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Taxon-specific recruitment of enzymes as major soluble proteins in the corneal epithelium of three mammals, chicken, and squid

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ABSTRACT **Studies of others have shown that class 3 aldehyde dehydrogenase is a major component of the epithelial cells of the mammalian cornea. Here we demonstrate by peptide sequencing that other major proteins of the corneal epithelium are also identical or related to enzymes in the human, mouse, kangaroo, chicken, and squid. Aldehyde dehydrogenase class 3 was found to be the major protein of human, mouse, and kangaroo corneal epithelial cells. Peptidyl prolyl cis–trans isomerase (cyclophilin) or a homologue thereof is strikingly abundant in the corneal epithelial cells of chicken, but not mammals, and appears to be absent from the cornea of squid. By contrast, enolase or its homologue is relatively abundant in both the mammalian and chicken corneal epithelial cells. In some instances, abundant enzymes are common to cornea and lens in the same species—for example, argininosuccinate lyase/δ1-crystallin in the chicken and glutathione *S*-transferase-like protein in the squid; in other cases, the abundant proteins in the cornea have not been found as lens crystallins in any species—for example, aldehyde dehydrogenase class 3 and cyclophilin. These data suggest that enzymes and certain enzyme-crystallins have been recruited as major corneal proteins in a taxon-specific manner and may serve structural rather than, or as well as, enzymatic roles in corneal epithelial cells.**

The cornea and lens are both transparent structures responsible for refraction in the eye. The lens is composed entirely of anterior cuboidal epithelial cells layered on a posterior array of fiber cells. The optical properties of the lens are associated with the accumulation of a diverse group of soluble proteins called crystallins (1), many of which have been recruited from metabolic enzymes or stress proteins (2–5) by a process called gene sharing (6, 7). There appears to have been considerable neutrality in the recruitment of crystallins, since they often differ among species in a taxon-specific fashion, even when the different species appear to have common visual requirements (2, 8).

In contrast to the lens, the cornea has an external epithelial layer, a central stroma enriched in collagen and other macromolecules, and an internal single-cell-thick endothelial layer regulating water and electrolyte balance (9). Although the stroma makes up the bulk of the cornea and is primarily responsible for its physical properties, it is possible that the thinner epithelial layer on the anterior surface of the cornea requires a sufficiently high concentration of structural proteins to minimize concentration fluctuations so that transparency will be assured (10). In this connection, we have been impressed by the fact that the tumor-inducible (class 3) aldehyde dehydrogenase (ALDH; EC 1.2.1.3) isoenzyme (11, 12) is a major component of the cornea in the rat (13), opossum (14), cow (15, 16), and baboon (17). Although the reasons for the striking accumulation of this enzyme in the cornea are not established, it appears reminiscent of the recruitment of enzymes in the lens to act as crystallins (see above). We reasoned that if the cornea recruited enzymes as structural proteins, as did the lens, other major proteins of the corneal epithelium might also be identical or related to enzymes and that the recruited enzymes might differ among species as do the enzyme-crystallins. We show here that major proteins of the corneal epithelium of three mammals (mouse, kangaroo, and human), a bird (chicken), and an invertebrate (squid) are in fact identical, or related, to enzymes or to proteins homologous to enzymes. In addition, as with the lens enzyme-crystallins, the distribution of these major corneal epithelial cell proteins is taxon-specific. It thus appears that the concept of gene sharing may extend to the corneal epithelium.

MATERIALS AND METHODS

SDS/Polyacrylamide Gel Electrophoresis (SDS/PAGE). Corneas from 18-day embryonic White Leghorn chickens (obtained from Truslow Farms, Chestertown, MD) were dissected anterior to the limbus. Under a dissecting microscope, a fresh blade was used to scrape either the epithelium or the endothelium from each cornea. Protein samples were then prepared by gentle homogenization with a few strokes of a Teflon pestle in 1% SDS/1% 2-mercaptoethanol from whole cornea, corneal epithelium, corneal endothelium, whole cornea minus epithelium, and whole cornea minus endothelium. After heating to 95°C for 10 min, the extract of protein equivalent to half a cornea was loaded per lane and examined by SDS/PAGE (Fig. 1).

Based on our finding that gentle homogenization of whole corneas by this technique gave an extract predominantly of proteins from the corneal epithelium, we then took corneas from 18-day embryonic White Leghorn chickens, 3-week-old mice (NIH, outbred), and adult humans and kangaroos (*Macropus fuliginosus*), dissected them anterior to the limbus, and briefly homogenized them as above. After heating to 95°C for 10 min, ≈5 μg of protein was loaded per lane and examined by SDS/PAGE.

Western Immunoblot Analysis. The extracted corneal proteins were electroblotted from the SDS/polyacrylamide gel onto nitrocellulose and incubated with polyclonal antibodies to duck δ-crystallin (8) and lamprey τ-crystallin (18), after a dilution of 1:2000 and 1:200, respectively. The Western blots were developed using standard techniques (19). The rabbit antiserum was prepared against purified S-III crystallin of the squid *Nototodarus gouldi* (20) and was diluted 1:2500 before use.

Peptide Sequencing. Major proteins of the corneal epithelial cells (indicated in Fig. 2) electroblotted to nitrocellulose membranes were subjected to *in situ* digestion with trypsin as described (21). The resulting peptides were separated by HPLC and optimal fractions were submitted to automated Edman degradation as described (22) (performed as a service by Harvard Microchemistry, Cambridge, MA). Comparisons

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Abbreviation: ALDH, aldehyde dehydrogenase.

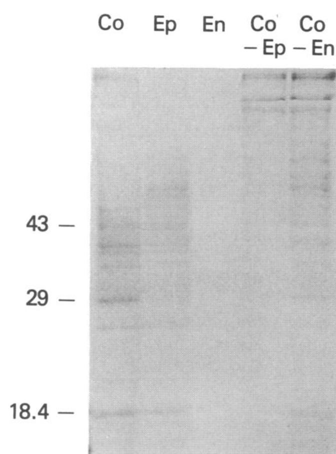

FIG. 1. Coomassie blue-stained SDS/10% polyacrylamide gel of soluble corneal proteins. Proteins were extracted by gentle homogenization of whole cornea (Co), corneal epithelium (Ep), corneal endothelium (En), whole cornea minus epithelium (Co-Ep), and whole cornea minus endothelium (Co-En) from 17-day chicken embryos. Proteins equivalent to one-half of a cornea were loaded per lane. The major proteins present in the whole cornea sample prepared by this method appear similar to those present in the isolated epithelial cells. The corneal endothelium, presumably due to its small relative volume, contributes little soluble protein using this procedure. As might be expected, the whole cornea denuded of epithelium (Co-Ep) shows only faint bands, which are much more intense in the epithelial sample (Ep), whereas the Co-En sample (which contains the epithelium) looks similar to that derived from the isolated epithelial cells (Ep). As expected, in both the Co-Ep and the Co-En lanes, bands of higher molecular mass proteins are visible that correspond to the collagens and other macromolecular components that were extracted by the gentle homogenization of the denuded corneal stroma. Molecular masses (in kDa) are indicated.

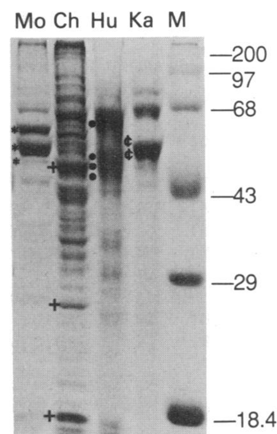

FIG. 2. Coomassie blue-stained SDS/10% polyacrylamide gel of soluble corneal proteins. Mo, 3-week-old mouse; Ch, 18-day embryonic chicken; Hu, adult human; Ka, adult kangaroo; M, protein size markers. The symbols mark regions of the gel from which, after blotting, peptide sequence was obtained. Bands: *, mouse; +, chicken; •, human; ¢, kangaroo. In some cases, more than one peptide was obtained per region, explaining why more peptides are listed in Fig. 4 than are shown in this figure. The origin of any peptide in Fig. 4 may be identified by noting the approximate molecular mass of the protein and its species of origin. Molecular masses (in kDa) are indicated.

were made using the TFASTA Version 7.0 GCG program (23) searching the translated total GenBank data base (Release 67.0).

RESULTS

We have been able to show that our method of gentle homogenization and extraction of whole dissected cornea yielded primarily proteins from the corneal epithelium. This is illustrated in Fig. 1. A similar result was obtained in another experiment with adult human cornea (data not shown). We thus conclude that the proteins studied by Western blot analysis and peptide sequencing in the present experiments are predominantly found in the corneal epithelial cells.

In our initial survey of the proteins of the corneal epithelial cells, the overall SDS/PAGE patterns were similar in the mouse, human, and kangaroo, whereas those of the embryonic and adult (data not shown) chicken were different and more diverse (Fig. 2). Immunoblot analysis showed that argininosuccinate lyase (EC 4.3.2.1)/δ-crystallin and α-enolase (EC 4.2.1.11)/τ-crystallin, both enzyme-crystallins in the lens (2, 3), were present in the chicken corneal epithelium (Fig. 3). δ-Crystallin has been detected by immunological criteria in the chicken cornea (24, 25). We show here by peptide sequencing the presence of the δ1 polypeptide in the embryonic chicken cornea (Fig. 4). The chicken δ-crystallin corneal peptide is also compared to the duck δ-crystallins in Fig. 4 to emphasize its similarity to the δ1 rather than the δ2 polypeptide. Sequencing of the δ-crystallin band from adult chicken cornea gave the same result (data not shown). Another prominent band, which by densitometric scanning represented about 10% of the soluble protein in the corneal epithelium of the embryonic chicken (Fig. 2) and about 5% of the protein in corneal epithelial cells of the adult chicken (data not shown), migrated with a molecular mass of about 18 kDa. Peptide sequencing indicated that this chicken corneal protein was homologous to type A peptidyl prolyl cis–trans isomerase, also known as cyclophilin (30) (Fig. 4). The relatively smaller amount of 18-kDa protein in the mammalian corneas is probably also cyclophilin or a cyclophilin homologue; however, this requires verification by sequencing. Still another abundant protein in the chicken corneal epithelium, which migrated with a molecular mass of about 27 kDa, had two peptide sequences that were very similar to chicken and human class alpha glutathione S-transferase (EC 2.5.1.18) (Fig. 4) (31). This protein also appears quite prevalent in the corneal stroma (Fig. 1).

As expected from previous studies (11–17, 32), peptide sequencing indicated that the abundant 54-kDa proteins in the corneal epithelial cells of the three mammalian species that we examined are all identical to, or homologues of, class 3

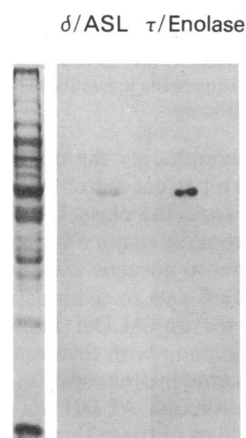

FIG. 3. Western immunoblot analysis of chicken soluble corneal proteins showing the presence of both δ-crystallin/argininosuccinate lyase (ASL) and τ-crystallin/α-enolase in the corneal extract. Approximately 5 μg of protein was loaded per lane. The left lane shows the Coomassie blue-stained proteins, and the two right lanes show the immunoblots of the total proteins after transfer of parallel lanes onto nitrocellulose filters.

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A

HUMAN

48 kD HCP                   YISPDQLADLYK
α-enolase           (H)     YISPDQLADLYK (a)
α-enol/τ-cry        (D)     YISPDQLADLYK (b)

52 kD HCP                   SLEDSLAEAEGDYCAQLSQVQQLIS
Acidic Ker 10       (H)     SLEASLAETEGRYCVQLSQIQAQIS (c)
Ker Type 1          (H)     SLENSLEETKGRYCMQLSQIQGLIG (d)
Epiderm Ker         (M)     SLEGSLAETENRYCVQLSQIQGLIG (e)

54 kD HCP1                        TPQTQQDELYIHSEXLGVVLVIG
ALDH III            (H)     DEPVEKTPQTQQ                    (f)
ALDH III            (R)     DEPVAKTRQTQQDDLYIHSEPLGVVLVIG   (g)

54 kD HCP2                  NEXNAYYEEVVYVLEWIEYMIQK
ALDH III            (R)     NEWTSYYEEVAHVLEELDTTIKE (g)

65 kD HCP                   ILATPPQEDAPSVDIANIR

MOUSE

48 kD MCP                   FVQNYPVVSIEDPFDQ
 -enolase           (H)     FVRDYPVVSIEDPFDQ (h)
α-enol/τ-cry        (D)     FVKNYPVVSIEDPFDQ (b)
β-enolase           (M)     FIKNYPVVSIEDPFDQ (i)
α-enolase           (H)     FIKDYPVVSIEDPFDQ (a)

54 kD MCP                   EKPLALYVFSNNDK
ALDH III            (R)     EKPLALYVFSNNEK (g)
ALDH I              (R)     PYGLAAGVFTKDLD (j)
ALDH II             (R)     KYGLAAAVFTKDLD (k)
ALDH I              (H)     FYGLSAGVFTKDID (l)
ALDH II             (H)     TYGLAAAVFTKDLD (m)

65 kD MCP                   ILATPPQEDAPSVDIANIR

B

KANGAROO

54 kD KCP1                  TAILLATLLPQYLDKDLFPVINGGVPETTELL
ALDH III            (R)     MADLLATLIPQYMDQNLYLVVKGGVPETTELL (g)

54 kD KCP2                  HLTPVTLELGGKNPCYIDKXCDLDIACR
ALDH III            (R)     HLTPVTLELGGKSPCYVDKDCDLDVACR (g)

57 kD KCP1                  KSFEDTLAETESGYCNQLSQ
Epiderm Ker V       (B)     QSLEASLAETEGRYCVQLSQ (n)
Epiderm Ker I       (M)     QSLEASLAETEGRYCVQLSQ (o)

57 kD KCP2                  NMQDTVEDFKNKYEE
Type II Ker         (M)     MMQDSVEDFKTKYEE (p)
Cytokeratin 4       (H)     TMQDSVEDFKTKYEE (q)

CHICKEN

18 kD CCP                   VTFELFADKVPITAENF
Cyclophilin A       (H)     VSFELFADKVPKTAENF (r)
Cyclophilin A       (R)     VCFELFADKVPKTAENF (s)
Cyclophilin B       (H)     VIFGLFGKTVPKTVDNF (t)

27 kD CCP1                  YFPVFEK
GST                 (H)     YFPAFEK (u)

27 kD CCP2                  QHGQDFLVGNR
GST                 (C)     DHGQDFLVGNR (v)

48 kD CCP1                  FVGSTDPIME
δ1-cry              (C)     FVGSTDPIME (w)
δ2-cry              (C)     FSGSTDPIME (x)
δ1-cry              (D)     FVGSTDPIMQ (y)
δ2-cry              (D)     FSGSTPPIME (z)

48 kD CCP2                  SISPLFASDVSQVFSVVNSVEQYXAV
δ1-cry              (C)     SISPLFASDVSQVFSVVNSVEQYTAV (w)
δ2-cry              (C)     SISPLFASDVSQVFNIVNSVEQYTAV (x)

48 kD CCP3                  LAMQEFMILPVGADNFK
Enolase             (X)     LAMQEFMILPVGADSFK (aa)
α-enolase           (H)     LAMQEFMILPVGAANFR (a)
α-enol/τ-cry        (D)     LAMQEFMIPPCGADSFK (b)
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FIG. 4. Comparison of corneal peptide sequences to known GenBank (Release 67.0) sequences. Note the identical sequences from the 65-kDa protein in both human and mouse; no homologues were found for these sequences in GenBank. The original protein bands are identified in Fig. 2 by symbols unique to each species. In each case we have identified the protein by its approximate molecular mass and the species—e.g., 48-kDa mouse corneal protein. HCP, MCP, KCP, and CCP stand for human, mouse, kangaroo, and chicken corneal proteins, respectively. CCP1, -2, and -3 were all derived from the 48-kDa doublet indicated by the cross in Fig. 2, lane Ch. GenBank accession names are indicated as follows, where the numbers that follow in parentheses are the amino acid positions when the entire entry is translated: a, HUMENOA (301–312); b, DUKCRYT (279–290); c, HUMKRT10A (259–283); d, HUMKERIA6 (11–35); e, MUSKTEPIC (95–119); f, ref. 26 (83–94); g, RATALD (HPC1 147–169; HPC2 112–134; KPC1 203–234; KPC2 260–287); h, HUMENOGA (257–272); i, MUSENOB (225–240); j, ref. 27 (425–438); k, ref. 28 (424–437); l, ref. 27 (425–438); m, ref. 27 (425–438); n, BOVKERVIC (1281–1300); o, MUSKTEPI2 (468–486); p, MUSKER57R (263–276); q, HUMKERC4 (103–117); r, HUMCYCR (24–40); s, RATCYCA (34–42); t, M60857 (52–68); u, HUMGST2 (150–156); v, CHKCL3 (150–160); w, CHKCRYD1 (521–530); x, ref. 29 (CD2, 14–23); y, DUKCRYD3A (235–244); z, ref. 29 (DD2, 14–23); aa, XELENOL (174–190). Species of comparison peptide sequences are as follows: B, bovine; C, chicken; D, duck; H, human; M, mouse; R, rat; X, *Xenopus*. References to names of species are in parentheses.

ALDH (Fig. 4). Although only the peptide from the mouse 54-kDa protein gave a perfect match with the corresponding published sequence from the class 3 ALDH of rat (11), the peptide sequences from the major 54-kDa soluble proteins in the human and kangaroo corneas were very similar both to the rat class 3 ALDH and to a human peptide sequence recently published for an ALDH isozyme isolated from stomach (26), in agreement with the conservation of ALDH isozyme sequences found in other species (33). It is important to underscore that although ALDH was the major soluble protein in the epithelium of these three mammalian corneas, it was not prominent in the chicken cornea. This is consistent with the preliminary report indicating that ALDH is limited to the mammalian cornea (34).

In contrast to the taxon-specific expression of ALDH in mammalian corneas, peptide sequencing established that the 48-kDa enolase-like polypeptide was present in the corneal epithelium of human, mouse, and chicken (Fig. 4). Our sequence data suggest that corneal enolase is probably the α-isoenzyme that is expressed in many different tissues. This is supported by a recent report of peptide sequencing of α-enolase from rabbit cornea (35). Further experiments are required to determine the presence or abundance of enolase in the kangaroo cornea. Several other prominent soluble protein bands were examined by peptide sequencing among the mammalian corneas. These included a protein of approximately 52 kDa that may be a member of the keratin family in humans and kangaroos, and a peptide, from an unidentified 65-kDa protein, that was identical in human and mouse corneas (Fig. 4).

Finally, we examined the squid to determine whether the cornea from an invertebrate also contains a few predominant soluble proteins identical or related to metabolic enzymes. The squid was of particular interest since its eye is a prototype of convergent evolution (36), its lens crystallins have been shown to consist of one major family of proteins (20, 37)

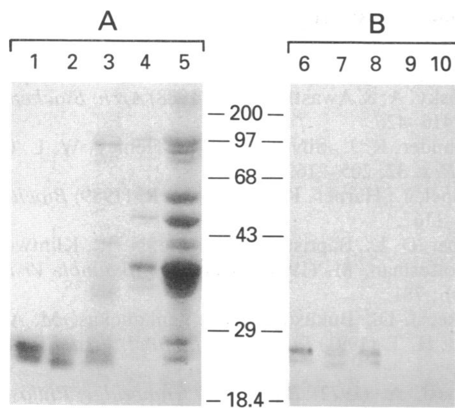

FIG. 5. Immunoblot analysis of squid soluble proteins demonstrating the major glutathionine *S*-transferase-like bands in cornea and lens. Lanes 1–5 are ponceau S-stained proteins, and lanes 6–10 are a peroxidase-stained immunoblot. Lanes: 1 and 6, lens from *Ommastrephes sloani pacificus*; 2 and 7, lens from *Sepioteuthis lessioniana*; 3 and 8, cornea from *Sepioteuthis lessioniana*; 4 and 9, muscle from *Ommastrephes sloani pacificus*; 5 and 10, muscle from *Ommastrephes sloani pacificus*. Protein (5 μg) was loaded on lanes 1–4 and 6–9; 50 μg of protein was loaded on lanes 5 and 10. Molecular masses (in kDa) are indicated.

related to glutathionine *S*-transferase (38, 39), and the ectodermal origin of the squid cornea is very different from its lens (40). Surprisingly, SDS/PAGE showed that the pattern of proteins in the squid cornea was essentially the same as that in the lens, with a few 27-kDa bands accounting for most of the soluble proteins in both cornea and lens (Fig. 5). Immunoblots showed that both the 27-kDa lens and corneal proteins reacted strongly with an antiserum developed previously from purified SIII-crystallin of the squid, whereas essentially none of the equivalent squid proteins from skeletal muscle reacted with the antiserum (Fig. 5).

DISCUSSION

The parallels between proteins identical or related to enzymes that are expressed as major constituents of the corneal epithelium and lens suggest that, as in the lens (1, 2), ubiquitously expressed enzymes have been recruited for their structural properties rather than their catalytic function *per se* in the corneal epithelial cells. This does not exclude them also serving enzymatic (or other) functions in the cornea, although the abundance of some of the corneal epithelial enzymes appears to exceed what might be expected for strictly enzymatic needs. This has been already noted for the corneas of the rat (12, 13), cow (15, 16), opossum (14), and baboon (17), where class 3 ALDH makes up between 5% (baboon) and 40% (bovine) of the soluble protein. It has been suggested that the high concentration of baboon ALDH may function to protect the cornea by absorption of UV irradiation and by oxidizing aldehydes generated by light-induced peroxidation (17). Interestingly, class 1 (cytosolic) ALDH and an unidentified isoenzyme of ALDH are used as lens crystallins in elephant shrews (41) and octopus (42), respectively.

The protein identical or related to cyclophilin in the chicken cornea is another example where its concentration is higher than can be easily explained by a possible enzymatic role in protein folding (43), although we cannot exclude that it (as well as other enzyme-related corneal proteins) may be fulfilling important metabolic roles in the epithelial cells. Indeed, previous highest estimates of cyclophilin concentration in tissues have ranged from 0.1 to 0.4% of the soluble protein (44), in contrast to our finding that it is approximately 10% of the soluble protein in the epithelial cells of the embryonic chicken cornea. Although α-enolase or its homologue appears to be in lower concentration than ALDH or the cyclophilin-like polypeptide, it still is a prominent protein of both chicken and mammalian corneal epithelial cells, reminiscent of the structural role played by α-enolase/τ-crystallin in the lenses of various species (2, 3).

Another indication that some of the major corneal epithelial enzymes, or enzyme-related proteins, serve a structural role is the abundance of δ1-crystallin. Of the two extremely similar chicken δ-crystallin genes, δ1 is specialized for lens expression, where it acts as a structural crystallin, whereas δ2 is preferentially expressed in nonlens tissues and encodes the active metabolic enzyme argininosuccinate lyase (6, 45, 46). Our finding of δ1-crystallin peptides in the corneal epithelial cells, rather than δ2-crystallin peptides, is consistent with the hypothesis that corneal δ-crystallin is fulfilling a structural, not an enzymatic, role. The actual ratio of the two δ-crystallin polypeptides in the cornea remains to be determined.

Finally, whereas some of the abundant proteins in the corneal epithelial cells are similar to those used as lens enzyme-crystallins (such as δ1-crystallin and α-enolase in the chicken), others are entirely different gene products than lens enzyme-crystallins (for example, mammalian class 3 ALDH and chicken cyclophilin), suggesting that these abundant proteins were recruited separately in lens and corneal epithelial cells. This appears to be true even in the squid, which has a very similar protein profile in the lens and cornea, since the lens and cornea are derived from different ectodermal tissues in this species (40).

Although different metabolic requirements may be responsible for the accumulation of the different enzymes or enzyme-related proteins in the corneal epithelial cells of mammals, chicken, and squid, it is also possible that different soluble proteins are able to satisfy the refractive needs of the corneal epithelium and that a considerable element of neutrality exists in the choice of which enzymes are used for this purpose.

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