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Metamorphic change in EP37 expression: members of the βγ-crystallin superfamily in newt

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Abstract EP37 is an epidermis-specific protein found in the developing embryo of the Japanese newt, *Cynops pyrrhogaster*. Our previous study predicted the presence of genes homologous to EP37, which show temporary shared expression at the turn of metamorphosis. In this study, we isolated and characterized three cDNAs encoding novel EP37 homologues; two from the skin of an adult newt and the other from swimming larva. Conceptual translation of the open reading frames of these cDNAs predicted proteins carrying βγ-crystallin motifs and putative calcium-binding sites, both of which are features shared by the originally identified EP37 (EP37L1), as well as a spore coat protein of *Myxococcus xanthus*, protein S. Immunoblot analyses and immunohistochemical studies indicated that two of the EP37 proteins, EP37L1 and EP37L2, are exclusively expressed in the epidermis (skein cells) including the figures of Eberth at premetamorphic stages. During and after metamorphosis, the expression of EP37 proteins was mainly observed in cutaneous glands, and a molecular transition to the adult types of EP37, EP37A1 and EP37A2, occurred. These observations suggest that EP37 proteins play an important role in construction of integumental tissues and adaptation to the aquatic or amphibious environment.

Key words Epidermis · Newt · Metamorphosis · βγ-crystallin superfamily · Cornea

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Introduction

The epidermis-specific protein, EP37L1, was originally identified as one of the eight embryonic epidermal proteins of the Japanese newt, *Cynops pyrrhogaster*, by two-dimensional gel electrophoresis (Takabatake et al. 1991). The cDNA, *ep*37L1, has been isolated and sequenced, with the predicted amino acid sequence showing slight similarity to β- and γ-crystallins (Takabatake et al. 1992). The β- and γ-crystallins are structurally stable proteins, including four Greek key motifs (Blundell et al. 1981). A killer toxin from *Williopsis mraki* is a predicted βγ-crystallin precursor structure since it has two Greek key motifs (Antuch et al. 1996). Recently Wistow et al. (1995), developing further on our previous findings (Takabatake et al. 1992), concluded that EP37L1 (which Wistow et al. named EDSP in their paper) is a novel non-lens member of the βγ-crystallin superfamily in vertebrates. The β- and γ-crystallins are evolutionarily related to stress-inducible proteins (Wistow and Piatigorsky 1988; de Jong et al. 1989) such as protein S, a sporulation-specific protein of the gram-negative bacterium *Myxococcus xanthus* (Inouye et al. 1983; Wistow 1985), and spherulin 3a, an encystment-specific protein of slime mould *Physarum polycephalum* (Bernier et al. 1987; Wistow 1990). Although proteins of this superfamily, including EP37, may have protective functions against stresses of developmental change (Wistow et al. 1995), little is known about the biological or biochemical role of EP37 protein.

Northern blot analyses using *ep*37L1 cDNA as a probe under low-stringency conditions revealed the presence of five transcripts at different developmental stages of the newt (Takahashi and Takeshima 1995). Two (2.3 and 1.9 kb) were detected at premetamorphic stages, another two (4.0 and 2.9 kb) were detected in adult tissues (skin, eye and tongue), and the other (3.5 kb) was detected in adult stomach. The transition of transcripts from the premetamorphic to adult types occurred during metamorphosis. These observations suggest the existence of an *ep*37 gene family, the temporal and spacial expressions of which are strictly regulated.

In this study, we isolated and characterized three cDNAs homologous to *ep*37 which are expressed in the integumental tissues. Their deduced amino acid sequences showed four putative βγ-crystallin motifs in their N-terminal regions, indicating that the so-called *ep*37 gene family is a sub-family of the βγ-crystallin superfamily. Furthermore, immunohistochemical studies showed drastic changes in the subcellular localization of EP37 proteins during metamorphosis as well as in the course of embryogenesis.

Materials and methods

Biological materials

Fertilized eggs of the Japanese newt, *Cynops pyrrhogaster*, were obtained by injecting human chorionic gonadotropin (Gonatropin; Teikoku Zoki, Tokyo, Japan) into adult females. Embryonic stages were determined according to Okada and Ichikawa (1947).

Isolation of cDNAs homologous to *ep*37

To isolate cDNAs homologous to *ep*37 in the adult newt, we prepared a λgt11 cDNA library from adult skin. Approximately 1×10^5 plaques were screened with a PvuII-HpaI fragment of pG3m11N138-48 cDNA (Takabatake et al. 1992), here designated as *ep*37L1. Eight positive clones were obtained and subcloned into the NotI site of pBluescript II SK(-) (Stratagene). These clones were classified into two types based on their restriction maps. The longest clone of each of the two groups was sequenced and designated as *ep*37A1 (2.7 kb) and *ep*37A2 (3.6 kb) respectively. To isolate homologous cDNA expressed during the larval stages, we used the polymerase chain reaction (PCR) amplification method. One of the primers was designed from the highly conserved nucleotide sequences located upstream of the open reading frame of *ep*37L1, A1 and A2; 5′-CCAG(CG)AGGAGT(CT)C(AT)-C(ACT)(AG)GAAGCC-3′. The other was a modified oligo dT primer; 5′-CCCGGATCCT$_{15}$(ACG)-3′. A template was prepared by synthesizing first strand cDNA from larval RNA using the modified oligo dT as a primer. Two kinds of fragments, 1.7- and 1.9-kb long, were amplified. Whilst the former had an identical nucleotide sequence to *ep*37L1, the latter showed a distinct sequence alignment, and was named *ep*37L2.

Whole mount in situ hybridization

Whole mount in situ hybridization was performed essentially as described by Harland (1991) except that the duration of each step was extended and treatment with proteinase K and RNase omitted. Stained specimens were bleached by placing them in hydrogen peroxide/methanol (10 and 70% respectively) for depigmentation of melanocytes. For thin sections, stained whole embryos were processed by the usual procedures and embedded in paraffin.

Preparation of anti-EP37 antibody

The plasmid encoding the EP37-His$_6$ protein was generated by inserting sequences corresponding to residues 70 to 335 of EP37L1 into the pET22b(+) expression vector (Novagen). Proteins were induced in BL21(DE3) *Escherichia coli* cells and purified by His-Bind Resin (Novagen) in the presence of 6 M urea. Anti-EP37 polyclonal antibody was raised by the subcutaneous injection of these proteins into a Japanese white rabbit. The antibody was affinity purified on a column of GST-EP37 fusion polypeptides, which were expressed from the cloned genes corresponding to either residues 46 to 227 or 230 to 335 of the EP37L1 protein, according to the manufacture's instructions (Pharmacia). These fusion polypeptides were synthesized from a plasmid constructed using a pGEX-3X expression vector (Pharmacia) in DH5α *E. coli* cells. The soluble fusion proteins purified with Glutathione Sepharose 4B (Pharmacia) were immobilized to AF-Trecyl Toyopearl 650 M (Tosoh, Japan) and used as affinity gel media.

Immunoblot analysis

Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed according to Laemmli (1970). Two-dimensional (2D) gel electrophoresis was carried out as described by O'Farrell et al. (1977). After electrophoresis, the proteins were transferred onto polyvinyliden difluoride (PVDF) membranes (Millipore) by electroblotting. The membranes were incubated in a blocking solution (Block Ace; Yukijirushi Nyugyo, Japan) and reacted with a 1:500 dilution of purified anti-EP37 antibody in 10% blocking solution containing 0.05% Tween 20 for 60 min at room temperature. After washing with phosphate-buffered saline (PBS) containing 0.05% Tween 20, the membranes were incubated with a 1:1000 dilution of horseradish-peroxidase-conjugated goat anti-rabbit IgG (Biosource International). Immunodetection was performed using chemiluminescence substrates (ECL™ Western blotting detection reagents, Amersham) according to the manufacture's instructions.

Immunohistochemistry

Specimens were fixed in fresh 4% paraformaldehyde in PBS for 12 h at 4°C, dehydrated and embedded in paraffin. Sections (6 μm) were incubated for 15 min with blocking solution (1% bovine serum albumin in PBS containing 0.02% Tween 20) and then for 60 min with a 1:2 dilution of purified anti-EP37 antibody in blocking solution. Subsequently, sections were incubated for 60 min with a 1:100 dilution of fluorescein isotriocyanate (FITC)-conjugated goat anti-rabbit IgG (Biosource International).

Results

Isolation and characterization of cDNAs homologous to *ep*37L1

We isolated three cDNAs homologous to *ep*37L1 (*ep*37A1, *ep*37A2 and *ep*37L2) and determined their complete nucleotide sequences. Their sizes (in nucleotides) and GenBank accession numbers are as follows: *ep*37A1: 2629nt., D84229; *ep*37A2: 3580nt., D84230; *ep*37L2: 1907nt., D84231. The open reading frames of newly isolated cDNAs (335 or 337 codons) were essentially the same length as that of *ep*37L1 (335 codons). Comparisons of nucleotide sequences indicated that these four genes share 77.5–97.6% sequence identity in the protein coding region (data not shown). Differences in primary structure indicated that these cDNAs (*ep*37L1, L2, A1 and A2) are not generated by alternative splicing, but are transcribed from independent genes. Alignment of the predicted amino acid sequences (Fig. 1) indicated that EP37A1 and A2 do not show the same level of homology (68%) that L1 and L2 show (97%), whilst L1 has moderate homology both to A1 (83%) and to A2 (74%). We counted more amino acid residues in the N-termini of EP37L2 and A1, but it is possible that the second ATG of each cDNA fits the Kozak consensus sequences better than does the first (Kozak 1991). Based on the deduced amino acid sequences, molecular weight and isoelectric point (pI) were calcu-

lated as follows; EP37L2: 37.7 kDa, pI = 5.63; EP37A1: 37.7 kDa, pI = 5.40; EP37A2: 37.9 kDa, pI = 5.89. Motif Finder (the online service applied by the Human Genome Centre, Institute of Medical Science, The University of Tokyo) identified a consecutive βγ-crystallin motif ([LIVMFYWA] -x- {DEHRKSTP} -[FY] - [DEQHKY] - x(3) - [FY] -×- G - x(4) - [LIVMFCST]) in the N-terminal half of the EP37 proteins. The program found only one motif in EP37L1, but found an additional motif both in EP37L2 and A1, and two additional motifs in EP37A2. Furthermore, it was found that the regions of Ile^{35} to Lys^{39} and Ile^{129} to Arg^{133} in EP37L1 and the corresponding regions in EP37L2, A1 and A2 (shaded boxes in Fig. 1), show a high level of homology to the proposed Ca^{2+}-binding sites in protein S of the gram-negative bacterium *Myxococcus xanthus* (Inouye et al. 1983), a non-lens member of the βγ-crystallin superfamily.

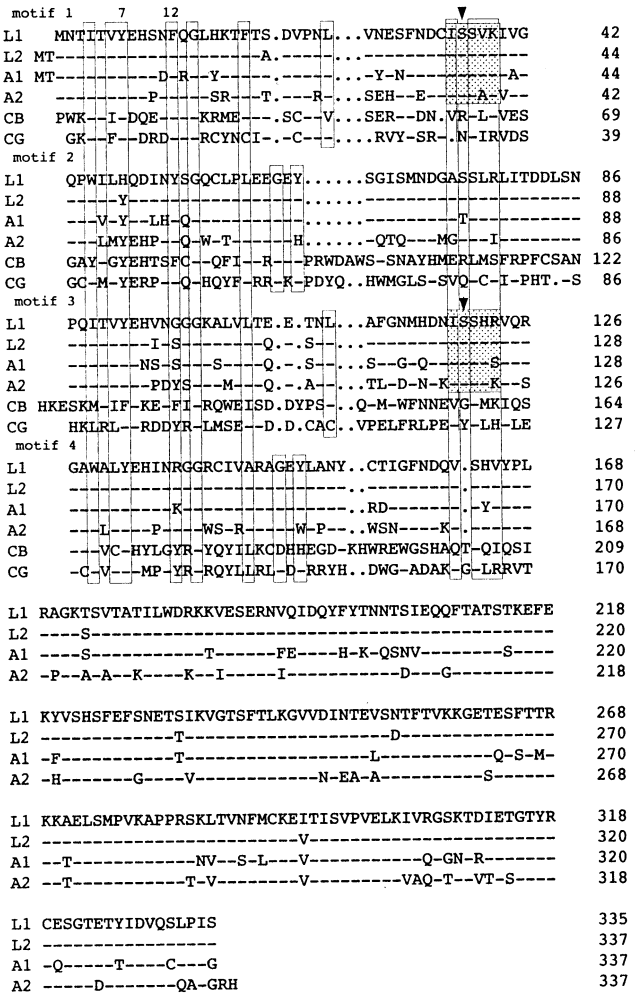


Fig. 1 Comparison of four types of EP37 proteins and βγ-crystallins. The deduced amino-acid sequences are shown in the *single letter code*. Sequences are aligned according to EP37L1 (Takabatake et al. 1992). The βγ-crystallin motifs of EP37 proteins, human β-crystallin A3 (*CB*; Hogg et al. 1986) and human γ-crystallin A (*CG*; Meakin et al. 1987) are aligned according to Wistow et al. (1995). *Shaded boxes* indicate sequences corresponding to putative Ca^{2+}-binding sites of protein S (Inouye et al. 1983). The *serines with an arrowhead* indicate important residues contributing to Ca^{2+}-binding (Teintze et al. 1988). *Open boxes* indicate structurally significant residues for the βγ-crystallin motif (Wistow et al. 1985, 1995)

Specificity of the anti-EP37 antibody

To study the subcellular distribution of EP37 proteins, we generated an affinity-purified rabbit polyclonal antibody against EP37L1 protein. The antibody detected proteins of ~40 kDa, which corresponded to the apparent molecular weight of EP37L1 (Fig. 2A). However, since the immunoreactivity was observed in skin, eye and tongue of the adult (Fig. 2A, lanes 2, 3 and 5), it seems that the antibody recognized other types of EP37 (i.e. EP37A1 and A2). The antibody could not detect protein

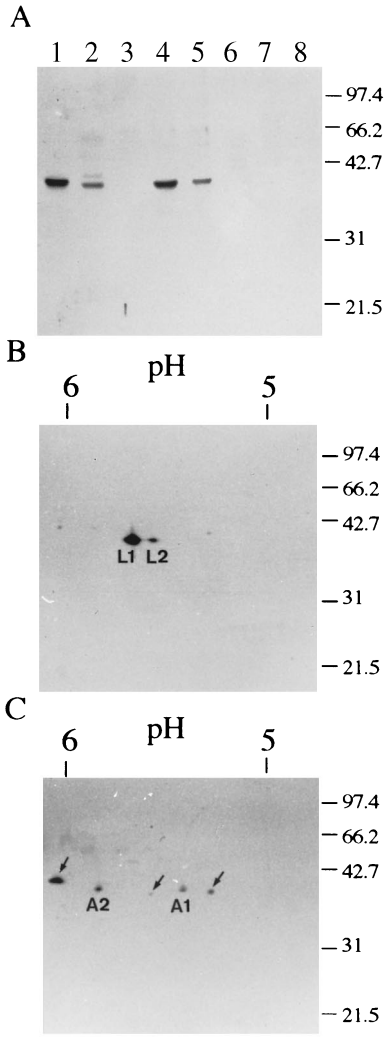


Fig. 2A–C Immunoblot analyses of EP37 proteins. Proteins from swimming larva (**A**; *lane 1*) and adult tissues (**A**; *lane 2–8*) were separated by SDS-PAGE, followed by immunoblot analyses using anti-EP37L1 antibody (*Lanes 1* swimming larva (stage 55), *2* adult skin, *3* adult muscle, *4* adult eye, *5* adult tongue, *6* adult stomach, *7* adult intestine, *8* adult liver). Proteins from swimming larva at stage 55 (**B**) and adult skin (**C**) separated by 2D-PAGE were analysed on immunoblots using anti-EP37L1 antibody. EP37L1, L2, A1 and A2 were designated according to their deduced pI. *Arrows* in panel **C** indicate three spots that reproducibly reacted with anti-EP37L1 antibody

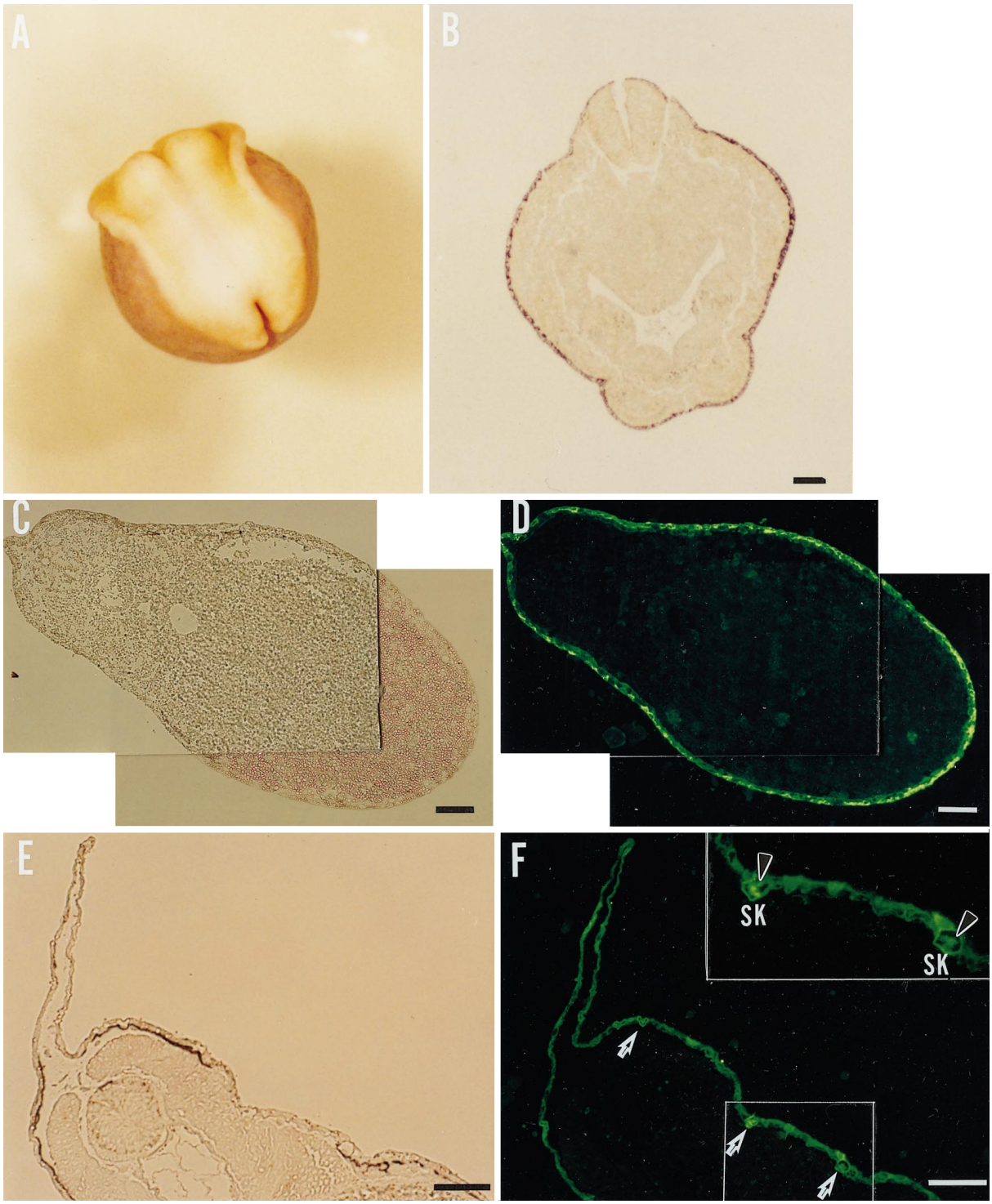
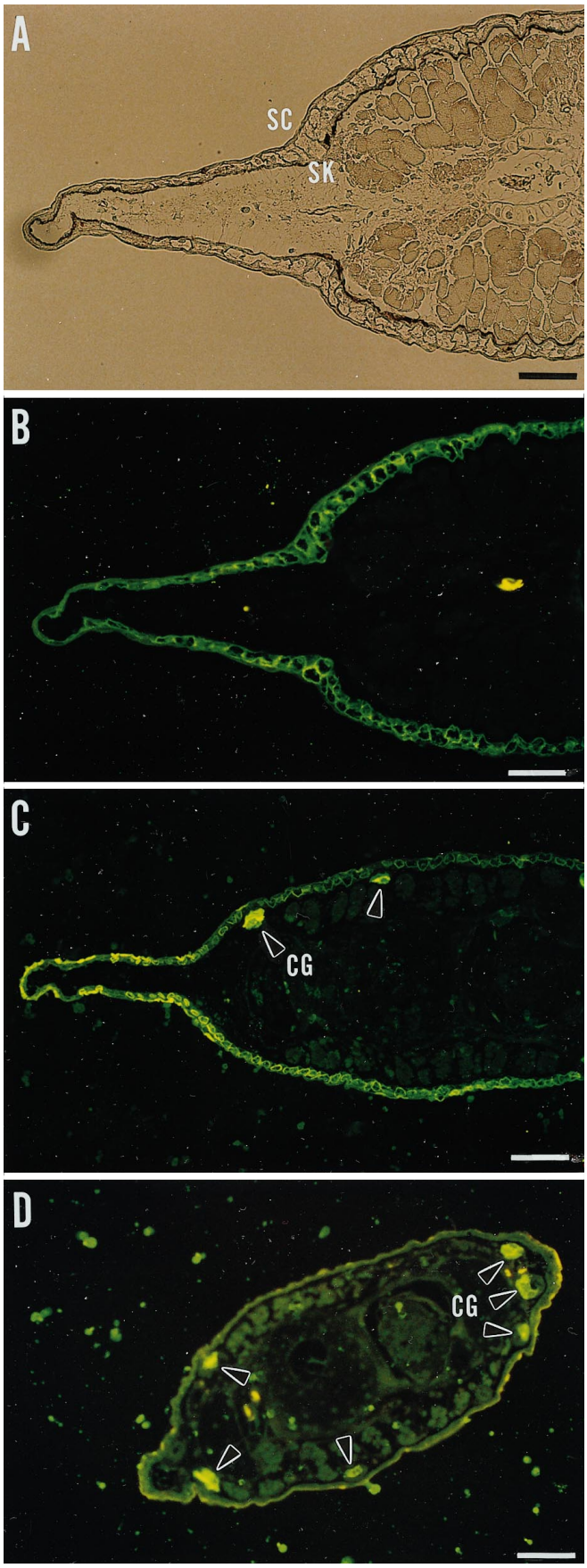


Fig. 3A–F Localization of *ep*37 mRNA and of EP37 proteins during the premetamorphic stages. Expression of *ep*37L1 mRNA in neurula embryo (**A**), as detected by whole mount in situ hybridization using antisense *ep*37L1 probe. **B** Transverse hybridization. **D**, **F** Transverse section of late tail-bud embryo (st. 32) and hatched swimming larva (st. 42) at tail region, probed with the anti-EP37L1 antibody. The sections are shown in bright field (**C** and **E**) and in dark field (**D** and **F**). The *inset* in **F** shows skein cells at a higher magnification (*Arrows* indicate the skein cells, *arrowheads* indicate figures of Eberth, *SK* skein cell, *bar* 100 μm)

in adult stomach, in which previous detection by northern blot analyses of a homologous transcript of *ep*37 occurred only at low stringency (Takahashi and Takeshima 1995). To exclude the possibility of co-existence of embryonic types in adult tissues, we carried out immunodetections against the proteins of adult skin separated by two-dimensional gel electrophoresis. Two proteins with pI values of 5.7 and 5.6 were discerned in the immunoblot of larval proteins (Fig. 2B). They corresponded to EP37L1 and L2, respectively. Five spots with similar



molecular weight but with different pI values (6.2, 5.9, 5.6, 5.4 and 5.3) were detected in adult skin (Fig. 2C). Two spots with pI values of 5.4 and 5.9 corresponded to EP37A1 and A2, respectively. We do not know whether the other three spots, which were reproducibly observed, are additional EP37 proteins or chemically modified versions of EP37A1 and/or A2. However, from the following observations, we deduce that there were no additional types of cDNAs homologous to *ep*37:

1. Only two kinds of transcripts were detected in RNAs of adult skin by northern blot analyses (Takahashi and Takeshima 1995).
2. We could not isolate any other types of *ep*37, other than *ep*37A1 and A2, from the cDNA library of adult skin even when screening under low-stringency conditions.

Expression of the *ep*37 gene in the early embryo

Northern blot analysis of RNA isolated from embryos has demonstrated that *ep*37 transcription begins at the late gastrula stage (st.15; Takabatake et al. 1992). To localize the site of *ep*37 expression in early embryos we performed whole mount in situ hybridization. Staining was detected as early as stage 17 of neural plate formation (Fig. 3A). The *ep*37 gene was expressed exclusively in the epidermal region and not in the cells of the neural plate or neural fold. The transverse section from the tailbud stage embryo (Fig. 3B) also demonstrates the localization of *ep*37 expression in the external surface of the embryo.

Developmental changes in EP37 protein localization

Specific antisera raised against EP37L1 were used to study the subcellullar localization of EP37 proteins during embryogenesis and metamorphosis of the Japanese newt. The presumptive epidermis is one cell-layer thick before the neural plate stage, shortly after which it becomes a bilayered structure. We could not detect immunoreactivity before the early neural plate stage (st. 16), but positive stainings were observed in the surface of the embryos as early as the mid neural plate stage (st. 17). At stage 32, staining in the cytoplasmic region of epidermal cells of the outer layer was stronger than in those cells of the inner layer (Fig. 3D). In the hatched swimming larva (stage 42), well-differentiated epidermal cells such as skein cells were observed (Fig. 3E, F). Figures of Eberth are the most prominent structure in the skein cells

Fig. 4A–D Localization of EP37 proteins during metamorphosis. Transverse sections were probed with anti-EP37L1 antibody. Tail region of stage 59 larva (**A**, bright field and **B**, dark field), stage 60 larva during metamorphosis (**C**) and young adult (**D**). Localization of EP37 proteins changed from skein cells to cutaneous glands during metamorphosis (*Arrowheads* indicate the cutaneous glands, *SC* stratum corneum, *SK* skein cell, *CG* cutaneous gland, *bar* 100 μm)

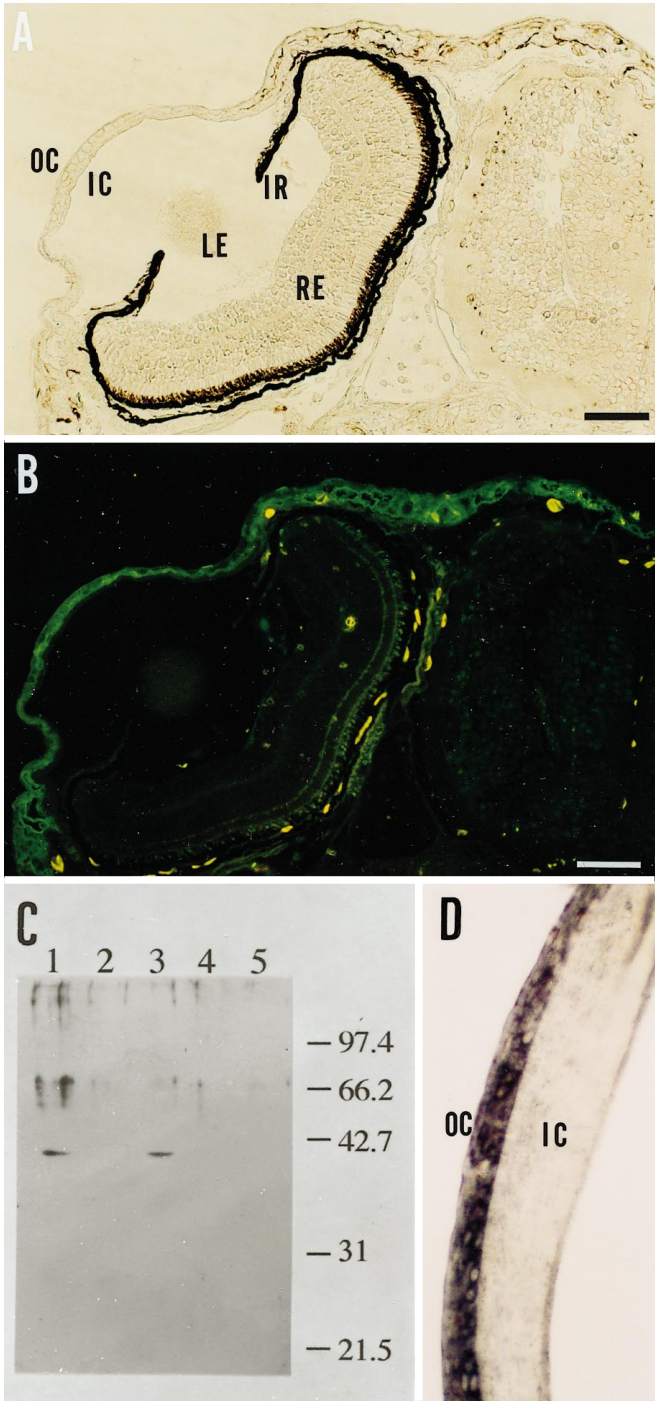


Fig. 5A–D Localization of EP37 proteins and *ep*37A1 mRNA in the eye. Immunostaining of a transverse section of larval eye at stage 59 (**A**, bright field and **B**, dark field). Outer corneas were stained with anti-EP37L1 antibody (*LE* lens, *OC* outer cornea, *IC* inner cornea, *IR* iris, *RE* retina, *bar* 100 μm). Proteins from the individual components of the adult eye were separated by SDS-PAGE and analysed by immunoblotting using anti-EP37L1 antibody (**C**; *Lanes 1* whole eye, *2* lens, *3* cornea, *4* iris, *5* retina). **D** In situ hybridization of a section of adult cornea using DIG-labelled antisense *ep*37A1 probe. Expression of *ep*37A1 was observed in the outer cornea

of the amphibian larval epidermis (Eberth 1866; Weed 1934) and they consist of numerous bundles of tonofilament (cytokeratin intermediate filaments) arranged in a sheaf in the cytoplasmic region (Fox and Whitear 1986). Immunostainings were observed in the skein cells as well as in the outer layer of the epidermis (Fig. 3F). Fine immunohistochemical analyses revealed that, in the skein cells, the plasma membrane and figures of Eberth were positive (insertion of Fig. 3F). In the swimming larva just before metamorphosis (st.59), most of the epidermal cells differentiated into skein cells (Fig. 4A, B), which stained strongly as in the larva at stage 42 (Fig. 3F). At this stage, stratum corneum, which is not recognized by the antibody, covers the outer most surface of the body (Fig. 4B). At the following stage (st. 60), additional strong stainings were observed in cutaneous glands which seemed to differentiate from epidermal cells during early metamorphosis (Fig. 4C, D). Shortly after metamorphosis, the mono-layer of skein cells was replaced with a multi-layer of small cells, which showed only faint immunoreactivity to anti-EP37L1 antibody. However, strong immunostainings remained in cutaneous glands (Fig. 4E).

Localization of EP37 proteins in the eye

Both northern blot (Takahashi and Takeshima 1995) and immunoblot analyses (Fig. 2A) showed that *ep*37(s) are expressed in the eye. We analysed the localization of EP37 proteins in transverse sections of the larval and adult eye by immunohistochemistry. In the larval eye, immunostaining was localized only in the outer cornea (Fig. 5B). In the adult eye, no immunostaining was detected even in the outer cornea (data not shown). However, immunoblot analyses of the dissected eye showed immunoreactivity in the adult cornea (Fig. 5C) and in situ hybridization of adult cornea reinforced that the *ep*37A1 gene was expressed in the outer layer (Fig. 5D). These observations indicated the possibility that EP37 proteins were confined to the well-packed structures of the adult cornea and that the antigenic epitope(s) was masked in adult cornea tissue. In fact, differentiation of the adult cornea is accompanied by compaction and dehydration (Carlson 1988).

Discussion

We have isolated and characterized three cDNAs homologous to the epidermis-specific cDNA, *ep*37L1. Sequence analyses of these cDNAs revealed that *ep*37L1, L2, A1, and A2 are independent but closely related genes. Although acceptable variations in the four βγ-crystallin motifs of *ep*37L1 have been demonstrated by Wistow et al. (1995), a protein-motif search using the available database program identified only one motif. However, sequence data of the homologous cDNAs directly showed the existence of additional consecutive βγ-

crystallin motifs in the N-terminal half of the EP37 proteins. Apart from some deviations found in motif 4 (Arg^{137} and Ala^{144}), all four motifs of EP37A2 satisfy the structural prerequisite for a βγ-crystallin motif. In addition to the βγ-crystallin motif, EP37 proteins have possible Ca^{2+}-binding domains in motifs 1 and 3 (shaded boxes in Fig. 1). Speculation of the existence of these regions was based on sequence alignment with protein S from the gram-negative bacterium *M. xanthus*. The residues (indicated with arrowheads in Fig. 1) correspond to the serine residues at which site-specific mutation caused a reduction in Ca^{2+}-binding affinity (Teintze et al. 1988). Such circumstantial evidence suggests that EP37 proteins may be associated with Ca^{2+} and that this may contribute to both their assembly and stability, as is the case in the developmental cycle of *M. xanthus* (Bagby et al. 1994). In contrast to other proteins of the βγ-crystallin superfamily, EP37 proteins have a unique sequence in the C-terminal half. We found two other specific sequences, G/S-x(2)-T-x-I-x(2)-G-T-x(5)-G, from residue 228 to 243 and from 307 to 322, in the C-terminal half. As the glycine residue participates in the extensive conformation of the secondary and tertiary structure of the protein (Branden and Tooze 1991), these sequences may shed some light on the function of the C-terminal half. At present the significance of these sequences is not known. However, different kinds of domains of EP37 proteins, including βγ-crystallin motifs and Ca^{2+}-binding domains, may participate in the construction of stable molecular assemblies as well as in other specific functions. Whole mount in situ hybridization and immunological observation suggests that expression of EP37 is principally associated with differentiating tissues of ectodermal origin. Immunohistochemical and immunoblot analyses showed no positive staining for EP37 in the eye lens, indicating that EP37 proteins are non-lens proteins. The 2D-PAGE immunoblotting showed that two and five kinds of EP37 proteins existed simultaneously in the larva and adult skin, respectively (Fig. 2B, C). Although we have not yet obtained the complete recombinant EP37 proteins by performing in vitro experiments for oligomeric association of EP37s, heterologous interaction of EP37 proteins may possibly result in a more stable structure as is the case with β-crystallins (Slingsby and Bateman 1990).

The immunoreactivity of the anti-EP37L1 antibody was restricted to the integumental tissues. However, the positive cell populations changed as development proceeded. During the early embryonic stages, immunoreactivity was relatively homogeneous in the cytoplasm of epidermal cells. Later, immunostaining was localized in the skein cells, especially around the plasma membrane and in the figures of Eberth. The epidermal cells of amphibian larvae are characterized by skein cells, which contain numerous bundles of cytokeratin filament (figures of Eberth). They appear early in larval life, reach peak size during the late premetamorphic stages and disappear after metamorphosis (Fox 1992). Deposition of EP37 proteins on the figures of Eberth and on the plasma membrane may contribute to the assembly of keratin intermediate filaments or in their anchoring to the plasma membrane, as is the case with cornified cell envelope proteins such as loricrin (Mehrel et al. 1990) and involucrin (Yaffe et al. 1992). After metamorphosis, localization of EP37 proteins was observed exclusively in cutaneous glands and not in other epidermal tissues (Fig. 4D) or cornea (data not shown). Immunoblot analyses, however, showed the presence of EP37 proteins both in the moulted skin of the adult (data not shown) and in the adult cornea (Fig. 5B). Since the βγ-crystallin superfamily proteins tend to aggregate compactly, it is conceivable that loss of immunoreactivity was due to a masking of the antigenicity caused by stable and compact assemblies of EP37 proteins. These structural changes may contribute to protection against imposition of terrestrial stimuli or osmotic stress.

The β- and γ-crystallins are abundant lens proteins and some of them are expressed at low level in non-lens tissues (Head et al. 1991; Smolich et al. 1994). EP37 proteins are expressed only in integumental tissues and not in lens. In that sense, EP37 proteins are the first example of non-lens members of the βγ-crystallin superfamily in vertebrates. EP37 proteins may retain more ancestral functions of the βγ-crystallin superfamily than β- and γ-crystallins, since lens differentiate from epidermal tissue in normal development. The pattern of *ep*37 gene expression during metamorphosis is complex and subject to tight spacial and temporal regulation. Thus the *ep*37 family serves as an excellent model system for elucidating the adaptation mechanisms to environmental changes at the molecular level.

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