Retroviral Expression of Connexins in Embryonic Chick Lens

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PURPOSE. To develop an in vivo model system in which exogenous proteins can be expressed in embryonic chick lens and to further understand the function of connexin-mediated gap junction intercellular communication in lens cell biology.

METHODS. RCAS(A) is a replication-competent chicken retrovirus that infects dividing cells. Retroviral constructs were prepared containing alkaline phosphatase (AP) and FLAG-tagged connexins. Chick lenses were infected in situ by injecting virus into the lumen of lens vesicles at stage 18, cultures were taken at various periods. The lenses were then dissected, and the expressed proteins were visualized by AP histochemical examination and immunostaining.

RESULTS. Twenty-four hours after infection, alkaline phosphatase could be seen in epithelia and fibers. As lens fiber maturation progressed, however, the alkaline phosphatase staining was lost as the fibers matured, presumably because of the proteolytic removal of the enzyme. By 72 hours, alkaline phosphatase staining could still be observed in epithelial cells and in differentiating fibers in the bow region but not in the mature lens fibers. FLAG-tagged exogenous lens connexins were also abundantly expressed by viral infection. The exogenous connexins were localized at the cell surfaces in junctional maculae and showed the same cell-type specific distribution as that of their endogenous connexin counterparts.

CONCLUSIONS. An in vivo model system has been developed in the chick that provides opportunities to study the expression of wild-type and mutant proteins during lens differentiation. Expression of wild-type connexins has revealed that the characteristic distribution of the three different lens connexins is maintained even when expression is driven by a viral promoter. (Invest Ophthalmol Vis Sci. 1998;39:537-543)

For osmotic and metabolic homeostasis, the lens depends on a highly developed network of gap junction-mediated intercellular communication to facilitate the exchange of ions and metabolites throughout the organ. As in other organs, gap junctions in the lens are macular aggregates of intercellular channels, each composed of a pair of hexameric assemblies, called connexons, of integral membrane proteins belonging to the connexin (Cx) family. The axial channel joining the cells permits the cell-to-cell diffusion of ions and molecules with molecular masses of less than 1000 Da. The chick lens is known to express three different connexins. Cx43 is a component of gap junctions present between lens epithelial cells and differentiating fibers in the bow region. Cx56 and Cx45.6 are abundantly expressed in lens fiber junctions, although they are also found to a lesser degree in junctions between epithelial cells.

We and others have characterized the chick lens as an ideal system to study connexin phosphorylation. To experimentally probe the functions played by connexin phosphorylation in chick lens physiology and development, an in vivo model system is required because the systems of cultured lens cells that have been developed only recapitulate the initial stages in lens fiber development.

Although the αA-crystallin promoter has been used to drive high-level, nonlethal expression of a variety of transgenes in the mouse lens, transgenic approaches in the chicken have had limited success because of their long generation time and poor efficiency for achieving stable transgenic lines by microinjection. The RCAS(A) chicken retrovirus has been successfully used for expression of the alkaline phosphatase gene in developing chick embryos and for misexpression of the Hox-4.6 gene in chick limb bud.

Lens development offers a unique time frame in which to specifically infect lens cells with retrovirus. During development, the lens differentiates within the embryonic ectoderm overlying the optic cup by first thickening to form the lens placode. That invaginates to form the lens pit, which separates from the ectoderm as the lens vesicle. At this stage, the lens is a hollow sphere of cells, and virions can be injected directly into the vesicle lumen. Because the apical surfaces of the lens cells face the lumen, there is no basement membrane to interfere with virion binding to the cell surface and infection. After this transient stage, the posterior cells of the lens vesicle elongate anteriorly to form the primary lens fibers, eventually occluding the lumen of the vesicle on contact with the anterior lens epithelium. As the primary fibers mature further, they synthesize large amounts of soluble protein, the
crystallins, and destroy cellular organelles and other proteins to increase the refractive index and to minimize light scattering of the fiber cytoplasm. This process is continuously recapitulated at the equator of the lens as mitotically active stem cells give rise to new lens fibers that differentiate superficially to the older cells, resulting in organ growth from the surface.

In this study, we expressed exogenous proteins in the embryonic chick lens through RCAS(A) infection. We injected RCAS(A) virions carrying the alkaline phosphatase gene directly into the lens vesicle of stage 18 chick lenses and measured alkaline phosphatase expression during subsequent stages of lens development. We observed that the lenses formed normally after virus infection and that after 24 hours the alkaline phosphatase was expressed in a subset of all lens cell types. Alkaline phosphatase staining did not accumulate in the central lens fibers after 72 hours, however, indicating that the protein is able only in the epithelium and in more superficial differentiating cells. Virions were also constructed containing cDNA coding for epitope-tagged chick lens connexins, which were injected into the lumen of lens vesicles. We describe here the pattern of expression of the different lens connexins during subsequent lens development.

**Methods**

Reagents

Fertilized eggs from standard specific pathogen-free White Leghorn chickens and P27 (anti-gag) antibody were obtained from SPAFAS (Norwich, CT). The fertilized eggs were incubated for the desired times in a humidified 37°C incubator. Tissue-Tek embedding medium was purchased from Miles Scientific (Naperville, IL). Formaldehyde (16% stock solution) was obtained from Electron Microscopy Science (Fort Washington, PA). Anti-Cx43 monoclonal antibody was from Zymed Laboratories (San Francisco, CA). Rhodamine-conjugated goat anti-mouse immunoglobulin G (IgG) was from Cappel (West Chester, PA). Fluorescein isothiocyanate-conjugated, goat anti-rabbit IgG was from Boehringer Mannheim (Indianapolis, IN). Mouse anti-FLAG antiserum was from Kodak (New Haven, CT). A staining kit (ABC) was from Vector Laboratories (Burlingame, CA). Nitrocellulose membrane was from Schleicher & Schuell (Keene, NH). Chicken embryonic fibroblast (CEF) cells, QT-6 cells, RCAS(A) viral vector, and RCAS(A) containing alkaline phosphatase were generous gifts from Constance Cepko’s laboratory (Harvard Medical School). DNA polymerases and restriction enzymes were from New England Biolabs (Revere, MA). All other chemicals were obtained from either Sigma Chemical (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Making Retroviral Constructs Containing Lens Connexins

Cloning of connexins to viral vectors included two steps modified from a published procedure25: The connexin was first cloned to an adapter plasmid to facilitate the proper restriction sites and then to the viral vector. DNA fragments containing connexins with their carboxyl termini in-frame with the FLAG epitope sequence were prepared by polymerase chain reaction. Oligonucleotide primers were synthesized on a DNA synthesizer (ABI model 391; Applied Biosystems, Foster City, CA), in which the sense primers contained the 5’ coding sequence, including a start codon and a restriction enzyme site for Ncol. The anti-sense primers contained the 3’ coding sequence plus the FLAG sequence followed by a stop codon and a restriction site for EcoRI. The cDNA template for Cx56 was a generous gift from Eric Beyer (Washington University, St. Louis, MO). Polymerase chain reactions contained 0.5 μg each connexin cDNA. 100 pmol sense and anti-sense primers, 200 μM dNTP, 50 mM Tris(hydroxymethyl)aminomethane, pH 8.3, 50 mM KCl, and 50 μl Vent (New England Biolabs, Revere, MA) polymerase (1 U) was added after the reaction reached 94°C to minimize undesired priming during the initial cycle. Thirty cycles were performed as follows: 94°C for 1 minute, 58°C for 1 minute, and 72°C for 1 minute. This was followed by a final extension for 10 minutes at 72°C. Reaction products were separated on 1% agarose gel, excised, and electroeluted in a dialysis bag as described previously.24 Eluted DNA was phenol extracted and ethanol precipitated with 10 μg glycogen as carrier. The purified polymerase chain reaction products were digested by restriction enzymes Ncol on the 5’ end and EcoRI on the 3’ end. The above restriction enzyme–treated, polymerase chain reaction products were subcloned into an adapter plasmid, Cla12NCO, using a sticky-end ligation reaction, in which Cla12NCO had been pretreated with Ncol and EcoRI and gel purified. The constructs with the correct DNA inserts of correct size were selected, and a large quantity of DNA was purified using a DNA isolation kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. To ensure the correct sequence of these constructs, DNA was sequenced by an in-house sequencing facility (Department of Biochemistry and Molecular Pharmacology, Harvard Medical School). The Cla12NCO, containing connexins, were then digested with Cla1, and the resultant fragments were gel isolated. The DNA fragments with Cla1 sites on both ends were subcloned to Cla1-linearized RCAS(A), and the constructs with correct inserts were selected. Finally, large quantities of RCAS(A) constructs containing the connexins were prepared and the concentrations determined.

Preparation of High-Titer Viruses and Titering

Preparation of high-titer retrovirus from host cells was modified from the procedures of Brasier and Fortin.25 CEF cells were plated at 2.5 × 10⁵ cells in a 35-mm culture dish the day before transfection to reach 50% to 75% cell confluence for transfection. In 200 μl OPTI MEM (Life Technologies; Gaithersburg, MD), 1.5 μg DNA was mixed with 12 μg lipofectamine and incubated for 20 minutes at room temperature. After incubation, the samples were diluted with 800 μl OPTI MEM and added to the CEF cells, which had been prewashed with OPTI MEM. These cells were incubated at 57°C for 6 hours before 1 ml Dulbecco’s modified Eagle’s medium, supplemented with 20% fetal calf serum and 4% chick serum, was added. Two days after transfection, when confluent, the cells were passaged to occupy four or five 15-cm dishes. After the cells again became confluent, the culture medium was collected consecutively for 3 days. The culture medium, containing virions, was centrifuged at 72,000g for 2 hours. The supernatant was carefully decanted, and a residual amount of medium (~50 μl) was used to resuspend the viral pellets on a shaker for 2 hours at 4°C. The resuspended viruses were then stored at −80°C. This step concentrated the virus up to 500-fold.
and four embryos for the 72-hour time point. A total of eight chick embryos were examined for RCAS(A)-Cx43 infection, nine for RCAS(A)-Cx45.6, and seven for RCAS(A)-Cx56.

Histologic Examination

Tissue-cultured cells and lens sections were initially fixed with paraformaldehyde (4% for QT-6 cells; 2% for lens sections) for 20 minutes and washed three times with phosphate-buffered saline (PBS). Alkaline phosphatase histochemical examination was carried out as described.\(^5\) For immunohistochemical examination, the samples were incubated in blocking solution containing 2% normal goat serum, 2% fish skin gelatin, 0.5% Triton X-100, and 1% bovine serum albumin in PBS for 30 minutes.

For viral titration, the QT-6 cells were incubated with anti viral protein, gag antibody, and P27 (1:150 dilution) for 1 hour and were washed three times with PBS. The cells were then incubated with biotinylated anti-rabbit antibodies (1:200 dilution) for 1 hour and washed three times with PBS. The cells were further treated with two solutions, one containing avidin-DH and the other biotinylated enzyme, each dissolved into PBS containing 1% normal goat serum for 30 minutes according to the manufacturer's instructions. After washing three times in PBS, the cells were stained with PBS containing 3,3′diaminobenzidine tetrahydrochloride and 0.01% peroxide for approximately 5 minutes until they turned brown.

RCAS-infected lenses were immersed in Tissue-Tek (Miles Scientific) compound and frozen in nitrogen-cooled propane slush. Sections (10-15 µm) were collected and prepared according to Paul et al.\(^28\) After permeation and blocking, lens sections were incubated with an anti-FLAG monoclonal antibody (10 µg/ml) and detected by rhodamine-conjugated, goat anti-mouse IgG (1:500 dilution). Fluorescence microscopic examination was performed using an Axioskop microscope (Zeiss, Thornwood, NJ) and recorded on film (Tmax 400; Kodak, Rochester, NY).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis and Immunoblot

A 60-mm culture dish with virus-infected, confluent CEF cells was lysed and used to prepare crude membranes as described.\(^4\) These were subsequently analyzed on 10% sodium dodecyl sulfate-polyacrylamide gels according to the method of Laemmli.\(^29\) Western blots were performed as previously described\(^28\) by probing with monoclonal antibodies for anti-FLAG (10 µg/ml) and Cx43 (1:50 dilution) or with the polyclonal anti-Cx56 and -Cx45.6 antibodies (both at 1:500 dilution). Preparation and immunoaffinity purification of anti-Cx56 and -Cx45.6 antisera were performed as described.\(^3\) The primary antibodies were detected with alkaline phosphatase-conjugated, goat anti-rabbit IgG (1:5000 dilution) for Cx56 and Cx45.6 or goat anti-mouse IgG (1:1000 dilution) for anti-FLAG and -Cx43 monoclonal antibodies.

RESULTS

Expression of Exogenous Alkaline Phosphatase in Embryonic Chick Lens

The expression of exogenous proteins in the embryonic lens was initially examined by introducing RCAS(A) containing the alkaline phosphatase reporter gene.\(^27\) The expression of alka-
Expression of RCAS(A) alkaline phosphatase constructs in chick embryonic lens examined 1 day after viral injection. (A) The lens is visible after the histochemical reaction for alkaline phosphatase (arrow) in this wholemount preparation viewed with a dissecting microscope. (B) A lens injected with a low dose of RCAS(A)-alkaline phosphatase shows only a single positive cell in this wholemount specimen. (C) A wholemount preparation of a lens injected with a high dose of virus. Positive cells are seen around the equator, although the center of the lens with its primary fibers is negative. (D) A section of the lens in C shows positive cells in the epithelium and bow regions. E represents lens epithelium; F represents lens fibers. Differentiating fibers are also positive, but the central primary fibers are not. The positive cells in the overlying ectoderm (presumptive cornea) occasionally seen are the result of leakage of virus from the pipette on approaching the lens (open arrows). Bar, 40 μm.

Alkaline phosphatase in infected CEF cells (data not shown) was detected by the alkaline phosphatase color reaction as described previously. In retrovirally infected lens cells, Figure 2A shows a wholemount chick head 24 hours after infection. The lens is seen as a dark-staining circle (arrow) caused by alkaline phosphatase activity. Dissection of the lens from the embryo and inspection in wholemount preparations or in frozen sections permitted diagnosis of the lens cell types expressing alkaline phosphatase. Figure 2C is a wholemount and Figure 2D a frozen section of the lens in Figure 2A, which was injected with 4 nl virus stock. Figure 2B is a wholemount photograph of a lens from a different embryo injected with 4 nl of a 1:50 dilution of the viral stock, in which only one cell has been infected. Alkaline phosphatase-expressing cells in the wholemount lens are not only seen in a ring around a nonstaining central zone (Fig. 2C). This central zone corresponds to the original primary lens fibers that elongated but did not divide after the lens vesicle stage; hence, they were not infected with virus. All other cells could be infected with virus; no clear pattern of infection was discernible, indicating that infection was a nonselective process. Figure 2D is a section of the lens shown in Figure 2C and shows that, at this early developmental stage, epithelial cells, bow cells, and early lens fibers could all express alkaline phosphatase, although the central primary fibers are unlabeled. In rare cases, alkaline phosphatase staining was also seen in the overlying ectoderm, presumably a result of virus leakage from the pipette during approach to the lens. This ectodermal staining is visible in Figures 2C and 2D (empty arrows).

Figure 3 shows chick lenses 72 hours after infection. Figures 3A and 3B are wholemounts of infected (above) and control contralateral (below) lenses after the alkaline phosphatase expression in lens 3 days after viral injection. Seventy-two hours after viral infection, the expression of alkaline phosphatase was detected either in whole lens (A, B) or in lens sections (C, D, E). In A and B, the injected lenses (top) show alkaline phosphatase staining not seen in the uninjected contralateral lenses (bottom). The alkaline phosphatase-positive cells are seen in groupings from single cells to large clusters of cells. In C–E, alkaline phosphatase-positive cells are evident in the epithelium and in the bow regions, although no positive cells are seen in the superficial or deep cortex, indicating that the positive cells seen in Figure 2 have lost their alkaline phosphatase activity during fiber maturation. Bar, 30 μm.
were transfected with RCAS(A) vectors containing Cx43, Cx45.6, and Cx56. After reaching confluence, cells were lysed and crude membranes were isolated. These membrane preparations were analyzed on sodium dodecyl sulfate-polyacrylamide gels and nitrocellulose replicas probed with anti-FLAG (lanes 1-3) or anti-connexin antibodies (lanes 4-6) and visualized with the alkaline phosphatase reaction.

Large regions of the lens surface are stained. Cryosections of this specimen (Figs. 3C, 3D, 3E) show that epithelial cells and bow cells are positive. However, the infected early lens fibers, which were visibly stained in Figure 2, could no longer be seen, indicating that the exogenous alkaline phosphatase had been lost from these fibers as lens development proceeded, most likely because of proteolysis.

Taken together, patterns of retroviral expression of alkaline phosphatase in the chick lens infected at stage 18 showed that all lens cell types could express alkaline phosphatase except for the primary lens fibers, which had left the cell cycle. The alkaline phosphatase expression patterns revealed a high concentration of infected cells near the lens equator, at which stem cells are known to be mitotically active. Lens epithelial cells, alone or in clusters, expressed alkaline phosphatase, indicating that continued cell division exists within the epithelium during the developmental stages examined. With increasing development time, more cells express alkaline phosphatase, suggesting that the initially infected cells expand as clones and that this replication-competent virus continues to infect additional cells after the initial injection. Finally, differentiating lens fibers in the equatorial region, seen to be expressing alkaline phosphatase at 24 hours, were negative at 72 hours. This indicated that previously expressed alkaline phosphatase may be proteolytically degraded during lens fiber maturation.

**Expression of Exogenous FLAG-Tagged Connexins in Embryonic Chick Lens In Situ**

Viral constructs were prepared containing each of the three full-length lens connexins, in which each C terminus was tagged with the FLAG sequence to distinguish them from the endogenous connexins. Retroviral constructs were transfected into CEF, and the expression of the connexins was detected by western blot analysis (Fig. 4). The connexins expressed in CEF were recognized either by anti-FLAG antibody (lanes 1-3) or by anti-connexin antibodies for Cx43, Cx45.6, and Cx56 (lanes 4-6). There were some degradation products from connexins revealed by anti-connexin antibodies and higher molecular weight cross-reacting species, which were not investigated further. The results confirmed that each RCAS(A) contained correct connexin inserts with C terminus-tagged FLAG sequences and that these constructs could be expressed in host CEF cells.

RCAS(A) preparations containing each of three lens connexins were individually injected into stage 18 embryonic chick lenses, as illustrated in Figure 1. The results with alkaline phosphatase suggested that more lens cells were infected if injected eggs were incubated an additional 3 days after viral injection (72 hours). This same time frame was used for lenses injected with FLAG-tagged connexin, and the lens sections were stained with FLAG antibody to detect the expression of exogenous connexins. Injected RCAS(A) viruses containing connexins were abundantly expressed in embryonic chick lens, and a portion of the signal was punctate, indicating incorporation into gap junctional plaques (Fig. 5, 6). Expression of exogenous connexins in a large quantity had no detectable effect on lens morphology and did not interfere with lens development.

The expression of FLAG-tagged Cx43 in the lens (Fig. 5) was found abundantly in the bow region (Figs. 5a, 5b), in which lens cell mitosis and differentiation are active. Cx43 was also found in the lens epithelium (Figs. 5c, 5d). However, there was no staining in the more mature lens fibers. FLAG-tagged Cx56 and Cx45.6 were abundantly expressed in the lens, and their localizations were similar. FLAG-tagged Cx56 (Fig. 6a) and Cx45.6 (Fig. 6b) were expressed in the lens bow region as well, although the staining extended deeper into the cortex than that seen for Cx43 (compare Fig. 5b with Figs. 6a, 6b [B]). In addition, the exogenous Cx56 and Cx45.6 were also expressed in superficial (Figs. 6a [C, D]) and in deep (Figs. 6a, 6b [B]).
jectives and smaller areas. Neither Cx56 nor Cx45.6 staining was found not to alter lens development visibly in 1 week, the longest time studied. RCAS(A), a replication-competent avian retrovirus,23'30'31 has been used to express exogenous proteins in the embryonic chick lens in situ. Specificity of viral targeting to the lens was achieved by injection of the virus into the lumen of the lens vesicle at developmental stage 18. Viral infection and spread achieved by injection of the virus into the lumen of the lens epithelium. RCAS(A)-Cx56 and -Cx45.6 were injected into stage 18 embryonic chick lenses in situ. After 72-hour incubation, frozen sections were prepared and stained with anti-FLAG antibody. The expression of FLAG-tagged Cx56 (a) and Cx45.6 (b) was found to be in junctional maculae in fibers found near bow regions (Cx56 and Cx45.6: A and B) and in the superficial (Cx56: C and D) and deep cortex (Cx56 and Cx45.6: E and F). Bar, 10 μm.

DISCUSSION

RCAS(A), a replication-competent avian retrovirus,23'30'31 has been used to express exogenous proteins in the embryonic chick lens in situ. Specificity of viral targeting to the lens was achieved by injection of the virus into the lumen of the lens vesicle at developmental stage 18. Viral infection and spread was found not to alter lens development visibly in 1 week, the longest time studied.

In this study, we have introduced two types of proteins by retroviral injection into embryonic chick lens in situ: alkaline phosphatase, a soluble cytosolic protein, and three lens connexins, the insoluble integral membrane proteins of gap junctions. Twenty-four hours after viral infection at stage 18, alkaline phosphatase was found in all lens cell types except the primary lens fibers at the posterior surface of the lens. Because the primary fibers were slowly growing or postmitotic,22'25 they were refractory to viral infection. The distribution of alkaline phosphatase was seen in all lens cell types, indicating that there was no cell type-specific selectivity in the regulation of expression. However, the alkaline phosphatase signal was lost as lens fibers matured, suggesting that the alkaline phosphatase was proteolytically removed from the fibers as part of the maturation program.

In contrast to these alkaline phosphatase data, lens connexins introduced by retroviral infection accumulated only in the same cellular locations as their endogenous counterparts. Cx45 accumulated only in lens epithelium but not in lens fibers; Cx56 and Cx45.6 accumulated mainly in lens fibers and not in the epithelium. These observations clearly suggested cell- and connexin-specific mechanisms for controlling protein accumulation. It is possible that there are targeting signals residing in the coding region of lens fiber connexins because exogenous retroviral constructs containing connexin cDNA, which lack their noncoding regulatory regions, nonetheless show endogenous cellular distributions. The opportunity to create chimeric molecules may help identify these targeting domains.34'35

The control of connexin expression in the lens may involve translational and posttranslational mechanisms, which have been documented in lens biology studies.8'35'36 Previous reports have shown that there is posttranslational processing of ovine Cx50,39'40 notably involving calpain.42 The experimental model described here will allow the design and expression of dominant negative mutant connexins to address the function of lens gap junction in lens physiology and development. In addition, lens connexins are phosphorylated proteins.46'47 This experimental model offers the opportunity to express connexins with deleted or mutated phosphorylation sites in the intact lens and to study the functional relevance of these posttranslational modifications.

References


