Xenopus laevis Lectin Is Localized at Several Sites in Xenopus Oocytes, Eggs, and Embryos

MARIE M. ROBERSON and SAMUEL H. BARONDES
Department of Psychiatry, University of California, San Diego, La Jolla, California 92093; and Veterans Administration Medical Center, San Diego, California 92161

ABSTRACT The endogenous lectin of Xenopus laevis oocytes, unfertilized eggs, and blastula-stage embryos was immunohistochemically localized using a highly specific antiserum. Each tissue was examined with several techniques, including paraformaldehyde or glutaraldehyde fixation, frozen or plastic sections, and immunofluorescence or immunoperoxidase staining. In oocytes and unfertilized eggs, lectin was detected in association with yolk platelets, cortical granules, and the vitelline envelope. In embryos, cortical granules had disappeared and lectin was found in the cleavage furrows between the embryonic cells. The distribution of the lectin suggests that it plays more than one role in this developing system.

Lectins are a class of carbohydrate-binding proteins originally identified in plants, but recently isolated from many animal tissues (1, 2). Little is presently known about their functions, but there is evidence that they are often localized extracellularly in association with glycoconjugates (3-4, and Cerra, R. F., P.L. Haywood-Reid, and S. H. Barondes, manuscript submitted for publication). For example, chicken-lactose-lectin-I, a lectin found in embryonic chicken muscle, is secreted by developing muscle cells into the extracellular matrix (3). A related beta-galactoside binding lectin in rat has been localized in the extracellular matrix of lung (Cerra, R. F., P. L. Haywood-Reid, and S. H. Barondes, manuscript submitted for publication). Another chicken lectin, chicken-lactose-lectin-II is concentrated in the secretory granules of the intestinal mucosal goblet cells, and is apparently externalized onto the mucosal surface of the intestine along with mucins to which it can bind (4). These findings suggest that lectins play a role in storage and/or secretion of intracellular glycoconjugates as well as in the organization of extracellular glycoconjugates at various sites. Since the same lectin may be expressed in different tissues and at different stages of development (5), it may have more than one function.

Among vertebrate tissues, frog eggs are a prominent source of lectins (6-10). Of these, the lectin from Xenopus laevis has been highly purified from both oocytes and early embryos, using both affinity chromatography and preparative isoelectric focusing (10). In an attempt to determine the function of this lectin, we raised an antiserum that reacts with it and localized the lectin immunohistochemically. We show here association of the lectin with yolk platelets, cortical granules, and the vitelline envelope of both oocytes and unfertilized eggs. In embryos, it is also found extracellularly in the cleavage furrows.

MATERIALS AND METHODS

Lectin Purification: Lectin from X. laevis oocytes was purified by affinity chromatography followed by isoelectric focusing, as described previously (10).

Preparation of Antiserum: Initial immunization in rabbits was carried out by subcutaneous injection of 100 μg of lectin in incomplete Freund's adjuvant (Difco Laboratories, Detroit, MI). Rabbits were subsequently injected with 10 μg of lectin in incomplete Freund's adjuvant at 4 and 6 wk after the initial injection. Serum was collected 7 d after the final injection.

In experiments in which absorbed antiserum was used as a control, 0.2 ml of antiserum was mixed with an equal volume of a 1 mg/ml solution of lectin that had been denatured by boiling for 10 min. After shaking for 1 h, this mixture was centrifuged at 10,000 g for 5 min and the supernatant was reacted with another aliquot of the boiled lectin and centrifuged. The final supernatant is the absorbed antiserum.

Identification of Antigens in Crude Extracts: Antigens in oocytes, eggs, and embryos that react with the antiserum were identified by electrophoresis and immunoblotting (11). In preliminary experiments in which extracts were made in SDS, the solubilized yolk platelet proteins interfered with migration of other proteins. We, therefore, used 0.5% Triton X-100 to extract the tissues because the yolk proteins are largely insoluble in this detergent. We also established that virtually all the tissue antigen that reacts with our antiserum is present in the Triton extracts by using a “dot immunobinding” procedure (12) in which serial dilutions of the extract were bound to nitrocellulose sheets, then reacted with antiserum.

For “dot immunobinding” experiments, each tissue was homogenized in a Sorvall Omni-Mixer at 4°C in 9 vol (wt/vol) of 10 mM Tris hydrochloride, pH 7.6, 10 mM CaCl2, 150 mM NaCl (TCS) containing 0.3 M galactose plus 0.5% Triton X-100. After centrifugation at 100,000 g for 1 h, twofold serial dilutions of the supernatant were prepared in the same medium and 2 μl of each dilution
was directly blotted onto nitrocellulose sheets (Bio-Rad Laboratories, Richmond, CA) and air-dried. The particulate fraction was rehomogenized in an equal volume of 1% SDS containing 2% TCA/chloroform, and serial dilutions of this mixture were also applied to nitrocellulose. The "dot immunobinding" sheets were incubated in 150 mM NaCl, 50 mM Tris-HCl, pH 7.2 (TBS) containing 5% normal goat serum (Colorado Serum Co., Denver, CO), 1% BSA (Fraction V, Sigma Chemical Co., St. Louis, MO) and 0.05% Tween-20 (Sigma Chemical Co.; this mixture is designated TBS-WASH) for 1 h at room temperature to block nonspecific binding sites. This was followed by a 1-h incubation in a 1:50 dilution (in TBS-WASH) of immune or preimmune antiserum. The sheets were then washed for 30 min with two changes of TBS plus 0.05% Tween-20 before a 1 h-incubation in a 1:200 dilution of peroxidase-conjugated goat anti-rabbit IgG (Cappel Laboratories, Cochranville, PA). The sheets were again washed for 30 min in TBS plus 0.05% Tween-20 before development in a fresh solution of TBS containing 0.2% H2O2 and 0.16 mg/ml 4-chloro-l-napthol (Polysciences, Inc., Warrington, PA). The highest dilution that showed a clear reaction product is a measure of the antigen content of the sample.

To identify the antigens in each extract, an immunoblotting procedure was used, based on that of Towbin et al. (11). After polyacrylamide gel electrophoresis in SDS under reducing conditions (10), the separated proteins were transferred to nitrocellulose sheets using an electrophoretic blotting chamber (CBS Scientific, Del Mar, CA) at 2 vol/cm, 50 mA for 30 min followed by 24 vol/cm, 400 mA for 2.5 h. Sheets were cut into strips and either stained with amido black or reacted with antiserum as described above for the "dot immunobinding" procedure. Visualization of bound antigen by the peroxidase procedure was performed as described above.

**Tissue Preparation:** X. laevis were obtained from Nasco, Ft. Atkinson, WI. Stage V (13) oocytes were obtained by laparotomy and the theca was removed by hand with watchmaker's forceps. Unfertilized eggs were obtained by injection of 1,000 u of human choronic gonadotropin (Sigma Chemical Co.) 6-7 h before manually stripping the unfertilized eggs from the animals. Blastula-stage embryos were a gift of Dr. Nicholas Spitzer, University of California, San Diego. In some circumstances, jelly coats were removed from embryos and unfertilized eggs by treatment with 0.1% mercaptoethanol, as previously described (14).

For immunofluorescence studies the tissue was fixed for 6 h at room temperature in a solution containing 3% paraformaldehyde and 0.025% saponin in 75 mM phosphate buffer, pH 7.2, 75 mM NaCl (PBS). The tissue was transferred to a solution of 0.3% paraformaldehyde, 30% sucrose, 0.025% saponin in PBS and stored overnight at 4°C before sectioning. For immunoperoxidase staining the tissue was fixed for 90 min with 3% glutaraldehyde, 0.025% saponin in PBS, then transferred to a solution of 30% sucrose, 0.025% saponin in PBS and stored overnight at 4°C before sectioning.

To prepare frozen sections, specimens were mounted on microtome chucks using OCT compound (Miles Laboratories, Inc., Elkhart, IN) and 1-2 mm sections were cut at ~35°C in an H/I Bright Cryostat (Hacker Instruments, Inc., Fairfield, NJ) fitted for sectioning with glass knives. Tissue used for plastic sectioning was dehydrated in a graded series of ethanol followed by two 10-min incubations in propylene oxide. The tissue was then infiltrated with a 1:1 mixture of propylene oxide and Epon overnight at room temperature under low vacuum. This mixture was replaced with fresh Epon and incubated for 3-4 h at room temperature under low vacuum before curing at 60°C for 18 h. Sections of 0.5-2 μm were obtained with an American Optical ultratome microtomed with glass knives.

**Immunoperoxidase Staining:** Before staining the slides were washed for 1 h in two changes of PBS containing 25% normal goat serum (PBS + GS). All dilutions of antiserum were made in PBS + GS and all incubations were done at room temperature. Incubations in a 1:100 dilution of immune or control antiserum were done for 30 min followed by a 10-min wash in PBS + GS. Next, slides were incubated in a 1:40 dilution of goat antiserum raised against purified oocyte lectin for 30 min followed by a 10-min wash in PBS + GS. Slides were transferred to a 1:40 dilution of rabbit peroxidase-antiperoxidase (Cappel Laboratories, Inc., Cochranville, PA) followed by two 10-min washes in TBS. The slides were incubated for 15 min in a reaction mixture prepared by combining 8 mg of 4-chloro-l-napthol (Polysciences, Inc.) dissolved in 250 μl of 75% ethanol to 50 ml of TBS containing 0.2% H2O2 immediately before use. Slides were washed for 10 min in TBS, drained and mounted with coverslips using 90% glycerol.

**Immunofluorescence Staining:** The slides were washed in normal or control serum as described above. After a 30-min wash in two changes of PBS + GS, the slides were incubated in a 1:50 dilution of rhodamine-conjugated goat anti-rabbit IgG (Cappel Laboratories, Inc.) for 30 min. After a 30-min wash in PBS, slides were mounted as above.

**Microscopy:** A Leitz Dialux epifluorescence microscope with a Leitz X100 (NA 1.3) oil immersion objective was used for examining sections stained with the immunofluorescence technique. Immunoperoxidase-stained frozen sections were examined with a Zeiss x40 (NA 0.9) Plan-neofluar oil immersion lens. Immunoperoxidase-stained plastic sections were examined with a Leitz x100 phase (NA 1.3) oil immersion lens. Photomicrographs were taken with a Wild MPS 45 camera using Kodak Tri-X film for the fluorescence studies and Pan-X film for the peroxidase studies. Experimental and control slides were exposed and printed under identical conditions.

**RESULTS**

**Specificity of Antiserum**

The specificity of the antiserum raised against the purified oocyte lectin was examined by immunodiffusion. A single precipitin line that merged without spurring was formed when purified oocyte lectin or crude extracts of oocytes or embryos were examined (Fig. 1a). This supports the biochemical evidence (10) that the lectins isolated from oocytes and embryos are very similar.

To evaluate the possibility that the tissues we wished to study contained immunologically cross-reactive materials, we used an immunoblotting technique (11). In initial experiments, we found that yolk platelet proteins, which comprise 70% of the total egg protein (15) interfered with the migration of others. Since yolk platelet proteins were largely insoluble in 0.5% Triton X-100, we used extracts prepared in this detergent for further studies. This was justified since >95% of the immunoreactive material in each of the tissues was solubilized by Triton X-100, as determined by the "dot immunobinding" procedure described in Materials and Methods. Immunoblots of the Triton extracted materials showed only lectin and no other reactive bands (Fig. 1b). The diffuse band of staining corresponds exactly with the pattern seen when only purified lectin had been electrophoresed. The staining pattern reflects the two broad glycoprotein bands found in the purified lectin (10).

**Alternative Fixation and Staining Procedures**

To properly evaluate lectin localization, we found it necessary to use various techniques. These included paraformaldehyde and glutaraldehyde fixation, frozen and plastic sections, and immunofluorescence and immunoperoxidase procedures. To obtain a clear picture of lectin localization, it is necessary to consider the results found with all these procedures.

**Lectin Localization in Oocytes**

Lectin in oocytes was found in yolk platelets, cortical granules, and the vitelline envelope. Localization in each of these structures was apparent in paraformaldehyde-fixed frozen sections stained by the immunofluorescence technique (Fig. 2a). Sections reacted with preimmune serum (Fig. 2c) or absorbed immune serum (not shown) showed no staining. In plastic sections of glutaraldehyde-fixed tissue stained by the immunoperoxidase procedure, localization in cortical granules was very clear (Fig. 3a), but lectin in yolk platelets and the vitelline envelope was difficult to detect. However, vitelline envelope localization was obvious when frozen sections of glutaraldehyde fixed tissue were examined with the immunoperoxidase technique (Fig. 3c). With this procedure, the cortical granules were difficult to resolve. Sections stained

Abbreviations used in this paper: TBS, 150 mM NaCl, 50 mM Tris-HCl, pH 7.2; PBS GS, PBS containing 25% normal goat serum.
FIGURE 1 Characterization of antiserum and tissue antigens by immunodiffusion and immunoblotting. (a) Immunodiffusion studies with antiserum raised against purified oocyte lectin. The center well contained 10 µl of antiserum. The labeled wells contained 5 µg of purified lectin (L), or crude extract from oocytes (O) or embryos (E); each contains 200 µg protein × 2. (b) Identification of tissue proteins that react with the antiserum. Extracts of the indicated tissues were prepared in a solution containing 0.5% Triton X-100 as described in Materials and Methods, electrophoresed on a 10% SDS polyacrylamide slab gel, and electrophoretically transferred to nitrocellulose sheets. Three lanes of each sample were cut apart and lane 1 was stained with 0.2% amido black to visualize the polypeptide bands; lane 2 was exposed to immune serum; and lane 3 was exposed to preimmune serum. Bound antibody was visualized by treatment with peroxidase conjugated goat anti-rabbit IgG, as described in Materials and Methods. Values at left represent molecular weight markers (× 10^3).

FIGURE 2 Localization of *X. laevis* lectin in oocyte sections by immunofluorescence. Frozen sections were stained with immune serum (a) or preimmune serum (c) and bound antibody was detected with rhodamine-conjugated goat anti-rabbit IgG. b is the bright field photomicrograph of a. YP, yolk platelet; CG, cortical granule; VE, vitelline envelope; P, pigment granule. Bar, 1 µm. × 6,250.

with preimmune serum (Fig. 3, b and d) or absorbed immune serum (not shown) showed no staining.

It is not clear why only the cortical granules stained prominently in plastic sections. One possibility is that the cortical granules stand out because the lectin is dispersed throughout their entire width whereas the lectin in the vitelline envelope and around the yolk platelets is in very thin deposits that are difficult to visualize. Since there is essentially no diffusion of reagents into these plastic sections, only the broader zones of staining in the cortical granules might be apparent. In contrast, in frozen sections that permit diffusion of reagents, the wider distribution of reaction product through the depth of
FIGURE 3  Localization of X. laevis lectin in oocyte sections by the immunoperoxidase procedure. Sections were stained with immune serum (a and c) or preimmune serum (b and d) and bound antibody was detected by the peroxidase method. a and b are plastic sections and c and d are frozen sections. PL designates the plasma membrane. Other symbols are as in Fig. 2. Bar, 1 μm. x 6,250.

Another possibility is that the cortical granules contained an especially high concentration of the lectin. In plastic sections like those used here, only the protein at the surface of the section can react with antibody, since the plastic is essentially impermeable. Only regions with high concentrations might be apparent. This should not, however, be taken as evidence that lectin is primarily concentrated in the cortical granules, since those structures are very scarce by comparison with yolk platelets.

The immunoperoxidase reaction product over the vitelline envelope in frozen sections is not due to release of lectin from cortical granules, since the tissue was well fixed with glutaraldehyde. Nor is it due to diffusion of excess immunoperoxidase reaction product, since the same pattern is seen under conditions that generate less staining. Furthermore, localization of lectin in the vitelline envelope by immunofluorescence (Fig. 2 a) does not involve a diffusible reaction product. Therefore, despite failure to detect lectin in the vitelline envelope in plastic sections, its localization at this site seems clear.

Lectin Localization in Unfertilized Eggs

As in oocytes, lectin was demonstrated in cortical granules in plastic sections (Fig. 4 a) and in the vitelline envelope in frozen sections (Fig. 4 c) stained by the immunoperoxidase technique. Lectin was also associated with yolk platelets, as demonstrated by immunofluorescence (Fig. 5 a). In all cases, absorbed serum (Fig. 5, b–d) and preimmune serum (not shown) did not stain.

Lectin in Blastula-state Embryos

The novel finding in the embryo was the presence of lectin in extracellular materials in the cleavage furrow region (Fig. 6 a). The immunoperoxidase reaction product is a very prominent purple color, but does not photograph vividly in black and white. Nevertheless, the gray clumps of stain throughout the cleavage furrow are apparent in the bracketed region upon staining with immune serum (Fig. 6 a) compared with preimmune serum (Fig. 6 b). The furrow shown appears to be in the process of lengthening during cytokinesis, but reaction product was also seen in clefts between two separate blastomeres (not shown).

Cortical granules had disappeared with fertilization, but lectin remained associated with the surrounding envelope, now called the fertilization envelope (Fig. 6 c). As with unfertilized eggs, localization in the surrounding envelope was prominent in frozen sections (Fig. 6 c) but not detectable in...
plastic sections of identical samples (Fig. 6a). Prominent lectin staining continued to be found in association with the yolk platelets of the embryo, as determined by immunofluorescence (not shown, but indistinguishable from Fig. 5a). As in other experiments, preimmune serum (Fig. 6, b and d) and absorbed serum (not shown) did not stain the sections.
DISCUSSION

Using a highly specific antiserum raised against the purified lectin from *X. laevis*, we immunohistochemically localized this protein to several structures. Of these, the yolk platelets were the most abundant, suggesting that the vast majority of the cellular lectin is associated with them. How the lectin is associated with these structures cannot be resolved by light microscopy. Immunofluorescence staining suggests that it is near the surface of the yolk platelet, either internal to or around the surrounding membrane.

The abundant lectin associated with the yolk platelets is presumably a storage form, like the remainder of the yolk materials. It is possible that the lectin found in the cleavage furrows of the embryo is derived from that associated with the yolk platelets through an externalization process that is not understood. Externalization of intracellular lectin has previously been described in other developing systems, including embryonic chicken muscle (3) and *Dictyostelium discoideum* (16). In all cases, the externalized lectin may play a role in organizing the extracellular matrix of the developing organism.

*Xenopus* lectin was also found associated with the cortical granules and vitelline envelope of oocytes and unfertilized eggs, and with the fertilization envelope of fertilized eggs. Although these structures are much less abundant than yolk platelets, they represent a significant site of lectin accumulation. Since cortical granules release their contents upon fertilization, it seems likely that lectin is one of the materials that is released. This possibility has, indeed, been proposed by Hedrick and co-workers (9, 17). They identified a lectin activity in *Xenopus* extracts, raised an antiserum to it, and localized immunoreactive material in cortical granules of unfertilized eggs (17). However, in contrast with our results,
they found no lectin either in yolk platelets or in association with the vitelline envelope of unfertilized eggs. They presented evidence that, with fertilization, lectin becomes associated with the F layer of the fertilization envelope, the perivitelline space, and the innermost jelly layer. They suggested that a complex formed between lectin and these other materials plays a role in formation of a block to polyspermy.

It is difficult to directly compare our work with that of Hedrick and colleagues, since it is not clear that we are working with the same lectin. Their material (9) and ours (10) share a similar Ca$^{2+}$ dependence, and bind α-galactosides, but the biochemical properties of their lectin have not been reported. Because of this ambiguity, it is possible that they are dealing with a specific single form of *Xenopus* lectin whereas we are dealing with a mixture of several forms. Indeed, the *Xenopus* lectin we isolated contains two protein subunits and shows some heterogeneity on isoelectric focusing (10), raising the possibility of functionally distinct forms. There is evidence that chicken (5) and *D. discoideum* (16) tissues contain more than one lectin, and that they localize differently (2, 16).

The disagreement about localization could also be due to differences in immunohistochemical techniques. Greve and Hedrick (17) performed all of their immunohistochemical studies with the immunoperoxidase procedure and plastic sections. In unfertilized eggs, the only place where they found lectin was in cortical granules. When we used plastic sections, we confirmed their results. However, using other procedures, we found lectin associated with the vitelline envelope prior to fertilization. It is difficult to argue that release of cortical granule lectin onto this envelope with fertilization plays a special role, since lectin is already present at this site before fertilization. Nevertheless, it remains possible that the lectin released from cortical granules has a functionally significant effect on some material surrounding the fertilized egg, which is not associated with lectin before fertilization. It is also possible that the cortical granules release a specific ligand in association with the intragranular lectin, and that this complex plays a role in blocking polyspermy. Therefore, the general idea of Hedrick and colleagues (9, 17) that release of cortical granule lectin with fertilization has functional significance remains appealing and is not negated by our findings.

Because *Xenopus* lectin is present at so many sites, it is especially difficult to draw specific conclusions about its function from these immunohistochemical studies. However, when these results are combined with those in other systems (2–4, and Cerra, R. F., P. L. Haywood-Reid, and S. H. Barondes, manuscript submitted for publication), they support the general conclusion that soluble intracellular lectins are ultimately externalized into the extracellular sites where they exert their functional effects.

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