ULTRASTRUCTURAL LOCALIZATION OF
LECTIN-BINDING SITES ON THE ZONAE PELLUCIDAE AND
PLASMA MEMBRANES OF MAMMALIAN EGGS

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ABSTRACT

Receptors for Ricinus communis agglutinin I (RCA₁), concanavalin A (Con A),
and wheat germ agglutinin (WGA) were localized on the zonae pellucidae and
plasma membranes of hamster, mouse, and rat eggs with ferritin-lectin conjugates.
Intact eggs labeled with the ferritin conjugates showed dense concentrations of
RCA₁ and WGA receptors in the outermost regions of their zonae pellucidae and
sparse distributions of Con A receptors throughout the zonae. Ferritin-lectin
labeling was specific, since inhibitory saccharides effectively blocked labeling. The
asymmetric density of RCA₁ receptors across the zona was confirmed by
ferritin-RCA₁ and fluorescein-RCA₁ labeling of mechanically isolated zonae
pellucidae, indicating that the RCA₁-binding sites are more densely distributed in
the exterior zona regions.

Plasma membranes of rodent eggs contained RCA₁, WGA, and Con A
receptors. These receptors were found to be more or less randomly distributed on
surfaces of aldehyde-fixed eggs or on eggs labeled near 0°C. However, eggs
incubated at 25°C showed aggregated WGA- and Con A-binding site distributions
on their plasma membranes. This indicates that lectin-induced receptor redistribu-
tion occurs at this temperature. The possibility that plasma membrane receptor
mobility is a requirement for sperm-egg fusion is discussed.

The mammalian egg is a complex structure con-
sisting of a cell body encapsulated by a plasma
membrane and surrounded by a moderately thick
transparent coat called the zona pellucida. The
zona pellucida is separated from the egg plasma
membrane by a fluid-filled region called the perivi-
telline space. Before fertilization the spermatozoa
must attach and bind to the exterior of the zona
pellucida (1–6) and subsequently penetrate through
it and bind to the egg plasma membrane (1–3,
6–10). Immediately after spermatozoa bind to the
egg plasma membrane, fusion of this membrane
with the sperm plasma membrane begins and
fertilization proceeds (2, 7, 10–12).

Because it is difficult to obtain adequate num-
bers of eggs, little is known about the molecular
structure of the zona pellucida and the egg plasma
membrane. Fragmentary evidence suggests that
the zona pellucida is composed of protein and carbohydrate (8, 13–19), possibly in the form of glycopeptide units stabilized by disulfide bonds (20, 21), hydrophobic interactions, or salt bridges.

During our studies on the structure of the zona pellucida of the hamster egg, we found that a lectin isolated from *Triticum vulgaris* (wheat germ agglutinin) (22) bound to the exterior surface of the zona pellucida and caused it to intensely scatter visible light (23). Wheat germ agglutinin (WGA)\(^1\) also prevented dissolution of the zona pellucida by trypsin and \(\beta\)-mercaptoethanol. After the eggs were treated with WGA, capacitated hamster spermatozoa failed to bind to or penetrate the zona pellucida; thus, fertilization was effectively blocked (23). Other lectins such as those isolated from *Ricinus communis* (*R. communis* I agglutinin or RCA\(_I\)) (24, 25), *Canavalis einsformis* (concanavalin A or Con A) (26), and *Dolichos biflorus* (27) bound to the hamster zona pellucida and prevented fertilization, but did not prevent sperm from binding to the zona (28).

In order to localize the lectin-binding sites, we have employed electron-dense ferritin conjugates (29, 30). We present here data on the localization and dynamics of the lectin receptors on the zonae pellucidae and plasma membranes of hamster, mouse, and rat eggs.

**MATERIALS AND METHODS**

**Biochemicals and Hormones**

Bovine testicular hyaluronidase (300 USP U/mg) and polyvinyl pyrrolidone (K-90, average mol wt 360,000) were obtained from ICN Nutritional Biochemicals Div., International Chemical & Nuclear Corp., Cleveland, Ohio. Bovine serum albumin was obtained from Reheis Division of Armour Pharmaceutical Co., Chicago, Ill., \(\beta\)-mercaptoethanol from Sigma Chemical Co., St. Louis, Mo., BioGel A 1.5 m (200–400 mesh) from Bio-Rad Laboratories, Richmond, Calif., and Sephadex G-75 and G-100 (fine) from Pharmacia Fine Chemicals, Inc., Piscataway, N. J. Mono- and disaccharides were produced of Sigma Chemical Co. or Calbiochem, San Diego, Calif. Pregnant mare serum gonadotropin and human chorionic gonadotropin were obtained from Ayerst Laboratories, New York.

**Plant Lectins and Ferritin-Conjugated Lectins**

Most of the plant lectins were purified by affinity chromatography. Con A, obtained as a twice-crystallized product (Calbiochem) was further purified by absorption onto a 2.5 x 30-cm column of Sephadex G-75. After washing with 0.5 M NaCl-0.05 M sodium phosphate buffer, pH 6.5, the agglutinin was eluted with 0.2 M sucrose in the same buffer (26) and extensively dialyzed to remove bound saccharides. *R. communis* agglutinins were purified by the procedures of Nicolson and Blausenstein (24). The diazyl ammonium sulfate-precipitated preparation (0–60% ammonium sulfate fraction) of the *R. communis* agglutinins in 0.2 M NaCl-0.05 M sodium phosphate buffer, pH 7.2, was applied to a 4 x 40-cm column of Bio-Gel A-0.5 m agarose (Bio-Rad Laboratories). After the column had been washed with buffer, the agglutinins were eluted in a single peak with the same buffer containing 0.2 M d-galactose. The 120,000 dalton RCA\(_I\) was separated from the 60,000 dalton RCA\(_H\) on a 2 x 50-cm column of Sephadex G-100 or Bio-Gel P-100 (25). WGA was affinity purified on an ovomucoid-Sepharose column (31). Crystallized ovomucoid (Sigma Chemical Co.) was coupled to Sepharose by the cyanogen bromide activation method of Cuatrecasas (32). After extensive washing to remove noncovalently bound protein, the ovomucoid-Sepharose was used to make a 2 x 20-cm affinity column. A crude wheat germ alkaline phosphatase preparation (Worthington Biochemical Corp., Freehold, N. J.) was heat inactivated at 58°C for 10 min and then quickly cooled to 5°C. After centrifugation to remove precipitated protein, the partially purified WGA preparation was applied to a 2 x 100-cm Sephadex G-75 column and eluted with 1 mM Tris-HCl, pH 7.4. The agglutinating fractions were pooled, concentrated, and applied to the ovomucoid-Sepharose column. After a washing with buffer, 0.1 M acetic acid, pH 2, was used to elute the agglutinin, and the eluted fractions were immediately neutralized.

Lectins were conjugated to ferritin by the techniques of Nicolson and Singer (29, 30) using the following final concentrations: ferritin, 4–5%; lectins, 1.5–2%; glutaraldehyde, 0.02–0.03%; saccharide inhibitors, 0.1 M. Some of the ferritin conjugates were affinity purified before use of Bio-Gel A 1.5 m (ferritin-RCA\(_H\)) or Sephadex G-100 (ferritin-Con A) (30). Ferritin-WGA was diazylated after conjugation with 0.01 M Tris- HCl-0.9% NaCl, pH 7.5, and chromatographed on a 1 x 100-cm column of Bio-Gel A 1.5 m. Ferritin-lectin conjugate activities were monitored by agglutination of rabbit erythrocytes (30). Lectins were conjugated to fluorescein as described previously (33).
Collection and Labeling of Eggs

Mature unfertilized eggs of the golden hamster, mouse, and rat were obtained as follows. Adult hamster females were injected intraperitoneally with 20-30 IU pregnant mare serum gonadotropin (PMSG) the morning after ovulation. This was followed by an intraperitoneal injection of 20-30 IU human chorionic gonadotropin (HCG) 54-60 h later. Adult mice were injected intraperitoneally with 5-10 IU PMSG, regardless of the stage of their estrous cycle, followed by an injection of 5-10 IU HCG 48 h later. Prepubertal rats were injected intraperitoneally with 20-30 IU PMSG followed by an injection of 20-30 IU HCG 48-56 h later. Between 15 and 18 h after injection of HCG, the animals were killed and their oviducts were flushed with albumin-saline (0.01 M Tris- HCl-0.9% NaCl containing 0.2-0.5% crystalline bovine serum albumin, pH 7.4). The eggs were freed from surrounding cumulus cells by being treated for 15 min at 25°C with 0.1-0.2% bovine testicular hyaluronidase in albumin-saline, and were then thoroughly rinsed with albumin-saline. Cumulus cells surrounding rat eggs were more difficult to remove, but could be removed by repeatedly sucking the eggs in and out of a small-bore pipette while they were in the hyaluronidase solution. Zonae pellucidae were dissolved by treating the cumulus-free eggs with either (a) 0.1 M 3-mercaptoethanol in 0.01 M Tris-HCl-0.9% NaCl-0.4% polyvinyl pyrrolidone, pH 7.4, for 5-8 min (hamster), or 30-40 min (mouse and rat), or (b) acidic Tyrode's solution, pH 2.5, adjusted with N/5 HCl, containing 0.4% polyvinyl pyrrolidone for 30-60 s (hamster) or 5-10 s (mouse and rat). Zonae pellucidae of some eggs were removed mechanically with a pair of sharp, stainless steel needles. Preliminary experiments showed that the pattern and intensity of binding of the ferritin-lectin conjugates to the egg plasma membrane were not noticeably affected by the various methods for removing the zona. After removal of the zonae pellucidae, the eggs were rinsed three times with albumin-saline.

Eggs with and without zonae pellucidae were incubated in a mixture of ferritin-conjugated lectin solution (1 part) and albumin saline (1 part) for 5-10 min at 0°C or 25°C. During this incubation the dish was agitated occasionally. Some eggs were prefixed with 3% formaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for 30 min at 4°C before being labeled with ferritin-conjugated lectins. The eggs were then rinsed three times with albumin-saline (0°C or 25°C), fixed for 1 h at 4°C in 3% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4, rinsed with the buffer again, and postfixed for 1 h at 4°C in 1% osmium tetroxide in 0.1 M sodium phosphate buffer, pH 7.4. After the eggs were washed with the buffer, they were dehydrated with a graded acetone series, and embedded in Epon. Thin unstained sections were examined by transmission electron microscopy. Controls were processed in the same manner except that lectin saccharide inhibitors (e.g., 0.1 M lactose for ferritin-RCA, 0.1 M N-acetyl-D-glucosamine for ferritin-WGA, or 0.1 M sucrose or α-methyl-D-mannoside for ferritin Con A) were included in the labeling as well as in the washing solutions.

Labeling of intact eggs or isolated zonae pellucidae with fluorescein-conjugated lectins was performed as follows. Fluorescent-lectin (10-100 µg/ml) in albumin-saline was mixed with several eggs or isolated zona at 25°C. The suspension was agitated occasionally for 3-4 min, and excess fluorescein-lectin was removed by three albumin-saline washes (25°C). Controls contained 0.1 M of the appropriate saccharide inhibitor in labeling and wash solutions. Eggs and isolated zonae pellucidae were observed with a Leitz UV microscope with BG-38 and BG-12 excitation filters and a K-510 barrier filter.

RESULTS

Hamster, mouse, and rat eggs have similar gross structures, but the size of the eggs and the thickness of their zonae pellucidae and perivitelline spaces are distinctly different for each species.
FIGURE 4  Hamster egg labeled with ferritin-RCA₁ at 25°C. zp, zona pellucida; m, microvilli. Arrows indicate deepest penetration of ferritin-conjugate. × 32,600; bar equals 0.2 μm.

FIGURE 5  Mouse egg labeled with ferritin-RCA₁ at 25°C. For symbols see Fig. 4. × 54,000; bar equals 0.2 μm.

FIGURE 6  Rat egg labeled with ferritin-RCA₁ at 25°C. For symbols, see Fig. 4. × 40,000; bar equals 0.2 μm.

FIGURE 7  Hamster egg lactose control for ferritin-RCA₁ labeling. × 33,600; bar equals 0.2 μm.
Figure 8  Hamster egg labeled with ferritin-WGA at 25°C. × 30,000; bar equals 0.2 μm.

Figure 9  Mouse egg labeled with ferritin-WGA at 25°C. × 61,300; bar equals 0.2 μm.

Figure 10  Hamster egg N-acetyl-D-glucosamine control for ferritin-WGA labeling. × 32,000; bar equals 0.2 μm.

Figure 11  Tangential section through a hamster egg zona pellucida labeled with ferritin-RCA1. × 13,000; bar equals 0.2 μm.

Figure 12  Mechanically isolated hamster egg zona pellucida labeled with ferritin-RCA1. The outer region of the zona (out) is heavily labeled, whereas the inner region of the zona (in) is lightly labeled. × 11,800; bar equals 0.2 μm.
When intact hamster, mouse, and rat eggs were labeled with ferritin-RCA₁, the outer region of the zona pellucida was densely labeled with little penetration to the perivitelline space or to the plasma membrane surface (Figs. 4–6). The labeling was specific, since the inclusion of the RCA₁ saccharide inhibitor lactose blocked all labeling of the egg (Fig. 7, only hamster control shown). Ferritin-WGA labeling of intact eggs gave similar results. The lectin-binding sites were predominately localized in the outer regions of the zona pellucida (Figs. 8, 9). Labeling with ferritin-WGA was also specific as N-acetyl-D-glucosamine inhibited binding (Fig. 10, only hamster control shown). The surface of the zona pellucida is not uniform in its appearance; it has many deep invaginations. In glancing or tangential sections through the zona, these appear as surface “holes” (Fig. 11). For examination of the possibility that poor penetration of the ferritin conjugates through the zona pellucida accounts for the low level of labeling of the inner zona region, mechanically isolated zonae were labeled with ferritin-RCA₁. When diluted ferritin-RCA₁ was used, the outer regions of the isolated zonae were more heavily labeled than the inner regions, even though the inner regions were just as accessible to the ferritin conjugate (Fig. 12). When the isolated zonae were labeled with high concentrations of (undiluted) ferritin-RCA₁ and examined at an “edge” formed by the mechanical breakage of the zonae, outer zona regions were still more densely labeled, and it appeared that the outer “half” of the zonae contained a higher density of RCA₁ receptors than did the inner “half” (Fig. 13). Similar results were obtained with ferritin-WGA (not shown).

For ascertaining whether the ferritin-lectin conjugates were unique in differentially labeling the outer regions of intact and isolated zonae pellucidae, fluorescein-conjugated lectins were employed. When intact hamster eggs were incubated with a low concentration (10–30 µg/ml) of fluorescein-RCA₁, only the outer region of each zona was labeled (Fig. 14 a). However, if a high concentration (≥ 100 µg/ml) of fluorescein-RCA₁ was used, the inner zona region was also labeled (Fig. 14 b). Similar results were obtained with isolated hamster zona pellucidae (Fig. 14 c), indicating that simple penetration of the lectin does not account for the lower level of fluorescein-RCA₁ labeling to inner zona regions when low concentrations of fluorescein-lectin were used.
Fluorescein-lectin labeling was specific, because inclusion of the appropriate inhibitor in labeling and wash solutions blocked lectin binding (Fig. 14d). These results indicate that there are higher densities of RCA$_1$ receptors in outer zona regions or that the affinities for RCA$_1$ in outer zona regions are higher.

In contrast to the distribution of RCA$_1$- and WGA-binding sites, the receptors for Con A were more uniformly and sparsely distributed throughout the zonae pellucidae of hamster (Fig. 15), mouse (Fig. 16), and rat eggs (Fig. 17). The deep penetration and labeling of the zonae by ferritin-Con A was not due to simple trapping of the ferritin conjugate, because the Con A inhibitor a-methyl-D-mannoside completely blocked labeling (Fig. 18, only hamster control shown).

Mammalian eggs also possess lectin-binding sites on their plasma membranes. Zona-free eggs labeled with ferritin-RCA$_1$ showed dense uniform labeling of their plasma membranes (Figs. 19–21). This labeling was specific and did not occur when lactose was present in the labeling solution (Fig. 22, only rat control shown). The sparse labeling of ferritin-RCA$_1$ on the plasma membranes of intact eggs (with zonae) suggests that ferritin-RCA$_1$ conjugates cannot easily penetrate through lectin-labeled zonae pellucidae. Plasma membrane lectin-binding sites for WGA (Fig. 23) and Con A (Figs. 24, 26, and 27) were discontinuously distributed and present in small clusters on egg plasma membranes labeled at 25°C. These clusters were not the result of nonspecific binding of polymerized conjugates because control preparations were unlabeled (Fig. 25, only hamster control shown). The clusters appear to be due to lectin-induced aggregation of surface receptors at room temperature. This was subsequently demonstrated by labeling zona-free eggs at 0°C or fixing the eggs with 3% formaldehyde before labeling at room temperature (Fig. 28). In both cases, more uniform distributions of the lectin receptors were found, indicating that the inherent distributions of these lectin-binding sites are relatively uniform on the egg plasma membrane surface, consistent with localization data on other cell types (33–36). The lower apparent level of ferritin-lectin labeling to unfixed naked eggs is probably the result of lectin-induced endocytosis (36).

DISCUSSION

Using ferritin-lectin conjugates of RCA$_1$, Con A, and WGA, we found that the lectin-binding sites on hamster, mouse, and rat eggs were localized in the zona pellucida (more densely in the exterior regions) and also on the surface of egg plasma membrane. In cross section the asymmetric distribution of RCA$_1$- and WGA-binding sites in the zona pellucida could be due to lower densities of lectin-binding sites or to low affinity lectin-receptors in the interior regions. The asymmetry of zona lectin binding was independently demonstrated with hamster eggs and isolated zonae stained with fluorescein-labeled RCA$_1$. At low fluorescent RCA$_1$ concentrations (10–30 µg/ml), only the outer region of the zona showed fluorescence. At high lectin concentrations (>100 µg/ml) the outer zona region showed intense fluorescent-RCA$_1$ labeling but the remainder of the zona was also stained, indicating the presence of RCA$_1$-binding sites deep inside the zona pellucida (Fig. 14). Thus, it appears either that there are relatively more lectin-binding sites in the exterior zona regions or that these exterior zona receptors bind the lectins more avidly than the lectin receptors in the inner zona regions. There are lectin sites buried deep within the zona that are not labeled with ferritin-RCA$_1$. However, ferritin-Con A was...
FIGURE 15 Hamster egg labeled with ferritin-Con A at 25°C. a, Outer region of zona pellucida; b, inner region of zona pellucida. Arrows indicate deepest penetration of the ferritin-conjugate. × 52,000; bar equals 0.2 μm.

FIGURE 16 Mouse egg labeled with ferritin-Con A at 25°C. For symbols, see Fig. 15. × 41,500; bar equals 0.2 μm.

FIGURE 17 Rat egg labeled with ferritin-Con A at 25°C. For symbols, see Fig. 15. × 75,000;
able to label its receptors deep within the zona, indicating the presence of Con A receptors in these regions.

The zona pellucida of the hamster egg contains a high concentration of acidic residues which may be responsible for impeding the penetration of large macromolecules. Using colloidal iron hydroxide (CIH) labeling techniques, Yanagimachi et al. (37) found that the outer half or outer two-thirds of the zona was intensely labeled with CIH at pH 1.8, indicating the presence of low pKₐ anionic groups such as sialic acid or its derivatives. The CIH particles did not penetrate as well to the inner zona surface or to the egg plasma membrane, although these structures possessed CIH-binding sites. This was demonstrated by neuraminidase treatment of eggs which drastically lowered the number of CIH particles bound to the exterior region of the zona, but at the same time allowed better penetration and binding of CIH to the zona interior and inner surface regions (37). The zona pellucida may consist of a compact matrix of glycopeptide-glycoprotein units polymerized enough to act as a charged molecular sieve which may prevent some large macromolecules from easily penetrating the zona and gaining access to the egg plasma membrane surface. However, Hastings et al. (38) found that horseradish peroxidase and ferritin easily penetrated the zona pellucidae of rat and mouse eggs, and it was found here that ferritin-Con A could penetrate the zona. Thus the poor penetration of some ferritin-lectin conjugates may depend more on their specificity, reactivity, and ability to cross-link oligosaccharides in the zonae rather than on their size.

Lectin receptors on the egg plasma membrane surface were found to be inherently distributed in a more or less random state on aldehyde-fixed eggs or on unfixed eggs labeled near 0°C, but were rapidly aggregated by the ferritin lectins into receptor “patches” or “clusters” on unfixed cells labeled at room temperature. This indicates that the egg plasma membrane lectin receptors are capable of rapid lateral mobility. Since membrane fluidity has been shown to be an essential requirement for fusion in a variety of cell systems (39), the lateral mobility of certain egg plasma membrane components may be important in the eventual fusion of egg and sperm plasma membranes. It is interesting in this regard that surface receptors in the sperm plasma membrane of the postacrosomal region which is involved in sperm-egg fusion show relatively high rates of lateral mobility compared to membrane receptors in other regions (reference 40, and G. L. Nicolson, N. Usui, and R. Yanagimachi, manuscript in preparation).

We previously found that low concentrations of WGA (10 μg/ml for 30 min at 25°C) caused a change in the light-scattering properties of the exterior surface of the zona pellucida. This change was prevented by the WGA inhibitor N-acetyl-d-glucosamine (23). When hamster eggs were treated with WGA concentrations of approximately 50 μg/ml, fertilization was completely blocked due to the inability of spermatozoa to penetrate the zona pellucida. When eggs were treated with high concentrations of WGA (≥250 μg/ml), spermatozoa failed to bind effectively to the zona pellucida and could be easily removed by agitation. We found that other lectins such as Con A, RCA₁, and Dolichos biflorus agglutinin (recognizing N-acetyl-d-galactosamine-like residues [27]) also change the light-scattering properties of the zona and inhibit sperm penetration through the zona pellucida. However, these lectins, unlike WGA, do not block sperm binding to the zona (28). It was proposed that the failure of spermatozoa to penetrate the zonae pellucidae of lectin-treated eggs was due to cross-linking of adjacent oligosaccharide chains preventing destabilization or depolymerization of the zona material by sperm-borne zonae lysin(s) (23, 28). Ownby and Shivers (41) found that antibodies against hamster ovaries produced a precipitation effect on the exterior of the zona pellucida of the hamster egg. These antibodies also blocked sperm attachment to and penetration into the zona pellucida (42, 43). It is reasonable to assume that the lectin-mediated and antibody-mediated inhibitions of hamster fertilization occur by similar mechanisms and are dependent upon high densities of the appropriate receptors on the zona pellucida. The cross-linking of adjacent receptors on the zona surface may prevent an essential step in sperm penetration through the zona. Use of high concentrations of WGA, but not RCA₁, Con A, or D. biflorus agglutinin, blocks sperm binding to the zona pellucida; thus, the WGA receptor on the zona pellucida may be structurally related, or sterically close to the sperm receptor sites. We also found that the lectin-induced block to fertilization does not occur by lectin interaction at the egg plasma membrane. Treatment of zona-free hamster eggs with up to 250 μg/ml RCA₁, WGA, or Con A, or sequential treatment with 250 μg/ml each of RCA₁, WGA, Con A, and D. biflorus...
agglutinin failed to prevent sperm-egg fusion (R. Yanagimachi and G. L. Nicolson, unpublished observations). Thus, the blocking or cross-linking of the lectin receptors of the zona pellucida appears to be the primary mechanism by which lectins prevent fertilization. Since WGA prevents sperm binding to the zona pellucida, the use of Sepharose-coupled WGA for affinity chromatography of a solubilized zona pellucida preparation may provide a rational approach to purification of the mammalian sperm receptor.

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