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Authors: Ohta, Kazumasa, and Nakazawa, Tohru

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Expression of Cyclophilin during the Embryonic Development of the Sea Urchin

Kazumasa Ohta[1†] and Tohru Nakazawa[2*]

[1]*Department of Biology, Faculty of Science, Toho University, Funabashi 274-8510, Japan*
[2]*Biological Laboratory, The University of the Air, Chiba 261-8586, Japan*

ABSTRACT—The spatial and temporal expression pattern of cyclophilin (Cyp) was examined during the embryonic development of the sea urchins *Anthocidaris crassispina* and *Hemicentrotus pulcherrimus* using Western blot analysis and indirect immunofluorescence microscopy. In this study, affinity-purified anti-human Cyp A antibody was used as the primary antibody. Western blot analysis revealed that a single 17.5 kDa immunoreactive band of Cyp was present in unfertilized eggs, in embryos during several stages of development, and in ovaries and testes of adult sea urchins. Cyp was also recognized in unfertilized eggs and embryonic sea urchin cells by indirect immunofluorescence microscopy, but its concentrations within the embryonic tissues varied significantly during embryogenesis. Expression of Cyp during the cleavage stage was thought to be attributable to maternal Cyp products, with zygotic expression appearing after gastrulation. Cyp expression appears to increase depending on the Cyp concentration in the vegetal and apical plates and primary mesenchyme cells in mesenchyme blastulae, and in the oral ectodermal ridge, gut and skeletogenetic mesenchyme cells in pluteus larvae. These results suggest that widespread embryonic distribution and an increased Cyp content occur during the gastrulation in sea urchin development.

INTRODUCTION

The members of the cyclophilin (Cyp) family of protein are highly conserved among various organisms and several forms of Cyp can be identified in different intracellular compartments in various mammalian cells (review by Heitman *et al*., 1992). The most abundant and ubiquitous form of Cyp in the cytosol is known as Cyp A in humans (Handschumacher *et al*., 1984; Harding *et al*., 1986; Haendler *et al*., 1987) and as Cyp-1 in *Drosophila* (Stamnes *et al*., 1991). Cyp has been found to be a binding protein for the immunosuppressant cyclosporin A (CsA) (Handschumacher *et al*., 1984) and to be identical to peptidyl prolyl *cis-trans* isomerase (PPIase), which catalyzes the *cis-trans* interconversion of proline-containing peptides (Fischer *et al*., 1989; Takahashi *et al*., 1989). In the human immune system, the Cyp-CsA complex interferes with the production of cytokines in T-cells by inhibiting calcineurin (CaN), a calcium- and calmodulin-dependent protein phosphatase (protein phosphatase Type 2B) (Liu *et al*., 1991; Friedman and Weissman, 1991). Previous studies have also shown that Cyp/PPIase is involved in protein folding *in vitro* and *in vivo* (reviews by Gething and Sambrook, 1992; Heitman *et al*., 1992; Schmid, 1993), and that the isomerization produced by Cyp/PPIase is inhibited by CsA without the relation to immunosuppression (Sigal *et al*., 1991). However, because the intrinsic ligand(s) of Cyp have not yet been determined, the physiological function of Cyp is not well understood.

In a previous study using embryos of the sea urchin *Hemicentrotus pulcherrimus*, a Cyp cDNA, designated HPCyp-1, was cloned and its nucleotide sequence was determined (Ohta and Nakazawa, 1996). The deduced amino acid sequence shows a high degree (about 75-80%) of homology with Cyp A and other Cyp A members. Northern blot analysis revealed that Cyp mRNAs originally accumulate in early blastulae and that marked accumulation occurs thereafter.

In the present study, in order to establish the spatio-temporal distribution of this protein during embryonic development, Western blot analysis and indirect immunofluorescent microscopy were performed to characterize and localize Cyp in sea urchin embryos at various stages during their development. We believe that this is the first description of the spatio-temporal distribution of Cyp during embryonic development of animals.

MATERIALS AND METHODS

Sea urchin embryos

Sea urchins of the species *Anthocidaris crassispina* and *Hemicentrotus pulcherrimus* were collected in Kumamoto and Chiba Prefectures, respectively, during their breeding seasons and were

* Corresponding author: Tel. +81-43-298-4181;
FAX. +81-43-298-4181.
† Present address: Department of Biochemistry, Tokyo Dental College, Chiba 261-8502, Japan.

kept in sea water until use. Their gametes were obtained by intracoelomic injection of 0.5 M KCl. The eggs were washed several times with artificial sea water (ASW; Jamarin U, Jamarin Laboratory, Osaka, Japan), then inseminated by adding diluted dry sperm. The fertilized eggs were allowed to develop at 22°C for *A. crassispina* and 18°C for *H. pulcherrimus* in ASW with constant agitation.

Western blot analysis

The proteins of unfertilized egg and embryo extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the methods of Laemmli (1970) and electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon P; Millipore, Bedford, MA, USA) according to the methods of Towbin *et al*. (1979). After blocking with 5% skimmed milk in phosphate buffered saline (PBS; 0.15 M NaCl, 10 mM sodium phosphate, pH 7.4) containing 0.05% Tween 20 (PBS-T), the membrane was incubated in PBS-T containing the primary antibody (anti-cyclophilin A; Affinity BioReagents, Neshnic Station, NJ, USA), which was purified according to the method of Weigel *et al*. (1989) using the recombinant glutathione-s-transferase (GST)- fused *H. pulcherrimus* Cyp (GST-HPCYP) affinity column (Ohta and Nakazawa, 1996), then washed several times with PBS-T. The blot was incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody (Vector Laboratory, Burlingame, CA, USA), washed several times with PBS-T and detected using the ECL western blotting analysis system (Amersham international plc, Buckinghamshire, England) according to the manufacturer's procedures.

Indirect immunofluorescence

Embryos at various stages of development were fixed in 5% formalin in ASW for 60 min at 4°C, then attached to poly-L-lysine-coated cover slips. In order to dissociate their blastomeres, the embryos were treated with 1.1 M glycine-HCl (pH 8.0), then washed with ASW to remove the hyalin layer (Mizuno *et al*. 1993). The specimens were immersed in 0.1% Nonidet P-40 in PBS for 15 min at room temperature and then in 1% bovine serum albumin (BSA) in PBS for 30 min at room temperature. The specimens were incubated with the affinity-purified anti-cyclophilin A antibody for 3 hr at room temperature, washed with PBS and incubated with a fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG antibody (Vector Laboratory, Burlingame, CA, USA) for 60 min at room temperature. The specimens were washed several times with PBS, mounted in 80% glycerol in PBS, and observed under a Nikon microscope equipped with fluorescence optics.

RESULTS

Cyp was present in the eggs, embryos and reproductive organs of the sea urchin

Extracts from the unfertilized eggs and embryos (early blastulae, hatched blastulae, mesenchyme blastulae, mid-gastrulae, prism larvae and pluteus larvae) of *A. crassispina* and *H. pulcherrimus*, and the testes and ovaries of *A. crassispina* were subjected to SDS-PAGE using 12.5% gel and equal amounts of total protein. In order to examine the temporal expression of Cyp protein during the embryonic development of the sea urchins *A. crassispina* and *H. pulcherrimus*, Western blot analysis was performed using an anti-human recombinant Cyp A antibody as shown in Fig. 1. Immunoreactive bands appeared in the extracts from eggs, all embryo stages that we examined, testes and ovaries. When the primary antibody was not purified using the GST-HPCYP affinity column, the blots from both species showed an intense band with a molecular mass of 17.5 kDa, together with faint bands at 47 kDa and 70 kDa in *A. crassispina*, and 66 kDa in *H. pulcherrimus*. When affinity-purified anti-human Cyp A antibody was used as the primary antibody, only a single immunoreactive band at 17.5 kDa was detected. In further experiments, therefore, we used affinity-purified anti-Cyp A antibody as the primary antibody. The intensity of the immunoreactivity of the 17.5 kDa band decreased slightly during the cleavage stages, then increased during gastrulation and reached a plateau at the prism larva stage.

Cyp concentrations within embryonic tissues varied during embryogenesis

Analysis of whole mounted embryos by indirect immunofluorescence microscopy was carried out using anti-human Cyp A antibody which has been affinity-purified against GST-HPCYP as the primary antibody. This recognized a single protein, as described above.

Immunofluorescent signals were observed in the cytoplasm of unfertilized eggs, in blastomeres and every embryonic cells at every stage tested, as shown in Figs. 2 and 3. Differences in the distribution patterns of the fluorescent signals were, however, found between the two sea urchin species in unfertilized eggs, blastulae and gastrulae. Maternal sea urchin Cyp was expressed in the cytoplasm of unfertilized eggs (Figs. 2a, 3a) and in the blastomeres of all cleavage stage embryos (data not shown). In *A. crassispina*, fluorescent signals were only detected in the cytoplasm of unfertilized eggs (Fig. 2a), but in the unfertilized eggs of *H. pulcherrimus*, intense fluorescent signals were also detected around the pronuclear membrane and in the pronucleus (Fig. 3a). At the blastula and gastrula stages of *A. crassispina*, fluorescent signals were detected on the apical side and were significantly apparent on the basal surface of the blastomeres (Fig. 2b, c), whereas in *H. pulcherrimus*, the signals on the basal surface of the blastomeres were faint (Fig. 3b, c). To determine the intracellular distribution of Cyp, the blastomeres of *H. pulcherrimus* embryos were dissociated at the blastula and gastrula stages. The fluorescent signals were concentrated in two batches and detected around the peripheral zone of the cells (Fig. 4). During gastrulation in both species, intense fluorescence appeared in the gut, in the vegetal and apical plates, and in the primary and secondary mesenchyme cells (Figs. 2c, 3c). In pluteus larvae, signals were localized in the guts, skeletogenetic mesenchyme cells and oral ectoderm in both species (Figs. 2d, 3d).

DISCUSSION

Immunophilins, the Cyps and FK506 binding proteins (FKBPs) which bind the immunosuppressants CsA and FK506, respectively, have PPIase activity which may be involved in protein folding and/or refolding (reviews by Gething and Sambrook, 1992; Heitman *et al*., 1992; Schmid, 1993). We here present the first description of the spatial and temporal expression pattern of Cyp protein during embryonic develop-

ment. To investigate the temporal expression of Cyp protein in sea urchin embryos, Western blot analysis was performed using an anti-recombinant human Cyp A antibody which recognizes the most abundant and ubiquitous 17-18 kDa cytosolic member of Cyp family, Cyp A, in various vertebrates. The embryonic stages used for the assay were the unfertilized eggs, early blastulae, hatched blastulae, mesenchyme blastulae, mid gastrulae, prism larvae and early pluteus larvae of *A. crassispina* and *H. pulcherrimus*. When primary antibody was not purified using the GST-HPCYP affinity column, an intense immunoreactive band was detected at 17.5 kDa together with other faint immunoreactive bands, as shown in Fig. 1. The primary antibody was therefore purified by affinity column chromatography using GST-HPCYP as a ligand, after which no faint bands were detected on the blots. This indicates that the affinity-purified antibody specifically reacted with the Cyp family proteins of sea urchins. The amino acid sequence of sea urchin Cyp as deduced from HPCyp-1 is homologous with the cytosolic Cyp, Cyp A (Ohta and Nakazawa, 1996). Furthermore, Western blot analysis using the affinity-purified anti-human Cyp A antibody as the primary antibody revealed that the molecular mass of the immunoreactive band (17.5 kDa) is consistent with that of HPCYP as estimated from HPCyp-1 (17,630 Da). These results suggest that the Cyp expressed in the unfertilized eggs, embryos and reproductive organs of sea urchins is one of the most well-known and abundant cytosolic members of the Cyp family, Cyp A. Many organisms express several forms of Cyp (review by Heitman *et al*., 1992) and

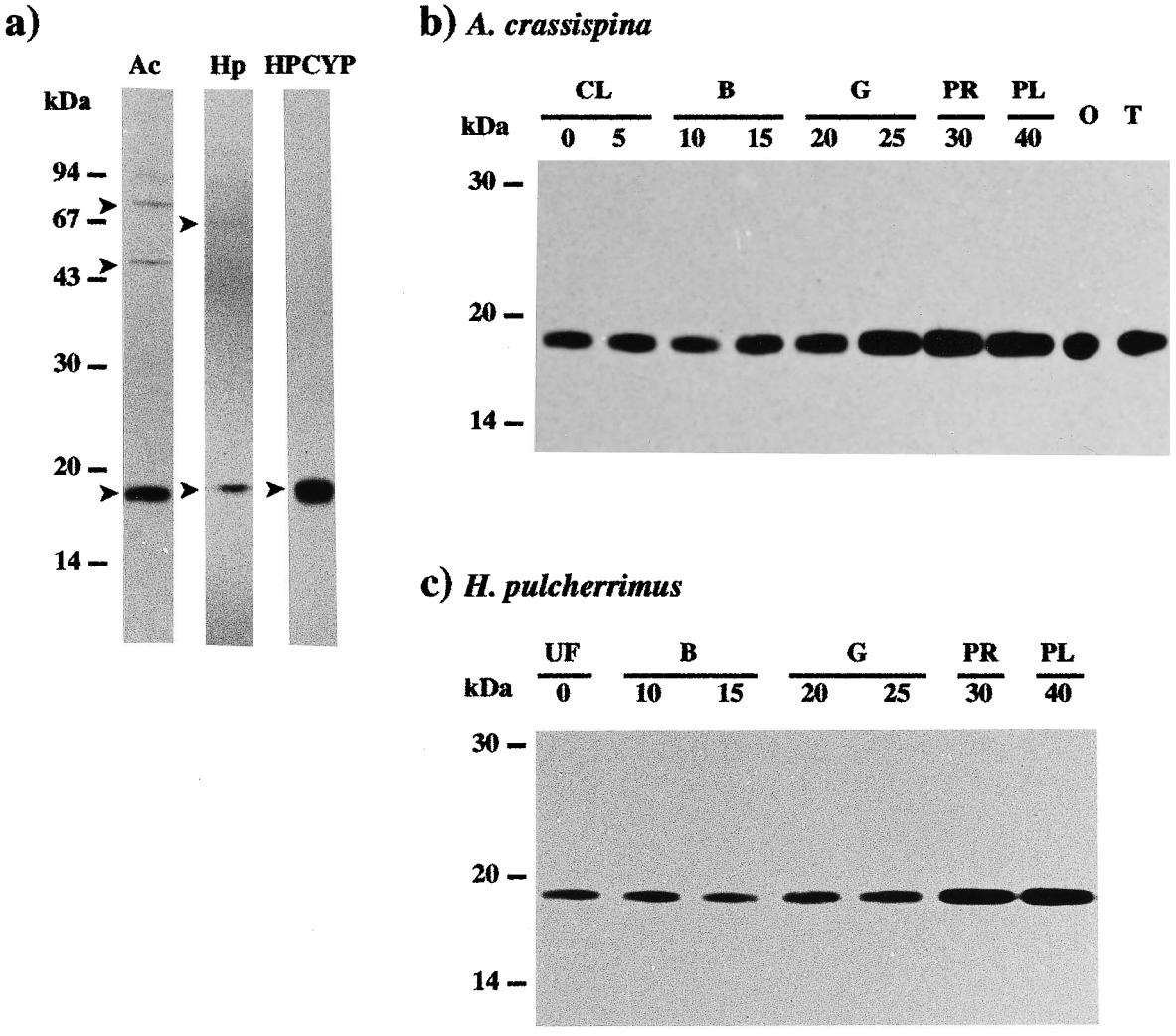

Fig. 1. Western blot analysis of Cyp in sea urchin embryos. (**a**) Unfertilized egg extracts from *A. crassispina* (Ac) and *H. pulcherrimus* (Hp), and HpCyp-1 (Ohta and Nakazawa, 1996) protein expressed in *E. coli* (HPCYP), were prepared for SDS-PAGE followed by immunoblotting using anti-human Cyp A. The antibody recognized 17.5 kDa, 47 kDa and 70 kDa proteins in *A. crassispina* eggs and 17.5 kDa and 66 kDa proteins in *H. pulcherrimus* eggs. Protein extracts from *A. crassispina* (**b**) and *H. pulcherrimus* (**c**) at 0 hr (unfertilized eggs), 5 hr (64-128 cells), 10 hr (early blastulae), 15 hr (mesenchyme blastulae), 20 hr (early gastrulae), 25 hr (mid-late gastrulae), 30 hr (prism larvae) and 40 hr (pluteus larvae) after fertilization were analyzed by immunoblotting using affinity-purified anti-human Cyp A as the primary antibody. UF, unfertilized eggs; CL, cleavage stage embryo; B, blastula; G, gastrula; PR, prism larva; PL, pluteus larva; O, ovary; T, testis.

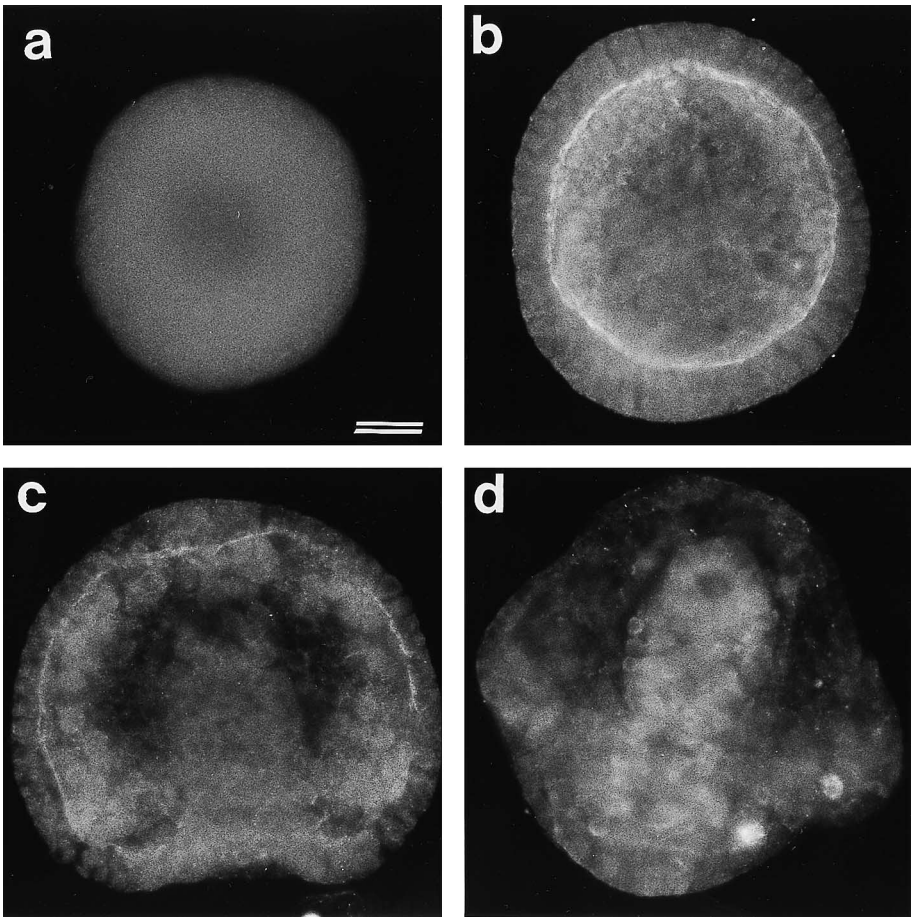

Fig. 2. Distribution of Cyp in the unfertilized eggs and embryos of *A. crassispina*. Whole mounted specimens of eggs and embryos were prepared for indirect immunofluorescent microscopy at the following stages: unfertilized egg (**a**), hatching blastula (**b**), mid-gastrula (**c**) and early pluteus (**d**). Affinity-purified anti-human Cyp A antibody was used as the primary antibody. Bar indicates 20 μm.

Cyp A shows a high degree of homology with the other Cyps, Cyp B (Price *et al*., 1991) and Cyp C (Friedman and Weissman, 1991). The faint bands revealed when the unpurified primary antibody was used may indicate proteins homologous with Cyp, but it is unknown whether they represent other members of the Cyp family or non-specific proteins. The weak immunoreactivity of these bands suggests the presence of non-specific proteins, because their molecular masses are quite different from those of other Cyp family members.

The protein expression pattern of Cyp is dependent on the stage of embryonic development in sea urchins. It is thought that maternal Cyp products may be responsible for its presence during the early stages and that a nascent product arises during the later stages of embryonic development. This possibility is consistent with previous data from a northern blot analysis which revealed that Cyp mRNA originally accumulates at the blastula stage (Ohta and Nakazawa, 1996).

Cyp localization was also studied in the embryonic tissues/cells of the sea urchins, *A. crassispina* and *H. pulcherrimus*. Indirect immunofluorescence microscopy using whole mounted preparations revealed that Cyp was present in unfertilized eggs, all blastomeres and embryonic cells during early development. This suggests that Cyp is an essential protein for all living cells throughout embryogenesis. At the mesenchyme blastula stage, Cyp begins to concentrate in the vegetal and apical plates and in mesenchyme cells, while the other regions of the embryo retain their previous signal intensity. The same regions were significantly stained with the fluorescent dye in pluteus larvae. It is thought that the increases in Cyp expression at the gastrulation are caused by concentration of Cyp protein in these embryonic regions.

The function of Cyp during the embryonic development of sea urchins and other organisms is not clear. However, its widespread tissue and ontogenetic distribution suggest a fundamental role in the metabolism of embryogenesis. Marked increases in the concentration of Cyp occurred in the vegetal and apical plates and primary mesenchyme cells at the mesenchyme blastula stage and in the oral ectodermal ridge, gut and skeletogenetic mesenchyme cells at the pluteus stage. Previous reports have suggested that various types of Cyp are involved in cytosolic or mitochondrial protein folding and/or refolding (Fischer *et al*., 1989; Steinmann *et al*., 1991; Kern *et al*., 1995; Matouschek *et al*., 1995; Rospert *et al*., 1996). It has also been suggested that Cyps act as chaperones

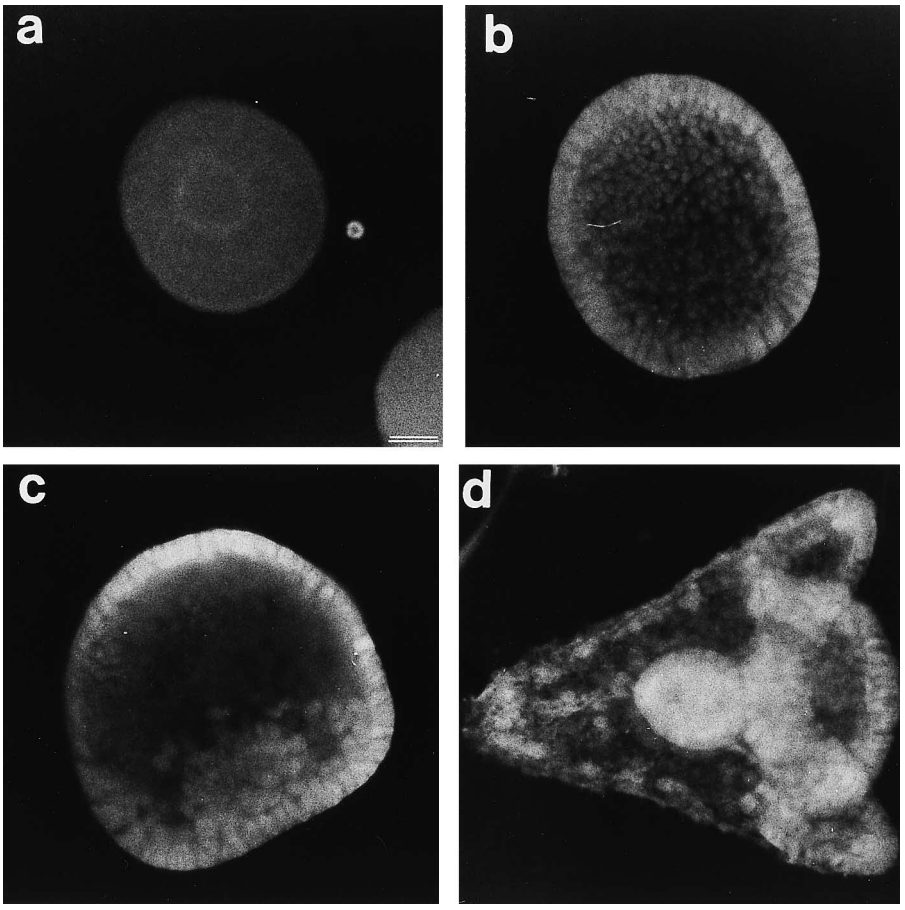

Fig. 3. Distribution of Cyp in the unfertilized eggs and embryos of *H. pulcherrimus*. Whole mounted specimens of eggs and embryos were prepared for indirect immunofluorescent microscopy at the following stages: unfertilized egg (**a**), hatching blastula (**b**), early gastrula (**c**) and early pluteus (**d**). Affinity-purified anti-human Cyp A antibody was used as the primary antibody. Bar indicates 20 μm.

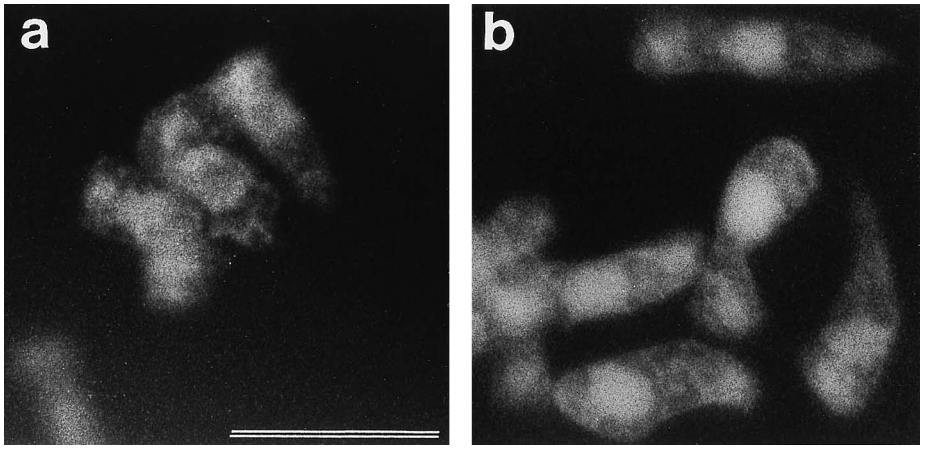

Fig. 4. Distribution of Cyp in the blastomeres of *H. pulcherrimus*. Blastomeres dissociated at the mesenchyme blastula (**a**) and mid-gastrula (**b**) stages as described in Materials and Methods were probed with anti-human Cyp A antibody which had been purified using the GST-HPCYP affinity column. Bar indicates 10 μm.

(Freskgard *et al*., 1992; Baker *et al*., 1994). By catalyzing the folding and/or unfolding of its target protein(s), Cyp may be responsible for the biochemical pathways leading to the formation of the arms, gut and spicules in sea urchin embryos. However, from our present results, we cannot identify its natural target protein(s).

REFERENCES

Baker EK, Colley NJ, Zuker CS (1994) The cyclophilin homolog NinaA functions as a chaperone, forming a stable complex *in vivo* with its protein target rhodopsin. EMBO J 13: 4886–4895

Fischer G, Wittmann-Liebold B, Lang K, Kiefhaber T, Schmid FX (1989) Cyclophilin and peptidyl-prolyl *cis-trans* isomerase are probably identical proteins. Nature 337: 476–478

Freskgard PO, Bergenhem N, Jonsson BH, Svensson M, Carlsson U (1992) Isomerase and chaperone activity of prolyl isomerase in the folding of carbonic anhydrase. Science 258: 466–468

Friedman J, Weissman I (1991) Two cytoplasmic candidates for immunophilin action are revealed by affinity for a new cyclophilin: One in the presence and one in the absence of CsA. Cell 66: 799–806

Gething M-J, Sambrook J (1992) Protein folding in the cell. Nature 355: 33–45

Haendler B, Hofer-Warbinek R, Hofer E (1987) Complementary DNA for human T-cell cyclophilin. EMBO J 6: 945–950

Handschumacher RE, Harding MW, Rice J, Drugge RJ, Speicher DW (1984) Cyclophilin: A specific cytosolic binding protein for cyclosporin A. Science 226: 544–547

Harding MW, Handschumacher RE, Speicher DW (1986) Isolation and amino acid sequence of cyclophilin. J Biol Chem 261: 8547–8555

Heitman J, Movva NR, Hall MN (1992) Proline isomerases at the crossroads of protein folding, signal transduction, and immunosuppression. New Biol 4: 448–460

Kern G, Kern D, Schmid FX, Fischer G (1995) Akinetic analysis of the folding of human carbonic anhydrase II and its catalysis by cyclophilin. J Biol Chem 270: 740–745

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head bacteriophage T4. Nature 227: 680–685

Liu J, Farmer JD Jr, Lane WS, Friedman J, Weissman I, Schreiber SL (1991) Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. Cell 66: 807–815

Matouschek A, Rospert S, Schmid K, Glick BS, Schatz G (1995) Cyclophilin catalyzes protein folding in yeast mitochondria. Proc Natl Acad Sci USA 92: 6319–6323

Mizuno N, Uemura I, Yamasu K, Suyemitsu T, Ishihara K (1993) Localization of an exogastrula-inducing peptide (EGIP) in embryos of the sea urchin *Anthocidaris crassispina*. Dev Growth Differ 35: 539–549

Ohta K, Nakazawa T (1996) Nucleotide sequence of a cDNA coding for cyclophilin of the sea urchin *Hemicentrotus pulcherrimus*. Zool Sci 13: 133–136

Price ER, Zydowsky LD, Jin MJ, Baker CH, McKeon FD, Walsh CT (1991) Human cyclophilin B: A second cyclophilin gene encodes a peptidyl-prolyl isomerase with a signal sequence. Proc Natl Acad Sci USA 88: 1903–1907

Rospert S, Looser R, Dubaquie Y, Matouschek A, Glick BS, Schatz G (1996) Hsp60-independent protein folding in the matrix of yeast mitochondria. EMBO J 15: 764–774

Schmid FX (1993) Prolyl isomerase: enzymatic catalysis of slow protein-folding reactions. Annu Rev Biophys Biomol Struct 22:123–142

Sigal NH, Dumont F, Durette P, Siekierka JJ, Peterson L, Rich DH, Dunlap BE, Staruch MJ, Melino MR, Koprak SL, Williams D, Witzel B, Pisano JM (1991) Is cyclophilin involved in the immunosuppressive and nephrotoxic mechanism of action of cyclosporin A? J Exp Med 173: 619–628

Stamnes MA, Shieh B-H, Chuman L, Harris GL, Zuker CS (1991) The cyclophilin homolog ninaA is a tissue-specific integral membrane protein required for the proper synthesis of a subset of Drosophila rhodopsins. Cell 65: 219–227

Steinmann B, Brucker P, Superti-Furga A (1991) Cyclophilin A Slows Collagen Triple-helix formation in vivo: indirect evidence for a physiologic role of peptidyl-prolyl cis-trans-isomerase. J Biol Chem 266: 1299–1303

Takahashi N, Hayano T, Suzuki M (1989) Peptidyl-prolyl *cis-trans* isomerase is the cyclosporine A-binding protein cyclophilin. Nature 337: 473–475

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins form polyacrylamide gels to nitrocellulose sheet: procedure and some applications. Proc Natl Acad Sci USA 76: 4350–4354

Weigel NL, Schrader WT, O'Malley BW (1989) Antibodies to chicken progesterone receptor peptide 523-536 recognize a site exposed in receptor-deoxyribonucleic acid complexes but not in receptor-heat shock protein-90 complexes. Endocrinology 125: 2494–2501

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