Subunit structure of a cortical granule lectin involved in the block to polyspermy in *Xenopus laevis* eggs

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The cortical granule lectin of *Xenopus laevis* eggs is a large molecular mass glycoprotein involved in the post-fertilization block to polyspermy. We have investigated the subunit structure of this lectin and found that the native molecule contains 10–12 monomers, each of which has considerable charge and size heterogeneity due to glycosylated side chains. In addition, significant amino acid sequence homology is indicated by peptide mapping of subunits separated by isoelectric focusing.

Fertilization *Crosslinking* *Deglycosylation* *Glycoprotein* *Lectin* *Peptide mapping*

1. INTRODUCTION

The *Xenopus laevis* egg integument has two components, a jelly coat and a vitelline envelope, separated from the egg plasma membrane by the perivitelline space. Following fertilization, polyspermy is prevented by at least two processes which appear to act in concert; membrane depolarization and the formation of a fertilization envelope (review [2]).

The process which forms the fertilization envelope involves a lectin [1], sequestered within cortical granules, which is released upon fertilization [2–4]. This cortical granule-derived lectin (CGL) interacts with a carbohydrate ligand in the innermost layer of the surrounding jelly coat, forming a structure known as the fertilization or F layer on the outer surface of the vitelline envelope [4,5]. Another cortical granule component, as yet uncharacterized, acts on two vitelline envelope glycoproteins via limited hydrolysis to form a structurally modified vitelline envelope [6]. The combination of this modified envelope with the F layer produces the fertilization envelope, a composite extracellular structure which is impenetrable to sperm [7].

CGL from eggs of *X. laevis* has been purified to homogeneity [1] and has been shown to be an oligomeric glycoprotein of high molecular weight. We present here further characterization of the CGL subunit structure.

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2. MATERIALS AND METHODS

2.1. *Cortical granule lectin monomers*

CGL was purified from a cortical granule exudate using a jelly agarose affinity column as described [1]. Protein concentrations were determined by the method of Lowry et al. [8], or, when protein was immobilized on Sepharose, by that of Marciani et al. [9]. Both protein methods used a bovine serum albumin standard. CGL was also detected by intrinsic fluorescence (291 nm excitation/328 nm emission) and by absorbance using an $A^{1\%}_{279\,nm,1\,cm} = 15.4$.

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Native CGL was dissociated in 2% SDS or 8 M urea, in the presence of mercaptoethanol. Subunits were then separated in the first case by SDS-PAGE [10] and in the second by isoelectric focusing, using a modification of the method of O'Farrell [11] with vertical slab gels. SDS-PAGE and isoelectric focusing gels were stained with Coomassie blue [12,13]. Subunits of CGL separated by isoelectric focusing in 8 M urea (first dimension PAGE) were compared by peptide mapping [16] after *S. aureus* V8 [19] proteolysis using SDS-PAGE in the second dimension.

2.2. *Deglycosylation*

Glycoproteins were reduced with 10 mM mercaptoethanol in 6 M guanidine-HCl and alkylated with iodoacetic acid [17]. Carboxymethylated glycoproteins (1.5 mg) were deglycosylated by treatment with Endo-F (1.5 IU) in 0.5 ml of 0.1 M sodium phosphate, 0.05 M EDTA, 1% Nonidet P-40, 0.1% SDS, 1% 2-mercaptoethanol, pH 6.1, for 16 h at 37°C. Reactions were continued for a total of 32 h after addition of a second enzyme aliquot (1.5 IU). Alternatively, CGL was carboxymethylated and deglycosylated by treatment with trifluoromethanesulfonic acid (TFMS) [18].

2.3. *Crosslinking of CGL monomers*

CGL was iodinated [14] and crosslinked [15] as described, except that 0.5 mM glutaraldehyde was used instead of dimethyl suberimidate as crosslinking reagent. Crosslinked products were separated electrophoretically [10] in a linear gradient gel of 3–10% polyacrylamide. Autoradiograms of dried gels were scanned using a Zeineh soft laser scanning densitometer Model SL-504-XL (Biomedical Instruments, Fullerton, CA).

2.4. *Materials*

Sexually mature *X. laevis* were imported from South Africa or collected in Orange County, California, USA. Chemical reagents were purchased from commercial sources and were of high purity.

3. RESULTS

3.1. *Separation of CGL subunits by isoelectric focusing*

In our previous work, electrophoresis of the dissociated oligomer in the presence of SDS generated a broadly staining region, suggesting that CGL monomers were microheterogeneous in size and charge. Resolution of monomers was significantly improved by isoelectric focusing of the dissociated oligomer in urea and Nonidet P-40, in which approximately eleven components with p*I* values which ranged from 4.30 to 5.00 (fig.1) were observed. Eight prominent bands were clustered from p*I* 4.40 to 4.70, each separated by 0.05 pH unit. In addition, three less intensely staining bands of p*I* 4.70–5.00 were present.

3.2. *Peptide mapping*

To ascertain if CGL subunits were composed of identical polypeptides, we attempted to determine the N-terminal sequences of the subunits by Edman degradation. The results were indeterminate, presumably due to blockage of the N-terminal amino acids. As an alternative approach, we took advantage of the heterogeneous isoelectric points of the component subunits and generated peptide maps. Separated by isoelectric focusing in the first dimension, components were partially digested with protease (*Staphylococcus aureus* V8), and the digestion products resolved in the second dimen-

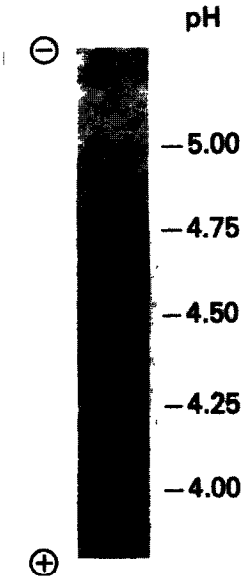

Fig.1. Isoelectric focusing of CGL subunits. Isoelectric focusing (pH 3–7) of CGL (75 μg) in urea and Nonidet P-40 after mercaptoethanol reduction.

sion by SDS-PAGE (fig.2). Peptides from the eight prominent components (p*I* 4.40–4.70; see fig.1) appeared in the center of the gel; apparent toward the basic end of the gel were peptides of less prominent components (p*I* 4.70–5.00). With respect to the former, each component produced the same peptide map when digested with *S. aureus* V8 protease. In contrast, species of p*I* 4.70–5.00 appeared to produce fewer peptides after cleavage with the enzyme. Because the staining intensities of these four species in fig.1 suggest that they are present in lower abundance than are the seven more acidic species (p*I* 4.40–4.70), we assume that this is due to inadequate sensitivity of the visualization method employed to detect all peptide species present. In either case, peptides unique to a single component were absent. These results suggest that the monomers of CGL contain polypeptide chains of significant sequence homology.

3.3. *Deglycosylation of CGL*

We further investigated the heterogeneity of the CGL subunits by removing carbohydrate moieties with either endo-β-*N*-acetylglycosaminidase F (Endo-F) or TFMS and analyzing the deglycosylated polypeptides by SDS-PAGE. Prior to treatment, carboxymethylated CGL monomers had apparent molecular masses of 46–58 kDa (fig.3, lane 1). After treatment with each reagent, CGL monomers displayed a single band with a molecular mass of 44500 Da (fig.3, lanes 2 and 3), which was of lower molecular mass and less diffuse than that of the untreated glycoprotein. While deglycosylation by TFMS effectively removes both

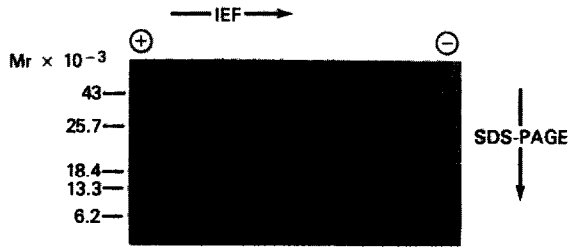

Fig.2. Peptide mapping of separated monomers. CGL monomers (40 μg total) were separated in an isoelectric focusing gel and transferred to an SDS-PAGE stacking gel where they were digested with V8 protease (1 μg). Digestion products were then analyzed in a 15% SDS-PAGE gel and visualized by Coomassie staining.

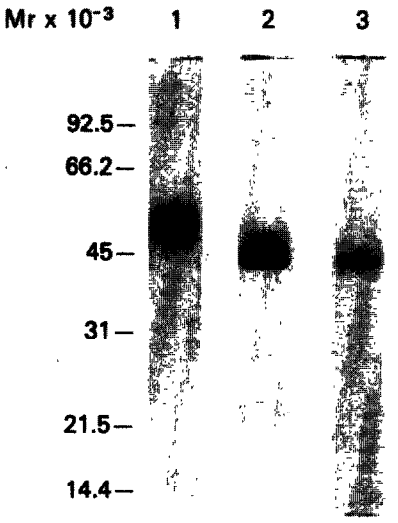

Fig.3. Deglycosylation of CGL. Coomassie blue-stained SDS-PAGE of carboxymethylated CGL after (lanes): (1) no treatment; (2) digestion with Endo-F; (3) treatment with TFMS. Lanes 1 and 2 each contain 20 μg CGL; lane 3,5 μg CGL.

O-linked and *N*-linked sugar side chains [20], Endo-F removes only *N*-linked oligosaccharides [21], suggesting only *N*-glycosylation of CGL monomers. Consistent with this result is the fact that, upon β-elimination, no dehydroamino acids could be detected (unpublished), indicating the probable absence of *O*-linked carbohydrates. Since deglycosylation significantly reduced the size heterogeneity of CGL monomers, the observed heterogeneity is likely due to structural differences in *N*-linked oligosaccharides rather than in polypeptides, a result consistent with that observed in the peptide mapping experiments described above.

3.4. *Limited crosslinking of CGL*

Earlier investigations from our laboratory had indicated that CGL exists primarily as an oligomeric glycoprotein with a molecular mass of 539 kDa. Therefore, the stoichiometry of the monomers within the native oligomer was investigated in crosslinking experiments. The conditions of crosslinking were adjusted so that partial reaction would generate a series of aggregates containing from one to the total number of monomers in the native molecule. ^{125}I-labeled CGL in dilute

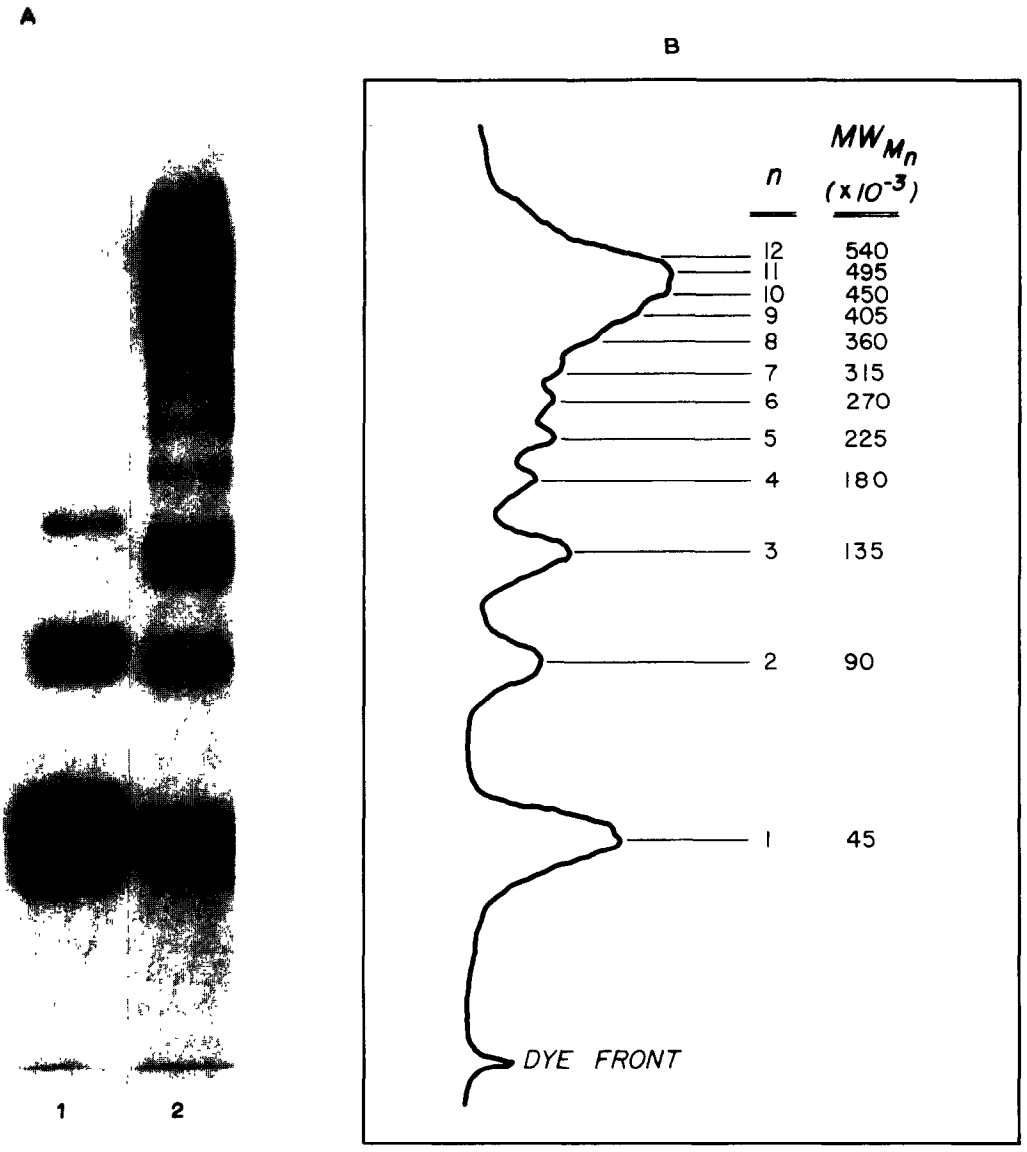

Fig.4. Crosslinking of CGL. (A) Autoradiograph of an SDS-PAGE gel using a 3–10% gradient of polyacrylamide (lanes): (1) radioiodinated CGL (23 μg, 1 × 10^6 dpm) incubated with glutaraldehyde for 0 min; (2) for 40 min. (B) A densitometer tracing of A, lane 2.

solution was crosslinked with 0.5 M glutaraldehyde for 0 min (fig.4A, lane 1) and 40 min (fig.4A, lane 2) at 25°C. Crosslinked aggregates were separated by SDS-PAGE, visualized by autoradiography, and a densitometer tracing was made of the autoradiogram (fig.4B). The crosslinked glycoproteins present indicated that oligomeric CGL was comprised of 10–12 monomers.

4. DISCUSSION

The cortical granule lectin from *X. laevis* eggs was found to be an oligomer of 10–12 non-

covalently bound monomers. Although the monomeric subunits have considerable size and charge heterogeneity, peptide maps suggest that the monomers have identical polypeptide backbones.

Previous work in our laboratory had shown that the predominant isomer of CGL has a native molecular mass of 539 kDa [1]. We predicted that the number of monomers (molecular mass average of 45 kDa by SDS-PAGE; T. Nishihara and J.L. Hedrick, in preparation) composing the native CGL oligomer (539 kDa) was approximately twelve. The data which we present here is consistent with this prediction. Eleven components were observed upon isoelectric focusing of the dissociated oligomer, while limited crosslinking suggested that 10–12 monomers comprise the native molecule. Two factors which make it difficult to assign a more precise monomer number to CGL are (i) isoelectric focusing may not have resolved all the monomers, and (ii) resolution of the glutaraldehyde crosslinked high molecular mass aggregates by SDS-PAGE was limited. Our estimate that CGL contains 10–12 monomers is contingent upon these considerations. From results of the deglycosylation experiments, the size heterogeneity of CGL monomers is apparently due to the presence of *N*-linked oligosaccharides.

The hypothesis by Wyrick et al. [1,2] proposes that CGL functionally performs a role in formation of the F layer and the block to polyspermy. If, as has been shown for a variety of lectins [22], CGL contains one carbohydrate binding site per monomer, then each subunit may be capable of binding to a ligand. An oligomer containing 10–12 subunits would crosslink with proportionally greater avidity than would a monomer. Our data suggest that CGL monomers have homologous polypeptide chains. Whether or not these monomers all have ligand binding sites in the oligomer and, therefore, similar biological functions in forming the F layer, remains to be determined.

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