Molecular Vision **3: 12, 1997** <http://www.emory.edu/molvis/v3/phillips>
Received 23 Sep 1997 | Accepted 18 Nov 1997 | Published 21 Nov 1997

© Molecular Vision

Antisense Inhibition of R-Cognin Expression Modulates Differentiation of Retinal Neurons In Vitro

Johanna L. Phillips, Dean R. Tolan, and Robert E. Hausman

Department of Biology, Boston University, Boston, MA 02215

Purpose: Retina cognin (R-cognin) is a 50 kDa membrane-associated polypeptide expressed during retinogenesis where it is involved in mediating tissue-specific cell-cell interactions. In addition to its intercellular role in aggregation, R-cognin may act as a cell surface signaling molecule. An antisense oligonucleotide was used to inhibit R-cognin expression and to investigate the effects of this inhibition on subsequent neuronal differentiation.

Methods: Cultures of retina cells were prepared from 6 day (E6) and 8 day (E8) chicken embryos and were incubated with a deoxyoligonucleotide complEmentary to 20 bases of the sequence encoding R-cognin or random oligonucleotides. The levels of choline acetyltransferase (ChAT) and glutamic acid decarboxylase (GAD), markers of cholinergic and GABAergic differentiation, respectively, were detected by Western blots on protein extracts from treated cultures.

Results: The antisense treatment inhibited ChAT levels at E6 and GAD levels at E8. The treatment resulted in no decrease in the level of the enzyme glyceraldehyde 3-phosphate dehydrogenase. A random oligonucleotide did not affect the levels of any of the proteins.

Conclusions: These results confirm the cell recognition role of R-cognin and suggest that it is important in intracellular signaling cascades necessary for normal retina development.

Retina cognin (R-cognin) is a 50 kDa protein which is expressed as a cell adhesion/recognition molecule on the surface of retina cells in the developing chick embryo. R-cognin was originally identified and isolated from the conditioned medium of embryonic chick retina cells in culture (1). Immunohistochemical studies have shown that the protein is present on the surface of neural retina cells in association with the cell membranes (2). Purified R-cognin enhances the reaggregation of trypsin-dissociated retina cells but has no similar effect on cells from other tissues (1,3). In keeping with these results, antibody against R-cognin blocks cell reaggregation in a tissue-specific fashion (4).

Previously, studies in which retina cells were treated with antibody against R-cognin were done to determine whether the disruption of normal cognin-mediated cell interactions would disrupt processes associated with neuronal differentiation. These studies assayed the activities of the biosynthetic enzymes for the neurotransmitters acetylcholine and γ-aminobutyric acid: choline acetyltransferase (ChAT) and glutamic acid decarboxylase (GAD), respectively. The results showed that treatment of retina cells with R-cognin antibody inhibited ChAT activity during embryonic days 5-8 (5) and GAD activity during embryonic days 7-11 (6). Based on these results we hypothesized that R-cognin was affecting subsequent events of neuronal differentiation by participating in signaling from the cell surface.

Molecular studies aimed at understanding how R-cognin functions have shown that the cDNA encoding R-cognin (7) closely resembles the cDNA encoding protein disulfide isomerase (PDI) (8,9). PDI is a 57 kDa multifunctional protein found primarily in the endoplasmic reticulum of secretory cells. However, there are reports that PDI can be secreted (10,11), and thioreductase (PDI-like) activity has been found at the surfaces of mammalian cells (12-14). The same polypeptide catalyzes the formation of disulfide bonds, is the ß-subunit of prolyl 4-hydroxylase (15), is a subunit of a triacylglycerol transfer protein (16), is a cellular thyroid hormone binding protein (17), and has chaperone-like affinity for peptides that is independent of its role in catalysis (18).

Each of PDI's functions depends on a specific amino acid motif. R-cognin shares some of the same motifs; thus, those shared motifs involved in protein-protein binding could be the basis for its action as a cell recognition factor or a signal molecule during retinogenesis. Our recent evidence implicates the thioreductase site (19, unpublished data).

In the present study, we designed a synthetic deoxyoligonucleotide, GSP1 complementary to 20 nucleotides shared by the chicken PDI and in R-cognin cDNAs (7). Antisense oligonucleotides have become valuable tools for selectively inhibiting gene expression to analyze a gene product's function (20). Our strategy was to introduce the antisense oligonucleotide directly into the media of cultured retinal neurons where it would be taken up by the cells and hybridize with its target molecule, R-cognin mRNA, thereby inhibiting R-cognin translation. We found significant inhibition of R-cognin expression with as little as 20 μM antisense oligonucleotide.

*To whom correspondence should be addressed: Robert E. Hausman, Department of Biology, Boston University, 5 Cummington Street, Boston, MA, 02215, Tel: (617) 353-2470, Fax: (617) 353-6340, email: hausman@bu.edu

We investigated the effects of antisense inhibition of R-cognin in subsequent developmental events in embryonic chick retinal cells. The levels of ChAT and GAD proteins were measured, since R-cognin is expressed in the cells which follow these differentiation pathways (21) and the product of ChAT, acetylcholine, plays an important role in retinal morphogenesis (22). We found inhibitory effects on both these enzymes consistent with previous antibody studies. We found that the antisense treatment led to slight increases in expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and heat shock protein 70 (Hsp70) but this result was obtained also with the control oligonucleotides. This antisense inhibition of ChAT or GAD was tissue-specific in that it was limited to retina, no effect was observed in forebrain or tectum. Taken together, our findings indicate that R-cognin is important for establishing and maintaining tissue-specific and developmental stage-specific aspects of differentiation.

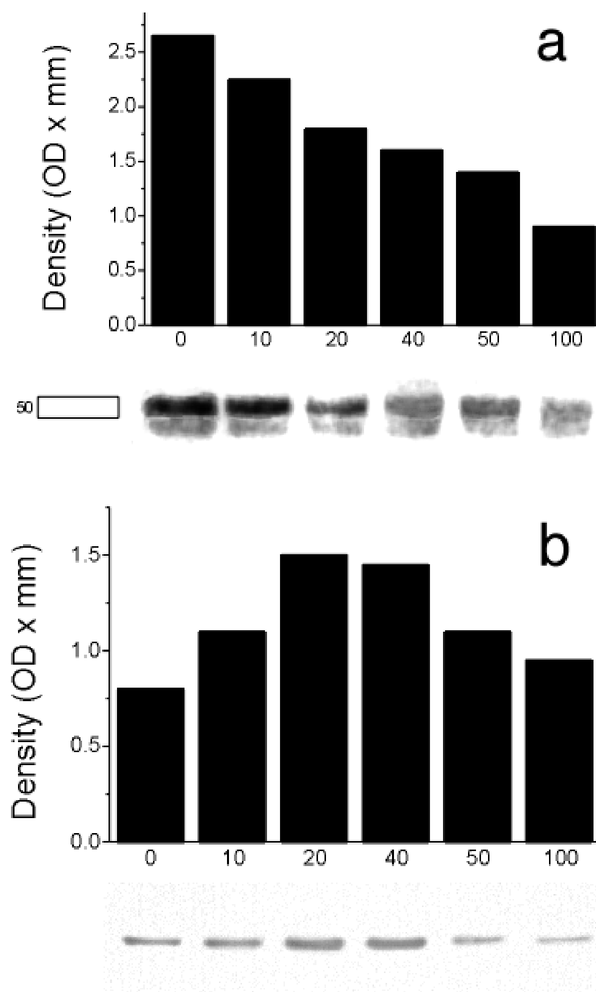

Figure 1. R-cognin and GAPDH protein levels in E8 retina cell cultures after treatment with increasing concentrations of antisense oligonucleotide to R-cognin. (a) Density scan and Western blot with R-cognin antibody (dil 1:100) and exposed for 1 min. The amount of R-cognin antisense oligonucleotide added is shown in µM underneath the density scan. (b) Density scan and Western blot with GAPDH antibody (diluted 1:250) and exposed for 10 min. Each lane contained 20 µg total protein. The amount of R-cognin antisense oligonucleotide added is shown in µM underneath the density scan.

METHODS

Fertilized eggs were obtained from Hardy's Hatcheries (Essex, MA). An antisense deoxyoligonucleotides (GSP1, 5'-TTCACTGGAATCCACCAACG-3') to a sequence found in both R-cognin and chicken protein disulfide isomerase, and one to a sequence found in chicken disulfide isomerase (OKR3, 5'-TCCACGAGCAGGTGGCGGTG-3') were synthesized at the Boston University DNA Synthesis Facility on Milligen 6500 or 7500 DNASynthesizers. A random 20 base deoxyoligonucleotide (dN20) was purchased from Quality Controlled Biochemicals, Inc. (Hopkinton, MA). Nitrocellulose blotting membranes were purchased from Bio-Rad (Hercules,CA). The LumiGLO Chemiluminescence System was purchased from Kirkegaard and Perry Laboratories, Inc. (Gaithersberg, MD). All other chemicals were purchased from Sigma (St. Louis, MO) or Fisher Scientific (FairLawn, NJ).

Antibodies— Polyclonal antisera against R-cognin was prepared as described previously (Dobi et al., 1986). Polyclonal antibodies against ChAT and GAD and monoclonal antibody against GAPDH were purchased from Hemicon International, Inc. (Temecula, CA). Horseradish peroxidase

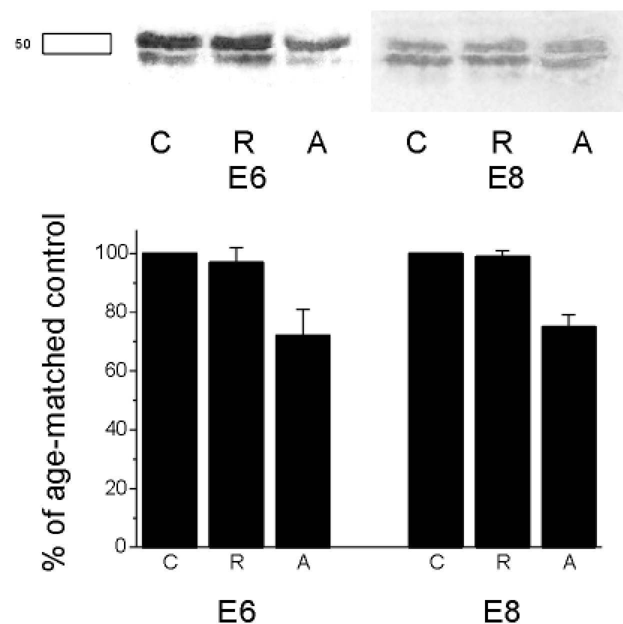

Figure 2. Effects of antisense treatments on R-cognin protein levels in E6 and E8 retinal cell cultures. For the representative Western blots, each lane contained 14 µg total protein for the E6 samples and 24 µg total protein for the E8 samples. The blots were probed with polyclonal antiserum against R-cognin and exposed for 2 sec. The 50 kDa R-cognin band that was quantitated is indicated by the box. The density scan is of cumulative data from Western blots of three separate experiments probed with anti-R-cognin. Levels of R-cognin in untreated cultures have been normalized and protein levels are expressed as percentages of the control. (C = control not treated with any oligonucleotide; R = treatment with 20 µM random oligonucleotide; A = treatment with 20 µM antisense oligonucleotide).

(HRP)-conjugated goat anti-rabbit secondary antibody was purchased fromSanta Cruz Biotechnology, Inc. (Santa Cruz, CA). HRP-conjugated goat anti-mouse secondary antibody was purchased from Sigma (St. Louis, MO).

Preparation of cell cultures— Eggs were incubated at 37° C to appropriate stages (day of embryonic development, designated E6, E8). For each experiment, 4-30 embryos were used. Tissue was dissected from retina, forebrain, or tectum for preparation of primary cultures. The tissue was washed twice with three volumes of calcium- and magnesium-free Tyrode's solution (CMF). The CMF was removed and an equal volume of trypsin (0.25% in CMF) was added to the tissue. The mixture was gassed with 5% CO_2 for 30 sec and was incubated 10-15 min in a 37° C rotary incubator. A suspension of single cells was obtained by repeated pipetting for 1 min with a Pasteur pipet. The trypsin was inactivated with 1.5 volumes soybean trypsin inhibitor (0.2 mg/ml in Tyrode's solution) and the mixture was gently pipetted to obtain a uniform cell suspension. The cell suspension was centrifuged 5 min in a clinical centrifuge, the supernatant fraction was removed, and the cells were resuspended in 5-15 ml Dulbecco's Modified Eagle's Medium (DMEM) containing 100 U penicillin, 0.1 mg streptomycin (small pellets required less volume). Cells were counted using a hemocytometer and plated onto polyornithine-coated 24 well plates, 2 x 10^6 cells per well in 0.5 ml DMEM. Cultures were incubated at 37° C in a sterile 5% CO_2 incubator.

Antisense treatments— After 1 hr of incubation at 37° C, antisense oligonucleotide (500 µM stock solution in sterile ddH_2O) was added directly to the well. Cultures were incubated 12-14 hr. Treated cells were washed with cold Tyrode's solution. Using a rubber spatula, cells were scraped from the surface of the plate. Identical treatments were pooled together in microcentrifuge tubes and centrifuged to pellet the cells. Cell pellets were resuspended in Suspension Buffer (0.1 M NaCl; 0.01 M Tris-HCl, pH 7.6; 1 mM EDTA, pH 8.0; 1 µg/ml aprotinin) and phenyl methyl sulfonyl fluoride (PMSF) was added to a final concentration of 122 µg/ml. The suspension was subsequently sonicated and the protein content measured by the Bradford assay (23).

Western blotting— In each experiment, equal amounts of protein (15-30 µg per lane) were separated by SDS-polyacrylamide gel electrophoresis as described by Sambrook et al. (24). Proteins were electrophoretically transferred to a nitrocellulose membrane in a buffer containing 2.5 mM Tris, 192 mM glycine (pH 8.3), 1% SDS, and 20% methanol. Transfers were carried out at 4° C, 200 mA for 2 hr or 40 mA overnight. Following transfer, the membranes were placed in Blocking Buffer (BB) consisting of 5% calf serum, 3%

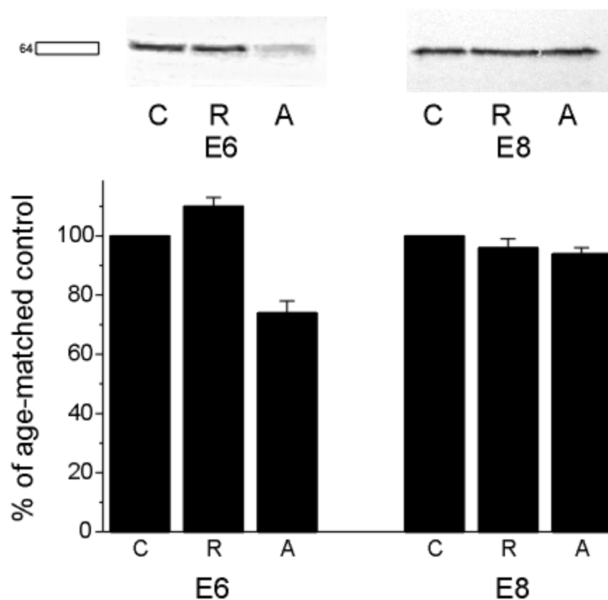

Figure 3. Effects of antisense treatments on choline acetyltransferase (ChAT) protein levels in E6 and E8 retinal cell cultures. For the representative Western blots, each lane contained 14 µg total protein for the E6 samples and 24 µg total protein for the E8 samples. The blots were probed with polyclonal antiserum against ChAT (diluted 1:1000) and exposed for 20 sec. The area used to quantitate the 64 kDa ChAT band is indicated by the box. The density scan is of cumulative data from Western blots of three separate experiments. Levels of ChAT in untreated cultures have been normalized and protein levels are expressed as percentages of the control. (C = control not treated with any oligonucleotide; R = treatment with 20 µM random oligonucleotide; A = treatment with 20 µM antisense oligonucleotide).

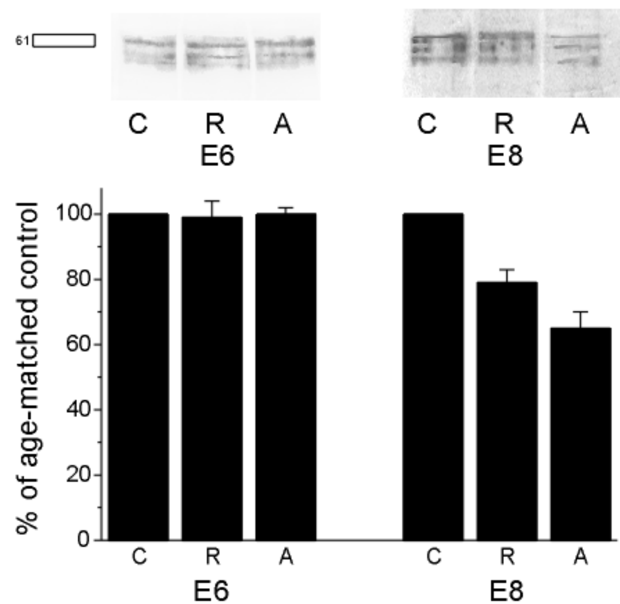

Figure 4. Effects of antisense treatments on glutamic acid decarboxylase (GAD) protein levels in E6 and E8 retinal cell cultures. For the representative Western blots, each lane contained 14 µg total protein for the E6 samples and 24 µg total protein for the E8 samples. The blots were probed with polyclonal antiserum against GAD (diluted 1:1000) and exposed for 1 min. The area used to quantitate the 61 kDa GAD band is indicated by the box. The density scan is of cumulative data from Western blots of three separate experiments probed with anti-GAD. Levels of GAD in untreated cultures have been normalized and protein levels are expressed as percentages of the control. (C = control not treated with any oligonucleotide; R = treatment with 20 µM random oligonucleotide; A = treatment with 20 µM antisense oligonucleotide).

Carnation milk, in Tris-buffered saline (TBS; 125mM NaCl, 25 mM Tris pH 8.0) or TBST (0.05% Tween in TBS) for 30-60 min at room temperature. The membranes were incubated overnight with the primary antibody diluted in 0.1% BSA in TBS. Following incubation in primary antibody, the membranes were washed 3 times in BB at room temperaturefor 15 min each wash. The membranes were incubated 1.5-2 hr at room temperature with the appropriate secondary antibody diluted 1:2000 in TBS with 0.1% BSA. Following incubation with secondary antibody, the membranes were washed in TBS or TBST once for 15 min and four times for 5 min each. The Western blots were developed using LumiGLO Chemiluminescence according to the manufacturer's instructions, and then the membranes were exposed to X-ray film.

Densitometer scanning and data analysis— The Western blot films were analyzed using a 420oe optically enhanced laser densitometer and Quantity One version 2.5 software (pdi, Inc.,Huntington Station, NY). The entire film was digitized and the relevant band scanned. The lane scanning width was 4 mm (80-90% of the total lane width). This scanning width was adjusted slightly in some lanes to compensate for artifacts. Protein levels correspond to density absorbance units (OD x mm). For each experiment measuring the level of a given protein at a given age, the intensity (in OD x mm) for the untreated control was normalized to 100%, and the intensities for the treated samples were compared to that normalized level as percentages of the control. Protein standards of known concentration were used to determine the linear range of chemiluminescence technique and the densitometer. Data were averaged for triplicate sets of experiments and the means and standard deviations calculated.

RESULTS

Dose dependent effect of the antisense oligonucleotide— Retina cell cultures were prepared from E8 chick embryos and treated with increasing doses of the antisense oligonucleotide (GSP1) to determine if it inhibited R-cognin and to determine the optimal dose for subsequent experiments (Figure 1). The polyclonal antibody against R-cognin recognized a band of 50 kDa, consistent with the size of R-cognin, as well as a fainter band of 45 kDa band. The identity of the 45 kDa antigen and its relationship to R-cognin is not known, although it typically reacts with polyclonal antibody preparations against R-cognin (21,25). Because only the 50 kDa band shows cell aggregation-enhancing activity (1) it alone was analyzed. The western blot and density scan (Figure 1a) shows that as the concentration of the antisense oligonucleotide increased, the level of R-cognin protein decreased. Treatment of the retinal cells with 10 µM antisense oligonucleotide inhibited R-cognin protein by 28% compared with the untreated control. Treatment with 20 µM antisense oligonucleotide inhibited R-cognin by 35%, 40 µM by 38%, 50 µM by 48%, and 100 µM by 71%. In a parallel western blot of the same samples, the levels of GAPDH did not reflect a dose dependence on increasing levels of antisense

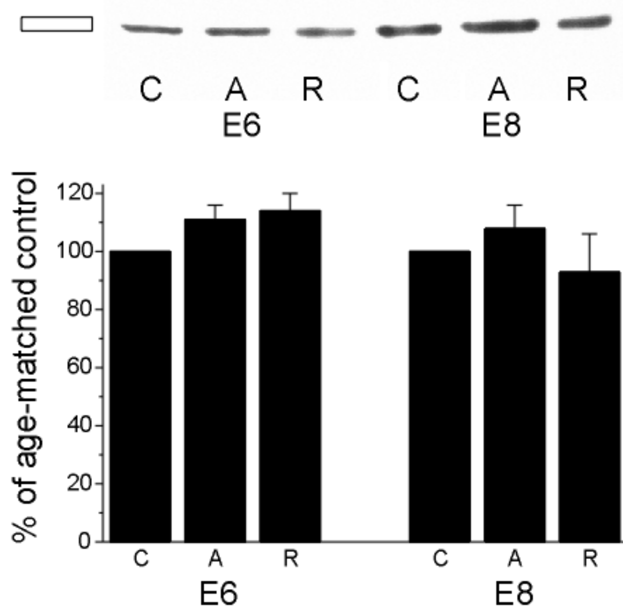

Figure 5. Effects of antisense treatments on glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein levels in E6 and E8 retinal cell cultures. For the representative Western blots, each lane contained 12 µg total protein. The blots were probed with polyclonal antiserum against GAPDH (diluted 1:250) and exposed for 15 min. The area used to quantitate the GAPDH band is indicated by the box. The density scan is of cumulative data from Western blots of three separate experiments probed with anti-GAPDH. Levels of GAPDH in untreated cultures have been normalized and protein levels are expressed as percentages of the control. (C = control not treated with any oligonucleotide; R = treatment with 20 µ M random oligonucleotide; A = treatment with 20 µM antisense oligonucleotide).

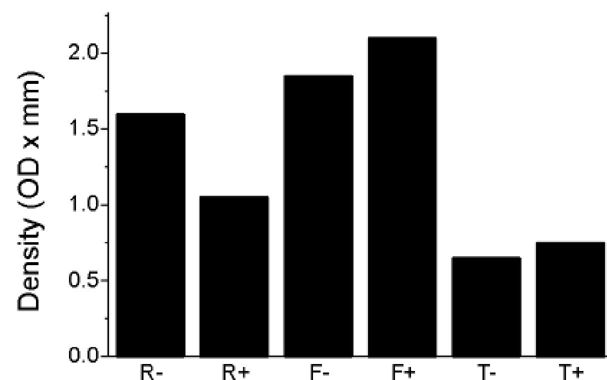

Figure 6. The effect of treatment with antisense oligonucleotide to R-cognin on ChAT in E8 retina, forebrain, and tectum. For the Western blot, each lane containing 15 µg total protein was probed with anti-ChAT (diluted 1:1000) and was exposed for 20 sec. The area used to quantitate the 64 kDa ChAT band is indicated by the box. The notation on the bottom of the bar graph is as follows: R- (retina without antisense oligonucleotide), R+ (retina treated with antisense oligonucleotide), F- (forebrain without antisense oligonucleotide), F+ (forebrain treated with antisense oligonucleotide), T- (optic tectum without antisense oligonucleotide), T+ (optic tectum treated with antisense oligonucleotide).

oligonucleotide (Figure 1b). These results indicated the specificity of the antisense oligonucleotide for R-cognin. Treatments with any of the oligonucleotides used here had slight stimulatory effects on GAPDH (Figure 1b & Figure 5). Subsequent experiments were performed with 20µM antisense oligonucleotide which inhibited the level of R-cognin protein 25-40%, as this is the range of antisense oligonucleotide used in most such experiments (20). In addition to GSP1, the antisense oligonucleotide, two other oligonucleotides were used, a random 20-mer and OKR3. Treatment of cells with the random 20-mer did not affect the level of R-cognin protein or the levels of other proteins that were assayed. OKR3 is complementary to nucleotides 136-155 of chicken PDI (numbering bases on sequence in ref. 8). This upstream sequence is not within the known R-cognin message. Treatment with OKR3 had no effect on the level of R-cognin protein itself and essentially identical results to the random oligonucleotide on the other proteins being assayed.

Age dependent effect on R-cognin protein— Stationary retinal cell cultures were prepared from E6 and E8 chick embryos. The cells were subjected to treatment with: ddH_2O (labeled C for control), 20 µM antisense oligonucleotide, GSP1 (labeled A for antisense), and 20 µM of either the random oligonucleotide or OKR3 (labeled R for random). Representative western blots and a quantitative summary of multiple experiments for R-cognin at each embryonic age are shown in Figure 2. At E6, antisense treatment resulted in a 30(±12)% inhibition of the level of R-cognin protein compared with the untreated control. At E8, the level of R-cognin protein was inhibited 23(±8)%. At both E6 and E8 treatment of the cells with the random oligonucleotides did not alter the level of R-cognin protein significantly from that of the control.

Effect on choline acetyltransferase (ChAT)— Our previous work had shown that exposure to polyclonal antibodies to R-cognin interfered with a limited but significant number of key proteins in the neuronal differentiation of retinal cells, among them the neurotransmitter synthetic enzymes ChAT (5) and GAD (6). While powerful probes, antibodies to cell surface-associated adhesion or recognition proteins can have effects that are not easily controlled for even with the use of antibodies to other cell adhesion molecules for comparison (5). Antisense experiments such as those described here have different problems thus similar results with the two different techniques would lend considerable validity to a relationship between these enzymes and R-cognin. To test for effects of antisense oligonucleotide to R-cognin on ChAT, western blots of protein extracts from antisense-treated retinal cells were probed with antibody againstChAT. The ChAT antibody recognized a band of 64 kDa in chick retinal cell extracts (26). Representative western blots and a quantitative summary of multiple experiments for ChAT at each embryonic age are shown in Figure 3. There was 23(±7)% inhibition in the level of ChAT protein in E6 retinal cells treated with antisense oligonucleotide. However, in E8 retinal cells, the level of ChAT did not differ significantly from control levels after antisense oligonucleotide treatment.

Effect on glutamic acid decarboxylase (GAD)— Previous work showed that the normal developmental increase in GAD protein level was sensitive to antibody to R-cognin only at later stages of development than ChAT (6). To determine the effects of the antisense treatment on GAD, western blots of protein extracts from treated retina cells were probed with antibody against GAD. The GAD antibody recognized a set of bands between 61 and 67 kDa. Based on other findings with GAD antibody on chick retinal extracts (27), the 61 kDa band was chosen for analysis of GAD protein levels. Representative western blots and a quantitative summary of multiple experiments for GAD at E6 and E8 are shown in Figure 4. The level of GAD protein was not affected by the antisense treatment at E6. However, in E8 retinal cells GAD levels were reduced to 73% of control levels with random oligonucleotide treatment and further to 57(±5)% of control levels with antisense treatment.

Effect on GAPDH— To demonstrate that the reduced ChAT and GAD protein levels were caused by the antisense inhibition of R-cognin and not by a general inhibition of protein synthesis in the retina, an antibody against GAPDH was used for western blotting of extracts from treated cells. This antibody recognized a 36 kDa band. Representative western blots and a quantitative summary of multiple experiments for GAPDH at E6 and E8 are shown in Figure 5. In fact, levels of GAPDH increased slightly after antisense treatment at both ages. Opsin, which is expressed in the outer retina, was not affected by treatment of the tissue with the antisense oligonucleotide to R-cognin (data not shown).

Retina-specificity of the effect— One of the important characteristics of R-cognin is the tissue-specificity of its effects.

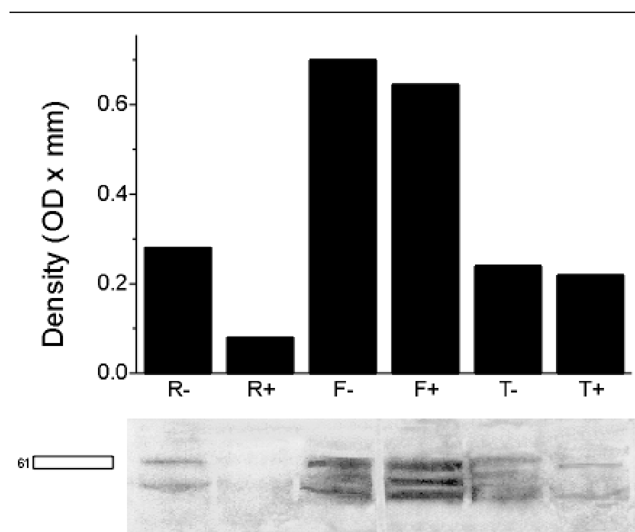

Figure 7. The effect of treatment with antisense oligonucleotide to R-cognin on GAD in E8 retina, forebrain, and tectum. For the Western blot each lane containing 15 µg total protein was probed with anti-GAD (diluted 1:1000) and was exposed for 20 sec. The area used to quantitate the 61 kDa GAD band is indicated by the box. The notation is as in Figure 6: R- (retina without antisense oligonucleotide), R+ (retina treated with antisense oligonucleotide), F- (forebrain without antisense oligonucleotide), F+ (forebrain treated with antisense oligonucleotide), T- (optic tectum without antisense oligonucleotide), T+ (optic tectum treated with antisense oligonucleotide).

It is only obtained from retina tissue(1), only enhances the reaggregation of retina cells (2) and its constituent thioreductase activity appears only to affect the aggregation of retina cells (19). In order to demonstrate that the inhibitory effect on R-cognin and on cognin-mediated differentiation pathways was retina-specific, cell cultures were prepared from two other tissues of the chick embryo CNS, forebrain and optic tectum. Previous immunofluorescence studies have shown that very little R-cognin, if any, is present in these tissues. Western blots were probed with antibodies against ChAT and GAD. Figure 6 shows the effect on ChAT of the antisense inhibition in E8 retina, forebrain, and tectum. Consistent with previous results, the treatment resulted in a decreased ChAT level in retinal cells; however, in forebrain and tectum the treatment resulted in slightly increased ChAT levels. Figure 7 shows the effect on GAD of antisense inhibition in E8 retina, forebrain, and tectum. Slight decreases in GAD were seen in forebrain and tectum; however, the GAD level in retina decreased nearly 60% below the control level.

DISCUSSION

The use of antisense oligonucleotides to inhibit gene expression either at the level of transcription or translation has become a valuable way to analyze gene function (20). The oligonucleotides are either applied to the media of cultured cells or injected directly into tissue. Typically, treatments of 5-20 μM result in 30-50% inhibition of target proteins (28-30). Treatment of retinal cell cultures with 20 μM of antisense oligonucleotide targeting R-cognin expression, resulted in similar levels of R-cognin inhibition. The inhibition of R-cognin protein presumably disruptedR-cognin-mediated cell-cell interactions and any signaling pathways that depend on such interactions. As a result, there were measurable effects on both ChAT and GAD expression. These effects were identical to those obtained previously with antibodies to R-cognin acting at the retina cell surface, demonstrating that the same effect on neuronal differentiation could be shown by two entirely different techniques. As previously, the antisense effect was most evident at the ages when the normal developmental increase in synthesis of these proteins is initiated: E6 for ChAT and E8 for GAD (5,6). It was concluded that R-cognin's role as a mediator of differentiation is specific for cells of the inner retina (amacrine, displaced amacrine, and ganglion cells) some of which are known to be cholinergic and GABAergic in their differentiated state (31,32). This is consistent with the finding that R-cognin expression becomes localized to the inner retina during development (21).

Cholinergic differentiation in the embryonic chick retina occurs in two phases, an initial triggering phase (E5-E9), followed by a maintenance phase (from E10)(33). Antisense inhibition of R-cognin affected ChAT levels at E6, but not significantly at E8. Therefore, it would seem that R-cognin is necessary for some part of the initial phase of cholinergic differentiation. This is consistent with our previous finding (5) that the initial developmental rise in ChAT activity was suppressed by treatment with antibody against R-cognin. Interestingly, at this stage of retinogenesis (E6-E7) R-cognin is distributed across the retina binding to the surfaces of most cells (34) and is not limited to future cholinergic neurons. However, the early dependence on R-cognin occurs at the same time that the future ganglion cells and amacrine cells, which become cholinergic (31), stop dividing and begin to differentiate (35,36).

There is also an initial triggering phase for GABAergic differentiation in the embryonic chick retina, although it occurs later (E8-E11) (6). The antisense effect on GAD also occurred later in retinogenesis. GAD levels were not affected at E6 but were significantly reduced at E8. These results are consistent with our previous finding (6) that the normal developmental increase in GAD activity was reduced by treatment with antibody against R-cognin. Like ChAT, GAD is found in chick retinal amacrine cells (32), so by the time GAD levels are increasing at E11-13 (27), R-cognin has localized to the same cells. Thus, R-cognin could affect the initiation of GABAergic differentiation as well as the maintenance of the GABAergic phenotype.

Functional synapses do not appear in the chick retina before E13 (37), so the neurotransmitters, acetylcholine and γ-aminobutyric acid, that are synthesized earlier must serve another function. Others (38) have proposed developmental roles for such neurotransmitters. Recent findings that curare blocks indigenous waves of depolarization in the unborn mouse retina and development of the normal columnar cellular organization of the tissue may be the basis for this effect (22). Such a critical role for acetylcholine might explain the complex regulation of ChAT and its early embryonic appearance and activity. In effect ChAT is more than simply a structural gene but plays an important, albeit indirect, role in retinal development.

How might R-cognin function as a mediator of neuronal differentiation? Early studies, that established R-cognin as a cell adhesion protein, led to the hypothesis that R-cognin carried out its role in differentiation by forming and mediating cell-cell contacts which made intercellular signaling possible. However, R-cognin itself might act as a signal transduction molecule, triggering intracellular signaling cascades to ultimately result in changes in gene expression of target proteins such as ChAT and GAD.

Sequence analysis of R-cognin cDNA (7) has revealed a high degree ofsimilarity between R-cognin and the 57 kDa multifunctional enzyme, PDI(8,9). While PDI may be present in all tissues it is highest in secretory tissues such as liver and pancreas and present at extremely low levels in adult neural tissue such as brain (39). A low level of authentic PDI may be present in embryonic chick retina (21) where it probably functions as the β-subunit of prolyl 4-hydroxylase (8). However, the PDI-related protein R-cognin is one of the more abundant proteins in the embryonic chick retina. The antisense oligonucleotide used here is complementary to the sequence shared by both R-cognin and PDI, as we do not yet have nucleotide sequence that we can be sure is unique to R-cognin (21). In retina this oligonucleotide targets R-cognin expression as there is little evidence of PDI protein(21). Our observation that the inhibition is seen in retina but not forebrain

or tectum supports the conclusion that R-cognin is a tissue-specific PDI-like protein with developmental functions that are distinct from those of PDI. These conclusions are supported by similar tissue-specific findings on the protein level (19, unpublished data).

Other enzymes have been implicated in neuronal maturation and differentiation. Two enzymes in the ubiquitin pathway, the activating enzyme (E1) and the carboxyl terminal hydrolase (PGP 9.5) appear in chick neural crest and dorsal root ganglion cells as they differentiate and may be involved in regulating gene expression (40). The receptor tyrosine kinase (Cek5) is activated and becomes concentrated in the plexiform layers during chick neural retina development (41). Like R-cognin, each of these enzymes binds to protein substrates. Possibly, R-cognin plays a similar developmental role in the retina.

ACKNOWLEDGEMENTS

This study was supported in parts by Fight for Sight grants from the Society for the Prevention of Blindness to J.L.P. and R.E.H., by research grants from the National Eye Institute to R.E.H., from the National Institute of Diabetes and Metabolic Diseases to D.R.T, and by a Clare Booth Luce Predoctoral Fellowship to J.L.P.

REFERENCES

1. Hausman RE, Moscona AA. Purification and characterization of the retina-specific cell-aggregating factor. Proc Natl Acad Sci U S A 1975;72:916-920.

2. Hausman RE, Moscona AA. Immunologic detection of retina cognin on the surface of embryonic cells. Exp Cell Res 1979; 119:191-204.

3. Ben-Shaul Y, Hausman RE, Moscona AA. Visualization of a cell surface glycoprotein, the retina cognin, on embryonic cells by immuno-latex labeling and scanning electron microscopy. Dev Biol 1979;72:89-101.

4. Hausman RE, Moscona AA. Isolation of retina-specific cell-aggregating factor from membranes of embryonic neural retina tissue. Proc Natl Acad Sci U S A 1976; 73:3594-3598.

5. Sagar GD, Rao AS, Ren Y, Hausman RE. The cell recognition molecule, cognin, mediates choline acetyltransferase activity in embryonic chick retina. Brain Res 1992; 585:63-70.

6. Shah BH, Rao AS, Hausman RE. Role of the cell recognition molecule, cognin, in GABAergic differentiation in chick retina. Brain Res 1992; 589:268-274.

7. Krishna Rao AS, Hausman RE. cDNA for R-cognin: homology with a multifunctional protein. Proc Natl Acad Sci U S A 1993; 90:2950-2954.

8. Kao WW, Nakazawa M, Aida T, Everson WV, Kao CW, Seyer JM, Hughes SH. Isolation of cDNA clones and genomic DNA clones of beta-subunit of chicken prolyl 4-hydroxylase. Connect Tissue Res 1988; 18:157-174.

9. Parkkonen T, Kivirikko KI, Pihlajaniemi T. Molecular cloning of a multifunctional chicken protein acting as the prolyl 4-hydroxylase beta-subunit, protein disulphide-isomerase and a cellular thyroid-hormone-binding protein. Comparison of cDNA-deduced amino acid sequences with those in other species. Biochem J 1988; 256:1005-1011.

10. Akagi S, Yamamoto A, Yoshimori T, Masaki R, Ogawa R, Tashiro Y. Localization of protein disulfide isomerase on plasma membranes of rat exocrine pancreatic cells. J Histochem Cytochem 1988; 36:1069-1074.

11. Terada K, Manchikalapudi P, Noiva R, Jauregui HO, Stockert RJ, Schilsky ML. Secretion, surface localization, turnover, and steady state expression of protein disulfide isomerase in rat hepatocytes. J Biol Chem 1995; 270:20410-20416.

12. Yoshimori T, Semba T, Takemoto H, Akagi S, Yamamoto A, Tashiro Y. Protein disulfide-isomerase in rat exocrine pancreatic cells is exported from the endoplasmic reticulum despite possessing the retention signal. J Biol Chem 1990; 265:15984-15990.

13. Mandel R, Ryser HJ, Ghani F, Wu M, Peak D. Inhibition of a reductive function of the plasma membrane by bacitracin and antibodies against protein disulfide-isomerase. Proc Natl Acad Sci U S A 1993;90:4112-4116.

14. Ryser HJ, Levy EM, Mandel R, DiSciullo GJ. Inhibition of human immunodeficiency virus infection by agents that interfere with thiol-disulfide interchange upon virus-receptor interaction. Proc Natl Acad Sci U S A 1994; 91:4559-4563.

15. Kivirikko KI, Myllyla R, Pihlajaniemi T. Protein hydroxylation: prolyl 4-hydroxylase, an enzyme with four cosubstrates and a multifunctional subunit. FASEB J 1989; 3:1609-1617.

16. Wetterau JR, Combs KA, Spinner SN, Joiner BJ. Protein disulfide isomerase is a component of the microsomal triglyceride transfer protein complex. J Biol Chem 1990; 265:9801-9807.

17. Cheng SY, Gong QH, Parkison C, Robinson EA, Appella E, Merlino GT, Pastan I. The nucleotide sequence of a human cellular thyroid hormone binding protein present in endoplasmic reticulum. J Biol Chem 1987; 262:11221-11227.

18. Noiva R, Freedman RB, Lennarz WJ. Peptide binding to protein disulfide isomerase occurs at a site distinct from the active sites. J Biol Chem 1993; 268:19210-19217.

19. Pariser HP, Hausman RE. Structural characterization of the peripheral neural retina protein, retina cognin, reveals high structural and functional homology to protein disulfide isomerase. Mol Biol Cell 1996; 7:110a.

20. Brysch W, Schlingensiepen KH. Design and application of antisense oligonucleotides in cell culture, in vivo, and as therapeutic agents. Cell Mol Neurobiol 1994; 14:557-568.

21. Phillips JL, Holdengreber V, Ben-Shaul Y, Zhang J, Tolan DR, Hausman RE. Developmental localization of retina cognin synthesis by in situ hybridization. Brain Res Dev Brain Res. In press 1997.

22. Feller MB, Wellis DP, Stellwagen D, Werblin FS, Shatz CJ. Requirement for cholinergic synaptic transmission in the propagation of spontaneous retinal waves. Science 1996; 272:1182-1187.

23. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72:248-254.

24. Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor (NY): Cold Spring Harbor Press; 1989.

25. Troccoli NM, Hausman RE. Retina cognin does not bind to itself during membrane interaction in vitro. Cell Differ 1988; 22:225-231.

26. Ren Y, Holdengreber V, Ben-Shaul Y, Shah BH, Varanasi J, Hausman RE. Causal role for jun protein in the stimulation of choline acetyltransferase by insulin in embryonic chick retina. Biochem Biophys Res Commun 1997; 232:788-793.

27. Shah BH, Hausman RE. Effect of insulin on GABAergic development in the embryonic chick retina. Brain Res Dev Brain Res 1993; 72:151-158.

28. Dragunow M, Tse C, Glass M, Lawlor P. c-fos antisense reduces expression of Krox 24 in rat caudate and neocortex. Cell Mol Neurobiol 1994; 14:395-405.

29. Majocha RE, Agrawal S, Tang JY, Humke EW, Marotta CA. Modulation of the PC12 cell response to nerve growth factor by antisense oligonucleotide to amyloid precursor protein. Cell Mol Neurobiol 1994;14:425-437.

30. Sainio K, Saarma M, Nonclercq D, Paulin L, Sariola H. Antisense inhibition of low-affinity nerve growth factor receptor in kidney cultures: power and pitfalls. Cell Mol Neurobiol 1994; 14:439-457.

31. Millar T, Ishimoto I, Johnson CD, Epstein ML, Chubb IW, Morgan IG. Cholinergic and acetylcholinesterase-containing neurons of the chicken retina. Neurosci Lett 1985; 61:311-316.

32. Yazulla S, Studholme KM, Vitorica J, de Blas AL. Immunocytochemical localization of GABAA receptors in goldfish and chicken retinas. J Comp Neurol 1989; 280:15-26.

33. DeMello FG, DeMello MC, Hudson R, Klein WL. Selective expression of factors preventing cholinergic dedifferentiation. J Neurochem 1990;54:886-892.

34. Dobi ET, Troccoli NM, Hausman RE. Distribution of R-cognin in late embryonic and post-hatching chicken retina. Invest Ophthalmol Vis Sci 1986; 27:323-329.

35. Reichman EF, Beebe DC. Acetylcholinesterase activity as a marker for the development of the ciliary epithelium in the chicken embryo. Exp Eye Res 1990; 51:249-255.

36. Spence SG, Robson JA. An autoradiographic analysis of neurogenesis in the chick retina in vitro and in vivo. Neuroscience 1989; 32:801-812.

37. Dobi ET, Naya FJ, Hausman RE. Distribution of R-cognin and choline acetyltransferase in the ganglion cell layer of developing chick neural retina. Cell Differ 1988; 22:115-123.

38. Meier E, Hertz L, Schousboe A. Neurotransmitters as developmental signals. Neurochem Int 1991; 19:1-15.

39. Edman JC, Ellis L, Blacher RW, Roth RA, Rutter WJ. Sequence of protein disulphide isomerase and implications of its relationship to thioredoxin. Nature 1985; 317:267-270.

40. Smith-Thomas LC, Kent C, Mayer RJ, Scotting PJ. Protein ubiquitination and neuronal differentiation in chick embryos. Brain ResDev Brain Res 1994; 81:171-177.

41. Pasquale EB, Connor RJ, Rocholl D, Schnurch H, Risau W. Cek5, a tyrosine kinase of the Eph subclass, is activated during neural retina differentiation. Dev Biol 1994; 163:491-502.