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Retinal in the Eggs of Phylum Chordata: A Novel Storage Mode of Retinoid

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The presence of retinals (retinal and 3,4-didehydroretinal) has been known in the eggs of wide range of oviparous vertebrates, but the biological significance of the egg retinals has yet to be clarified. We here show that retinals are the major components of retinoids in the eggs of all species of chordate animals we examined. The egg retinals were commonly bound to egg yolk proteins, the storage proteins, *via* a Schiff base linkage. The Schiff base linkage, which protects the reactive aldehyde group, would negate the toxicity of aldehyde, and enable to accumulate much amount of retinals. The retinals in chordate eggs are considered to be the precursor of functional retinoids, such as photoreceptive pigment chromophores and retinoic acid, during development. The results of the present research strongly suggest that retinals in the eggs of oviparous chordates are the common and essential mode of retinoid storage.

Key words: ascidian, chordate, egg, retinal, retinoid, vertebrate, yolk protein

INTRODUCTION

Retinoids are known to be involved in two different functions: retinal (RAL1) and 3,4-didehydroretinal (RAL2) for the photoreception, and retinoic acid (RA) for gene regulation. As retinoids play important roles for the visual pigment formation and embryonic morphogenesis during development, the precursors of the functional retinoids ought to be stored in the eggs.

The occurrence of RAL1 or both RAL1 and RAL2 was shown to be distributed in the eggs or ovaries of a wide range of oviparous vertebrates: lampreys, elasmobranches, teleosts, amphibians, reptiles and birds [1-4]. Recently, it is reported that retinals in the eggs of *Xenopus laevis* and some teleosts were major components of retinoids [5-8], and that the egg retinals were shown to be bound to egg yolk proteins [6,8]. However, it still remains to be elucidated whether the egg retinals (RALs) contribute to the retinoid storage as the precursors of the functional retinoids.

In the present study, we analyzed the egg retinoids of oviparous vertebrates, which were different species from the previous studies, to compare with the retinoid composition in eggs. We also examined the retinoids in the eggs and the larvae of the ascidian, an urochodate, which is one of the closest relatives of vertebrates. On the basis of the results, we discuss the biological significance of RALs in the eggs of chordate animals.

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MATERIALS AND METHODS

Animals and eggs.

The eggs of Japanese toad (*Bufo japonicus*) were collected in Okunoike pond, Osaka, Japan. Bastard halibut (*Paralichthys olivaceus*) eggs were supplied from the Osaka Prefectural Foundation for Fishery Promotion, Sea Farming Center, Osaka, Japan. The eggs of the solitary ascidian, *Halocynthia roretzi*, were laid in the Marine Biological Station of Tohoku University, Aomori, Japan. To obtain the ascidian larvae, the eggs were fertilized and incubated in a flat pan until the hatching was almost completed.

Extraction and analysis of retinoids.

The retinoid extraction was performed with organic solvents by using the oxime method [5]. The normal phase high performance liquid chromatography (HPLC) was carried out using a column of silica gel. The eluent was 5% *tert*-butylmethyl ether, 0.04% ethanol, 25% benzene in *n*-hexane. To determine retinyl esters (REs), the void fraction was collected, saponified to retinols with ethanolic KOH and rechromatographed.

Gel electrophoresis and protein fractionation

The sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 12.5% slab gels, principally according to the method described by Laemmli [9]. After the electrophoresis, the gels were stained with Coomasie Brilliant blue R-250 to visualize the protein bands.

The egg yolk proteins of the Japanese toad were solubilized in 20 mM Tris-HCl buffer containing 0.6 M NaCl and fractionated with ammonium sulfate at 60% saturation. The precipitate was treated with sodium borohydride ($NaBH_4$) in the presence of 2% SDS, and chromatographed with a Sephacryl S-300 HR column (2.5×86 cm) equilibrated with 20 mM Tris-HCl buffer containing 0.1% (SDS) and 0.02% dithiothreitol.

RESULTS AND DISCUSSION

Occurrence of retinals in the eggs

As shown in Table 1, RALs were the major or exclusive components of retinoids in the eggs of every species we examined. These results are consistent with those of other studies [1-8]. Except for the Japanese toad eggs, RALs were the almost exclusive retinoids in the eggs without lipid bodies, whereas REs were detected in the eggs with lipid bodies. In the teleost eggs, the amount of ROLs and REs correlated with the developmental degree of lipid bodies. It is already described that RALs and REs in chum salmon eggs were localized in aqueous and lipid part, respectively [8]. In the eggs of Japanese toad, retinyl esters were clearly detected, although lipid bodies were not found on microscopic observations. The localization of RALs and REs in Japanese toad eggs is still unknown.

The retinal isomers detected in vertebrate eggs were mostly all-*trans* form, similar to previous results of teleosts [1,7,8,12] and amphibians [5,6]. In the, ascidian eggs, several peaks, which correspond to *cis* isomers in their retention time, were observed. However, this point was required to be certified for further study.

Table 1. Amount and composition of retinoids in the eggs.

Species	Lipid bodies	Amount (ng) / egg		RALs / RET (%)
		RALs	ROLs+REs	
Clawed toad [6]	–	14.5	Trace	~100
Japanese toad	–	34.3	6.67	83.7
Chum salmon [8]	++	729	329	68.9
Black porgy [8]	+	0.65	0.047	93.3
Bastard halibut	+	0.67	0.009	98.7
Marbled flounder [8]	–	1.10	Trace	~100
Stingfish [8]	–	0.69	Trace	~100
Ascidian	–	17.0*	n.d.	~100

RALs, retinals; ROLs, retinols; REs, retinyl esters; RET, total retinoids; n.d., not defined; *Amount (ng) / mg of protein

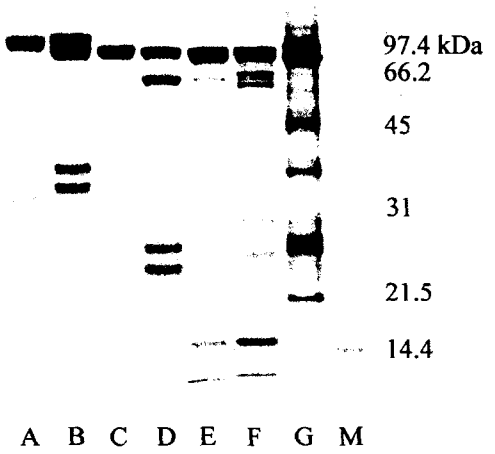

Fig. 1. SDS-polyacrylamide gel electrophoresis of the protein fractions, in which retinals were detected. The patterns of every species corresponded to egg yolk proteins.
A, clawed toad; B, Japanese toad; C, chum salmon; D, black porgy; E, bastard halibut; F, marbled flounder; G, ascidian; M, marker proteins

Retinal binding proteins

Retinals in the eggs of every species were detected in the aqueous part of the eggs. The electrophoretic examinations revealed that the egg RALs were always detected in the fractions of proteins, which consisted of egg yolk proteins, the storage proteins (Fig. 1). RALs were not extracted after the treatment with $NaBH_4$, indicating that the egg RALs are bound *via* a Schiff base linkage. This result is consistent with that of our previous examinations [6,8]. Such existing state, which the reactive aldehyde group is protected by the Schiff base linkage, would negate the toxicity of aldehyde, and enable to accumulate much amount of RALs in eggs. ROLs were not increased after the $NaBH_4$ treatment, suggesting the absence of free RALs, which would have been reduced to retinols by $NaBH_4$.

On the gel chromatography, retinals in the eggs of the Japanese toad were shown to be bound to the protein in the first peak, which showed the absorption of the retinyl product at 330 nm (Fig. 2A). SDS-PAGE revealed that the proteins in the peak were lipovitellin1, the main components of yolk proteins (Fig. 2B), as we have already shown in the eggs of clawed toad [6] and of chum salmon [8]. The precursor of yolk proteins, vitellogenin, is known to be synthesized in the liver in oviparous vertebrates. Egg retinals in clawed toad eggs were suggested to be synthesized in the liver, and

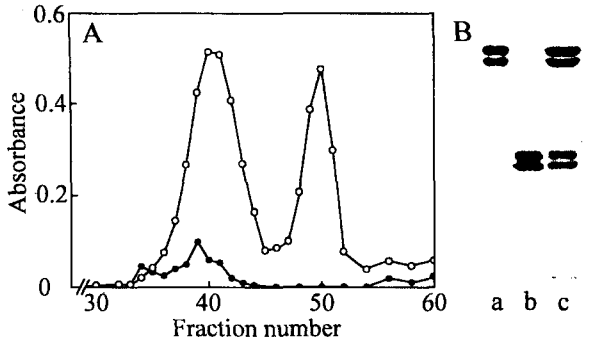

Fig. 2. A, Gel chromatography of Japanese toad egg yolk proteins treated with $NaBH_4$ and SDS, using a Sephacryl S-300 HR column equilibrated with 20 mM Tris HCl buffer (pH 7.4) containing 0.1% SDS and 0.02% dithiothreitol. Absorbances at 280 nm (protein, ○) and 330 nm (retinyl products, ●, five-times enlargement) of each fraction were measured after the chromatography.
B, SDS-polyacrylamide electrophoresis of the peak fractions (Fr. 39, lane a; Fr. 50, lane b) of the gel chromatography and yolk proteins (lane c) of Japanese toad eggs.

transported in a vitellogenin-bound form in the blood to the eggs where they accumulate [10]. In the eggs of oviparous chordates, the existing state of RALs appears to closely resemble each other.

Changes of egg retinoids during development

In the ascidian larvae, the amount of retinal detected was 9.7 ng/mg of protein. The value is apparently smaller than the retinal amount in the eggs (17.0 ng/mg of protein) shown in Table 1. The decrease in the amount of retinal might be due to the retinoic acid formation. The retinals in the egg of clawed toad were shown to be metabolized after the neurulation, and 11-*cis* retinals were produced with eye development, suggesting that the egg retinals were utilized for the visual pigment formation [11,12]. The egg RALs were also shown to be the precursor of retinoic acids of zebrafish [13] and *X. laevis* [14]. These results indicate that the egg retinals are the precursors of the functional retinoids, such as visual pigment chromophores and retinoic acid.

The storage mode of retinoid in chordate eggs

The every experimental result regarding egg RALs supports the idea that the RALs are the essential retinoid storage in eggs of chordate animals. It is strongly suggested that the accumulation of the retinal-protein complex into eggs is a common characteristic in oviparous chordates.

In adult vertebrates, REs are well known to be the predominant mode of retinoid storage. The stored mode of retinoids is different between eggs and adults. It is apparent that the stored mode of retinoids changes from retinals into retinyl esters during development. In conclusion, therefore, we insist that the new physiological role of RALs, which are the common and essential mode of retinoid storage in eggs of oviparous chordates, should be perceived.

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