Cloning and expression of a *Xenopus laevis* oocyte lectin and characterization of its mRNA levels during early development

Jin Kyu Lee¹, Phillip Buckhaults¹, Christopher Wilkes¹, Meredith Teiheipt¹, Mary Lou King, Kelley W.Moremen¹, and Michael Pierce¹²

Department of Cell Biology and Anatomy, University of Miami School of Medicine, Miami, FL 33101, USA and ²Department of Biochemistry and Molecular Biology and the Complex Carbohydrate Research Center, University of Georgia, Athens, GA 30602, USA

To whom correspondence should be addressed

Introduction

Studies by two laboratories have demonstrated that *Xenopus laevis* oocytes and embryos contain soluble, calcium-dependent, lectins which form multimers of about 500 kDa (Roberson and Barondes, 1982; Nishihara et al., 1986). On reducing SDS-PAGE gels these lectins migrate as overlapping diffuse bands of about 45 and 43 kDa. (Roberson and Barondes, 1982; Outenreath et al., 1988). These proteins are contained within the cortical granules of the oocytes, as well as in several other locations, and they are released from the cortical granules at fertilization (Wyrick et al., 1974; Nishihara et al., 1986). Several lines of evidence suggest that the released multimeric lectins bind to oligosaccharide targets on glycoproteins in the egg jelly coat where they participate in the formation of the fertilization envelope. We have affinity-purified the oocyte lectins on immobilized melibiose (Roberson and Barondes, 1982). Moreover, a polyclonal antibody prepared against the oocyte lectin preparation showed binding to cleavage furrows and to areas of active cell migration (Roberson and Barondes, 1983; Monk and Hedrick, 1986). Proteins purified from the oocytes by melibiose affinity chromatography agglutinate trypsinized rabbit erythrocytes in the presence of Ca⁺², and this reaction is strongly inhibited by α-galactosides such as melibiose, suggesting that the lectins bind to the abundant glycolipids on these erythrocytes terminated by α-galactose residues (Clark et al., 1987).

After fertilization, lectins with very similar physical properties and ligand specificity have been purified from blastulae (Roberson and Barondes, 1982). Moreover, a polyclonal antibody prepared against the oocyte lectin preparation showed binding to cleavage furrows and to areas of active cell migration (Roberson and Barondes, 1983), including the blastopore region and the roof of the blastocoel (Outenreath et al., 1988). At these locations, it has been suggested that the lectins are involved in cell adhesion and migration. In order to define in detail the structure and function of the lectins during fertilization and early development, we have affinity-purified the oocyte lectins on immobilized melibiose (Roberson and Barondes, 1982; Roberson et al., 1985) and shown that after N-glycanase treatment, only one major polypeptide of 35 kDa could be detected. We obtained peptide sequences from the purified protein and used PCR techniques to isolate cDNA clones which encode this polypeptide. Expression of the cDNA in *E.coli* yielded lectin, which after purification demonstrated specific agglutination activity. Southern and Northern blot analyses demonstrated a single genomic sequence and expression of the lectin mRNA in the embryo up to at least the tail-bud stage, respectively.

Results and discussion

Characterization of the melibiose-binding oocyte lectin

The lectin was purified by affinity chromatography on melibiose-sepharose (Roberson and Barondes, 1982), revealing a diffuse pair of bands at about 45 and 43 kDa after SDS-PAGE. Exhaustive treatment of the preparation with N-glycanase, which cleaves Asn-linked oligosaccharides, and subsequent SDS-PAGE revealed a single major band at 35 kDa (Figure 1). These results demonstrate that the oocyte lectin, termed XL35,
is expressed as a single polypeptide and that the diffuse protein bands observed after affinity purification differ primarily in their Asn-linked oligosaccharide structures.

Isolation of XL35 cDNAs
The protein preparation was blotted onto nitrocellulose and subjected to trypsinization and peptide separation on reverse-phase HPLC. Several peptides were sequenced, and two of them (Materials and methods) were used to design degenerate PCR primers, as depicted in Figure 2. Two degenerate oligonucleotides, 29 and 26 basepairs in length, were synthesized and utilized in an RT-PCR reaction with total ovary RNA. The 159 bp amplimer generated (Figure 2) was subcloned and sequenced. This amplimer was then used to screen a λgt11 cDNA library prepared from Xenopus laevis ovary. The insert from a partial clone was isolated as a 401 bp EcoRI restriction fragment, subcloned, sequenced, and used as a probe for Southern and Northern blot analyses. The inserts from the remaining five clones were PCR-amplified using either vector primers flanking the cloning site or lectin-specific primers flanking the coding region. The respective coding region amplimers were subsequently subcloned and sequenced. Each of these clones contained a complete open reading frame, and the consensus sequence is shown in Figure 2.

Expression of XL35 cDNA in E.coli
The sequence encoding XL35 was modified by PCR to introduce a SalI restriction site at the predicted signal sequence cleavage site, and cloned into the pQE-9 QIAexpress vector. After harvesting, the cells were disrupted in guanidinium hydrochloride, and the extract was chromatographed on a Ni²⁺-NTA column, to which the 35 kDa protein bound. After exchanging the guanidinium HCl buffer to one with 8 M urea, elution of the recombinant lectin from the affinity column was accomplished by lowering the pH to 4.5. These eluent fractions contained a single major band at 35 kDa, whose N-terminal 25 amino acid sequence demonstrated it to be XL35. The purified recombinant lectin was assayed for its ability to agglutinate trypsinized, fixed rabbit erythrocytes. When all urea was removed from the solution containing the recombinant lectin, it slowly precipitated, perhaps due to its lack of glycosylation. In 2 M urea, however, as summarized in Table I, the recombinant lectin was active in agglutinating the erythrocytes, similar to the activity observed with the affinity-purified oocyte lectin. Moreover, the recombinant XL35 agglutination activity was completely inhibited by EDTA and by melibiose, but not by sucrose. These characteristics are similar to those observed for the affinity-purified oocyte lectin (Table I) (Roberson and Barondes, 1982). The purified oocyte lectin was also active in 2 M urea with the same specificity of binding, but with significantly lower specific activity. That the recombinant, nonglycosylated lectin shows agglutinating specificity similar to that observed for the oocyte lectin demonstrates that the XL35 cDNA we have isolated and expressed does indeed encode the oocyte lectin purified by melibiose chromatography.

Southern and Northern blot analyses
The 401 bp cDNA probe (Figure 2) was used to probe Southern and Northern blots. Analysis of the Southern blot shows that the probe hybridized with one major band in five genomic DNA restriction digestions (Figure 3), although small amounts of incomplete digestion were observed with EcoRI and PstI. These results argue against the existence of multiple, homologous genes in Xenopus.

To examine the expression patterns of lectin mRNA at fertilization and during embryo development, Northern analysis was performed on total RNA purified from Stage VI oocytes and from embryos at various stages of development. Northern analysis (Figure 4a) shows that relatively high levels of XL35 mRNA were present in the Stage VI oocytes and persisted through gastrulation, after which it declined. Compared to the levels of expression in gastrulae, very little XL35 mRNA was present in hatching tadpoles. The same blot was then probed with a cDNA encoding a fragment of mitochondrial rRNA in order to normalize these results to the amount of RNA in each lane of the blot (Figure 4b), and this normalization confirmed the results shown in Figure 4a. Since it is highly unlikely that maternal mRNAs have persisted until tadpole stages, together with the observation of a slight increase of RNA levels at gastrulation, it appears that XL35 mRNA is most likely newly transcribed at the mid-blastula transition along with many other zygotic RNAs. The fact that these RNAs are transcribed zygotically, as well as maternally, would strongly support the hypothesis that XL35 displays multiple functions during fertilization and development, as suggested by the immunolocalization experiments.

Characterization of XL35 and possible functions in morphogenesis
XL35 activity is assayed by agglutination of trypsinized, fixed rabbit erythrocytes (Roberson and Barondes, 1982). This ag-
Fig. 2. Nucleotide and amino acid sequences of XL35. The position in the lectin coding region that was used as a hybridization probe for Southern and Northern blots is indicated by an underline. The positions in the coding region corresponding to the initial primers designed from peptide sequence are denoted by a dotted underline. Asterisks below the peptide sequence indicate the three potential Ñ-glycosylation sites.

Agglutination activity requires calcium and can therefore be inhibited by EDTA. Many animal lectins have been shown to require calcium ions for activity ("C-type" lectins) and a sequence motif for the calcium-binding site of these lectins has been defined (Drickamer, 1993). Interestingly, XL35 does not contain this sequence motif. The activity of recombinant XL35 does, however, demonstrate that the TV-linked oligosaccharides on the native lectin are not required for ligand binding. The precise structures of the glycoconjugates in the egg jelly to which XL35 binds remain to be determined.

Experiments which examined the binding of polyclonal antibodies prepared against the oocyte lectin demonstrated that populations of the lectin are localized in cortical granules, are released at fertilization, and participate in formation of the fertilization envelope to prevent polyspermy (Nishihara et al., 1986). The conclusion that XL35 mRNA is expressed in developing embryos is consistent with the results of two previous studies: one that demonstrated significant amounts of a lectin with similar structure and specificity to the oocyte lectin in blastulae (Roberson and Barondes, 1982), and another that used a polyclonal antibody to the oocyte lectin to localize cross-reacting material to the blastopore region and to extracellular locations on the roof of the blastocoel (Outenreath et al., 1988). Taken together, these results suggest strongly that the XL35 functions not only in formation of the fertilization membrane, but also in cell adhesion and migration during early morphogenesis. The nature and localized expression of the glycoconjugate ligands in the embryo are unknown, although there are recent reports of a blood group-B active determinant expressed on glycoconjugates from *Xenopus* blastulae. These determinants are expressed in areas of cell-cell contact, and it seems very possible that XL35 or lectins similar to it could be binding to these glycoconjugates and regulating intercellular adhesion in the embryo (Nomura et al., 1995). Structural analyses of the oligosaccharide ligands of the lectin, as well as in situ hybridization and immunocytochemical experiments to refine the locations of the lectin biosynthesis and secretion are in progress to investigate this hypothesis.

Sequence similarity searches using the XL35 DNA and pep-
Fig. 3. Southern blot analysis of XL35. Twelve micrograms of genomic DNA derived from Xenopus laevis ovaries was digested for 24 h with BamHI, lane 1; EcoRI, lane 2; HindIII, lane 3; PstI, lane 4; and XhoI, lane 5. The blot was then subjected to hybridization using the 401 bp lectin restriction fragment (Materials and methods) as the radiolabeled probe.

The lectin sequences detected no similarity to any known published sequences, including plant or animal lectins. A human EST sequence was detected with >80% DNA sequence similarity to the XL35 lectin. Using this EST sequence, we have recently isolated cDNAs from several human and mouse tissues which show similar sizes to XL35, as well as high degrees of identity to the Xenopus lectin (60% at the amino acid level; J.-K. Lee, K.W. Moremen, and M. Pierce, unpublished observations). These results suggest that a family of lectins with similarity to XL35 exists in vertebrates, and that members of this family perform physiological functions in adult organisms as well as in Xenopus oocytes and embryos.

Materials and methods

Materials

Restriction enzymes, Thermus aquaticus DNA polymerase, agarose, and other chemicals were purchased from major chemical suppliers. Nitrocellulose filters, DNA labeling kits, and [α-32P]dCTP were purchased from Amersham. Nick and N-glycanase desalting columns were purchased from Pharmacia. Ultrafree-MC Low Binding Durapore Membranes were purchased from Millipore and used for gel purification of DNA fragments. Zeta-Probe GT nylon membranes for Southern analysis were purchased from Bio-Rad. N-Glycanase was from Boehringer. BSA, IPTG, melibiose, and immobilized melibiose were from Sigma. Sephaglas reagents were from Pharmacia. N2-NTA columns and vectors used to construct the 6-His tag were from Qagen and were used according to the manufacturer’s instructions.

Lectin purification and generation of PCR primers

The oocyte lectins were purified using a procedure previously described (Roberson and Barondes, 1982), followed by a C4 reverse phase HPLC step using acetonitrile-TFA buffers to desalt and concentrate the lectins. An aliquot of the protein peak from the column was dried, and the sample was sent to the Harvard Microchemistry Facility (Cambridge, MA), where it was then reduced with dithiothreitol, alkylated with iodoacetamide, resuspended in urea, and subjected to exhaustive tryptic digestion. The digested sample was chromatographed on a reverse phase HPLC column and two of the resolved peaks were subjected to gas phase Edman degradation. The isolated peptides yielded the following sequences: Peak 43-ESCNAEHVC10GCGGYYPEADPR and Peak
The amplimer obtained using the 5' primer CCCCGTCGACAGACATGATGAGGTTGTATCC and the 3' primer CCCCTGCAGTTGTAATAGTAGCCTCAAAA was designed into the 5' and 3' primers, respectively: 5' primer CCCGTCGACAGACATGATGAGGTTGTATCC, 3' primer CCCCTGCAGTTGTAATAGTAGCCTCAAAA; 3' primer CCCCTGCAGTTGTAATAGTAGCCTCAAAA; 5' primer CCCCGTCGACAGACATGATGAGGTTGTATCC. Two restriction enzyme cleavage sites, PstI, and XbaI, were found within amino acids 18 and 19 (von Heijne, 1985). PCR primers were designed to anneal at pH 7.0 at room temperature for 10 min and data collected using a Molecular Dynamics phosphorimager. The hybridization buffer was prepared by mixing 52.5 ml of 20% SDS, 1.5 g of fatty acid free BSA, 1.5 ml of a 10 mg/ml solution of heat-denatured herring sperm DNA, and 20.7 ml of water. The hybridization buffer was used for control hybridizations. All blots were probed using the same labelled 401 basepair probe (depicted in Figure 2) was generated and radiolabeled using the Amersham megaprime DNA labeling system according to manufacturer's instructions. The majority of the major protein band which migrated at 35 kDa on SDS-PAGE bound to the column. After washing the column with 8 M urea, the 0.1 M NaH2PO4, 0.01 M Tris, pH 8.0, the column was step-eluted with the same buffer at pH 6.3, 5.9, and finally the 35 kDa protein band was eluted using the same buffer at pH 4.5. The yield of purified recombinant peptide was about 250 mg/l of culture after purification on the nickel column.

**Digestion with N-glycanase**

Lyophilized affinity purified oocyte lectin (6 μg) was brought to 100 μl with a solution of 0.1 M β-mercaptoethanol/0.1% SDS. The sample was heated at 100°C for 30 min, after which 25 μl of 0.5 M Tris-HCl, pH 7.5, was added, followed by 10 μl of 0.1 M phenanthroline, 10 μl of 10% Triton X-100, and 3 μl of 0.1 M EDTA (200 U/μl). The sample was then incubated for 18 hr at 35°C, after which an additional 3 μl of enzyme was added and the incubation continued for 4 hr. The protein was then precipitated with trichloroacetic acid and resuspended in reducing sample buffer for SDS-PAGE. The control incubation was handled exactly as described, except no enzyme was added. SDS-PAGE was performed using gradient 4–15% polyacrylamide gels (Bio-Rad), which were stained with Coomassie blue.

**Expression of lectin cDNA and purification of recombinant protein**

The predicted signal peptide cleavage site was determined to be between amino acids 18 and 19 (von Heijne, 1985). PCR primers were designed to amplify the coding sequence excluding the signal peptide sequence (amino acids 19–313). Two restriction enzyme cleavage sites, Sall and PstI, were designed into the 5' and 3' primers, respectively. 5' primer: CCCCGTCGACAGACATGATGAGGTTGTATCC; 3' primer: CCCCTGCAGTTGTAATAGTAGCCTCAAAA. The amplimer obtained using these primers and the XL-1-2 cDNA clone as a template was cloned directly into the QIAexpress Vector (pQE-9) (Qiagen Corp.) using Sall-PstI restriction sites. This procedure appended the sequence, MRGSHis6GS, to the N-terminus. The resulting plasmid was transfected into E.coli host strain SB13009(pREP4). Recombinant protein production was induced with 1 mM IPTG. The purification of recombinant protein was performed by the method described in the QIA expression instructions. Briefly, the cells were harvested and subjected to one freeze-thaw, after which 10 g of the pellet (wet weight) were resuspended in 50 ml of 0.6 M guanidine hydrochloride, 0.1 M NaH2PO4, 0.01 M Tris, pH 8.0. After stirring for 1 h, the solution was centrifuged (10,000 x g, 20 min, 4°C) and the clear supernatant loaded on a 4 ml Ni-NTA chromatography column which was equilibrated and eluted using the manufacturer's instructions. The Agglutination assay was performed essentially as described using trypsinized, glutaraldehyde-fixed rabbit erythrocytes (Barondes and Roberson, 1987) in TCS buffer (10 mM Tris, pH 7.6, 10 mM CaCl2, 150 mM NaCl). To compare the purified and recombinant lectins, two lectins were assayed under similar conditions. Because the eluted recombinant lectin was in 8 M urea, a solution of purified nonrecombinant lectin was also brought to 8 M with a urea solution. The agglutination assay was performed using 10 μg of either recombinant or native lectin in 25 μl TCS and 8 M urea was added to wells of a 96-well microtiter plate. Next, 25 μl of TCS alone or TCS containing 0.4 M EDTA or 1.0 M melibiose or 1.0 M sucrose was added to the wells and mixed with the lectin solution. To this solution, 50 μl of a 1:1 suspension of trypsinized, glutaraldehyde-fixed rabbit erythrocytes in TCS was added and the contents of the wells were mixed by trituration. The final concentration of urea in the wells was 2 M, while that of EDTA was 0.10 M and that of melibiose and sucrose was 0.25 M. Agglutination activity was scored after 1.5 h at room temperature using an inverted microscope on low power, based on the criteria listed in the legend to Table 1. After 1.5 h in TCS plus 2 M urea, the purified oocyte lectin retained about 25–30% of the agglutination activity observed when it was assayed in TCS alone, but the relative abilities of various sugars to inhibit the agglutination reaction was similar in the presence or absence of urea. The recombinant lectin precipitated in the absence of urea; therefore, at least 2 M urea was used in the agglutination assay.

**Acknowledgments**

We thank Candice Johnson for performing the N-glycanase experiments, Frank Comer and Sergio DiVirgilio for assistance with the reverse-phase chromatography, and Gerardo Manilla-Alverez for assistance with illustrations. This research has been supported by a grant from the NCRR Resource Center for Biomedical Complex Carbohydrates to K.M. and M.P.

**Abbreviations**

XL35, the calcium-dependent melibiose-binding lectin from Xenopus laevis oocytes; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; IPTG, isopropyl-β-D-thiogalactopyranoside; TCS, buffer containing 10 mM Tris, pH 7.6, 10 mM CaCl2, 150 mM NaCl; BSA, bovine serum albumin.
References


Received on August 23, 1996; revised on September 25, 1996; accepted on September 30, 1996.