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# Distribution and localization of galectin purified from *Rana catesbeiana* oocytes

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**Galectins are a family of lectins that recognize β-D-galactosides independently of calcium ions, and are widely distributed in animals. To characterize a galectin previously purified from oocytes of *Rana catesbeiana* (American bullfrog), we studied its distribution and localization in several tissues from this frog. Hemagglutination assay and western blotting showed that this lectin is present in many tissues including the liver, skin, kidney, skeletal muscle, and sciatic nerve, but is particularly concentrated in the ovary. Light microscopic immunohistochemistry showed that this lectin is localized in such places as cell–cell junctions, basement membranes, extracellular matrix, or secretory substances in several organs, indicating that this galectin is mainly distributed extracellularly. However, in the ovary, light microscopy showed that this lectin is present in or associated with the yolk platelet. Electron microscopy further revealed that it is localized in the periphery of the yolk platelet (the yolk plasm), but not in the cortical granule. These results indicate that *Rana* oocytes contain abundant galectin in their yolk platelets in contrast to *Xenopus laevis* oocytes, which have been found not to contain galectins but other classes of lectins in their yolk platelets and cortical granules.**

*Key words:* bullfrog/galectin/immunohistochemistry/oocyte/yolk platelet

## Introduction

Galectins (Barondes *et al.*, 1994a) are a family of lectins that bind β-D-galactosides independently of calcium ions, and are widely found in animals from sponges to humans (Hirabayashi and Kasai, 1993; Barondes *et al.*, 1994b). Higher vertebrate galectins can be divided into three structural types. The "proto" type has a basic polypeptide structure of 14–16 kDa with a carbohydrate recognition domain (CRD). Galectin-1 (about 30 kDa) is a noncovalent dimer of such subunits, while galectin-5 is a monomer. Galectin-3 (about 30–35 kDa) is one of the "chimera" type, in which a nonsugar binding domain precedes the N-terminus of a prototype polypeptide, and is a monomer. Galectin-4 (about 32–36 kDa) is also a polypeptide monomer, but is divalent with two serial prototype structures, and is an example of the "tandem-repeat" type. The galectin family contains still more members, including galectin-2, -6 to -8 (Barondes *et al.*, 1994a), so typing of galectins, with all these members plus those of lower animals, is not very simple. Ahmed and Vasta (1994) classified the CRDs of galectins into "conserved" (type I; galectin-1) and "variable" (type II; galectins -2 to -8).

Various lectins have been purified from amphibian, especially frog, tissues. For instance, a calcium-dependent lectin with 43 and 46 kDa subunits, specific for both α-D- and β-D-galactosides, has been purified from *Xenopus* oocytes and embryos (Roberson and Barondes, 1982, 1983) and its complete structure has recently been elucidated (Lee *et al.*, 1997). A 69 kDa lectin with similar carbohydrate specificity but dissimilar amino acid composition has been purified from *Xenopus* serum (Roberson *et al.*, 1985). Also, a lectin secreted from the cortical granule to strengthen the jelly coat upon fertilization has been found in *Xenopus* oocytes (Greve and Hedrick, 1978; Nishihara *et al.*, 1986; Mozingo and Hedrick, 1996). Similar lectins are purified from *Xenopus* yolk platelets (Yoshizaki, 1990). An 11 kDa hemagglutinating factor that binds sialic acids has also been purified from *Rana* oocytes (Nitta *et al.*, 1987), as has a 210 kDa lectin specific for N-acetyl-galactosamine and N-acetyl glucosamine (Sakakibara *et al.*, 1976). These lectins are definitely different from galectins in their sugar specificity and calcium-dependency. On the other hand, galectins are also found in amphibian tissues. A β-D-galactoside-binding lectin with two identical 16 kDa subunits is present in the granular and mucous glands of *Xenopus* skin (Bols *et al.*, 1986), and its cDNA sequence has been determined (Marschal *et al.*, 1992) . Bufo ovary has a β-D-galactoside-specific lectin with two 15 kDa subunits (Fink de Cabutti *et al.*, 1987). Its complete amino acid sequence has recently been elucidated, and it has been proven to be highly homologous to mammalian galectin-1 (Ahmed *et al.*, 1996).

We previously purified a lectin consisting of two identical 14 kDa subunits, that agglutinates trypsinized rabbit erythrocytes, from *Rana catesbeiana* (American bullfrog) oocytes (Ozeki *et al.*, 1991). The partial amino acid sequence of this lectin showed it to be one of the prototype galectins. In the present study, we first determined the amino acid sequence of three peptide fragments derived from this lectin, then investigated the distribution of this lectin using hemagglutination assay and western blotting. The results showed that this lectin is present in many tissues, but is particularly concentrated in the ovary. Localization of the lectin was examined by immunohistochemistry in several tissues. In the ovary, galectin was shown to be present in the superficial layer of the yolk platelet but not in the cortical granule by electron microscopy. These findings contrast with those in Xenopus oocytes, which do not contain galectins (Marschal *et al.*, 1994), but other type of lectin(s) in their yolk platelets and cortical granules (Greve and Hedrick, 1978; Roberson and Barondes, 1983; Yoshizaki, 1990).

## Results

### *Partial primary structure of* Rana *galectin*

Three new partial amino acid sequences of *Rana* galectin were obtained by the primary structure analysis on each peptide

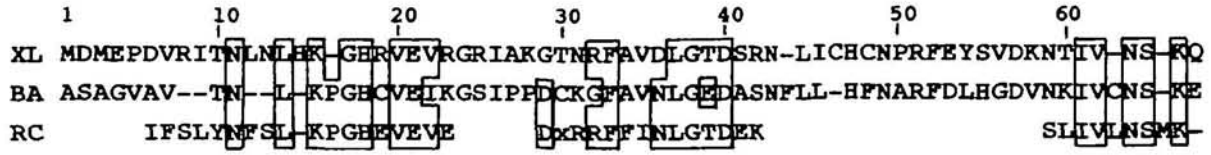


**Fig. 1.** Amino acid sequence alignment of three frog galectins. XP, *Xenopus laevis* galectin (Marschal *et al.*, 1992); BA, *Bufo arenarum* galectin (Ahmed *et al.*, 1996); RC, *Rana catesbeiana* galectin. Identical amino acid residues to those of *R.catesbeiana* galectin are boxed. x, Unidentified residue. Residue number is according to *Xenopus* galectin numbering system.

digested with Acromobacter protease I. The sequences were identified to be IFSLYNFSLKPGHEVEVE, DxRRFFINLGTDEK, and SLIVLNSMK, respectively (x denotes for unidentified residue) (Figure 1). Adding the previously determined 54 amino acid residues (Ozeki *et al.* 1991), the sequence of *Rana* galectin of 93 amino acid residues showed 48 and 49 % identity with those of *Xenopus* galectin (Marschal *et al.*, 1992) and *Bufo* galectin (Ahmed *et al.*, 1996), respectively.

### *Hemagglutination assay*

To determine the tissue distribution of $Ca^{2+}$-independent D-galactoside specific lectin(s) in adult *Rana,* various tissues were homogenized with excess lactose, centrifuged and dialyzed. A serial 2-fold dilution series of the supernatant was then made on a V-shaped multititer plate, and subjected to hemagglutination assay under presence of 10 mM EDTA. The activity was expressed as a titer, the reciprocal of the highest dilution causing hemagglutination (Table I). The detection limit of this hemagglutination assay was 1.5 ng/ml of the final concentration of galectin determined as purified *Rana* oocyte galectin. The ovary exhibited very high hemagglutination activity. The liver and kidney showed medium activities, while the oviduct, skin, heart, skeletal muscle, small intestine, and stomach showed weak activities. The testis and brain produced no activity. The activities in the liver and kidney are probably not a result of blood contamination because serum showed no activity. The activities of these tissues were completely canceled by the copresence of 100 mM lactose, suggesting that these activities resulted from D-galactoside-specific lectin(s).

### *Western blotting*

To examine the distribution of galectin in *Rana* tissues, Western blotting of tissue homogenates was performed using anti-*Rana* oocyte galectin antiserum. As a result, 14–15 kDa signals were found in all tissues tested (Figure 2). The signals in liver, kidney and heart were rather broad, suggesting a doublet of bands with slightly different mobilities. The signal from the ovary was by far the strongest, since we used only 1/12 of the amount of proteins per lane compared with the other tissues. A band of different size (24 kDa) was also detected in ovarian homogenates. Several other faint bands were recognized by the antibody, but were also found in the control blot, in which preimmune serum was used (data not shown), indicating that these bands were nonspecific. Thus, anti-*Rana* oocyte galectin antiserum was monospecific in all tissues tested.

**Table I.** D-Galactoside binding activities of lectins in crude bullfrog tissue homogenates

| | Titer |
|---|---|
| Ovary | 1024 |
| Oviduct | 4 |
| Liver | 64 |
| Kidney | 32 |
| Skin | 4 |
| Heart | 4 |
| Skeletal muscle | 4 |
| Small intestine | 4 |
| Stomach | 4 |
| Brain | 4 |
| Testis | nd[a] |
| Serum | nd[a] |
| *Rana* oocyte galectin (1.5 ng/ml) | 1[b] |

[a]nd, not detected.
[b]The lowest final concentration of purified bullfrog oocyte lectin producing hemagglutination was 1.5 ng/ml.

### *Immunohistochemistry*

To examine the localization of oocyte galectin within tissues, light microscopic immunohistochemistry was performed (Figures 3, 4). In the ovary, signals were observed in the cytoplasm of the growing oocytes (Figure 3A). This signal was absent when preimmune serum was used, indicating specificity of staining (Figure 3B). In the oocyte, the signal was observed in or around the yolk platelets, and became stronger as the oocyte grew (Figure 4A,B). The inner ovarian wall including the blood vessel was weakly positive, but neither the germinal vesicle of the oocyte nor the follicle cells were stained. In the liver, parenchymal cells were negative while the interlobular connective tissue was positive (Figure 4C). In the kidney, basement membranes surrounding the glomerulus, Bowman's capsule, and proximal and distal convoluted tubules were positive (Figure 4D). In addition, cytoplasmic membranous structures were also stained in the proximal and distal convoluted tubules. The antibody also stained the pronephros of the tadpole (data not shown). In the skin, signals were apparent around each keratinocyte, and also in the dermal extracellular matrix (Figure 4E). Smooth muscle layer beneath the dermal extracellular matrix and the contents of the epidermal granular gland were

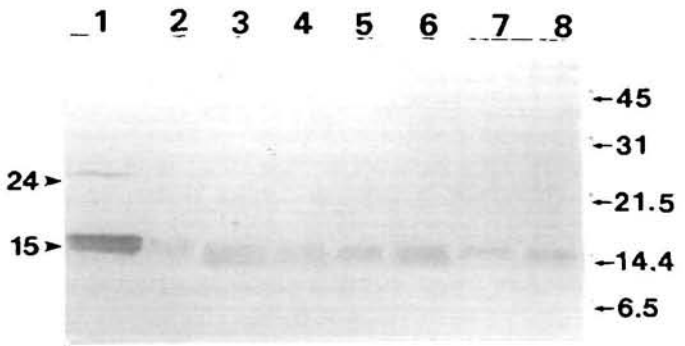


**Fig. 2.** Western blotting of tissue extracts using anti-*Rana* oocyte galectin antiserum. Lane 1, ovary; lane 2, oviduct; lane 3, liver; lane 4, kidney; lane 5, skin; lane 6, heart; lane 7, thigh muscle; lane 8, small intestine. Mobilities of size markers are shown on the right.

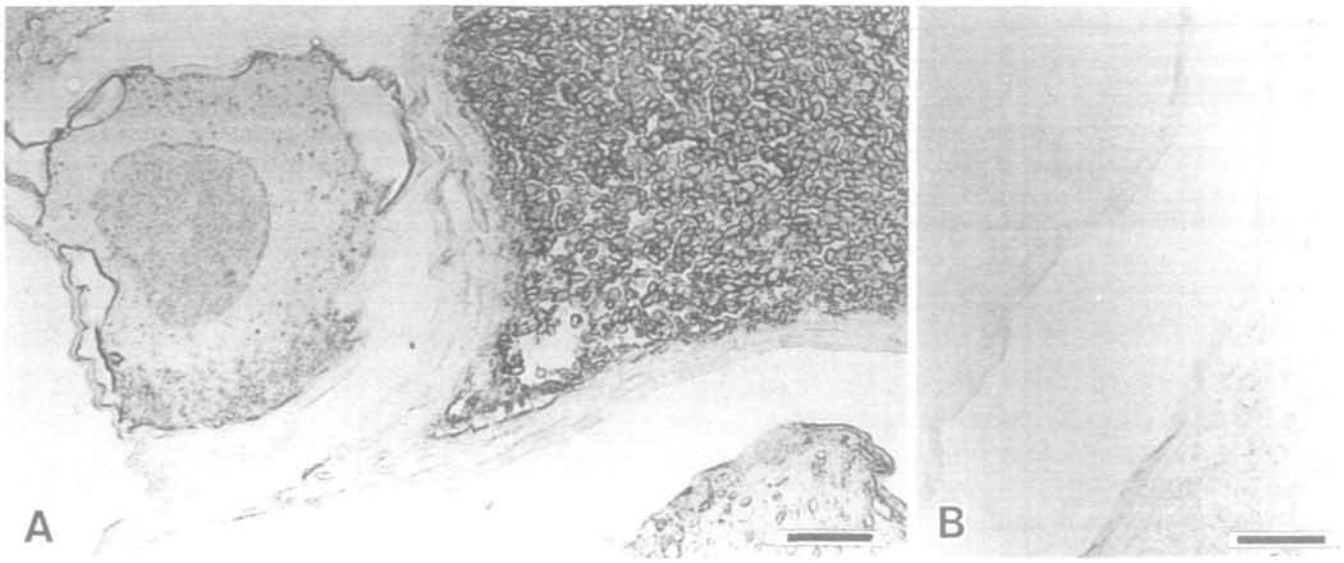


**Fig. 3.** Staining of the ovary with anti-*Rana* oocyte galectin antiserum **(A)** or preimmune serum **(B)**. Signals were visualized using diaminobenzidine with no counterstaining. **(A)** signals on a Stage II oocyte (left) appear to be granular in the periphery of the oocyte, whereas signals on a Stage IV oocyte (right) appear to be heavily concentrated in the yolk platelet. **(B)**, preimmune serum did not stain the oocytes. Scale bars, 25 μm.

stained, but the mucous gland was not (data not shown). In the thigh muscle, the signal was observed in endomysium (Figure 4F). In the small intestine, mucus in the lumen and in the goblet cell contents was stained (Figure 4G). The lamina propria and smooth muscle cells were also stained. The staining of smooth muscles was confined to the contours of the muscle fibers seen in cross section. The antibody weakly stained almost all regions of the brain as well as the neural tube of the tadpole (data not shown), but the signal was more pronounced in the sciatic nerve. The signal was observed in the endoneurium surrounding each nerve fiber (Figure 4H). In the heart, weak granular signals were observed in the cardiac muscle, and more prominent signals occurred in the blood vessel walls of the ventricle (data not shown). In all these tissues, preimmune serum did not generate definite signals (not shown).

*Electron microscopic observation of ovary*

In the ovary, the activity of the D-galactoside-binding lectin was remarkably high, and Western blotting showed the presence of abundant galectin in this tissue. Furthermore, galectin was localized in the oocyte, where extracellular or juxtacellular localization is common with this protein species. To understand more about the nature of this lectin in the oocyte, electron microscopic immunohistochemistry was performed. This revealed that galectin was present in the periphery of the yolk platelet (Figure 5A,B), sometimes known as the yolk plasm (Yoshizaki, 1990) or electron dense layer (Komazaki and Asashima, 1987). This signal was apparent in almost all yolk platelets, and there was no apparent gradient of the signal in the oocyte. The main body of the yolk platelet was completely negative, but the cytosol and other organelles were faintly stained (Figure 5A,B). Cortical granules were clearly not stained (Figure 5A). Preimmune serum did not stain any part of the section (Figure 5C).

## Discussion

Galectins have been purified from various animal tissues, and extensive structural, chemical and histochemical studies have been performed. There is also some information on the whole-body distribution of one kind of galectin in animals. In the chicken embryo, the presence of a 14 kDa galectin in skin, muscle, bone, eye, heart, liver and brain has been detected by radioimmunoassay (Oda and Kasai, 1983). In *Xenopus laevis*, a detailed immunohistochemical study was performed using anti-14 kDa skin galectin antibody (Marschal *et al.*, 1994). However, considering the diversity of the galectin family and the variety of the animal kingdom, it is still useful to investigate the distribution of galectins.

In the present study, we investigated the distribution of galectin in *Rana* catesbeiana by hemagglutination assay and Western blotting using anti-oocyte galectin antibody. As a result, it became clear that galectin is distributed in a wide variety of tissues. However, the level of D-galactoside-specific lectin activity in the ovary was remarkable, being 16 times that of the liver, 32 times that of the kidney, and 256 times that of muscle. On the other hand, little lectin activity was detected in testicular homogenate, suggesting that the activity in the ovary is not high because it contains germline tissue.

We detected quite high D-galactoside-binding lectin activities in the liver and kidney, although the signals from these tissues on Western blotting were no stronger than those of other tissues. It is probable that the liver and kidney contain other type of galectin along with the oocyte galectin. It is known that MDCK-II canine kidney cells (Lindstedt *et al.*, 1993) and baby hamster kidney cells (Sato *et al.*, 1993) have a "chimera" type galectin-3 diffusely distributed in the cytosol and secrete it by a nonclassical pathway. In our study, *Rana* oocyte galectin was not diffused in renal tubular cells, but was located mainly on basement membranes or associated with cytoplasmic membranous structures. Such localization seems reasonable because laminin and fibronectin were found to be endogenous ligands for *Rana* oocyte galectin when human placenta was used as the study material (Ozeki *et al.*, 1995).

Bols *et al.* (1986) reported that a 16 kDa lectin is present in the granular and mucous glands of *Xenopus* skin. Anti-*Rana* oocyte lectin antibody also stained the granular but not the mucous gland. The intense staining observed around the keratinocytes and dermis of *Rana* skin agrees with the findings in

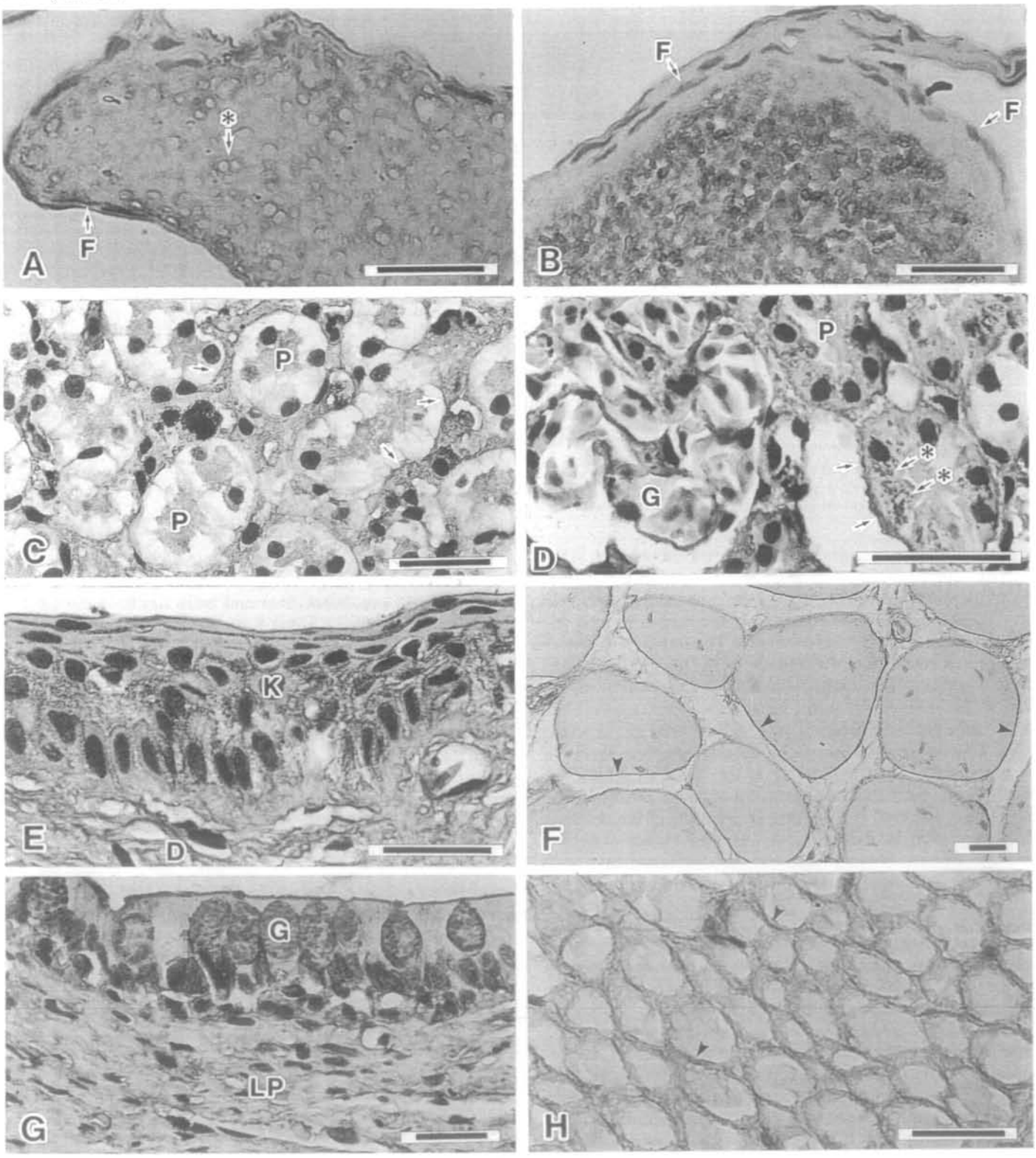


**Fig. 4.** Staining of tissue sections with anti-*Rana* oocyte galectin antiserum. **(A)** Stage III oocyte; F, follicle cell layer; an arrow with an asterisk indicates the signal associated with membranous structures; **(B)** Stage V oocyte; F, follicle cell layer; **(C)** liver; P, parenchymal cell; arrows indicate interlobular connective tissue; **(D)** kidney; G, glomerulus; P, proximal convoluted tubule; arrow indicates signals on the basement membrane while arrow with asterisk indicate signals in cytoplasmic membranous structures. **(E)** Skin; K, keratinocyte; D, dermis; **(F)** thigh muscle; arrowhead indicates endomysium; **(G)** small intestine; G, goblet cell; LP, lamina propria; **(H)** sciatic nerve; arrowhead indicates endoneurium. Scale bars, 25 μm.

chicken skin analyzed by anti-14 kDa and anti-16 kDa skin galectin antibodies, respectively (Akimoto *et al.*, 1992, 1993). In thigh muscle, small intestine, sciatic nerve, and heart, the localization of staining was similar to that described in *Xenopus laevis* (Marschal *et al.*, 1994).

In Western blotting experiment, anti-oocyte galectin antibody detected two bands, 15 kDa and 24 kDa in the ovary. A 15 kDa band is slightly bigger than the 14 kDa purified oocyte galectin (Ozeki *et al.*, 1991). This is probably caused by the presence of large amount of yolk proteins that affect mobilities

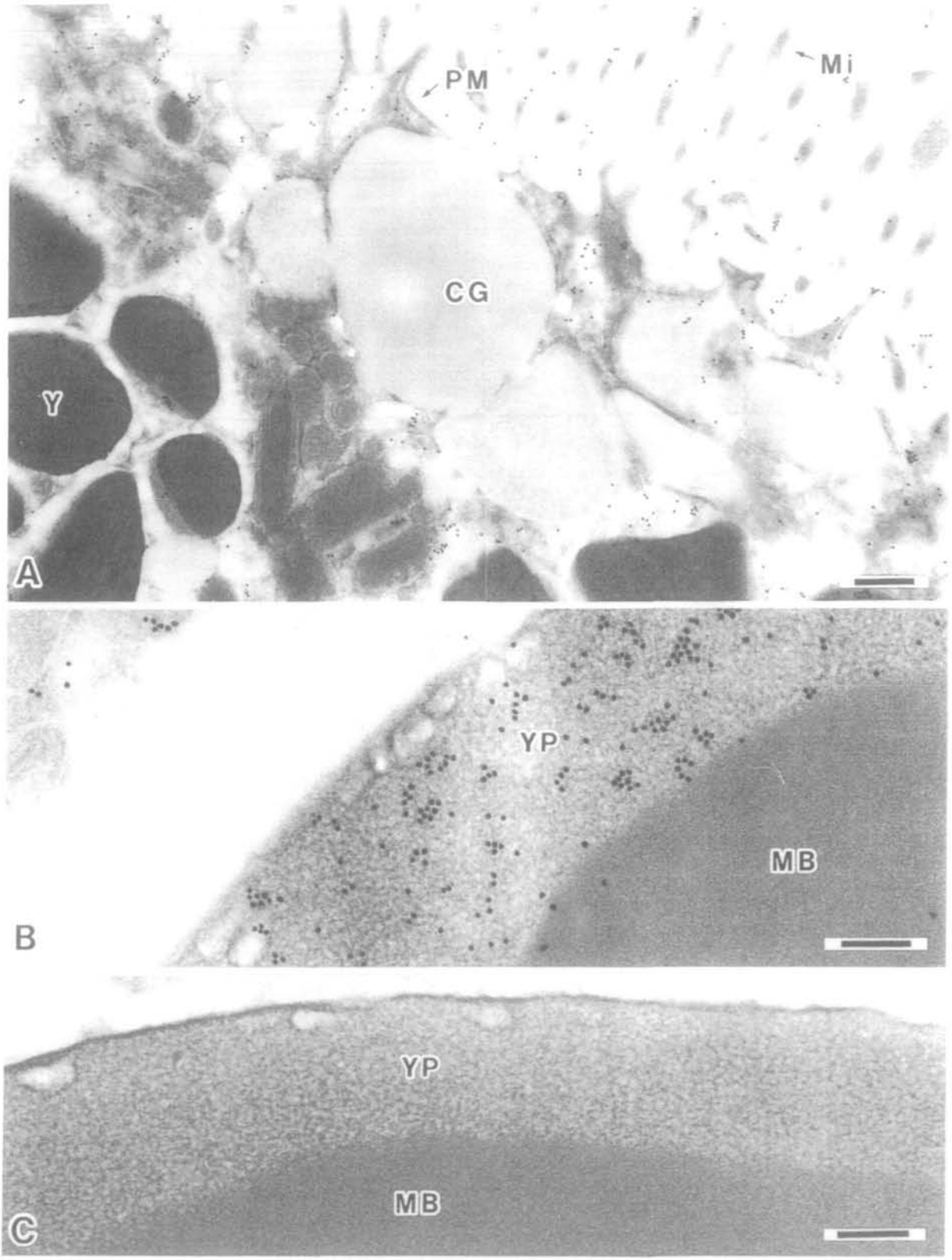


**Fig. 5.** Electron micrograph of a fully grown (Stage VI) oocyte section stained with anti-*Rana* oocyte galectin antiserum (**A, B**) or with preimmune serum (**C**). (**A**) Low magnification surface view of an oocyte. Colloidal gold signals are prominent in the yolk platelet (Y) but not in the cortical granule (CG). Mi, microvillus of oocyte; PM, plasma membrane of oocyte. Scale bar, 400 nm. (**B**) Enlarged view of a periphery of a yolk platelet. The signals are located in the yolk plasm (YP), but not in the main body (MB). Scale bar, 200 nm. (**C**) Preimmune serum did not stain any part of the yolk platelet. Scale bar, 200 nm.

of smaller proteins. The identity of 24 kDa band is unknown, but we detected similar band in female, but not male serum (data not shown). The Western blotting signals of the liver, kidney, and heart seemed as a doublet of 14–15 kDa. The chick possesses two galectins of 14 kDa and 16 kDa, each with 134 amino acids (Sakakura *et al.*, 1990). The homology of the amino acid sequence between these isolectins is about 50%, and anti-16 kDa antibody does not cross-react with the 14 kDa species. At present, it is not clear whether there are immunologically cross-reactive isolectins in *Rana* catesbeiana. In *Xenopus laevis*, two 16 kDa galectins from skin are encoded by almost identical mRNAs and differ by only three amino acids out of 135. The homology of the partial amino acid sequence showed almost identical divergence of *Rana* galectin from *Xenopus* and *Bufo* galectins. However in *Xenopus*, anti-galectin antibody did not react with oocytes or embryonic tissue (Marschal *et al.*, 1994). In this respect, the properties of *Rana* oocyte galectin are more akin to those of *Bufo* ovary galectin.

It is interesting that *Rana* oocyte galectin occurs only in the yolk plasm of the yolk platelet. During *Xenopus* oogenesis, it has been suggested that the TGF-β-like hormone activin associates with vitellogenin and is incorporated into the yolk platelet by endocytosis of the oocyte. In this situation, activin is distributed homogeneously within the yolk platelet (Uchiyama *et al.*, 1994). We investigated whether *Rana* oocyte galectin has any affinity for vitellogenin in a ligand blot experiment using biotinylated oocyte lectin as a probe. The results, although preliminary, suggested that galectin does not have affinity for vitellogenin (data not shown). Vitellogenin is taken up by the oocyte via a specific receptor-mediated endocytosis in *Xenopus* (Okabayashi *et al.*, 1996). In the chick, α2-macroglobulin (Jacobsen *et al.*, 1995), very low density lipoprotein, apolipoprotein B and vitellogenin (Stifani *et al.*, 1990) are all endocytosed via the same receptor, oocyte VLDL/VTG receptor (OVR). It is probable that galectin is also recognized directly by such a receptor, or associates with a factor(s) that is endocytosed by the oocyte in *Rana*. Another possibility is that the oocyte synthesizes galectin and transport it via a nonclassical pathway to the yolk platelet. This is also probable because galectin is secreted from the eukaryotic cell via a nonclassical pathway (Cooper and Barondes, 1990; Cleves *et al.*, 1996), and the limiting membrane of the yolk platelet has some common characteristics with its origin, the plasma membrane (Gallo *et al.*, 1995). In our RT-PCR assay using degenerate primers, a weak signal was detected from the ovary (data not shown), but it is uncertain that such level of galectin mRNA could explain the vast amount of this protein in the oocyte.

In amphibian eggs, sulfated glycosaminoglycans are present in the periphery and the limiting membrane of the yolk platelet (Robertson, 1979). A ganglioside M5 is present in the yolk granule of sea urchin eggs (Shogomori *et al.*, 1997). Thus, it is possible that galectin interacts with some sugar chains in the yolk platelet. However, it is apparent that galectin is present in great excess than its ligands in *Rana* yolk platelet because the lectin is extracted by homogenization from the eggs without addition of excess ligands (Ozeki *et al.*, 1991).

## Materials and methods

*Purification and structural characterization of* Rana *galectin*

Rana galectin was affinity purified from *Rana* catesbeiana unfertilized eggs as previously reported (Ozeki *et al.* 1991). Intact *Rana* galectin was digested with *Acromobacter protease* I (TaKaRa, Japan) , which specifically cleaves lysyl bonds, at 37 oC in 50 mM Tris–HCl (pH 9.0) in the presence of 2 M urea, at an enzyme-to-substrate ratio of 1:150 by weight. The digests were separated by reversed-phase high performance liquid chromatography on a Cosmosil 5C8 (4.6 × 100 mm; Nacalai Tesque, Japan) column with gradients of acetonitrile in dilute aqueous trifluoroacetic acid. Amino acid sequence of each separated peptide was determined with an Applied Biosystems model 470A protein sequencer.

*Hemagglutination assay*

Tissues from adult *Rana* catesbeiana were homogenized in a 10-fold volume of tris-buffered saline (TBS, pH 7.4) supplemented with 100 mM lactose. The homogenate was centrifuged at 20,000 × *g* for 1 h at 4°C. The supernatant was dialyzed against TBS four times for 2 days at 4°C, then the protein content was determined by the Bradford protein assay (Bio-Rad, USA) using bovine serum albumin as a standard. Protein concentration of the homogenate was adjusted to 1.5 mg/ml prior to the hemagglutination assay, which was performed as previously described (Matsui 1984). The assay was performed in the presence of 10 mM EDTA to eliminate the activity of any calcium-dependent lectins.

*Western blotting*

Tissues were homogenized in a 10-fold volume of phosphate-buffered saline (PBS) supplemented with 100 mM lactose, then centrifuged at 20,000 × *g* for 30 min at 4°C. The protein concentration of the supernatant was determined by the Lowry method using bovine serum albumin as a standard. Ovarian (2.5 μg) and other tissue (30 μg) proteins were subjected to SDS–PAGE under reducing conditions using 15% polyacrylamide gel (Laemmli, 1971). The proteins were blotted to a PVDF membrane (Bio-Rad, USA) using a semidry blotting apparatus (Nippon Eido, Japan). The membrane was blocked in 3% skim milk in PBS, then reacted with anti-*Rana* oocyte galectin rabbit serum (Ozeki *et al.*, 1995) or preimmune serum diluted 1:1000 in the blocking solution for 2 h at room temperature (RT), followed by washing in 0.05% Tween 20 in PBS (PBST). The membrane was further treated with 1:1000 biotinylated anti-rabbit IgG (Bio-Rad, USA) for 2 h at RT, washed in PBST and reacted with an ABC complex, consisting of 1 μl biotinylated peroxidase (Zymed, USA) and 5 μg streptavidin (Wako Chemicals, Japan) in 5 ml of PBST, for 30 min at RT. The membrane was washed in PBST and stained with 0.5 mg/ml 3,3′-diaminobenzidine tetrahydrochloride and 0.015% hydrogen peroxide.

*Immunohistochemistry*

Adult *Rana catesbeiana* were anesthetized in ice and pithed. The tissues were excised and fixed in 4% paraformaldehyde with or without 0.1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 2 h at 4°C, dehydrated using an ethanol series, cleared in Lemosol (Wako Chemicals, Japan) and embedded in Histoprep (Wako Chemicals, Japan). Sections (4 μm) were cut on a microtome and mounted on poly-L-lysine-coated slides. The sections were deparaffinized, treated with 0.45% hydrogen peroxide in methanol for 45 min at RT to inhibit endogenous peroxidase activity, and stained with anti-*Rana* oocyte galectin antiserum in a similar way to the blot membrane. Each section was counterstained with Delafield's hematoxylin, and mounted in Canada balsam. Oocytes were staged according to Dumont (1972).

For electron microscopy, a fully grown oocyte (Stage VI) was manually defolliculated, and fixed and dehydrated in an ethanol series as described above, then embedded in LR Gold resin (London Resin Co., UK) at –20°C for 2 days by UV irradiation. Ultrathin sections were cut with a diamond knife and mounted on collodion-coated nickel grids. The sections were blocked in PBS containing 20% (v/v) fetal calf serum for 30 min, then stained with anti-*Rana* oocyte galectin antiserum diluted 1:40 in 0.5% BSA in PBS for 30 min. They were then washed in PBS, and treated with 15 nm-colloidal gold-labeled anti-rabbit IgG (Ultra Biosols, UK) diluted 1:10 in 0.5% BSA in PBS for 30 min. The sections were finally washed with 0.05% Triton X-100 in PBS, then with distilled water, stained with uranyl acetate and observed under a JEOL-200 electron microscope (Nihon Denshi, Japan).

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