Identification of G-Protein α-Subunits During Distinct Stages of Lens Cell Differentiation

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Purpose. The purpose of this study was to identify α-subunits of heterotrimeric guanosine triphosphate-binding proteins in lens cell populations at various stages of terminal differentiation.

Methods. Crude cell membranes were isolated from the annular pad, cortical fibers, and nuclear fibers of adult chickens and subjected to cholera and pertussis toxin-mediated ribosylation reactions. Specific labeling of toxin substrates was visualized after SDS–PAGE and radioautography. In complementary experiments, cell membranes were first separated by SDS–PAGE, transferred to a nitrocellulose support membrane, and probed with a panel of commercially available antibodies that recognize various classes of G-protein α-subunits.

Results. A cholera toxin substrate was identified in cortical fibers whose labeling was dependent on a soluble factor. No cholera toxin substrates were labeled in annular pad cells. Two pertussis toxin substrates were seen in the relatively undifferentiated annular pad and the more differentiated cortical fibers. Relative abundance of the pertussis toxin substrates differed between the two cell types. Antibody staining revealed the presence of α-subunits belonging to the Gs, Gi, and Gt families throughout the lens. Molecular weight differences of G12 polypeptides were noted between annular pad and cortical fiber cells.

Conclusions. These results suggest that G-protein-based signal transduction pathways continue to function in lens cells during various stages of terminal differentiation. Differences noted between annular pad and cortical fibers provide additional evidence that dynamic alterations in receptor-mediated processes may be responsible for the accumulation of differentiated characteristics during fiber formation. This also indicates that ocular pharmacologic intervention could affect various aspects of lens physiology throughout the process of fiber formation.

proteins can be achieved with bacterial toxins, a more complete analysis requires the use of antibodies directed against the purified α-subunits or peptide sequences unique or common to the different α-subunits.

The vertebrate lens continues to grow throughout life as concentric shells of newly differentiating fiber cells are added peripherally to underlying generations of slightly more differentiated fibers. Because fibers are not turned over or shed, the lens presents a complete developmental record of nearly every cell that has gone into its formation, from epithelial cells newly committed to lens fiber formation to fully differentiated, aging lens fibers. This continuous process is known to be regulated by numerous receptor-mediated events. Thus, the lens offers a unique opportunity to study mechanisms that operate during the protracted process of lens fiber elongation and differentiation. The adult chicken lens, in particular, is amenable to these kinds of studies because of its unique organization. In this species, the anterior epithelium contains a large population of postmitotic cells known as the annular pad, which are in the very early stages of lens fiber formation. Therefore, large numbers of annular pad cells can be obtained routinely for examination of receptor-mediated processes.

Signal transduction during differentiation of lens cells and during regulation of basal metabolic activities is not well understood. It is also unknown whether dynamic alterations in receptor-mediated processes might control the temporal and spatial integration of the morphologic and biochemical alterations that highlight lens fiber formation. In support of the preceding notion are observations that indicate regional and developmental differences in receptor kinetics and abundance, and the effector molecule activities of adenyl cyclase and phospholipase C. The current study was undertaken to characterize more fully the G-protein-based signal transduction capacity of lens cells during various stages of terminal differentiation into lens fibers. We show that α-subunits of the G,, G,, and G, families can be detected at diminishing levels through-out the process of lens fiber formation. Distinct sets of G, proteins were identified in annular pad and cortical fibers. This indicates that discrete stages of lens fiber development may be characterized by unique signal transduction pathways.

MATERIALS AND METHODS

Membrane Preparation

Unless otherwise indicated, all reagents were obtained from Sigma (St. Louis, MO). Methods for securing tissues were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All pictured results were representative of at least five separate experiments. Lenses were enucleated from the globes of freshly slaughtered chickens and were placed in ice-cold buffer (16 mM Tris, 50 mM HEPES, pH 7.4, 120 mM NaCl, 10 mM MgCl2, 1 mM EGTA) supplemented with 1 μg/ml leupeptin, 1 μg/ml pepstatin, 80 μg/ml benzamidine, 4 μg/ml aprotinin, 100 μg/ml phenylmethylsulfonyl fluoride, and 100 μg/ml 1,10 O-phenanthroline. After 30 minutes, the lenses were decapsulated from their posterior pole while under buffer. The annular pad cells, which are the direct precursors of fiber cells, were teased gently away from the lens capsule, retrieved, and stored on ice. The fiber cell mass was separated into an outer cortical fiber mass and an inner nuclear fiber cell mass. Cortical fibers represented approximately half the total fiber mass (by wet weight) and included all the nucleated fiber cells still in the process of elongation. In a typical experiment, annular pad cells were obtained from 100 lenses, whereas cortical and nuclear fibers were obtained from 50 lenses. Cells were disrupted using tight-fitting glass homogenizers (100 mg wet weight/ml buffer). Homogenates were centrifuged at 4°C for 3 minutes at 750g to sediment nuclei and cell debris. Supernates were further subjected to centrifugation for 30 minutes at 40,000g to obtain a crude cell membrane preparation. The supernate from the second centrifugation was retained to investigate the presence of soluble ribosylation factors. Membranes also were prepared from chick forebrain (telencephalon) essentially as described above. Briefly, tissues were homogenized in the above buffer containing 0.32 M sucrose. After the initial low-speed centrifugation, the resultant supernate was subjected to high-speed centrifugation. The resultant pellet was rehomogenized in buffer without sucrose and centrifuged at high speed. Then that pellet was rehomogenized and centrifuged one more time.

Toxin Labeling

Cell membranes were sonicated in ribosylation buffer containing 10 mM HEPES, pH 8, 10 mM thymidine, 5 mM dithiothreitol, 1 mM EDTA, 0.2 mg/ml bovine serum albumin, and 0.025% sodium dodecyl sulfate (SDS) at a final concentration of 500 μg/ml. Before cholera toxin labeling, aliquots of sonicated membranes were incubated for 10 minutes at 37°C with the retained cytosolic fractions. After incubation, the membranes were repelleted in a refrigerated microfuge before resonication in ribosylation buffer. Cholera and pertussis toxins were purchased from List Biologicals (Campbell, CA) and were stored at −20°C as 50% glycerol stocks at 0.5 mg/ml and 1.0 mg/ml, respectively. Toxins were activated in a buffer (9 parts buffer:1 part toxin stock) consisting of 50 mM HEPES, pH 8, 20 mM dithiothreitol, 1 mg/ml bovine serum albumin.
albumin, and 0.125% SDS. Cholera toxin was activated for 10 minutes at 37°C, and pertussis toxin was activated for 30 minutes at 30°C immediately before use. Final concentrations were 10 μg/ml for cholera toxin and 20 μg/ml for pertussis toxin. All ribosylation reactions were incubated at 30°C for 30 minutes and contained 100 to 200 μg of crude cell membranes and $10^7$ dpm of $[^{32}P]NAD^+$ (specific activity, 250 μCi/mmol; ICN, Costa Mesa, CA). As indicated in the figures, controls for toxin specificity were performed in the absence of toxin, whereas controls for nonspecific binding of radiolabel were incubated with a 100-fold molar excess of unlabeled NAD$^+$. After incubation, samples were boiled for 5 minutes in 2x SDS sample buffer. Ribosylated samples were separated on 10% polyacrylamide gels. After staining, gels were dried between two sheets of cellophane and exposed to x-ray film to obtain autoradiographic images.

Antibody Labeling
Alternatively, cell membranes were immediately boiled in SDS sample buffer. Protein concentrations of samples were determined as previously described. Equal amounts of protein from annular pad, cortical fibers, nuclear fibers, and chick brain were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose paper. Replicas were blocked in 3% gelatin before they were exposed to commercially available primary antibodies that recognize various α-subunits of G proteins. Sources of primary antibodies (along with immunizing peptides–proteins) were as follows: Anti-G$_{\text{mi}}$ (purified bovine brain Glr40) and anti-G$_{\text{ui}}$ (C-terminal decapeptide common to both subunits in human, bovine, rat, and mouse) were obtained from Upstate Biotechnology (Lake Placid, NY); anti-G$_{\text{mi}}$ (internal decapeptide corresponding to amino acid residues 159–168 in human and bovine), anti-G$_{\text{rii}}$ (C-terminal decapeptide in human and rat), and anti-G$_{\text{mi}}$ (C-terminal decapeptide in human, bovine, rat, and mouse) were obtained from CalBioChem (La Jolla, CA). All antibodies were polyclonals raised in rabbit. Antibodies were used at the dilutions indicated in the figures. Partially purified mouse recombinant G-protein α-subunits expressed in Enterobacteria coli also were obtained from CalBioChem to assess antibody specificity. In addition, the recombinant α-subunits were included in excess amounts during reaction of primary antibodies with chick tissues to establish specificity further. Secondary antibodies conjugated to hors eradish peroxidase and enhanced chemiluminescence substrate mixtures were obtained from Amersham (Arlington Heights, IL) and were used according to the manufacturer’s specifications.

RESULTS
Toxin Labeling
As shown in Figure 1, a substrate for cholera toxin-mediated ADP ribosylation was identified in cortical fiber cell membranes, but only after preincubation with the retained cytosolic fraction. The requirement of a cytosolic extract during cholera toxin treatment indicates that a soluble ADP-ribosylation factor (ARF) is present in lens fibers. The toxin substrate in cortical fibers migrated with an $M_r$ of 42 kDa. Unexpectedly, a toxin substrate could not be labeled in annular pad cells under the same conditions described for the cortical membranes (data not shown). Experiments in which annular pad membranes were preincubated with cortical fiber cytosol similarly did not reveal a toxin substrate (data not shown). Pertussis toxin substrates were identified readily in annular pad cells and cortical fibers and did not require preincubation with cytosolic extracts (Fig. 2). When aliquots of the labeled samples were
Annular Pad Cells  Fiber Cells

45 36
66 45 36 29

PT  -  -  +  +  -  -  +  +
NAD+  -  +  -  +  -  +  +  +

**FIGURE 2.** Pertussis toxin substrates in lens cells. (A) Coomassie blue-stained 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and (B) corresponding autoradiogram of annular pad and cortical fiber membranes subjected to pertussis toxin (PT) labeling. Note that specifically labeled substrates were found in both cell types. Inclusions to the ribosylation mixture are indicated below (B). The positions of molecular weight markers are indicated in kilodaltons to the left of A.

In Figure 2 were electrophoresed on a higher percentage gel, two substrates of M, 41 and 40 kDa were resolved (Fig. 3). Although the annular pad substrates exhibited equal levels of label incorporation, the 40-kDa substrate in cortical fibers showed much less label incorporation. Less abundant levels of pertussis toxin substrates were observed in nuclear fibers (data not shown). The inclusion of GTP or nonhydrolyzable GTP analogs in the incubation mixture had no effect on the labeling of either cholera or pertussis toxin substrates (data not shown).

**Antibody Labeling**

The specificity of the α-subunit antisera is shown in Figure 4. A series of duplicate replicas was prepared by separating equivalent protein loads of the partially purified recombinant α-subunits in the adjoining wells of each gel. After electrophoresis and transfer to nitrocellulose, replicas were probed individually with the primary antisera indicated. In every instance, only the immunizing antigen was recognized by its respective antiserum. Figure 4E also shows the slightly different molecular weights of the α1 and α2 polypeptides (41 and 40 kDa, respectively).

This panel of commercially available antibodies was applied to Western blots of lens cell membranes to characterize more fully the signal transduction pathways of lens cells in discrete stages of fiber development. Samples of brain membranes were included on each blot to assure that the antisera were able to recognize the respective α-subunit in the chicken. This approach revealed a level of complexity of signal transduction pathways in lens cells not evident through the use of toxin labeling. As shown in Figures 5B and 5C, Gα and Gβ polypeptides were found throughout the lens as single immunoreactive bands with relative M, levels of 42 kDa and 39 kDa, respectively. Both polypeptides comigrated with their chick brain counterparts and diminished in abundance as a function of increasing cell age. Inclusion of the appropriate recombinant protein during primary antiserum incubations eliminated or greatly reduced the specific staining attributed to each antiserum (data not shown). Densitometric analysis of Figures 5B and 5C was performed for quantitative purposes. With the values for annular pad cells set as 1.0, immunostaining for Gα declined to 0.77 in cortical fibers and 0.11 in nuclear fibers, whereas immunostaining for Gβ declined to 0.63 in cortical fibers and 0.13 in nuclear fibers. With regard to the presence of Gβ in annular pad cells, although a cholera toxin substrate was not labeled in these cells (see above), antibody staining clearly identified the presence of this polypeptide. Similar to the pattern observed for Gα, and Gβ, the staining of Western blots with the Gα antisera showed that these related polypeptides declined in abundance as a function of cell age and terminal differentiation. The α1 antisera, although weakly immunoreactive with chick brain, stained 40- and 41-kDa bands in annular pad cells (Fig. 5D). Recombinant α1, however, migrated at a higher molecular intermediate to the 41- and 40-kDa bands identified in annular

**FIGURE 3.** Multiple pertussis toxin substrates in lens cells. Autoradiogram of specifically labeled samples shown in Figure 2 electrophoresed on a 12% gel to obtain better resolution of the labeled substrates. Note equal labeling intensity of annular pad (AP) substrates in cortical fiber cells (F), the slower migrating species is predominant.
G Proteins in Lens Cells

annular pad cells and was not detectable in nuclear fibers. The α1-2 antiserum identified bands of 40 and 41 kDa in annular pad and cortical fiber cells (Fig. 5E). Although the 40-kDa band was stained more intensely in annular pad cells, the 41-kDa band was most prominent in cortical fibers. Because the α1-

### FIGURE 4. Antibody specificity. (A) Silver-stained 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis of partially purified recombinant G-protein α-subunits. Each lane was loaded with 2 to 5 μg of the indicated partially purified α-subunit. (B to F) Western blots of the immunoreactive portions of duplicate gel pictured in A reacted with the following primary antisera: 1:1500 dilution of anti-Гα1 (B); 1:10000 dilution of anti-Гα3 (C); 1:1500 dilution of anti-Гα11 (D); 1:750 dilution of anti-Гα11-2 (E); and 1:6000 dilution of anti-Гα9 (F). Positions of molecular weight standards (in kilodaltons) are indicated to the left of A.

pad cells. In studies in which recombinant proteins were included during Гα11 primary antiserum incubations, only the α1 polypeptide was able to reduce the staining intensity of the two bands. Densitometric analysis showed that the immunoreactive 41-kDa band in cortical fibers was reduced to 0.14 compared to

### FIGURE 5. Immunologic identification of Гα polypeptides in lens cells. (A) Coomassie blue-stained 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis of crude membrane proteins from chick brain (B), annular pad (AP), cortical fiber (C) and nuclear fiber cells (N). Amounts separated were 100 μg for chick brain and 150 μg for lens samples. MW = molecular standards (in kilodaltons). (B to F) Western blots of the immunoreactive portions of duplicate gel pictured in A reacted with the following primary antisera: anti-Гα1 (B); anti-Гα3 (C); anti-Гα11 (D); anti-Гα11-2 (E); and anti-Гα9 (F). Primary antisera dilutions were the same as for Figure 4.
2 antiserum recognizes an epitope common to both subunits, competitive studies using recombinant αι1 and αι2 proteins during primary antiserum incubations were inconclusive. However, we think the 40-kDa band in annular pad cells and the 41-kDa band in cortical fibers represent the αι2 polypeptide for the following reasons: the generally accepted 40-kDa molecular weight for αι2; the comigration of 40-kDa band in annular pad cells with an immunoreactive band in chick brain; and the staining intensity of the 41-kDa band in the cortical fiber cells. If the 41-kDa band identified in cortical fibers by the αι1–2 antiserum was caused by the cross-reactivity of antisera with the αι1 polypeptide, one also would expect to see much greater immunoreactivity with the 41-kDa band in annular pad cells. This indicates, however, that the αι2 polypeptide in cortical fibers has a slightly higher molecular weight than its annular pad counterpart. Recombinant αι2 polypeptide, however, did comigrate with the immunoreactive band in chicken brain (data not shown). Total immunostaining with the Gαι1–2 antiserum was reduced to 0.72 in cortical fibers and 0.10 in nuclear fibers. Finally, the αι3 antiserum, which was also weakly immunoreactive with chick brain, stained a 41-kDa band in annular pad and cortical fiber cells (Fig. 5F). As with the other antisera tested, the abundance of αι3 was seen to decrease as more differentiated fibers were sampled. Recombinant αι3 also migrated at a slightly higher molecular weight than the chick brain component, making it appear to comigrate with the lens immunoreactive species (data not shown). Again, densitometric analysis showed that immunostaining was reduced to 0.44 in cortical fibers and 0.03 in nuclear fibers when compared to annular pad cells. In addition, only the αι3 recombinant protein was able to eliminate staining when it was included during the primary antiserum incubation (data not shown).

DISCUSSION

By using toxin-catalyzed ADP ribosylations and highly specific antibodies, we have identified multiple heterotrimeric GTP-binding protein α-subunits in distinct populations of terminally differentiating lens fiber cells. Although lens cells expressed many varieties of α-subunits, their pattern was probably no more complex than other differentiated cell types.6,29 To our knowledge, this is the first identification of any α-subunits in lens epithelial cells committed to the formation of lens fibers. These data more completely characterize the signal transducing capacities of lens cells and identify potential mechanisms that may affect early and late stages of fiber cell formation. It remains to be determined whether G-protein-based signal transduction mechanisms are linked to the expression or regulation of the many distinctive morphologic and biochemical features of lens fiber formation. Because the majority of lens cells have a complex set of receptor-mediated response mechanisms, even subtle changes in the ocular environment could have direct effects on lens growth and transparency.

Unexpectedly, we could not label Gαs in annular pad cells by cholera toxin-mediated ribosylation even though antibody staining clearly revealed the presence of this protein. Furthermore, the soluble ARF, which was necessary for toxin-mediated ribosylation in cortical fibers, did not support α labeling in annular pad samples. This finding indicates that the αs subunits in annular pad and cortical fibers are structurally distinct polypeptides, even though they comigrate on SDS gels and are recognized by the same antiserum. This may contribute to the differential production of cyclic adenosine monophosphate in these two cell types in response to β-adrenergic stimulation.30 Our findings also show that annular pad cells lack the ARF that supports ADP ribosylations of αs. Because the best-characterized in vivo functions of ARFs are to regulate vesicular transport in a GTP-dependent manner,24 annular pad cells probably use ARF-like proteins that do not activate cholera toxin during their extensive membrane trafficking.24,25 The presence of classic ARFs in cortical fibers would serve as yet another stage-specific characteristic of lens cell development.

As revealed by pertussis toxin-mediated ADP ribosylation and Western blot analysis, the profile of αι and αο subunits in lens cells is complex. Although αο subunits usually are also substrates for ADP ribosylations, we cannot say with certainty that this protein was identified by this technique owing to the lack of 32P-labeling at the appropriate molecular weight (see Fig. 3). There are no available protein sequence data available to determine whether chicken αο subunits have the specific sequence that supports toxin-mediated modification. Adenosine diphosphate–ribosylation studies showed two major 41- and 40-kDa polypeptides in annular pad cells, whereas cortical fibers exhibited a major 41-kDa polypeptide and a minor 40-kDa polypeptide (see Fig. 3). When combined with the Western blotting studies, these ribosylated proteins probably represent a cell type-specific mixture of the three αi polypeptides. The 41-kDa ribosylated band in annular pad cells would be composed of αι1 and αι3 subunits, and the 40-kDa band would be composed of the αι2 subunit. On the other hand, the 41-kDa ribosylated band in cortical fibers would be composed primarily of αι2 and αι3 subunits. The minor 40-kDa ribosylated band in cortical fibers probably represents some form of the αι2 subunit. In annular pad cells, it was not surprising that the αι1 and αι3 subunits in annular pad cells migrated as 41-kDa bands nor for the αι2 subunit to be found at 40 kDa because these are their generally accepted molecular
weights. An unusual feature of αι subunits in cortical fibers was the apparent 41-kDa molecular weight of the αι2 subunit (see Fig. 5). Similar to the findings concerning cholera toxin substrates and αι subunits, the data concerning αι2 indicates a second signal transducing pathway that distinguishes annular pad cells from the more differentiated cortical fibers. The minor differences noted in the relative migrations of the recombinant proteins and the immunoreactive bands identified in brain and lens could arise because of species–tissue peculiarities or posttranslation lipid modifications common to α-subunits.

Many aspects of lens formation and physiology are regulated by receptor-mediated processes. Growth factors such as fibroblast growth factor, insulin-like growth factor I, platelet-derived growth factor, epidermal growth factor, and transforming growth factor-β have roles in lens growth rates, cell elongation, cell migration, and altered gene expression during the terminal differentiation of lens fibers and the abnormal cellular responses of cataract formation. By regulating the production of cyclic adenosine monophosphate in the lens, β-adrenergic drugs may be involved in maintaining the postmitotic state of peripheral lens epithelial cells as well as in affecting the biochemical processing and membrane association of fiber cell-specific cytoskeletal proteins. Additional ligands, such as acetylcholine, bombesin, serotonin, and glucagon, elicit responses in lens cells, although the functional significance of these responses is not well understood.

Receptors for only some of the above ligands and second-messenger systems have been identified or characterized in lens cells. With regard to lens adenylyl cyclase, previous studies have shown enzyme activity to be affected by the interplay of β-adrenergic receptors, heterotrimeric G proteins, and Ca++/calmodulin. Therefore, one would anticipate the involvement of Gαo and Gαt proteins in the regulation of lens cyclic adenosine monophosphate levels. Although a previous study with bovine lens fibers identified multiple cholera toxin substrates and a single pertussis toxin substrate, it was proposed that the pertussis toxin substrate did not inhibit cyclase activity. This conclusion was based on results obtained from broken cell cyclase assays conducted in the presence of forskolin and nonhydrolyzable GTP analogs. Most likely, sample preparation and assay conditions were not optimal for maintaining critical associations of cyclase and G proteins with the cell membrane. Our results, particularly with the more sensitive and specific battery of antibodies tested, indicate that additional members of the Gα family are present throughout the lens and could contribute to cyclase regulation. If lens cyclase is regulated in this manner, our findings also would predict the presence of αβ-adrenergic receptors in lens cells. Another area of lens research to which the current data have relevance is the regulation of various cell membrane ion channels. It is well established that the isoforms of Gαo proteins influence the activity of K+ and Ca++ channels. In lens cells, these channels have been implicated in such diverse processes as the initiation of lens fiber elongation and the onset of lens opacities. Intuitively, such activities in the lens must be highly regulated to maintain normal lens growth and transparency. Although it remains to be determined what specific ligands affect the activity of lens ion channels, we have provided indirect evidence that some lens channels may be regulated by G-protein-dependent mechanisms. Finally, because compounds such as bombesin and acetylcholine elicit the release of intracellular calcium stores in lens epithelial cells, we can predict that members of the Gα family ultimately will be found in this tissue. Preliminary experiments in our laboratory have confirmed this possibility (data not shown).

In summary, we have identified distinct types of Gα, signal transducing proteins in variously differentiated lens cell populations. These data suggest that complex regulatory mechanisms operate during lens terminal differentiation, during the maintenance of normal basal metabolism, or both. Delineating the regulation of receptor-mediated events in the lens should be facilitated by these observations.

Key Words
differentiation, G proteins, lens, ribosylation, signal transduction

References


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