

Ubiquitous Lens α -, β -, and γ -Crystallins Accumulate in Anuran Cornea as Corneal Crystallins*

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Corneal epithelium is known to have high levels of some metabolic enzymes such as aldehyde dehydrogenase in mammals, gelsolin in zebrafish, and α -enolase in several species. Analogous to lens crystallins, these enzymes and proteins are referred to as corneal crystallins, although their precise function is not established in any species. Although it is known that after lenticectomy, the outer cornea undergoes transdifferentiation to regenerate a lens only in anuran amphibians, major proteins expressed in an anuran cornea have not been identified. This study therefore aimed to identify the major corneal proteins in the Indian toad (*Bufo melanostictus*) and the Indian frog (*Rana tigrina*). Soluble proteins of toad and frog corneas were resolved on two-dimensional gels and identified by matrix-assisted laser desorption ionization time-of-flight/time-of-flight and electrospray ionization quadrupole time-of-flight. We report that anuran cornea is made up of the full complement of ubiquitous lens α -, β -, and γ -crystallins, mainly localized in the corneal epithelium. In addition, some taxon-specific lens crystallins and novel proteins, such as α - or β -enolase/ τ -crystallin, were also identified. Our data present a unique case of the anuran cornea where the same crystallins are used in the lens and in the cornea, thus supporting the earlier idea that crystallins are essential for the visual functions of the cornea as they perform for the lens. High levels of lens α -, β -, and γ -crystallins have not been reported in the cornea of any species studied so far and may offer a possible explanation for their inability to regenerate a lens after lenticectomy. Our data that anuran cornea has an abundant quantity of almost all the lens crystallins are consistent with its ability to form a lens, and this connection is worthy of further studies.

Crystallins are structural proteins responsible for the transparency of the eye lens (1). These proteins have been grouped as ubiquitous lens crystallins and taxon-specific crystallins. α -, β - and γ -Crystallins are ubiquitous lens crystallins because they are generally present in the lenses of all vertebrates (1–3). However, some metabolic enzymes, such as α -enolase and argininosuccinate lyase, are found abundantly in the lenses of some specific species and are known as taxon-specific crystallins (4, 5).

Some enzymes and proteins have been found in unusually high concentrations in the corneal epithelium of many species (6). For example, aldehyde dehydrogenases 1 and 3 (7–9), transketolase (10), and isocitrate dehydrogenase (11) are expressed in human and bovine cornea, cyclophilin in the chicken cornea (6), and gelsolin in the zebrafish cornea (12), whereas α -enolase is expressed in the corneas of many species (6, 13, 14). Because their expression in corneal epithelium is significantly high, these proteins have been termed corneal crystallins, analogous to the multifunctional lens crystallins (9, 15–17). However, whether these abundant corneal proteins can legitimately be considered as crystallins is a matter of discussion. Because many examples of unusually high levels of enzymes in the cornea are now known (6–14), the idea of corneal crystallins has gained considerable support (9, 15, and 17), although the exact function of the corneal crystallins is not established for any species. The recognition that, akin to lens crystallins, corneal crystallins exist is a novel concept that has implications that involve the process by which multifunctional gene products have evolved. It is not known whether the corneal crystallins contribute to the optical properties of a cornea. Thus, the structural and functional relationship between the lens and corneal crystallins remains ambiguous. In some cases, corneal crystallins perform several functions; aldehyde dehydrogenase is shown to play a protective role against oxidative stress and in the regulation of cell cycle (18, 19).

Interestingly, some members of the aldehyde dehydrogenase family are lens as well as corneal crystallins (20). Although not many examples of crystallins common to the lens and cornea are known, there are a few examples such as τ -crystallin, which is present in the lenses of several species and is also a corneal crystallin (6, 14). Some invertebrates (e.g. squid and scallop) accumulate lens crystallins in their corneas, suggesting that common lens and corneal crystallins exist (21, 22). However, the validity of this concept still requires more verification. Vertebrate ubiquitous lens α -, β -, and γ -crystallins have not been found to accumulate in the cornea of any species studied so far. If it were true that these crystallins are also corneal crystallins, it would be really important and possible to correlate the optical functions of corneal crystallins. As mentioned, corneas of only a few species have been analyzed for the presence of abundant proteins. It is, therefore, important to identify the abundant proteins in the cornea of a number of diverse species in order to understand the nature of corneal crystallins.

We have attempted to identify the major soluble proteins expressed in anuran cornea. After lenticectomy, anuran such as *Xenopus laevis* exhibits a unique property of regenerating a

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lens by transdifferentiation of the outer corneal epithelium (23–26). This transdifferentiation process is known to be triggered by several factors such as fibroblast growth factors and vitamin A (27–30). In fact, when implanted into the vitreous chamber of a host larva, fragments of outer cornea transdifferentiate into lens fibers (31–34). However, the molecular basis of this lens-forming competence of the corneal epithelium is not understood and has been a subject of intense investigations. The focus has been largely on the identification of factors that could induce lens formation. However, a major unanswered question is: Why does only an anuran cornea have lens-forming competence? Is the composition of an anuran cornea different from that of others? Do anuran corneas have abundant transcripts for the α -, β -, and γ -crystallins? To the best of our knowledge, no study has been directed toward the identification of the abundant proteins in anuran cornea or understanding its protein composition and its comparison with other species that could throw light on this unique property of anuran cornea.

With these objectives, we undertook detailed analysis of the major corneal proteins from an Indian toad (*Bufo melanostictus*) as a representative of anuran amphibian. To our utmost surprise, we found that cornea of the Indian toad contains almost the full complement of vertebrate ubiquitous lens α -, β -, and γ -crystallins. The major protein spots observed on a two-dimensional gel of the toad cornea and identified by MALDI-TOF/TOF⁴ and ESI-Q-TOF belong to these ubiquitous lens crystallins, namely α A-, α B-, β B2-, β A1/A3-, β A2-, and γ -crystallins. Immunofluorescence analysis using mouse polyclonal antibodies for α B-, β B2-, and γ -crystallins demonstrates that these crystallins are largely localized in the corneal epithelium, thus supporting the hypothesis of corneal crystallins (15, 17) and suggesting that they are essential for the visual functions of cornea.

MATERIALS AND METHODS

Indian Toad and Frog Corneas—The adult toads and frogs were caught from the field and identified as *B. melanostictus* and *Rana tigrina*. The animals were handled according to Association for Research in Vision and Ophthalmology guidelines and approved by the institutional ethics committee for handling animals. The corneas were surgically removed, carefully avoiding the lens tissues, homogenized in 50 mM Tris buffer, pH 7.5, and centrifuged, and the supernatant was collected. The total water-soluble protein was estimated in the supernatant using rapid protein estimation reagents (Bio-Rad) and used for the experiment.

Two-dimensional Gel Electrophoresis—Two-dimensional gel electrophoresis was performed using immobilized pH gradient strips, pH 3–10 (Protean IEF cell; Bio-Rad). Proteins were resolved by isoelectric focusing in the first dimension and SDS-PAGE (12% acrylamide) in the second dimension. Protein spots were visualized by Coomassie blue R 250 staining and were identified based on their discrete presence in the gel and processed further.

⁴ The abbreviations used are: MALDI-TOF/TOF, matrix-assisted laser desorption ionization time-of-flight; ESI-Q-TOF, electrospray ionization quadrupole time-of-flight; RT-PCR, reverse transcription PCR; MS/MS, tandem mass spectrometry.

In-gel Tryptic Digestion—The selected spots of proteins were excised manually from gels and were subjected to in-gel digestion using sequencing grade bovine trypsin (Sigma) reconstituted in 25 mM ammonium bicarbonate at pH 8.0 at a molar ratio of the protein to trypsin between 10:1 and 30:1. After digestion, peptides from the gel were extracted twice using 100 μ l of 50% acetonitrile containing 5% trifluoroacetic acid.

Mass Spectrometry—Peptide mass fingerprinting of the reconstituted peptides was obtained using MALDI-TOF/TOF Analyzer (MDS SCIEX, Applied Biosystems) mass spectrometer. For determination of internal sequence tags of the protein, MS/MS fragmentation spectra were obtained using API Q-STAR Pulsar from PE SCIEX, Applied Biosystems, with nanospray source at 1000 V spraying voltage. Protein identifications by peptide mass fingerprinting were examined for the number of peptides matched (at least 5) or the sequence coverage (15–20%) using MASCOT search on the NCBI data base. Protein identification was confirmed with MS/MS data that were analyzed by the program Bio-Analyst from PE SCIEX, Canada, and homology searches were carried out using sequence tags. We have included only those protein identifications that were confirmed by MS/MS analysis.

Preparation and Validation of Anti- β B2- and Anti- γ -Crystallin Antibodies—Recombinant purified γ - and β B2-crystallins (~150 μ g) cloned from bovine lens were emulsified with Freund's complete adjuvant (Sigma) and injected into Balb/c mouse subcutaneously. Serum was separated, and antibody titer and specificity were assessed by Western blot analysis using recombinant-specific proteins.

Western Immunoblot and RT-PCR—Corneas of common Indian toad and frog were removed surgically and homogenized in 50 mM Tris buffer, pH 7.5, containing 100 mM NaCl, 0.02% sodium azide, 1 mM EDTA. ~60 μ g of protein was resolved on 12% SDS-PAGE and transferred onto a nitrocellulose (Hybond-P; Amersham Biosciences) membrane, probed with polyclonal mouse anti- β B2- or γ -crystallin antibodies (1:5000 dilution) and secondary antibody anti-mouse-horseradish peroxidase, and visualized using an ECL kit (Amersham Biosciences, Inc.). Polyclonal anti- α B-crystallin antibody raised against α B-crystallin-specific peptide (kindly provided by Dr. Mohan Rao, Centre for Cellular and Molecular Biology) was used to probe α B-crystallin. For RT-PCR, total RNA was extracted from the whole cornea using TRIzol reagent, and first-strand cDNA synthesis was performed using a kit from Invitrogen. The RT-PCR was performed using gene-specific primers designed from the crystallin sequences of frog for α B, β B2, and β A1-crystallins and γ II-crystallin from bovine.

Immunofluorescence Staining and Confocal Imaging—The frozen sections of cornea (10 μ m) were prepared by a cryomicrotome (Leica Microsystems), fixed in 100% methanol for 1 min, and washed with 1 \times phosphate-buffered saline, pH 7.2. The sections were incubated with appropriate antibody (mouse polyclonal) at 1:100 dilution. After three washes with phosphate-buffered saline, sections were visualized with fluorescein isothiocyanate-labeled sheep anti-mouse antibody (Jackson Laboratories) at a 1:200 dilution. The nucleus was stained with propidium iodide. Dual immunofluorescence microscopy was also carried out on a confocal microscope (model LSM 510

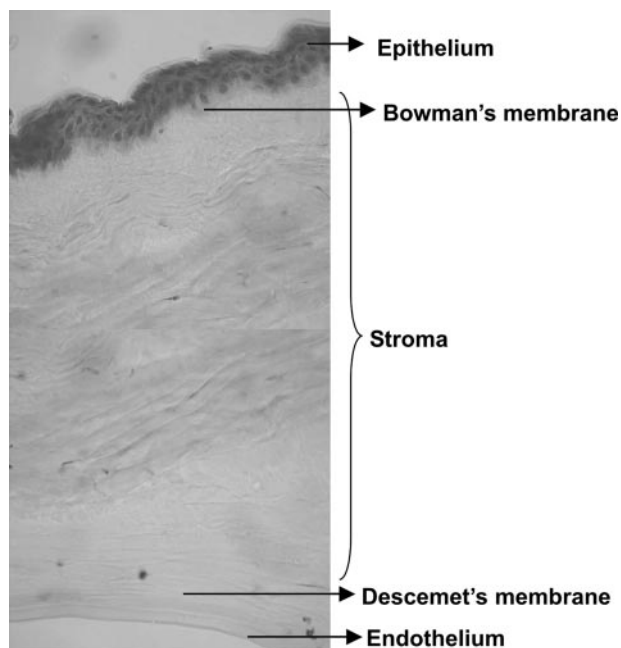


FIGURE 1. **Morphology of a toad cornea.** The cornea was sectioned and stained by hematoxylin and eosin. It was visualized by an imaging microscope (AxioPlan 2; Zeiss). Three major sections are labeled. The positions of Bowman's membrane and Descemet's membrane, although not clearly visible, are indicated.

META; Carl Zeiss) using a 488-nm excitation and 540-nm long pass emission filter.

RESULTS AND DISCUSSION

Cornea is a transparent tissue, composed of an outer layer called the corneal epithelium, middle stroma, and inner endothelial layer. The morphology of a toad cornea is shown in Fig. 1. The stroma, which forms a major part of the cornea, is largely made up of type II collagen and keratan sulfate proteoglycans. Corneal epithelium is made up of three layers of cells, whereas endothelium is a single cell layer.

Abundant proteins in the corneal epithelium from a few species (such as mammal, birds, and zebrafish) have been identified. Corneal epithelium of these species is known to accumulate several metabolic enzymes, such as aldehyde dehydrogenase (7–9), α -enolase (6, 13, 14), or gelsolin (12). In this study, we have selected the Indian toad as a representative of anuran amphibian and identified major soluble proteins in the cornea. This would be important not only because anuran corneas are known to have lens-forming competence but also to understand the nature of corneal crystallins in these amphibians. To analyze the abundant proteins expressed in toad cornea, we have resolved corneal soluble proteins on a 12% two-dimensional gel and identified the major protein spots by mass spectrometry.

High Accumulation of Ubiquitous Lens Crystallins in the Cornea of Common Indian Toad and Frog—Fig. 2 shows two-dimensional gel electrophoresis of toad (*Bufo*) total corneal extract. Most of the spots in the two-dimensional SDS-PAGE accumulated in the range of 20 and 50 kDa (Fig. 2). The major spots seen on a two-dimensional gel pattern of toad corneal homogenate were quite similar to those of the spots on two-dimensional gel of the homogenate of a toad lens (Fig. 3).

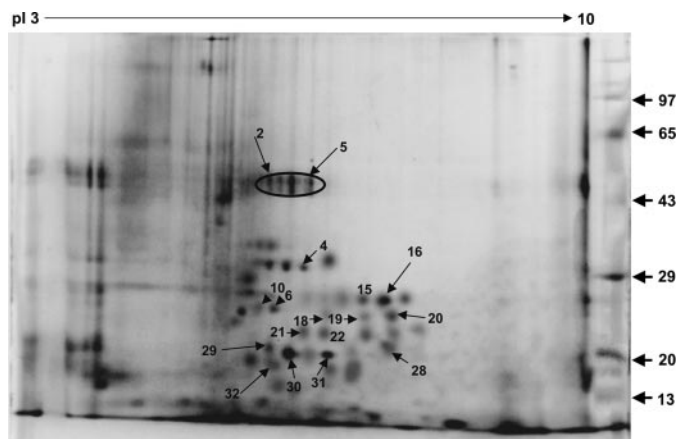


FIGURE 2. **Two-dimensional gel of (a) a toad (*Bufo*) soluble corneal extract; first dimension with the pI range between 3 and 10; second dimension with 12% SDS-PAGE.** 38 spots from two-dimensional gel were excised and analyzed by MALDI-TOF/TOF and ESI-Q-TOF. The results of protein identification are summarized in Table 1.

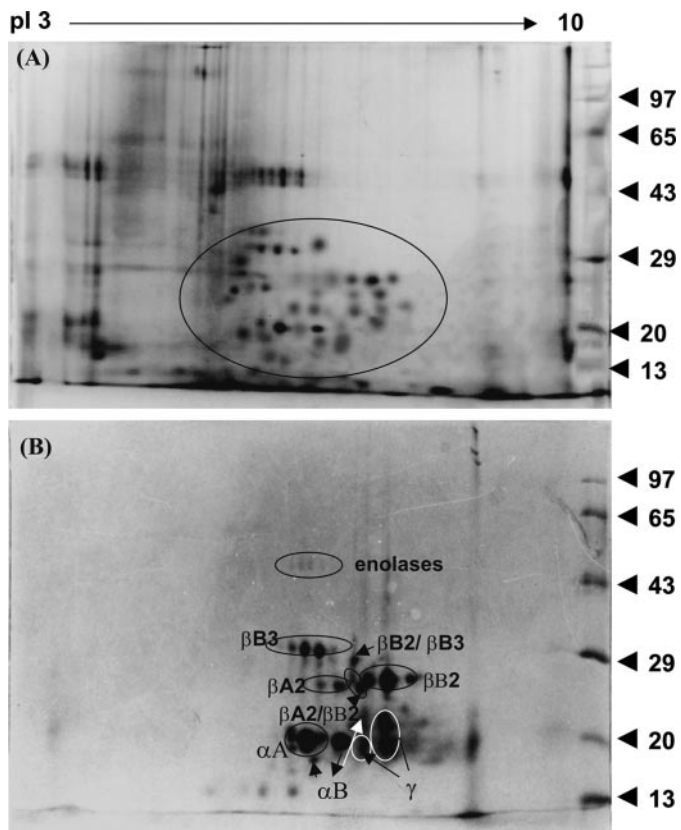


FIGURE 3. **A and B, two-dimensional analysis of proteins of *Bufo* cornea and *Bufo* lens for the sake of direct comparison depicting the separation and position of various crystallins in both tissues.** Spots on two-dimensional gel of *Bufo* lens were identified by MALDI-TOF/TOF only. Protein identification of the spots of *Bufo* cornea is shown in Fig. 2.

We selected the major spots (38 spots) of molecular mass between 15 and 50 kDa from one two-dimensional gel and analyzed by MALDI-TOF/TOF. All the protein spots were also subjected to ESI-Q-TOF for final confirmation. Of the 38 spots selected, 17 were confirmed by MS/MS analysis. Of them, 14 spots were confirmed as lens α -, β -, or γ -crystallins. Spots for α A-crystallin, α B-crystallin, and some β -crystallins, namely β B1-, β B3-, β B2-, β A1-, and β A2-, were identified. These

TABLE 1

Protein identifications for spots from two-dimensional gel of water-soluble fractions of *Bufo* corneal epithelium shown in Fig. 2 by MS/MS sequencing

MS/MS sequencing was done on one or two precursor ions, and the proteins were identified using internal sequence/sequence tags. Due to high internal sequence homology, two identifications were obtained for spots 15 and 16.

Spot number	Protein identification	Precursor ion MW	MS/MS sequence/tag
		<i>D_a</i>	
2	α/β -Enolase	1148.58	(244.1594)LPVGASSF(128.0220)
4	β B3	1334.62	(386.1279)GEQFVLE(128.0955)
		1453.70	(214.1011)LYELENFQG(128.0901)
5	α/β -Enolase	1148.62	(244.1062)L(773.3607),(768.3766)SF(128.0739)
6	Calgizzarin	1331.64	(294.1019)NTELVSFT(128.0956)
10	β B1 or β A4	1492.74	IILFEQENFQGR
15	β A4	1492.73	(226.1294)LFEQENFQG(156.0672)
	β B2	1393.68	(212.2126)LYENPNFTG(127.9907)
16	β B2	1393.69	(212.1364)LYENPNFTG(128.0214)
	β A4	1492.73	(226.1973)LFEQENFQG(156.0354)
	β A2	1411.64	(226.1973)LFEQENFQG(156.0354)
18	β A2	1411.63	(313.1989)YEGENFQG(156.0570)
19	β B2	1393.66	(212.1915)LYENPNFTG(127.9965)
20	β B2	1393.68	(325.2158)YENPNFTG(128.0715)
21	β B2	1393.69	(212.1201)LYENPNFTG(128.0932)
22	β B2	1393.69	(163.0722)TEWGAVTP(156.0931)
28	γ I	1178.57	(184.1320)GPFYPN(156.0758)
29	α A	1033.52	(184.1320)GPFYPN(156.0758)
30	α A	1033.52	(220.0900)MDSGLSEV(156.1109)
		1228.54	(113.2031)PSWLSEGMSEL(155.9630)
31	α B	1503.72	(184.1550)GPFYPN(156.0628)
32	α A	1033.53	(220.1030)MDSGLSEV(156.0991)
		1212.56	

TABLE 2

Major proteins expressed in *R. tigrina* corneal epithelium

Protein spots from two-dimensional gel shown in Fig. 4 were excised; protein was in-gel digested with trypsin, eluted, and identified using MALDI-TOF/TOF peptide mass fingerprinting.

Spot number	Protein identification	MASCOT search	Number of peptides matched	Sequence coverage	Peptide mass fingerprint span	Theoretical mass	Observed mass
				%		<i>kDa</i>	
1	α -Enolase	103	10	28	9–250	47.3	40.3
2	Phytanol-CoA hydroxylase	72	10	25	81–276	39	38.6
3	Solute carrier family 25	76	7	26	35–136	29	33.9
4	β B1-Crystallin	136	9	51	25–253	29.0	28.0
5	β B3-Crystallin	85	7	39	25–195	29.0	24.3
6	β A3-2 Crystallin	141	9	66	11–211	24.0	25.1
7	β A3-1 Crystallin	118	8	49	17–211	24.0	25.1
9	B-lymphocyte stimulator	86	6	38	22–175	24.3	22.7
10	γ -Crystallin I	81	5	23	22–122	17.9	16.5
11	Glutathione S-transferase	99	8	32	1–135	20.3	25.7
12	β B2-Crystallin short isoform	181	13	45	1–212	20.1	25.0
13	Tripartite motif protein	119	9	38	14–136	20.9	23.9

MS/MS identification data are presented in Table 1. Two spots for α A and α B-crystallin each were identified. Expression of α B-crystallin is not surprising because it is known to be expressed in a large number of tissues. However, the expression of α A-crystallin in cornea is important because it is considered a lens-specific crystallin. There were five spots for β B2-crystallins, suggesting different forms of this crystallin with similar molecular mass but different pI (Fig. 2, Table 1). Alternatively spliced forms of some β -crystallins have earlier been observed in the lens, where more than one protein spot was identified as β B2-crystallin (35, 36), suggesting a similar composition of lens and corneal crystallins. Due to high internal sequence homology, it was difficult to clearly distinguish some spots between various β -crystallins, such as MS/MS data of one spot (spot 15) matched to β A4-crystallin as well as to β B2-crystallin (Table 1). One spot was identified as γ I-crystallin (Table 1). These crystallins form >60% of the total proteins from corneal epithelium as approximately estimated by densitometry scan. Because these results were unexpected and could possibly be due to contamination with lens crystallins, we took extreme care dur-

ing surgical removal of a cornea to make sure that the iris and the lens were not damaged so that potential contamination from lens tissue was avoided. Additionally, before scraping the epithelium, corneas were washed thoroughly with phosphate-buffered saline. We obtained similar results analyzing the spots from several other two-dimensional gels by MALDI-TOF/TOF. Almost all the lens-specific crystallins mentioned above were also identified in these independent two-dimensional gels (data not shown).

To investigate whether these crystallins also accumulate in the frog cornea, the proteins expressed in the cornea of *R. tigrina* were analyzed. MALDI-TOF/TOF analysis of the major spots from a two-dimensional gel identified several lens crystallins in *R. tigrina* cornea, suggesting that the accumulation of α -, β -, and γ -crystallins is an inherent characteristic of anuran cornea (Fig. 4, Table 2).

Presence of Enzyme Crystallins (Taxon-specific Crystallins) in Anuran Cornea—As many taxa ranging from cephalopods to mammals have enzyme crystallins in their cornea, we expected the expression of some taxon-specific corneal crystallins in the

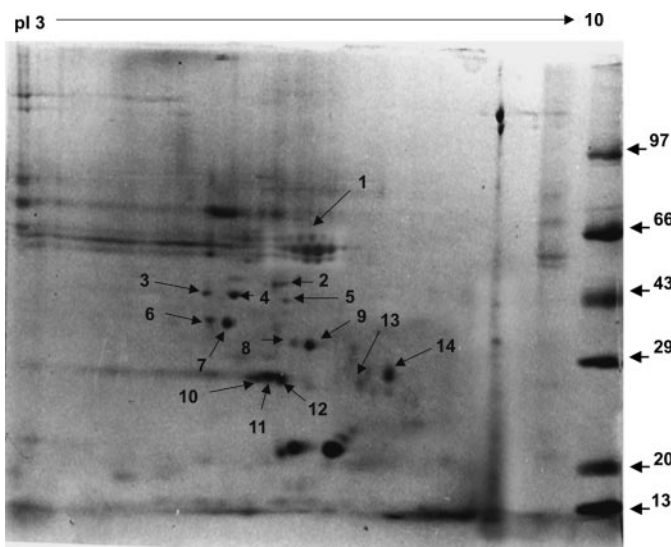


FIGURE 4. Two-dimensional gel of frog (*Rana*) cornea on a 10% gel. 14 spots were analyzed by MALDI-TOF/TOF. Protein identifications of these spots are presented in Table 2.

amphibian cornea too. Interestingly, we found several taxon-specific lens crystallins such as τ -crystallin/ α - or β -enolase in the toad cornea (Table 1). This taxon-specific lens crystallin is also corneal crystallin in several species (6, 19, 22, 37, 39), including *Bufo* lens (Fig. 3). Three spots of different pI were identified for τ -crystallin/ α -enolase, suggesting the presence of alternatively spliced or post-translationally modified forms (Fig. 2). Such isoforms of τ -crystallin have also been seen in crocodile cornea (14) as well as in *Bufo* lens. Our data demonstrate that toad cornea not only expresses lens α -, β -, and γ -crystallins but some taxon-specific lens crystallins too. We have also identified some proteins in the toad cornea, such as albumin and calgizzarin. However, we have not verified their identification by another method. It is interesting to note that this protein has not been reported in the corneas of bovine, crocodile, fish, and chicken. The function of these proteins in anuran cornea is yet to be ascertained. At this stage, we do not propose to classify them as corneal crystallins.

Western Blot and RT-PCR Analysis—We further confirmed by Western blot and RT-PCR analysis that *Bufo* and *Rana* corneas express ubiquitous lens crystallins. The proteins of the total corneal homogenate were resolved on SDS-PAGE. Using properly validated antibodies the expression of α B-, β B2-, and γ -crystallins in anuran cornea was confirmed by Western blot (Fig. 5A). The observed comparable intensities in the Western blot seen in the case of β B2- and γ -crystallins (Fig. 5A, lanes 3–5) could be due to underestimation of corneal proteins, because many of these proteins have no or little aromatic amino acids. We also used the RT-PCR strategy for confirmation of the expression of a few selected crystallins. Gene-specific primers were designed from different exons to avoid any amplification from genomic DNA contamination. Using gene-specific primers for α A, β B2, β A1, and γ -crystallins, we have demonstrated that transcripts for all these crystallins are present in the cornea (Fig. 5B), thus confirming the results of our proteomics analysis.

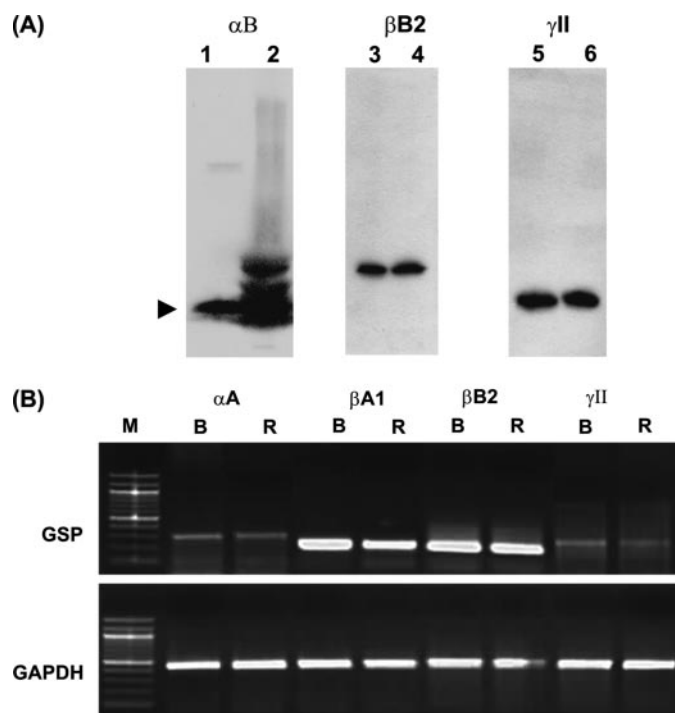


FIGURE 5. A, Western blot showing the presence of α B-crystallin in total corneal extract of toad cornea. Lane 1, *Bufo* total corneal extract (arrowhead indicates α B-crystallin). Lane 2, toad lens homogenate. Lanes 3 and 4, toad cornea and lens probed by β B2-crystallin antibody. Lanes 5 and 6, toad cornea and lens probed by γ -crystallin antibody. B, RT-PCR of the total corneal RNA using the gene-specific primers (GSP) for α A, β A1, β B2, and γ -crystallins. B, *Bufo* cornea, R, *Rana* cornea. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control.

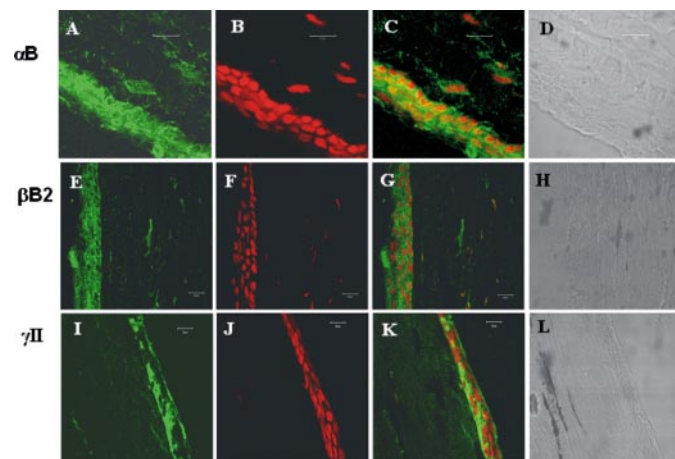


FIGURE 6. Immunofluorescence staining for α B-, β B2-, and γ -crystallins with mouse polyclonal antibodies. Antibody for α B was raised against a specific peptide selected from the sequence and against recombinant bovine β B2-crystallin and γ -crystallin. A, α B-crystallin. B, propidium iodide staining for nucleus. C, overlap of panels A and B. D, phase contrast image of cornea section. E, β B2-crystallin. F, propidium iodide staining for nucleus. G, overlap of panels E and F. H, phase contrast image of cornea section. I, γ -crystallin. J, propidium iodide staining for nucleus. K, overlap of panels I and J. L, phase contrast of corneal section. Scale bar, 20 μ m.

α B-, β B2-, and γ -Crystallins Localize in Corneal Epithelial Layers—As cornea has several distinct layers (Fig. 1), we probed the localization of these crystallins, selecting a crystallin from each group (α B, β B2, and γ -crystallins). Using properly validated antibodies for specificity against these crystallins, we performed the immunofluorescence localization of these proteins

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in an adult toad cornea. As seen in Fig. 6, all three crystallins are localized largely in the corneal epithelium (outer cornea). However, low levels of expression of these proteins are also seen in the corneal stroma. The tissue specimens were also stained with secondary antibody, leaving primary antibody to check the specificity. As expected, we did not observe any staining in these samples, indicating that there is no artifact due to non-specific staining (data not shown). These results are consistent with earlier studies in which high accumulation of several proteins has been reported in the epithelial cell layers in cornea (12). It has been considered a characteristic for defining a corneal crystallin (9, 15, 17).

Ubiquitous Crystallins as Crystallins in Lens and Cornea of Anurans—Accumulation of enzymes and proteins in the corneas of several taxa such as cephalopods, fish, crocodiles, and mammals raises the question as to whether the abundant corneal proteins in these species can logically be considered as crystallins (15–17). Some invertebrates (e.g. squid and scallop) have high concentrations of glutathione *S*-transferase and aldehyde dehydrogenase in their corneas that are also lens crystallins (21, 22). τ -Crystallin/ α -enolase is a known example of a lens and the corneal crystallin in some species (6, 14, 40, 41). The accumulation of ubiquitous lens α -, β -, and γ -crystallins in a cornea has never been reported. However, expression of γ - and α B-crystallins in some corneas could be detected by Western blot or PCR only, suggesting their weak or basal level expression (42, 43). Expression of α B-crystallin in a cornea is not surprising, as it is known to express widely in several tissues. Our data demonstrate for the first time that the cornea of a toad and frog has abundant ubiquitous lens α -, β -, and γ -crystallins, expressed largely in the outer cornea or corneal epithelium.

We now present the anuran case where the same crystallins are used in the lens and cornea. This supports the idea that crystallins are essential for the visual functions of the cornea just as they are for the visual functions of the lens, although their precise role in the two tissues is still not known. This new finding about the accumulation of lens crystallins in anurans cornea supports the idea of the “refracton,” that cornea and lens have many similarities and can be considered as a single refractive unit (17). Our data also support the earlier idea that diverse, abundant corneal proteins may have evolved for a crystallin-like role, in addition to their enzymatic or cytoskeletal functions, by a gene-sharing mechanism similar to the lens crystallins (17, 44). It is interesting to note that during evolution most species have recruited a different set of proteins in cornea; only anurans express ubiquitous lens crystallins in cornea.

Possible Implications of Abundant Lens Crystallins in Corneal Transdifferentiation—Our results demonstrate that *Bufo* and *Rana* corneas are rich in lens crystallins, unlike the corneas of many other species studied so far. Interestingly, it is well known that the outer cornea of *Xenopus* transdifferentiates into lens fibers (23–25, 27–29, 45), although we have not been able to analyze the cornea of *Xenopus*. There have also been few reports on the occurrence of corneal transdifferentiation in *Rana* and *Bufo* (25, 38). The mechanism of corneal transdifferentiation to lens is not clearly understood. The prevalence of lens crystallins in anuran cornea is consistent with the lens-forming competence of their cornea, and further studies are

necessary to establish the relationship between the presence of lens crystallins in the cornea and the mechanism of transdifferentiation from cornea to lens. It appears that anuran cornea could partly be a lens even before transdifferentiation, and therefore, the final conversion into lens is relatively easy to accomplish as the pathway for differentiation is already in motion. It is possible that the same crystallin transcripts are used during transdifferentiation of corneas into lens as are used in the development of cornea before transdifferentiation. We propose this as a possibility, and this connection is worthy of further studies in order to understand the mechanism of transdifferentiation, which would have significant clinical implications in regenerative biology.

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