Hetero-oligomerization-dependent Binding of Pig Oocyte Zona Pellucida Glycoproteins ZPB and ZPC to Boar Sperm Membrane Vesicles*

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The zona pellucida surrounding the pig oocyte contains two Mr 55,000 glycoproteins, pZPB and pZPC, which are orthologues of mouse zona proteins ZP1 and ZP3, respectively. We previously reported that isolated boar sperm membrane vesicles possess high affinity binding sites for partially purified pZPB, but not pZPC. Interestingly, co-incubation experiments also implicated pZPB-pZPC complexes as potential ligands. We now report that when depleted of a minor pZPC contaminant by size exclusion chromatography, pZPB lacks independent binding activity. In solid phase binding assays employing immobilized boar sperm membranes, pZPB failed to compete with biotin-(pZPB+pZPC) probe, and biotin-labeled pZPB yielded negligible binding. However, when co-incubated with pZPC prior to the binding assays, pZPB acted as a potent competitor, and biotin-labeled pZPB exhibited high affinity, saturable binding. Binding activity was attributed to pZPB-pZPC heterocomplexes, which were detected in co-incubation mixtures by size exclusion chromatography and Western blot analysis. In the pig, therefore, sperm membranes possess a zona-binding protein with high affinity sites for pZPB-pZPC heterocomplexes, but not free glycoprotein subunits. Consequently, associative interactions between zona molecules can contribute toward both the assembly of the zona matrix and generation of ligands important for sperm-zona interactions.

The zona pellucida is a morphologically discrete, extracellular matrix which envelopes and protects the oocyte and preimplantation embryo. This egg investment also actively participates in the fertilization process. The zona pellucida provides docking sites for species-specific sperm attachment and induces bound sperm to undergo the acrosome reaction, the preparatory event for zona penetration and eventual sperm-egg fusion. This intimate association of male and female gametes is mediated in part by sperm-adhesive glycoproteins within the zona matrix and complimentary zona-binding proteins on the sperm surface. Interestingly, although multiple gamete adhesion molecules have been characterized at the molecular level (1–11), the relative physiological importance of many remains a topic of considerable debate (12).

Zonae pellucidae contain a repertoire of glycoproteins encoded by three gene families that are conserved across species. Adopting the terminology of Harris et al. (13), the three cDNA-predicted precursor polypeptides in the pig (10, 13, 14) are herein designated pZPA (79 kDa), pZPB (59 kDa), and pZPC (46 kDa). Due to post-translational processing, the three mature glycoproteins from isolated zonae appear on nonreducing SDS gels as two diffuse bands (15): pZPA runs at Mr 90,000, whereas pZPB and pZPC co-migrate at Mr 55,000. Electrophoretic or chromatographic protocols permit isolation of copurified pZPB and pZPC glycoproteins (15–17) and such pZPB-pZPC preparations exhibit several important biological activities, including: stimulation of the acrosome reaction in capacitated boar sperm (18), inhibition of sperm-zona attachment (19, 20), binding to a sulfated polysaccharide-binding domain on boar sperm procasin (21), and binding to isolated boar sperm membrane vesicles (22).

Although intractable to further purification as fully glycansated entities, pZPB and pZPC elute as separate peaks during reverse phase HPLC when pretreated with endo-β-galactosidase (17). The pZPB glycoprotein (Mr 41,000 following endo-β-galactosidase digestion) elutes first and appears homogeneous on SDS gels. The pZPC glycoprotein (Mr 44,000 following endo-β-galactosidase digestion) follows and routinely contains small amounts (5–10%) of pZPC contaminant.

Purified pZPB and pZPC differ markedly in apparent biological activity. When tested in gamete adhesion assays, pZPB inhibits sperm-zona binding, whereas pZPC is without effect (20, 23). Likewise, when tested in solid phase binding assays, biotin-labeled pZPB binds to immobilized boar sperm membranes in a concentration-dependent, saturable manner, whereas biotin-pZPC does not (22). In competition assays, pZPB blocks binding of a biotin-pZPB (B+C) probe to membranes; pZPC fails to compete but, surprisingly, enhances the apparent affinity of pZPB for its cognate zona-binding protein. Collectively, the data suggest that (i) boar sperm possess a zona-binding protein with a pZPB-pZPC binding domain that recognizes both pZPB and putative pZPB-pZPC complexes, and (ii) interaction of pZPB with pZPC enhances pZPB binding affinity.

More recently, both pZPB- and pZPC-specific antibodies were unexpectedly found to block binding of biotin-pZPB to boar sperm membranes.2 This paradoxical data prompted us to

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§ This abbreviations used are: HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; TBS, Tris-buffered saline; BSA, bovine serum albumin; MES, 2-(N-morpholino)ethane sulfonic acid.

1 E. C. Yurewicz, unpublished results.
question whether the pZPB glycoprotein indeed binds independently to membrane-associated zona-binding proteins. Could minor amounts of putative pZPB-pZPC complexes account for the observed binding activity of our previous pZPB preparations? Here we conclusively demonstrate that whereas pZPC and more highly purified pZPB lack independent binding activity, reconstituted pZPB-pZPC heterocomplexes are novel, high affinity ligands for zona-binding molecules present in boar sperm membrane vesicles.

EXPERIMENTAL PROCEDURES

Materials—Three different pig zona protein preparations were used in this study. Their nomenclature conforms with that adapted by Harris et al. (13). pZPB(B+C) was purified from heat-solubilized porcine zonae pellucidae by gel filtration and hydroxyapatite chromatography and consists of fully glycosylated pZPB and pZPC glycoproteins (17). pZPB and pZPC were isolated from endo-p-β-galactosidase-digested pZPB(C) by reverse phase HPLC (17). pZPB1–C), pZPB, and pZPC correspond to preparations designated ZP3, ZP3α, and ZP3β in prior publications from this laboratory. Membrane vesicles were isolated from capacitated boar sperm by nitrogen cavitation and differential centrifugation and stored at −70 °C as described previously (22).

Antibodies—The rabbit polyclonal antiserum raised against the keyhole limpet hemocyanin-conjugated peptide CELQIAKDERYGS (residues 319–330 of the deduced pZPB precursor protein with an added N-terminal cysteine) was described previously (24). Affinity purification on a matrix consisting of the synthetic peptide coupled to AminoLink resin (Pierce) yielded pZPB-specific, site-directed antibodies (anti-pZPB12,123–330). For pZPC-specific immunodetection, we used an ascites fluid containing a previously described (25) mouse monoclonal antibody, MA-451, which recognizes an internal pZPC epitope (EEKLVF, residues 166–171 of cDNA-derived pZPC precursor protein) (26). Horse-radish peroxidase-conjugated secondary antibodies were obtained from Amersham.

Size-exclusion Chromatography—HPLC separations were performed on a Dionex instrument equipped with a TSK G3000SWXL column (7.8 × 300 mm, TosohHaas). The flow of high-salt PBS (50 mM sodium phosphate, pH 6.6, 300 mM NaCl) was maintained at 0.5 ml/min at ambient temperature. For preparative isolations, lyophilized proteins were reconstituted in high-salt PBS and centrifuged through Spin-X filter units (0.45-μm cellulose acetate, Costar) prior to injection. Individual peaks were collected manually, pooled from multiple runs, concentrated by ultrafiltration (Centricon-10, Amicon) to a final protein concentration of >500 μg/ml and stored at −20 °C. The TSK G3000SWXL column was calibrated daily using blue dextran 2000 and dinitrophenylglycine as void volume (V0) and total volume (Vt) markers, respectively. The elution position (Ve) of individual peaks was used to calculate Keq using the equation, Keq = (Ve − V0)/(Vt − Ve).

Mass Spectrometry—MALDI-MS of zona proteins was performed on a PerSeptive Biosystems Voyager Elite instrument equipped with a nitrogen laser emitting at 337 nm with a pulse duration of 2 ns. The instrument was operated at an accelerating voltage of 30 KV in the linear continuous extraction mode. Sinapinic acid (3,5-dimethoxy-4-hydroxyccinamic acid) was used as the MALDI matrix and dissolved in acetonitrile/water/trifluoroacetic acid (50:49.9:0.1, v/v/v) to make a solution of 10 mg/ml. Proteins were dialyzed against 25 mM ammonium acetate and lyophilized to dryness prior to analysis. To prepare the target, 1 μl of the protein sample dissolved in water was mixed with 1 μl of matrix solution on the sample plate cell and air-dried.

Immunoblotting—Zona proteins were separated by SDS-gel electrophoresis on nonreducing 10% polyacrylamide gels and transferred to nitrocellulose (Novex). After treatment with Blocker®BLOTTO (Pierce), membranes were incubated with either 0.5 μg/ml anti-pZPB12,123–330 or MA-451 diluted 1:5000. Bound antibodies were decorated with horseradish peroxidase-conjugated secondary antibodies diluted 1:5000 and visualized with ECL reagents and Hyperfilm-ECL (Amersham).

Solid Phase Binding Assays—Interactions between purified zona proteins and boar sperm membrane vesicles were investigated using a previously described solid phase binding assay (22) with minor modifications. Immulon-2 microtiter wells (Dynatech) were coated with 500 ng of sperm membranes and subsequently blocked with 1% BSA. For competition binding assays, wells were incubated in quadruplicate with 50 μl of 0.2 μg/ml biotin-pZPB + C) in TBS/TBSA (20 mM Tris, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.05% Tween 20, 0.1% BSA) in the presence and absence of unlabeled competitor. Competitors were pre-incubated overnight at 4 °C in high-salt PBS, diluted in TBS/TBSA to twice their final concentration, and mixed with an equal volume of 0.4 μg/ml biotin-pZPB + C) at least 30 min prior to transfer to wells. Binding of biotin-labeled probe was measured using strepavidin-alkaline phosphatase (Jackson ImmunoResearch) and p-nitrophenyl phosphate. IC50 values at 405 nm were recorded using a Bio-Tek microplate reader. All data in competition assays were corrected for nonspecific binding as determined in wells incubated with biotin probe in the presence of a 100-fold excess of pZPB + C). IC50 (mean ± S.E.) values are defined as the concentration of competitor required to inhibit binding of biotin-pZPB + C) by 50%; for co-incubates, IC50 values represent the concentration of the pZPB component only.

Direct binding assays followed a similar protocol, except that biotin-labeled proteins were preincubated overnight at 4 °C at ≥50 μg/ml, either alone or with added test protein, in high-salt PBS and then diluted into replicate tubes, one containing TBS/TBSA (total binding) and the other TBS/TBSA plus a 100-fold excess of pZPB + C) relative to the biotin-labeled ligand (nonspecific binding). Wells were incubated in triplicate. IC50 values (mean ± S.E.) were calculated by nonlinear regression analysis of saturation binding data using the one-site binding equation in PRISM software (GraphPad).

Miscellaneous—For biotin labeling, 450 μg of protein was reacted with 30 μg of N-biotinoyl-e-aminoacridine-N'-hydroxysuccinimide ester in 180 μl of 50 mM sodium borate buffer, pH 8.0. After a 1-h incubation, the reaction was quenched with ammonium chloride. Biotinylated protein was recovered by HPLC on a TSK G3000SWXL column in high-salt PBS and concentrated by ultrafiltration. Proteins were quantitated using a BCA Protein Assay kit (Pierce) in a microtiter plate format. BSA was used to construct standard curves. Protein concentrations were converted to molarities using molecular masses of 36,670 and 36,308 Da for the core polyptides of pZPB (cDNA-deduced residues 137–463) and pZPC (cDNA-deduced residues 23–348), respectively; N termini have been experimentally determined (17, 27) and C termini are positioned at consensus sites for furin cleavage (10, 13).

RESULTS

Purification of pZPB Glycoprotein by Size Exclusion Chromatography—To produce a preparation depleted of residual pZPC, we subjected pZPB to size exclusion chromatography on a TSK G3000SWXL column in high-salt PBS. Under these conditions, pZPB eluted as a series of incompletely resolved UV peaks (Fig. 1A). The first peak emerged in the column void volume (exclusion limit >700 kDa) and the final peak eluted between the 43- and 80-kDa molecular mass markers. Five subfractions, designated pZPB1–pZPB5 and containing 15, 17, 21, 26, and 26% of recovered protein, respectively, were collected (Fig. 1A) and concentrated by ultrafiltration. Macromolecular compositions were examined by SDS-polyacrylamide gel electrophoresis and immunoblotting. Protein staining revealed the pZPB glycoprotein as a predominant Mf, 44,000 band (≥95% of total Coomassie Blue staining) in the pZPB preparation used as starting material (Fig. 1B, lane B) and in each pZPB subfraction obtained by size exclusion chromatography (Fig. 1B, lanes 1–5). By comparison, purified pZPC glycoprotein migrates as a Mf, 41,000 band (Fig. 1B, lane C). Subfractions pZPB1–pZPB5 also contained a Mf, 81,000 band accounting for ≤5% of total Coomassie Blue staining.

Western blots were probed with anti-pZPB12,123–330 and MA-451 to visualize pZPB and pZPC glycoproteins, respectively. Immunostaining with anti-pZPB12,123–330 uniformly illuminated the Mf, 44,000 pZPB glycoprotein in all five subfractions (Fig. 1C, top). In contrast, MA-451 produced dramatically different staining intensities of pZPC bands and subfractions pZPB1–pZPB5 versus pZPB(C) (Fig. 1C, bottom). Subfractions pZPB1–pZPB5 possessed easily detected pZPB bands, whereas pZPB(C) bands were not visible on reducing gels and presumably represents a disulfide-bonded dimer of pZPC. Importantly, subfractions pZPB4 and pZPB5 yielded barely discernible pZPC bands, thus indicating negligible pZPC cross-contamination.

Competitive Binding Activity of pZPB Subfractions—To as-
Functional Activity of ZPB-ZPC Heterocomplexes

Fig. 1. Purification of pZPB by size exclusion chromatography. A, elution profile on TSK G3000SWg XL resin. 200 µg of pZPB in 100 µl of high-salt PBS was injected onto the TSK G3000SWg XL column running at 0.5 ml/min in high-salt PBS mobile phase. Five subfrations (pZPB1–pZPB5) were collected as indicated. Arrowheads mark the elution positions of the protein standards, thyroglobulin (669 kDa), glutamate dehydrogenase (330 kDa), IgG (160 kDa), transferrin (80 kDa), and ovalbumin (43 kDa). B, Coomassie Blue staining of proteins (1 µg/lane) separated on a 10% NuPAGE gel (Novex) in MES/SDS running buffer. Lane B, pZPB starting material; lanes 1–5, subfrations pZPB1–pZPB5; lane C, pZPC. M, molecular mass markers. Values are indicated on the right.

Fig. 2. Competitive inhibition of biotin-pZPB(B+C) binding to boar sperm membrane vesicles by pZPB subfractions. Microliter plate wells were coated with 500 ng of boar sperm membrane vesicles and challenged with 0.2 µg/ml biotin-pZPB(B+C) in the presence or absence of competing pZPB subfractions that had been preincubated (overnight, 4 °C) at 400 µg/ml in high-salt PBS. All data are corrected for nonspecific binding as determined from wells incubated with biotin-pZPB(B+C) in the presence of a 100-fold excess of unlaled pZPB(B+C) competitor. A, competition curves obtained using subfractions pZPB1–pZPB5. Note that subfractions pZPB1–pZPB3, which are devoid of pZPC, all five pZPB subfractions produced similar competitive binding curves (Fig. 2A); IC50 values (n = 2) were 11.4 ± 0.3, 8.2 ± 1.1, 7.1 ± 0.3, and 6.5 ± 0.5 nM for pZPB1–pZPB5, respectively. Taken together, these results implied the existence of a membrane-associated zona-binding protein with high affinity binding sites for putative pZPB-pZPC complexes, but not individual pZPB or pZPC glycoproteins. Further molecular and functional studies of pZPB-pZPC interactions focused on pZPB5.

Purified pZPB5 Is Predominantly Monomeric—We used MALDI-MS and size exclusion chromatography to evaluate the

were excellent competitors (Fig. 2). Subfractions pZPB1–pZPB5 blocked binding of biotin-pZP(B+C) in the presence of zona-binding proteins and biotin-pZP(B+C) as labeled probe. Significantly, only those subfractions possessing both pZPB and pZPC glycoproteins demonstrated competitor activity (Fig. 2A). Subfractions pZPB1–pZPB5 blocked binding of biotin-pZP(B+C) to immobilized membranes with respective IC50 values (n = 2) of 20.7 ± 7.1, 36.0 ± 4.9, and 103 ± 11 nm. In striking contrast, pZPB4 and pZPB5, which are devoid of pZPC, did not compete. The ability of pZPB1–pZPB3, but not pZPB4 or pZPB5, to displace biotin-pZPB(B+C) implied that competitor activity depends upon the coordinate action of pZPB and pZPC glycoproteins. To test this hypothesis, a fixed quantity of pZPB5 was preincubated (4 °C, overnight) with variable amounts of added pZPC and allowed to compete with biotin-pZPB(B+C) in the solid phase binding assay. Whereas pZPC added alone did not block binding of biotin-pZPB(B+C) to sperm membranes (Fig. 2C), co-incubates with pZPC:pZPB5 ratios of 1:5, 1:2, and 1:1 were excellent competitors (Fig. 2B).

Based on the above results, pZPB1–pZPB5 were tested in the solid phase binding assay after overnight preincubation with pZPC (pZPC:pZPB5 ratio = 1:2). In the presence of added pZPC, all five pZPB subfractions produced similar competitive binding curves (Fig. 2C); IC50 values (n = 2) were 11.4 ± 1.6, 11.4 ± 1.4, 8.2 ± 1.1, 7.1 ± 0.3, and 6.5 ± 0.5 nM for pZPB1–pZPB5, respectively. Taken together, these results implied the existence of a membrane-associated zona-binding protein with high affinity binding sites for putative pZPB-pZPC complexes, but not individual pZPB or pZPC glycoproteins. Further molecular and functional studies of pZPB-pZPC interactions focused on pZPB5.

Functional Activity of ZPB-ZPC Heterocomplexes

sessed relative ligand activity of pZPB subfractions toward boar sperm zona-binding proteins, we constructed competitive binding curves using immobilized membrane vesicles as the source of zona-binding proteins and biotin-pZP(B+C) as labeled probe. Significantly, only those subfractions possessing both pZPB and pZPC glycoproteins demonstrated competitor activity (Fig. 2A). Subfractions pZPB1–pZPB5 blocked binding of biotin-pZP(B+C) to immobilized membranes with respective IC50 values (n = 2) of 20.7 ± 7.1, 36.0 ± 4.9, and 103 ± 22 nm. In striking contrast, pZPB4 and pZPB5, which are devoid of pZPC, did not compete. The ability of pZPB1–pZPB3, but not pZPB4 or pZPB5, to displace biotin-pZPB(B+C) implied that competitor activity depends upon the coordinate action of pZPB and pZPC glycoproteins. To test this hypothesis, a fixed quantity of pZPB5 was preincubated (4 °C, overnight) with variable amounts of added pZPC and allowed to compete with biotin-pZPB(B+C) in the solid phase binding assay. Whereas pZPC added alone did not block binding of biotin-pZPB(B+C) to sperm membranes (Fig. 2C), co-incubates with pZPC:pZPB5 ratios of 1:5, 1:2, and 1:1 were excellent competitors (Fig. 2B).

Based on the above results, pZPB1–pZPB5 were tested in the solid phase binding assay after overnight preincubation with pZPC (pZPC:pZPB5 ratio = 1:2). In the presence of added pZPC, all five pZPB subfractions produced similar competitive binding curves (Fig. 2C); IC50 values (n = 2) were 11.4 ± 1.6, 11.4 ± 1.4, 8.2 ± 1.1, 7.1 ± 0.3, and 6.5 ± 0.5 nM for pZPB1–pZPB5, respectively. Taken together, these results implied the existence of a membrane-associated zona-binding protein with high affinity binding sites for putative pZPB-pZPC complexes, but not individual pZPB or pZPC glycoproteins. Further molecular and functional studies of pZPB-pZPC interactions focused on pZPB5.

Purified pZPB5 Is Predominantly Monomeric—We used MALDI-MS and size exclusion chromatography to evaluate the...
Fig. 3. MALDI mass spectrum of pZPB₅. The [M + H]⁺ peak indicates a mass centered about 43,643 Da for the multiple pZPB glycoforms.

oligomeric status of purified pZPB₅. The MALDI spectra of pZPB₅ (Fig. 3) and pZPB starting material (not shown) were highly similar and indicated masses centered about 43,643 and 43,577 Da for the constituent families of pZPB glycoforms, respectively. However, in contrast to the complex elution profile of pZPB starting material in Fig. 1A, size exclusion chromatography of pZPB₅ on the G3000SW XL column in high-salt PBS yielded a dominant peak at $K_{av} = 0.435$ (Fig. 4A); the molecular size relative to protein standards (Fig. 4B) was $M_r$ 55,400. As this approximates the molecular mass determined by MALDI-MS, pZPB₅ most likely exists as monomers in high-salt PBS. The presence of minor $M_r$ 124,000 and 230,000 peaks at $K_{av} = 0.313$ and 0.221, respectively, indicated a tendency of the pZPB glycoprotein to self-assemble.

pZPB₅ and pZPC Form Heteromultimeric Complexes—Size exclusion chromatography provided direct evidence that co-incubated pZPB and pZPC glycoproteins form heterocomplexes. Proteins were incubated overnight at 4 °C in 40 μl of high-salt PBS as follows: (i), pZPB₅, 16 μg; (ii), pZPB₅, 16 μg + fetuin, 12 μg; (iii), pZPB₅, 16 μg + pZPC, 12 μg; (iv), pZPC, 12 μg; and (v), pZPC, 12 μg + fetuin, 16 μg. Fetuin, a glycoprotein with physicochemical characteristics similar to pZPB and pZPC, served as a negative control. Twenty-μl aliquots were injected onto the TSK G3000SWXL HPLC column and 1-min fractions were collected. Elution of pZPB and pZPC glycoproteins was monitored by Western blotting using anti-pZPB₅ and anti-pZPC antisera. Protein eluted as a monomer in fraction 17 (cross-reference, Fig. 4A). In contrast, the pZPC glycoprotein eluted as multiple peaks ($M_r$ 300,000, $K_{av} = 0.178$; $M_r$ 160,000, $K_{av} = 0.274$; $M_r$ 68,000, $K_{av} = 0.404$) in fractions 14–17 whether preincubated alone (not shown) or in the presence of fetuin (Fig. 5C); the $M_r$ 160,000 peak (fraction 15) was predominant. Importantly, when pZPB₅ and pZPC were co-incubated, the chromatographic behavior of each changed dramatically (Fig. 5, B and D); a major percentage of each glycoprotein now eluted in the void volume (fraction 11). The elution shifts demonstrate that when incubated together, pZPB and pZPC glycoproteins assemble into high mass heterocomplexes.

High Affinity Binding of Biotin-labeled pZPB₅-pZPC Heterocomplexes to Boar Sperm Membrane Vesicles—To further test specificity of membrane-associated zona-binding proteins for pZPB₅-pZPC heterocomplexes and to estimate binding affinities, we performed direct binding experiments using biotin-labeled heterocomplexes generated by co-incubation of either biotin-pZPB₅ + pZPC or pZPB₅ + biotin-pZPC. In the first set of experiments, a fixed concentration (100 μg/ml) of biotin-pZPB₅ was preincubated (overnight, 4 °C) in high-salt PBS with or without variable quantities of pZPC. Co-incubates were diluted to yield 0.2 μg/ml biotin-pZPB₅ (5.4 nM) and incubated with immobilized membrane vesicles. Binding of biotin-pZPB₅ depended strongly upon prior incubation with pZPC. At pZPC: biotin-pZPB₅ ratios <0.3:1, binding of biotin-pZPB₅ was directly proportional to the concentration of co-incubated pZPC.

Fig. 4. pZPB₅ elutes as a monomer during size exclusion chromatography. A, after overnight preincubation at 400 μg/ml in high-salt PBS, an 8-μg aliquot of pZPB₅ was injected onto a TSK G3000SWXL HPLC column equilibrated with high-salt PBS mobile phase. The major peak had a $M_r$ 55,400 based on the calibration curve in B. Protein standards in B included, in order of elution, thyroglobulin (669 kDa), glutamate dehydrogenase (330 kDa), human IgG (160 kDa), aldolase (158 kDa), lactate dehydrogenase (140 kDa), transferrin (80 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20.1 kDa), horse myoglobin (17 kDa), and cytochrome c (12.4 kDa).

Fig. 5. Co-incubated pZPB₅ and pZPC form heteromultimeric complexes. Co-incubations were performed overnight at 4 °C in 40 μl of high-salt PBS: 16 μg of pZPB₅ + 12 μg of pZPC (B and D); 16 μg of pZPB₅ + 12 μg of fetuin (A); 12 μg of pZPC + 16 μg of fetuin (C). Samples were injected onto a TSK G3000SWXL HPLC column via a 20-μl sample loop and 1-min fractions (0.5 ml) were collected during elution with high-salt PBS. For immunoblot analysis, 8-μl aliquots of fractions 11–17 were separated under nonreducing conditions on 10% SDS gels, transferred to nitrocellulose, and probed with anti-pZPB₅ (MA-451) or MA-451 (C and D) to monitor elution of pZPB and pZPC glycoproteins, respectively. When co-incubated, pZPB and pZPC assembled into high mass complexes that eluted in the column void volume (fraction 11) (B and D). In contrast, elution profiles of pZPB and pZPC preincubated separately (not shown) were similar to those obtained following co-incubation with fetuin (A and C).
Using highly purified glycoproteins isolated from the pig oocyte zona pellucida, we demonstrate for the first time that pZPB and pZPC associate to form high mass heteromultimeric complexes. We also provide the first description of a zona-associated zona-binding proteins. Co-incubates with pZPC:biotin-pZPB5 complexes exhibited high affinity (EC50 = 4.3 nM), saturation binding of reconstituted (biotin-pZPB5–pZPC) complexes. C and D, experiments using biotin-pZPC. C, 1.0 μg of biotin-pZPC was titrated with 0–4.0 μg of pZPB5 in 10 μl of high-salt PBS (overnight, 4 °C). Binding of pZPB5–(biotin-pZPC) complexes to boar sperm membrane vesicles was measured at a final concentration of 0.1 μg/ml biotin-pZPB5 (2.8 nM). D, 1.5 μg of biotin-pZPC was co-incubated (overnight, 4 °C) in 15 μl of high-salt PBS with either 3.0 μg of pZPB5 (circles) or 1.5 μg of fetuin (triangles) and then incubated with immobilized membrane vesicles at the indicated final biotin-pZPB5 concentrations. Note the high affinity (EC50 = 4.3 nM), saturation binding of reconstituted (biotin-pZPB5–pZPC) complexes. C and D, experiments using biotin-pZPC. C, 1.0 μg of biotin-pZPC was titrated with 0–4.0 μg of pZPB5 in 10 μl of high-salt PBS (overnight, 4 °C). Binding of pZPB5–(biotin-pZPC) complexes to boar sperm membrane vesicles was measured at a final concentration of 0.1 μg/ml biotin-pZPC (2.8 nM). D, 1.5 μg of biotin-pZPC was co-incubated (overnight, 4 °C) in 15 μl of high-salt PBS with either 3.0 μg of pZPB5 (circles) or 1.5 μg of fetuin (triangles) and then incubated with immobilized membranes at varying final concentrations of biotin-pZPC. Reconstituted pZPB5–(biotin-pZPC) complexes exhibited high affinity (EC50 = 1.7 nM), saturation binding comparable to that observed with reconstituted (biotin-pZPB5–pZPC) complexes in B.

**DISCUSSION**

Using highly purified glycoproteins isolated from the pig oocyte zona pellucida, we demonstrate for the first time that pZPB and pZPC associate to form high mass heteromultimeric complexes. We also provide the first description of a zona binding activity in boar sperm membranes that possesses avid affinity for pZPB-pZPC heterocomplexes but not the free zona glycoprotein subunits. As a consequence, we propose that at least for the porcine system, pZPB-pZPC heteromers are important functional and structural units of this gamete-specific extracellular matrix.

Data presented earlier (22) suggested that pZPB binds directly to isolated boar sperm membranes and hinted that pZPB can associate with pZPC to form putative pZPB-pZPC complexes with enhanced binding affinity. Our new data obtained with pZPB depleted of residual pZPC contaminant by size exclusion chromatography confirm the heterotypic association of co-incubated pZPB and pZPC glycoproteins but conclusively demonstrate that free pZPB subunits fail to engage membrane-associated zona-binding proteins. Consequently we conclude that pZPB-pZPC complexes, although present in minor amounts, accounted exclusively for the ligand activity observed for earlier pZPB preparations (20, 22).

Size exclusion chromatography and Western blot analyses reveal that co-incubated pZPB5 and pZPC assemble into high mass heterocomplexes. Although the domains mediating this associative interaction are not yet identified, pZPB residues 142–187 encompass a cysteine-rich trefoil motif, or P-domain, which is expressed in mucins and secretory peptides and is postulated to mediate protein-protein and protein-carbohydrate interactions (28–30). In addition, pZPB (residues 190–454), pZPC (residues 45–306), and pZPA (residues 368–623) each contain a single ZP domain (31) consisting of a module with eight conserved cysteines and presumed characteristic three-dimensional structure which has been suggested to mediate binding activities between proteins that form filamentous extracellular matrices (32, 33). Native pig zona pellucida glycoproteins also possess N- and O-linked oligosaccharides bearing sulfated polylactosamines (34–36). As the pZPB and pZPC molecules have by necessity been exposed to endo-β-galactosidase...
ase during the purification protocol, we can conclude that the polyamino sugars associated with native glycoproteins are not required for assembly of pZPB-pZPC heterocomplexes or subsequent high affinity binding to membrane vesicles. Congruently, enzymatic removal of polyamino sugars does not diminish the biological activity of pZPB(p+B+C) preparations in competitive sperm-zona adhesion assays (20). Polyamino sugars nonetheless partially occlude a peptide epitope on pZPB (37) and attenuate binding affinity of pZPB(p+B+C) toward membrane vesicles (22). By analogy, in the native zona pellucida these highly extended and intensely anionic glycan may modulate (a) subunit interactions that drive assembly and maintain stability of pZPB-pZPC complexes and (b) affinity of pZPB-pZPC complexes toward zona-binding proteins on boar sperm.

Experiments performed with reconstituted pZPB-pZPC complexes in which one partner molecule bears a biotin label have permitted measurement of ligand binding affinity and confirmed obligate specificity of membrane-associated zona-binding proteins for pZPB-pZPC heteromers. Based on saturation binding curves (Fig. 6), binding affinities for (biotin-pZPB5)-pZPC complexes (Kd = 4.3 nM) and pZPB5-(biotin-pZPC) complexes (Kd = 1.7 nM) are comparable. The somewhat lower binding affinity of (biotin-pZPB5)-pZPC complexes may indicate an adverse affect of pZPB subunit biotinylation. Alternatively, the Kd values may mirror the stoichiometry of pZPB and pZPC subunits in membrane-binding heterocomplexes, i.e. a pZPB:pZPC molar ratio of approximately 2–3:1. Additional data support this hypothesis. Titration experiments produced:

(i) maximal receptor-binding activities at pZPB:pZPC molar ratios of 3:1 for biotin-pZPB5 (Fig. 3), and attenuation binding affinity of pZPB(p+B+C) toward membrane vesicles (22). By analogy, in the native zona pellucida these highly extended and intensely anionic glycans may modulate (a) subunit interactions that drive assembly and maintain stability of pZPB-pZPC complexes and (b) affinity of pZPB-pZPC complexes toward zona-binding proteins on boar sperm.

The present study raises important questions regarding the mechanism by which association of pZPB and pZPC glycoproteins creates a high affinity ligand and whether one or both subunits in this complex directly engage cognate zona-binding proteins. In one scenario, a conformational change concomitant with hetero-oligomerization may expose a cryptic adhesion domain in one subunit. Assuming that (i) domains responsible for subunit interactions are conserved across species and (ii) sperm adhesive determinants are species-specific, then experiments that test binding of chimeric ZPB-ZPC complexes to boar sperm membrane vesicles may provide insight into specificity of corresponding zona-binding proteins. In this context, a preliminary experiment has shown that Chinese hamster ovary expressed recombinant human pZPC promotes binding of biotin-pZPB5 to boar sperm membrane vesicles. Alternatively, pig sperm-zona interactions may mimic the gonadotropin ligand-receptor model (48) and both pZPB and pZPC may participate directly in binding, e.g. by creation of a high affinity adhesion domain consisting of oligosaccharides and/or peptide residues contributed by both molecules.

Zona carbohydrates play an essential role in mammalian gamete interactions (49), and several reports in the literature (18, 19, 23, 36, 50) provide evidence that the pig system conforms with this paradigm. The current study, where we demonstrate that boar sperm membranes exhibit an obligate specificity for heteromeric pZPB-pZPC complexes, emphasizes that attempts to identify bioactive carbohydrate ligands in the solid phase system by use of chemically or enzymatically liberated pig zona glycans are likely to prove futile. Alternative approaches, such as assembly of pZPB-pZPC complexes using either native pig zona proteins pretreated with glycosidases or recombinant pig zona proteins with foreign N- and O-glycans or ablated glycosylation sequons, are therefore required. By analogy to the gonadotropins (51), data from such experiments must be interpreted with caution as molecular modifications that alter the interaction between pZPB and pZPC subunits may in theory also diminish the avidity of reconstituted pZPB-pZPC complexes for cognate zona-binding proteins.

3 E. C. Yurewicz and G. K. Fontenot, unpublished results.
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Hetero-oligomerization-dependent Binding of Pig Oocyte Zona Pellucida Glycoproteins ZPB and ZPC to Boar Sperm Membrane Vesicles
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