Certain subunits of β-crystallin are highly specific substrates for transglutaminase, and the primary product is a cross-linked dimer X(β); with an apparent mass of about 55,000 (ie, 55K). In the rabbit material, the 55K species can be elicited either by reacting purified β-crystallin with the Ca\(^{2+}\)-activated enzyme\(^3\) or simply by immersing the whole lens in a Ca\(^{2+}\)-rich medium. \(^4\) X(β), though probably a heterogeneous product, by immunological cross-reactivities, is known to comprise the 29–30K\(^5\) and the 23K βBp subunits of β-crystallins.\(^5\)

We have previously shown that the formation of X(β) could be inhibited by adding histamine (75 mM) to the incubation mixture.\(^4\) Inasmuch as the Ca\(^{2+}\)-treated lens might be used as an experimental cataract model,\(^6\) it was desirable to search for inhibitors which might be effective in lower concentrations than histamine and to gather insights into the conditions necessary for blocking the generation of dimeric β-crystallin.

Materials and Methods. Each rabbit lens (supplied by Pel Freez, Rogers, AK) was incubated at 37°C for 1 hr in 0.5 ml of medium buffered with 50 mM Tris-Cl, pH 7.4. The solution contained 1 mM leupeptin (gift of the U.S.-Japan Cooperative Cancer Research Program), varying concentrations of amine inhibitors and 20–100 mM sodium chloride. The latter was used for adjusting osmolarity to approximately 300 mOsm/kg. Penicillin (0.2 mM) and streptomycin (0.01%) were included to prevent bacterial growth. Treatment with Ca\(^{2+}\), lasting 4 hr, was initiated by adding 0.1 ml of 50 mM CaCl\(_2\), dissolved in Tris-Cl and NaCl to match the concentrations of these constituents in the preincubation medium. To control specimens, 0.1 ml of 10 mM ethylenediaminetetraacetate (EDTA) was added. At the end of the 4 hr period, lenses were cooled to −20°C and were stored