Regulation of Extracellular Matrix Assembly: In Vitro Reconstitution of a Partial Fertilization Envelope from Isolated Components

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Abstract. At fertilization, the glycocalyx (vitelline layer) of the sea urchin egg is transformed into an elevated fertilization envelope by the association of secreted peptides and the formation of intermolecular dityrosine bonds. Dityrosine cross-links are formed by a secreted ovoperoxidase that exists in a Ca^{2+}-stabilized complex with proteoliaisin in the fertilization envelope. By using purified proteins, we now show that proteoliaisin is necessary and sufficient to link ovoperoxidase to the egg glycocalyx. Specifically, we have found that (*a*) ovoperoxidase can associate with the vitelline layer only when complexed with proteoliaisin; (*b*) proteoliaisin binds to the vitelline layer independently of its association with ovoperoxidase; (*c*) proteolytic modification of the vitelline layer is not required for this interaction to occur; (*d*) the binding of proteoliaisin to the vitelline layer is mediated by the synergistic action of the two major seawater divalent cations, Ca^{2+} and Mg^{2+}; (*e*) the number of proteoliaisin-binding sites on the vitelline layer of unfertilized eggs is equivalent to the amount of proteoliaisin secreted at fertilization; and (*f*) the binding of ovoperoxidase to the vitelline layer, via proteoliaisin, permits the in vitro cross-linking of these two in vivo substrates. The association of purified ovoperoxidase and proteoliaisin with the vitelline layer of unfertilized eggs reconstitutes part of the morphogenesis of the fertilization envelope.

SIGNIFICANT advances have been made in elucidating the nature of the diverse components that constitute extracellular matrices (ECM),[1] yet little is known about the coordination of their assembly, or how the combination of individual constituents defines the specialized functions of the ECM. Since the ECM may determine such processes as the cell–cell interactions and cell movements that modulate developmental pathways, there must be specific controls for glycocalyx assembly and modification. These controls, isolated from the cytosolic machinery, may involve novel regulatory mechanisms. The lack of information on this issue relates in part to the paucity of model systems where specific questions can be asked about discrete glycocalyx modifications.

In this regard the sea urchin egg at fertilization provides an ideal system for investigating the assembly and modification of a specific extracellular matrix. A secretory event that follows fertilization (the cortical reaction) releases 5% of the egg protein to modify the glycocalyx (vitelline layer) and convert it to an elevated, hardened fertilization envelope that protects the embryo until the blastula hatches (for review see reference 13). Modification of the vitelline layer occurs in discrete steps and is complete within 10 min of fertilization, thus providing a convenient system for biochemical investigation. Analysis is facilitated by the large quantities of eggs that can be synchronously induced to undergo these modifications.

We have been studying these modification reactions by using isolated components and attempting to effect a reconstitution of the system in vitro. Two components of the fertilization envelope, ovoperoxidase and proteoliaisin, have been purified (7, 20).[2] Proteoliaisin, which forms a Ca^{2+}-stabilized complex with ovoperoxidase, is thought to play a coordinating role in fertilization envelope assembly. This hypothesis was formed from the observations that the ovoperoxidase–proteoliaisin complex is found in uncross-linked fertilization envelopes and that proteoliaisin is a substrate for ovoperoxidase-catalyzed dityrosine formation in vivo (20). In this paper, we explore the possibility that proteoliaisin is responsible for integrating ovoperoxidase into complexes with vitelline layer components.

We have examined the association of ovoperoxidase with the vitelline layer by directly measuring the binding of purified, ^{125}I-labeled ovoperoxidase and proteoliaisin to the vitelline layers of unfertilized eggs. With this in vitro system, we have also tested the possibility that proteolytic modifica-

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1. *Abbreviations used in this paper*: ECM, extracellular matrix; SW, seawater.

2. Weidman, P. J., D. C. Teller, and B. M. Shapiro, manuscript submitted for publication.

Table I. Interaction of Ovoperoxidase with the Vitelline Layer

Incubation conditions	^{125}I-Ovoperoxidase bound
	%
A. Eggs with intact vitelline layers	
NSW	0
NSW + proteoliaisin	76
NSW + proteoliaisin + nonradioactive ovoperoxidase	2
EGTA-SW + proteoliaisin	0
B. Eggs with disrupted vitelline layers	
Dithiothreitol-treated eggs in NSW + proteoliaisin	2
Trypsin-treated eggs in NSW + proteoliaisin	23

The assay procedure is described in Materials and Methods. Each 250 μl sample contained 12 μg/ml ^{125}I-ovoperoxidase (8×10^4 cpm/ml) and 10% eggs (by volume). The concentrations of nonradioactive proteoliaisin and ovoperoxidase were 24 μg/ml and 120 μg/ml, respectively. Results are expressed as the percent of ^{125}I-ovoperoxidase in the incubation mixture that was bound to the egg surface.

tion of the vitelline layer is required for the binding interactions, since limited proteolysis of this glycocalyx occurs after fertilization (3). Our results indicate that ovoperoxidase binds to the vitelline layer via its interaction with proteoliaisin, that proteoliaisin binds to the vitelline layer independent of its association with ovoperoxidase, and that proteolytic modification of the vitelline layer is not a prerequisite for binding. We characterize this binding interaction and present evidence that it represents one step in the pathway of fertilization envelope assembly.

Materials and Methods

Gamete Preparation and Materials

Sea urchins (*Strongylocentrotus pupuratus*), collected on the Straits of Juan de Fuca, were maintained, spawned, and the eggs collected as previously described (10). The egg jelly coat was removed either by brief exposure to pH 5.0 or to EGTA artificial seawater (SW) (19). Either treatment gave quantitatively similar results in our egg binding assays. In some experiments, the egg vitelline layers were disrupted by dithiothreitol (DTT) treatment (9) or by trypsin treatment (8). Eggs were washed three times in excess artificial SW before use in the assays.

All artificial SWs contained 10 mM KCl and 10 mM Tris, pH 7.8–8.0 (at 4°C). In addition, normal seawater (NSW) contained 458 mM NaCl, 10 mM $CaCl_2$, and 50 mM $MgCl_2$; calcium seawater (CaSW) contained 530 mM NaCl and 10 mM $CaCl_2$; magnesium seawater (MgSW) contained 473 mM NaCl and 50 mM $MgCl_2$; EGTA seawater (EGTA-SW) contained 500 mM NaCl and 25 mM EGTA. The divalent cation dependence of the egg binding assay was determined in seawater containing 500 mM NaCl and the indicated concentrations of divalent cations.

Protein concentrations were determined using the method of Bradford (1). All spectrophotometry was performed with a DU-7 spectrophotometer (Beckman Instruments, Inc., Palo Alto, CA). Unless otherwise indicated, all proteins and reagents were purchased from Sigma Chemical Co. (St. Louis, MO) and were of the highest purity available.

Iodination of Ovoperoxidase and Proteoliaisin

Ovoperoxidase was purified as previously described (7, 20).[2] ^{125}I-Ovoperoxidase was prepared by autoiodination as follows. The enzyme was first dialyzed against 20 mM Tris methanesulfonate or Tris acetate, pH 8.0, (7, 19) and diluted to 140 μg/ml in the same buffer. KI (final concentration, 4.0 μM) and 10–100 μCi of ^{125}I (New England Nuclear, Boston, MA) were added and the reaction was initiated by adding H_2O_2 in three aliquots at 1-min intervals to give a final concentration of 0.1 mM. After 15 min most of the free ^{125}I was removed by 2–3 cycles of dilution into buffer A (20 mM 2-(*N*-morpholino)ethane sulfonic acid (MES), pH 6.0, 10% glycerol) and concentration using a Centricon centrifugal concentrator (Amicon Corp., Danvers, MA). The sample was then applied to a column containing 5 ml CM-Sephadex and washed with buffer A to remove free ^{125}I. When ^{125}I was no longer detected in the eluate, labeled ovoperoxidase was eluted with buffer B containing 200 mM NaCl (7). 95–99% of the ^{125}I associated with the ovoperoxidase at this stage was precipitable with trichloroacetic acid (TCA). Iodinated ovoperoxidase contained 0.3–1.0 mol of iodine/mol ovoperoxidase, retained 70–100% of its original peroxidase activity, and was indistinguishable from nonradioactive enzyme as a ligand for proteoliaisin in the in vitro assay described by Weidman et al. (20; data not shown).

Proteoliaisin was isolated from freshly prepared cortical granule exudate by chromatography on DEAE-Sephacel, omitting dialysis against Ca^{2+} and benzamidine, but otherwise as previously described (20). Hydrophobic chromatography was used as the final purification step (19). Proteoliaisin-enriched fractions from the DEAE-chromatography step (in chromatography buffer consisting of 20 mM MES, pH 6.0, 50 mM glycine ethyl ester, 1 mM EGTA, 10% glycerol) were adjusted to 500 mM NaCl and applied to a column of phenyl-Sepharose equilibrated with 500 mM NaCl chromatography buffer at room temperature (5 mg protein per ml packed bed volume). The column was washed with one column volume of chromatography buffer containing 300 mM NaCl. Eluate fractions containing proteoliaisin activity were pooled and concentrated, and protease inhibitors were added (soybean trypsin inhibitor, pepstatin, and leupeptin, 1 μg/ml each). This procedure gave a 10-fold purification of proteoliaisin from cortical granule exudate with ~50% recovery of activity (not shown). Purified proteoliaisin had a reduced molecular weight of ~250,000 (235,000 nonreduced) and was greater than 99% homogenous, as determined by SDS-PAGE (not shown).[2]

I^{125}-labeled proteoliaisin was prepared by ovoperoxidase-catalyzed iodination as follows. Purified proteoliaisin was dialyzed against 20 mM Tris methanesulfonate or Tris acetate, pH 8.0, and mixed with purified ovoperoxidase at a ratio of 100 mg proteoliaisin to 1 mg ovoperoxidase. The final protein concentration varied from 0.65 to 4.0 mg/ml in different preparations. Carrier free $Na^{125}I$ (New England Nuclear; 10–70 μCi/mg protein) was added and the reaction initiated by adding H_2O_2 at 1-min intervals to give a final concentration of 0.1 mM. In some preparations, the reaction mixture also contained 4 μM KI. After 15 min, most of the free ^{125}I was removed by two cycles of dilution with buffer A and concentration using a Centricon centrifugal concentrator (Amicon Corp., Danvers, MA). The sample was then diluted into buffer A containing 200 mM NaCl, applied to a column containing 10 ml DEAE-Sephadex, and washed to elute ovoperoxidase (20) and free ^{125}I. The column was then washed with the same buffer containing 600 mM NaCl to elute the iodinated proteoliaisin. Fractions were collected and assayed for TCA-precipitable ^{125}I. The latter fractions were pooled and the specific radioactivity of the protein was determined. In four separate labeling experiments, the average proportion of TCA-precipitable ^{125}I was 82%. Attempts to remove residual free ^{125}I by either repeated concentration and dilution or by dialysis failed to significantly reduce this amount. In the calculations, the specific radioactivity was taken to be the total cpm ^{125}I/mg proteoliaisin, which ranged from 6.7×10^6 to 5.8×10^7 cpm/mg. The average recovery of protein with this method was 80%, and the radiolabeled proteoliaisin retained 75–95% of its original ovoperoxidase-binding activity, even under conditions where 5–30 mol of I^- were incorporated per mole of proteoliaisin (data not shown). The radiolabeled proteins were concentrated and dialyzed against 500 mM NaCl, 10 mM KCl, and 20 mM Tris, pH 8.0

Egg Binding Studies

The binding of proteins to sea urchin eggs was measured by mixing radiolabeled ovoperoxidase or proteoliaisin with a washed egg suspension in the desired artificial SW. The final suspension contained 1–10% eggs (vol/vol) and the incubation volumes ranged from 0.2 to 0.5 ml. After a 1-h incubation on ice with periodic gentle agitation, the eggs were resuspended and an aliquot (10% of the suspension) was taken to measure the total ^{125}I per assay. Then 80% of the suspension was transferred to a 1.5 ml Eppendorf tube containing 0.3 ml silicon oil (General Electric Co., Wilmington, MA) overlaid with 0.5 ml of SW and centrifuged at 15,000 *g* for 30 s. The tubes were inverted to drain off the oil, the tip of the tube containing the egg pellet was cut off, and the associated ^{125}I measured in a gamma counter (Beckman Gamma 6000; Beckman Instruments, Inc.). Identical suspensions containing ^{14}C-sucrose as an impermeant marker were processed to determine the

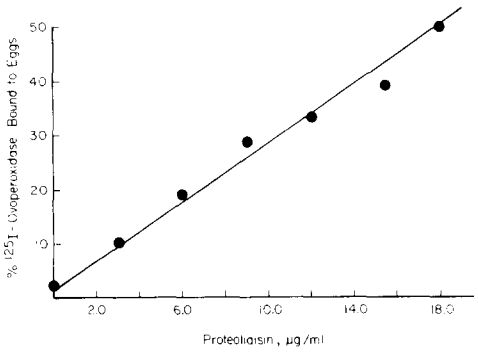

Figure 1. Binding of ovoperoxidase to the vitelline layer of unfertilized eggs as a function of proteoliaisin concentration. Assays were performed in NSW as described in Materials and Methods. Each 0.5-ml assay contained 4.5 μg/ml ^{125}I-ovoperoxidase (8×10^4 cpm/ml), 10% eggs (vol/vol), and the indicated concentrations of proteoliaisin. Results are expressed as the percent of ^{125}I-ovoperoxidase in the incubation mixture that was bound to the eggs.

external water volume of the pellet. All results were corrected to account for this volume which usually contained <5% of the counts associated with the pellet. Each assay was performed in triplicate and the results presented are the average of these values; the usual standard deviation was ±8%. In experiments where other proteins or compounds were added to the assay during incubation, these were either dissolved in or dialyzed against the indicated artificial seawater.

For the determination of the proteoliaisin egg binding parameters, K_d, and molecules per egg, the binding assay was modified as follows. The concentration of eggs was 1% (vol/vol) in a total assay volume of 200 μl. Samples were incubated in inverted 0.5 ml Eppendorf tubes that had been cut with a razor to remove the tip of the tube. Total radioactivity was determined by counting the incubation mixture before adding the eggs. At the end of the incubation, the entire mixture was quantitatively transferred to the silicon oil–containing 1.5 ml Eppendorf tube by setting the smaller incubation tube, open (cut) end down, into the larger. The stacked tubes were then centrifuged and the egg pellets processed as described above. The number of eggs per assay was determined by counting 5 × 10 μl aliquots of a control egg suspension on a clear plastic grid using a dissecting microscope. The values of K_d and N were derived by using an iterative routine, written by Dr. David Teller (University of Washington, Seattle, WA), to fit the data to a normal titration curve. Chi square analysis was used to test the probability of normality after each fit. The best fit was obtained when points lying greater than 2.5 standard deviations from the mean were omitted from the data set. The final data sets consisted of 20 out of 21, 19 out of 21, and 15 out of 16 original data points for NSW, CaSW, and MgSW, respectively. Using this method, the least square fit of the unweighted data constitutes a maximum likelihood estimate (MLE) (12).

Results

Binding of Ovoperoxidase to the Surface of Unfertilized Eggs

We began our investigation by examining the ability of ^{125}I-ovoperoxidase to bind to the vitelline layers of unfertilized eggs. The results of these experiments are summarized in Table I and Fig. 1. Ovoperoxidase alone did not bind to the surface of unfertilized eggs. However, substantial binding was observed when the incubation mixture included proteoliaisin. The binding of ^{125}I-ovoperoxidase showed a linear dependence on the concentration of proteoliaisin (Fig. 1) and was inhibited when a 10-fold excess of unlabeled ovoperoxidase was included in the incubation (Table I). Thus, ovoperoxidase appeared to bind to the egg surface only when complexed with proteoliaisin. As expected, no binding was observed in EGTA-SW, since the interaction of ovoperoxidase with proteoliaisin requires Ca^{2+} (20). These results also suggested that proteolytic processing is not obligatory for the binding of these two proteins to the vitelline scaffold, since eggs with intact vitelline layers were used. In fact, treatments which disrupt the vitelline layer (DTT and trypsin) decreased the binding of ovoperoxidase–proteoliaisin complexes to the egg surface (Table I). We conclude that proteolytic processing of the egg glycocalyx was not necessary for the binding of ovoperoxidase to the vitelline layer scaffold, and that proteoliaisin was necessary and sufficient to mediate this binding.

Table II. Interaction of Proteoliaisin with the Vitelline Layer

Divalent cation composition of medium		^{125}I-Proteoliaisin bound
mM	*mM*	*% of Control*
Ca^{2+}	Mg^{2+}	
10	50	(100)
10	50 + ovoperoxidase	98
10	0	73
0	50	7
0	0	3

The total compositions of the artificial seawaters used and the assay procedure are described in Materials and Methods. Each 0.5 ml of incubation mixture contained 50 μg/ml ^{125}I-proteoliaisin (2.4×10^5 cpm/ml) and 1% eggs by volume. The concentration of nonradioactive ovoperoxidase was 50 μg/ml. Results are expressed as percent of binding observed with ^{125}I-proteoliaisin in NSW (control), which was 1.4×10^4 cpm.

Binding of Proteoliaisin to the Vitelline Layer of Unfertilized Eggs

To determine whether the binding of ovoperoxidase to the vitelline layer, in association with proteoliaisin, is cooperative or indirect, we examined the binding of ^{125}I-proteoliaisin to the vitelline layer of unfertilized eggs. Proteoliaisin bound to the vitelline layer in the absence of ovoperoxidase (Table II), as long as divalent cations were present. Addition of ovoperoxidase had little effect on this binding. Changes in the divalent cation content of the seawater, however, did have significant effects on the association of proteoliaisin with the vitelline layer. Binding was optimal in NSW and decreased by 27% in CaSW. A small but significant amount of binding was observed in MgSW. Virtually no binding was observed in seawater lacking divalent cations. We conclude that proteoliaisin binds directly to the vitelline layer in a divalent cation–dependent fashion and that ovoperoxidase is not required for this interaction. Therefore, ovoperoxidase binds to the vitelline layer indirectly, by its association with proteoliaisin. In the experiments that follow, incubation in EGTA-SW was used to determine the nonspecific binding of proteoliasin to the vitelline layer.

Divalent Cation Requirements for the Proteoliaisin–Vitelline Layer Interaction

Divalent cations are known to mediate the association of structural proteins released from the cortical granules with the vitelline layer during fertilization envelope assembly (13). Although the exact requirements are not known, reducing the total divalent cation concentration below 1 mM

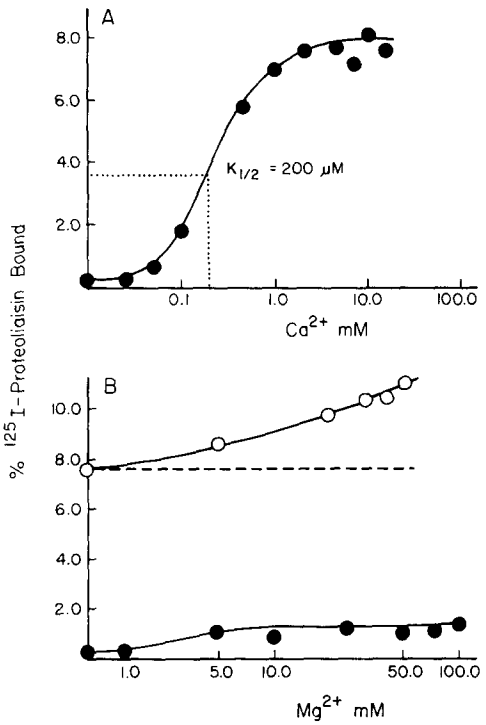

Figure 2. Divalent cation-dependence of proteoliaisin binding to the egg vitelline layer. Each 200-μl assay contained 50 μg/ml ^{125}I-proteoliaisin (2.4×10^5 cpm/ml) and 1% eggs by volume. Results are expressed as percent of ^{125}I-proteoliaisin in the incubation mixture that was bound to the eggs. (*A*) Binding as a function of Ca^{2+} concentration in Mg^{2+}-free seawater. (*B*) •, binding as a function of Mg^{2+} concentration in Ca^{2+}-free seawater; ○, binding as function of Mg^{2+} concentration in seawater containing 10 mM Ca^{2+}; *dashed line*, level of binding observed in Mg^{2+}-free seawater containing 10 mM $CaCl_2$ (from data in *A*).

allows uncross-linked fertilization membranes to partially disassemble without disrupting the interaction between ovoperoxidase and proteoliaisin (20). This suggests that the requirements for divalent cations in the interaction between ovoperoxidase–proteoliaisin complexes and other components of the fertilization envelope (i.e., the vitelline layer) must be different than the requirements for the formation of the ovoperoxidase–proteoliaisin complex, which is specific for Ca^{2+} with $K_{½}$ = 50 μM (20).

To explore this hypothesis, we examined the quantitative requirements for seawater divalent cations in proteoliaisin binding to the vitelline layer. As illustrated in Fig. 2 *A*, half-maximal binding in CaSW was observed at 200 μM Ca^{2+}. In MgSW, half-maximal binding occurred at ~2.5 mM Mg^{2+} (Fig. 2 *B*, closed circles). In CaSW, saturable binding was not observed at concentrations of Mg^{2+} up to 50 mM (Fig. 2 *B*, open circles). Note that the binding observed in seawater containing both 10 mM Ca^{2+} and 50 mM Mg^{2+} was greater than the sum of binding observed for each cation alone (Fig. 2 *B*), suggesting a synergistic effect of these cations on the binding interaction. This is supported by the observations presented in the next section. Thus, the divalent cation dependence of proteoliaisin binding to the vitelline layer is distinct from the Ca^{2+}-specific ovoperoxidase–proteoliaisin binding interaction.

Quantitative Measurements of Proteoliaisin Binding to the Egg Surface

To further characterize the proteoliaisin–vitelline layer interaction, we determined the dissociation constants and number of proteoliaisin-binding sites on each egg. The number of proteoliaisin molecules bound per egg as a function of proteoliaisin concentration is shown in Fig. 3. Saturation binding curves were fit to the observed data and the values of K_d and N were derived as described in Materials and Methods. For seawater containing either 10 mM Ca^{2+}, 50 mM Mg^{2+}, or both divalent cations, the shape of the curve was indicative of a single class of proteoliaisin-binding sites on the vitelline layer. Highest affinity binding was observed in MgSW (Fig. 3 C; K_d = 0.19 μM) with 8.3×10^7 molecules of proteoliaisin bound per egg at saturation. In CaSW (Fig. 3 *B*), over six times as many molecules of proteoliaisin (5.5×10^8 per

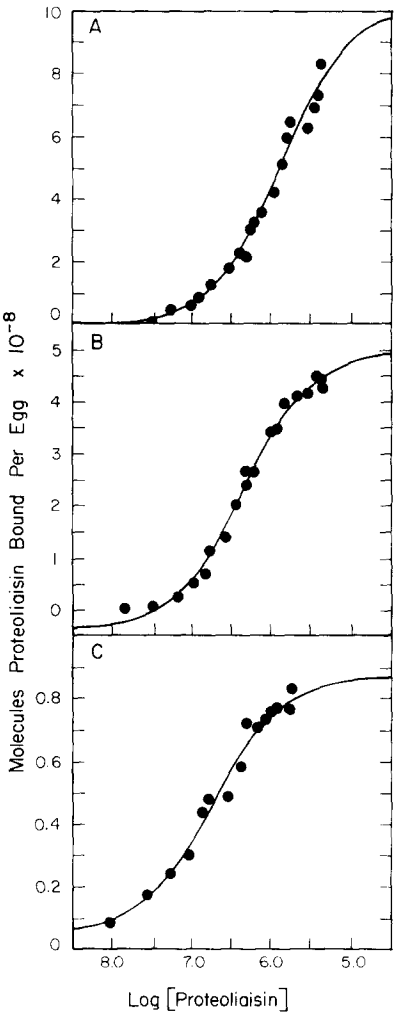

Figure 3. Saturation binding curves for proteoliaisin binding to the vitelline layer of unfertilized eggs. The composition of the artificial seawaters used and the modified assay procedure are described in Materials and Methods. The concentration of ^{125}I-proteoliaisin was varied over the range of 0.003 to 1.5 mg/ml. (*A*) Binding in NSW (10 mM Ca^{2+}, 50 mM Mg^{2+}); (*B*) binding in CaSW (10 mM Ca^{2+}); (*C*) binding in MgSW (50 mM Mg^{2+}). Note the difference in scales between *A*, *B*, and *C*. All curves were drawn with the use of a curve fitting routine (see Materials and Methods).

egg) were bound at saturation but the binding was of lower affinity (k_d = 0.47 μM). The lowest apparent binding affinity was observed in seawater containing both Ca^{2+} and Mg^{2+} in physiological concentrations (Fig. 3 *A*; K_d = 1.43 μM). However, at saturation, nearly twice as many molecules of proteoliaisin (1.03 × 10^9 per egg) were bound to the egg surface as were bound in CaSW. Although these are substantial quantities of bound proteoliaisin (see Discussion), these values represent minimum estimates since we have not corrected the data for the non-TCA-precipitable ^{125}I associated with the radiolabeled proteoliaisin (21% of the total ^{125}I) or for loss of proteoliaisin ovoperoxidase-binding activity after iodination (25% of the original ovoperoxidase-binding activity). There are significant differences in the binding observed in each of the seawaters, consistent with the observation (Fig. 2) that Ca^{2+} and Mg^{2+} have a synergistic effect on the binding interaction between proteoliaisin and the vitelline layer.

Inhibition of the Proteoliaisin–Vitelline Layer Interaction

Many compounds interfere in fertilization envelope assembly, but the mechanisms underlying the effects of most of these compounds are unknown (13, 19). We therefore tested the effects of several inhibitors of fertilization envelope assembly on the binding of ^{125}I-ovoperoxidase (via proteoliaisin) and ^{125}I-proteoliaisin to the egg vitelline layer. Three inhibitors were examined: glycine ethyl ester, which inhibits a characteristic morphological transition that occurs during fertilization envelope assembly, known as the I to T transition (18); glycine, which increases the amount of ovoperoxidase (and possibly other proteins) not incorporated into the fertilization envelope (11); and benzamidine, a competitive inhibitor of serine proteases that also binds to proteoliaisin and other fertilization envelope proteins, possibly at divalent cation-binding sites (19, 20). BSA and IgG, proteins that do not interfere in fertilization envelope assembly (Weidman, P. J., unpublished data), were also assayed to determine whether other nonspecific proteins were inhibitors of the binding interaction. Of these compounds, only benzamidine significantly inhibited binding of proteoliaisin to the vitelline layer in vitro, with $I_{0.5}$ for binding occurring at 20 mM benzamidine (data not shown). The effects of glycine and glycine ethyl ester can not be attributed to an inhibition of either the ovoperoxidase–proteoliaisin or proteoliaisin–vitelline layer interactions.

Evidence is accumulating that the carbohydrate elements of extracellular proteins frequently mediate specific cell surface interactions. In *Xenopus* eggs, assembly of the fertilization envelope proceeds in a manner similar to the sea urchin fertilization envelope and the assembly process involves a lectin-like association of secreted proteins with the egg glycocalyx (21). The vitelline layer of the sea urchin egg contains at least one cell surface receptor, the sperm receptor, in which the primary binding domain corresponds to the carbohydrate portion of a proteoglycan-like molecule (16). To determine whether carbohydrates play a role in the proteoliaisin–vitelline layer binding interaction, we examined the effects of various simple and complex carbohydrates in the in vitro assay. No significant inhibitory effects were found with the following monosaccharides, tested at concentrations up to 100 mM: α-methyl-D-xylose, β-methyl-D-xylose, α-methyl-D-mannoside, D-glucose, D-galactose, α-L-fucose, α-D-fucose, *n*-acetyl-D-glucosamine, *n*-acetyl-D-galactosamine, and sialic acid. Fetuin, a highly glycosylated protein that frequently inhibits carbohydrate-specific interactions, also had no effect on binding. Because of the proteoglycan-like nature of some of the vitelline layer components, we examined the effects of several complex carbohydrates on the proteoliaisin–vitelline layer interaction. Chondroitin sulfate and dextran sulfate had no effect on the binding of proteoliaisin to the egg vitelline layer, whereas inconsistent results were obtained with different preparations of heparin and heparin sulfate. Although we can not rule out the possibility that carbohydrates play an important role in the proteoliaisin–vitelline layer interaction, an inhibitory effect of specific carbohydrates has not been demonstrated for this interaction.

Ovoperoxidase-catalyzed Cross-linking of Proteoliaisin to the Egg Vitelline Layer

In addition to binding ovoperoxidase, proteoliaisin is also a substrate for ovoperoxidase-catalyzed cross-linking of the fertilization envelope (14; Kay, E. S., and B. M. Shapiro, unpublished data). We therefore examined the ability of pure ovoperoxidase, bound to the vitelline layer via proteoliaisin, to cross-link proteoliaisin to the surface of unfertilized eggs. In these experiments, we have taken advantage of the divalent cation dependence of the proteoliaisin–vitelline layer interaction. If proteoliaisin is noncovalently associated with the vitelline layer, then it should be possible to extract it from the egg by removing divalent cations. On the other hand, if proteoliaisin is covalently linked to the vitelline layer, it should not be extracted by this treatment. In these experiments, several different molar ratios of ovoperoxidase and proteoliaisin were incubated with eggs in CaSW. To prevent egg lysis during the extraction procedure, bound proteoliaisin was extracted by diluting the eggs into 30 vol of EGTA-SW containing 2 mM $MgCl_2$ and incubating for 30 min. In control eggs that were not treated with H_2O_2, the oxidative substrate of ovoperoxidase, extraction with EGTA-SW removed ~80% of the proteoliaisin bound to the eggs (Fig. 4). After an aliquot of the same eggs were exposed to H_2O_2, the amount of proteoliaisin bound in NSW did not change significantly (data not shown); however, as much as 70% of this bound proteoliaisin was not extractable. These results suggest that proteoliaisin was covalently cross-linked to the vitelline layer of the unfertilized egg by ovoperoxidase-catalyzed oxidation. That both ovoperoxidase and proteoliaisin must be bound to the vitelline layer for cross-linking to occur is suggested by the observations that no proteoliaisin was bound in seawater containing EGTA and H_2O_2, and that the maximum level of irreversible binding occurred only when stoichiometric amounts of ovoperoxidase were present (e.g., ovoperoxidase/proteoliaisin molar ratios greater than or equal to one; Fig. 4). These data support the hypothesis that binding of ovoperoxidase and proteoliaisin in our in vitro system reconstitutes one step in the fertilization envelope assembly process.

Discussion

In the more than a century that fertilization envelope assembly has been studied, certain mechanistic principles have emerged, which may have general validity (13). Before the

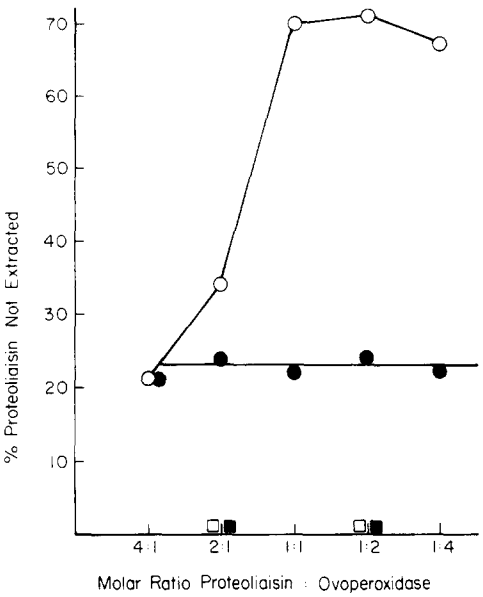

Figure 4. Ovoperoxidase-catalyzed cross-linking of proteoliaisin to the vitelline layers of unfertilized eggs. The incubation mixtures contained ^{125}I-proteoliaisin (200 μg/ml, 2.6 × 10^5 cpm/ml), sufficient unlabeled ovoperoxidase (14–112 μg/ml) to give the indicated molar ratios of ovoperoxidase to proteoliaisin, and 5% eggs by volume in CaSW. After the 1-h incubation, two 50-μl aliquots were removed to determine the amount of proteoliaisin bound. Identical aliquots were diluted into 1.5 ml of EGTA-SW containing 2 mM Mg^{2+}, allowed to incubate for 30 min, and then processed to determine the amount of proteoliaisin remaining bound. To the remainder of the incubation mixture, H_2O_2 was added to a final concentration of 100 μM. After 15 min, 50-μl aliquots were processed to determine the amount of proteoliaisin bound before and after EGTA extraction, as described above. Results are expressed as the fraction of proteoliaisin bound to the eggs that was not extracted by EGTA-SW as a function of the ovoperoxidase/proteoliaisin molar ratio. ●, Control eggs not exposed to H_2O_2; ○, eggs exposed to H_2O_2; ■, □, incubation in EGTA-SW with and without H_2O_2.

modification induced by fertilization, the interacting components are kept in separate cellular compartments: the secretory cortical vesicles and the vitelline layer. At fertilization, these compartments are mixed by a controlled wave of exocytosis induced by the sperm and using a rise in intracellular free Ca^{2+}. Secreted proteins are then stoichiometrically added to the vitelline layer in a reaction dependent upon divalent cations in the extracellular environment. One component inserted into the fertilization envelope, ovoperoxidase, catalyzes the covalent association of envelope peptides by ditryosine formation, using H_2O_2 formed in a respiratory burst after fertilization. This is an extreme example of extracellular matrix remodeling that may be instructive for a consideration of general mechanisms that obtain when other glycocalyces are altered in developmental pathways. As demonstrated in this report, the problems of assembly and its regulation in this system are approachable with conventional biochemical techniques because several key components have been purified and characterized. By using two purified fertilization envelope components and the glycocalyx of unfertilized eggs, we have shown that it is possible to reconstruct, in vitro, a pathway leading to the partial assembly of a fertilization envelope.

The presence of the peroxidative enzyme, ovoperoxidase, in the fertilization envelope presents an opportunity to examine a specific strategy for the regulation of enzymatic activity in the extracellular environment of a cell. Ovoperoxidase exhibits a pH-dependent hysteretic behavior that may act as a timing mechanism to delay its activity until it is assembled in the fertilization envelope (5, 6). When activated, however, ovoperoxidase is similar to most peroxidases and can oxidize a variety of substrates in addition to tyrosine (7). This suggests that part of the strategy for regulating ovoperoxidase involves rapidly sequestering the enzyme within the assembling fertilization envelope, in an orientation favorable for both interaction with specific substrates and induction of conformational changes that activate the enzyme. Our data indicate that the first step in this process is mediated by the association of ovoperoxidase with proteoliaisin and the subsequent interaction of this complex with the vitelline layer scaffold. Since ovoperoxidase binds to the vitelline layer of unfertilized eggs only when proteoliaisin is present, in amounts proportional to the amount of proteoliaisin added (Table I), proteoliaisin is probably not a component of the vitelline layer. Thus, both ovoperoxidase and proteoliaisin most likely reside in the cortical granules, possibly as a preformed complex, before fertilization. Preliminary results with antibodies to both proteoliaisin and ovoperoxidase verified this supposition (Somers, C., D. Battaglia, and B. M. Shapiro, unpublished data).

It is well known that some proteolytic modification of the vitelline layer occurs during fertilization envelope assembly. The best characterized of these modifications are the cleavage of attachment posts (15) between the vitelline layer and plasma membrane and the inactivation of sperm receptors (3). More extensive proteolysis has been proposed, since the morphology of the vitelline layer changes from thick fibers to thin strands during fertilization envelope assembly (4). Although this additional processing might be required for complete assembly to occur, it is clear that such processing is not obligatory for the binding of ovoperoxidase and proteoliaisin to the vitelline layer scaffold. Proteolysis might, however, enhance this binding interaction, for example, by increasing the accessibility of vitelline layer binding sites.

Divalent cations have been implicated in the assembly process for many years. In this context, two features of the divalent cation dependence of the proteoliaisin–vitelline layer interaction are interesting. First, this dependence is substantially different than that for the ovoperoxidase–proteoliaisin interaction (20), which is highly specific for Ca^{2+} and requires relatively low concentrations of this cation ($K_{½}$ = 50 μM). In the interaction of proteoliaisin with ovoperoxidase, Mg^{2+} can not substitute for Ca^{2+} nor does it enhance binding in the presence of Ca^{2+} (20; Weidman, P. J., unpublished observation). Second, the two major divalent cations found in seawater influence binding through a synergistic action. In the interaction of proteoliaisin with the vitelline layer, either Ca^{2+} or Mg^{2+}, can mediate binding, albeit with different efficiencies. Both cations are required for optimal binding. This suggests that proteoliaisin has at least two distinct, divalent cation–dependent binding domains, one for interacting with ovoperoxidase and one for interacting with the vitelline layer.

The synergistic effect of Ca^{2+} and Mg^{2+} on the binding of proteoliaisin to the vitelline layer was most striking in the saturation binding studies. There were three apparently

different classes of binding sites. Relatively low amounts ($N = 8.3 \times 10^7$ molecules per egg) of the highest affinity sites ($K_d = 0.19$ μM) were found in seawater containing only Mg^{2+}. Over six times as many sites ($N = 5.5 \times 10^8$ molecules per egg) were found in seawater containing only Ca^{2+}, but the affinity of these sites was approximately threefold lower ($K_d = 0.43$ μM). In seawater containing both cations, the number of binding sites increased even further ($N = 1.03 \times 10^9$ molecules per egg). This was a nearly 10- and 2-fold increase in number of sites over MgSW and CaSW, respectively, yet the apparent affinity of these sites was considerably lower ($K_d = 1.47$ μM). This very large number of binding sites on the egg compares favorably with the number of concanavalin A binding sites on the surface of the egg (4.0×10^8 sites per egg; reference 17). Although we can not directly measure the number of molecules of proteoliaisin per fertilization envelope, we can estimate the number of molecules of proteoliaisin released at fertilization. Since the structural proteins of the fertilization envelope released from the cortical granules comprise 3.6% of the total egg protein (2), and 10% of this protein is proteoliaisin (20)[2], we estimate that there are 1.4×10^9 molecules of proteoliaisin released per egg at fertilization. Thus, the number of proteoliaisin-binding sites on the egg surface in this in vitro system is reasonably close to the levels that might be expected to act during the in vivo assembly process.

One clue to the apparently cooperative effects of Ca^{2+} and Mg^{2+} in the proteoliaisin–vitelline layer interaction is suggested by the observation that the assembled fertilization envelope is a trilaminar structure. This results from the penetration of cortical granule proteins through the vitelline layer during assembly and the deposition of these proteins on both sides of this scaffold (4). In NSW, if we are indeed measuring the total binding capacity of the vitelline layer for proteoliaisin during the assembly process, then proteoliaisin must be binding to both sides of the vitelline layer, even though this structure is covalently attached to the plasma membrane in the unfertilized egg (15). The fact that almost half as many binding sites are observed in CaSW would be consistent with binding of proteoliaisin to only one side of the vitelline layer. Perhaps Mg^{2+} facilitates the transport of proteoliaisin across the vitelline layer, thereby doubling the number of binding sites available. Since there is a distinct class of binding sites in MgSW, such sites might play a role in allowing proteoliaisin to reach the other side of the vitelline layer during envelope assembly, and thus serve to prevent saturation of the plasma membrane–apposed face.

We have shown that purified ovoperoxidase can bind to the vitelline layer of unfertilized eggs when complexed with purified proteoliaisin. This supports our hypothesis that the function of proteoliaisin is to appropriately direct the insertion of ovoperoxidase into the fertilization envelope. The observations that ovoperoxidase can cross-link proteoliaisin to the vitelline layer of unfertilized eggs and that optimal cross-linking requires stoichiometric (rather than enzymatic) quantities of ovoperoxidase indirectly demonstrate that this insertion places ovoperoxidase in an orientation that allows cross-linking. Because ovoperoxidase is bound to proteoliaisin, it is likely that the binding of this complex to the vitelline layer results in highly specific and targeted cross-linking. This would serve as another mechanism for insuring specificity in the use of reactive oxygen intermediates by the egg at the beginning of its developmental program.

The data presented in this report show that it is possible to effect the in vitro assembly of a partial fertilization envelope on the surface of an unfertilized egg by using purified components. This type of in vitro morphogenesis has previously been possible only with genetically characterized systems, such as viral capsid assembly. By extending this approach, it should be possible to further elucidate the rules governing pathways and mechanisms that allow this specialized glycocalyx to be assembled in the absence of intracellular control mechanisms.

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