INTRODUCTION

Until now, there has been relatively little information available about how the mammalian egg extracellular coat, the zona pellucida (ZP), assembles during oogenesis. Recently, two experimental approaches were taken, in part, to address this question in mice. In one case, antisense oligonucleotides directed against ZP glycoprotein mRNAs were injected into mouse oocytes to prevent synthesis of the glycoproteins (Tong et al., 1995). In the other case, the mZP3 gene was disrupted by targeted mutagenesis using homologous recombination in mouse embryonic stem (ES) cells and null female mice (mZP3−/−) were produced (Liu et al., 1995, 1996). The outcome of these experiments is described here. It is consistent with the current view of ZP structure, provides some new insight into ZP assembly, and suggests further avenues of research on the ZP.

THE ZONA PELLUCIDA AND ZP3

The ZP is a thick extracellular coat that surrounds all mammalian eggs (Fig. 1). A sperm must bind to and then penetrate the ZP before it can fuse with the underlying egg plasma membrane; i.e. before it can fertilize an egg (Gwatkin, 1977; Yanagimachi, 1994). The ZP contains receptors for sperm that restrict binding of sperm from heterologous species to unfertilized eggs and that prevent binding of sperm from the homologous species to fertilized eggs. In addition, binding of sperm to the receptor causes sperm to undergo the acrosome reaction, a form of cellular exocytosis. The sperm receptor/acrosome reaction-inducer is a glycoprotein, called ZP3, that is a constituent of all mammalian egg ZP, from mice to human beings (Bleil and Wassarman, 1980a, 1983; Wassarman, 1990, 1995). Free-swimming sperm recognize and bind in a species-specific manner to particular serine/threonine- (O-) linked oligosaccharides located at the ZP3 combining-site for sperm (Florman et al., 1984; Florman and Wassarman, 1985; Rosiere and Wassarman, 1992; Miller et al., 1992; Kinloch et al., 1995; Wassarman, 1995; Litscher and Wassarman, 1996). In fact, certain O-linked related oligosaccharide constructs with defined structures can inhibit binding of mouse sperm to unfertilized mouse eggs in vitro (Litscher et al., 1995). Thus, structural differences among receptor oligosaccharides could account for the degree of species specificity of gamete interaction observed when mammalian eggs and sperm are cultured together in vitro (Gwatkin, 1977; Yanagimachi, 1994; Wassarman and Litscher, 1995). Following fertilization, free-swimming sperm are unable to bind to ZP3 (Bleil and Wassarman, 1980a). Conversion of ZP3 from an active to inactive receptor during the so-called zona reaction helps to prevent polyspermic fertilization.

EXPRESSION OF ZP3 DURING DEVELOPMENT

The mouse egg ZP is laid down late in oogenesis, when non-growing oocytes, arrested in the dictyate stage of meiosis, start to undergo tremendous growth (Wassarman and Albertini, 1994). ZP glycoproteins are synthesized and secreted by growing oocytes themselves during a two-to-three week period (Bleil and Wassarman, 1980b; Greve et al., 1982; Salzmann et al., 1983; Shimizu et al., 1983; Wassarman, 1988, 1993). As oocytes increase in diameter the ZP increases in thickness, culminating in a ~7 μm thick ZP surrounding a fully-grown mouse oocyte (~80 μm in diameter). The mouse ZP contains ~3 ng of protein which consists of three glycoproteins, called mZP1 (~200 kDa), mZP2 (~120 kDa), and mZP3 (~83 kDa) (Bleil and Wassarman, 1980c; Wassarman, 1988, 1993). It has been demonstrated that mZP2 and mZP3 are expressed concomitantly and exclusively by growing oocytes (Philpott et al., 1987; Roller et al., 1989; Kinloch et al., 1993; Epifano et al., 1995). Furthermore, oocyte-specific expression of mZP3 has been attributed to promoter elements very close to the transcription start-site of the mZP3 gene (Lira et al., 1990, 1993; Schickler et al., 1992; Millar et al., 1992; Kinloch et al., 1993).

ORGANIZATION OF ZONA PELLUCIDA GLYCOPROTEINS

Glycoproteins of the mouse ZP are organized in a very specific manner. The ZP is composed of long interconnected filaments which are polymers of mZP2 and mZP3 (Greve and Wassarman, 1997). Key words: Mouse, Oogenesis, Fertilization, Zona pellucida, Antisense oligonucleotide, Targeted mutagenesis, Null mutation.
An mZP2-mZP3 dimer is located every 140 Å or so along the filaments, thus imposing a structural periodicity that can be seen in electron micrographs of dissolved ZP (Fig. 2). The filaments, in turn, are crosslinked by mZP1 to create a three-dimensional matrix. Thus, in addition to their other functions, each of the ZP glycoproteins plays a structural role during ZP assembly. It has been suggested that the amino-terminal half of mZP3 is involved in dimer formation with mZP2 (Wassarman and Litscher, 1995).

TARGETED DEGRADATION OF mZP2 AND mZP3 mRNA

To target the degradation of mZP2 and mZP3 messenger-RNA, a large excess of complementary oligonucleotide was injected into the cytoplasm of isolated growing mouse oocytes (Tong et al., 1995). Within 16 hours of injection, the targeted ZP glycoprotein was no longer synthesized by the oocyte, whereas the non-targeted glycoprotein continued to be synthesized. Interestingly, little, if any, mZP2 or mZP3 was found in ZP isolated from oocytes injected with either mZP2 or mZP3 antisense oligonucleotides. That is, the absence of synthesis of either glycoprotein prevented incorporation of the other glycoprotein into the ZP.

TARGETED DISRUPTION OF THE mZP3 GENE BY HOMOLOGOUS RECOMBINATION

mZP3 null mutant mice were produced (Liu et al., 1995, 1996) using homologous recombination in ES cells by following standard gene targeting procedures (Wassarman and DePamphilis, 1993; Hogan et al., 1994). The targeting vector was constructed such that part of mZP3 exon-2 and exon-3, and all of mZP3 intron-2, were replaced by a positive selection marker (Fig. 3). Six positive ES cell lines were injected into mouse blastocysts and all were transmitted through the germ line. Heterozygous (mZP3+/−) and homozygous (mZP3−/−) female mutant mice were identified by polymerase chain reaction (PCR) assays and Southern blotting, and mZP3 messenger-RNA and glycoprotein levels were assessed by northern and western blotting, respectively. Whereas, mZP3 mRNA and glycoprotein were present in ovaries excised from mZP3+/+ and mZP3+/− mice, they were not present in ovaries excised from mZP3−/− mice. Despite these differences, mZP3−/− female mice were indistinguishable in appearance from wild-type and heterozygous littermates, and exhibited normal growth and development.

When growing oocytes were isolated from ovaries of juvenile mZP3−/− mice they were found to be about the same size as growing oocytes isolated from wild-type mice of the same age. On the other hand, growing oocytes from mZP3−/− mice always lacked a ZP. Consistent with this finding, fully-grown oocytes and unfertilized eggs recovered from adult mZP3−/− mice also lacked a ZP. These results strongly suggest that, in the total absence of mZP3 synthesis, a ZP fails to form around growing mouse oocytes. However, apparently this does not interfere with growth of the oocyte or with follicle development. Follicle development in mZP3−/− mice appeared to be very similar to follicle development in wild-type mice (Fig. 4). However, in null mice, the portion of the oocyte facing the follicular antrum was naked and the degree of contact between the rest of the oocyte and follicle cells was diminished. In other words, the oocyte does not appear to be intimately associated with follicle cells as in the wild-type case.

Fig. 1. Photomicrograph (Nomarski DIC) of mouse sperm bound to the zona pellucida of an unfertilized mouse egg in vitro.

Fig. 2. Transmission electron micrographs of mouse zona pellucida filaments. (A) Enzyme-solubilized zona pellucida preparation adsorbed to substrate-coated grid and negatively stained. (B) Enzyme-solubilized zona pellucida preparation freeze-dried and unidirectionally shadowed (courtesy of Dr John E. Heuser, Washington University School of Medicine, St Louis, MO). F, filaments.
Interestingly, female mZP3−/− mice are infertile and the infertility apparently is related to the absence of a ZP on ovulated eggs. Examination of superovulated mZP3−/− females revealed that, while a cumulus mass was always found in their oviducts, frequently no eggs, or only a few eggs, were present within the cumulus mass. This finding is consistent with the observation that mZP3−/− oocytes are not intimately associated with surrounding follicle cells in the ovary.

THE BOTTOM LINE

What do the results of antisense (Tong et al., 1995) and targeted mutagenesis (Liu et al., 1995, 1996) experiments tell us about ZP assembly? The major lesson learned is that failure to synthesize one of the two ZP filament glycoproteins prevents assembly of a ZP. This, despite the fact that the other glycoprotein continues to be synthesized by the growing oocyte. One interpretation of the evidence is that mZP2 and mZP3 can only be incorporated into a ZP as mZP2-mZP3 dimers. Thus, the inability to construct dimers would preclude incorporation of either glycoprotein into a nascent ZP.

The site of mZP2-mZP3 dimer formation remains an interesting question, although there is some evidence that dimers may form in the oocyte prior to secretion. For example, it has been found that secretory vesicles in growing oocytes contain both newly synthesized mZP2 and mZP3 (Wassarman and Mortillo, 1991). Therefore, it is tempting to suggest that a certain degree of ZP pre-assembly occurs within the growing oocyte. How extensive this pre-assembly is in the oocyte (e.g., whether short filaments form) remains to be determined. In this context, we have found that ZP filaments can form when purified mZP2 and mZP3 are incubated together under the proper conditions in vitro (M. Vazquez and P. Wassarman, unpublished observations). This suggests that all of the structural information required to form dimers and filaments is present in the glycoproteins themselves.

Results of the targeted mutagenesis experiments bear on other aspects of oogenesis in the mouse. Surprisingly, oocytes appear to grow normally and follicles reach the antral stage of development in the absence of ZP assembly. There is ample evidence in the literature to support the idea that gap junctions formed between follicle cell and oocyte plasma membrane are necessary for normal oocyte growth (Schultz, 1986; Wassarman and Albertini, 1994). Small metabolites are thought to flow through the follicle (all follicle cells are interconnected by gap junctions) and into the growing oocyte which is metabolically and ionically active.

![Fig. 3. Schematic representation of the mZP3 locus and the targeting vector used to produce null-mutation mice. Black boxes represent exons, stippled boxes represent introns, and lines represent upstream and downstream flanking sequences of the mZP3 gene. In the targeting vector, portions of exon 2 and exon 3, and the entire intron 2, were replaced by the pGKneoβ expression cassette in order to disrupt the mZP3 gene. The location of the negative selection marker, pMC1-HSV-TK cassette, and the restriction sites for EcoRI and ClaI are also shown. The numbered arrows indicate the relative positions of oligonucleotide primers used in PCR. Primers 1 and 2 were used to screen targeted ES cell clones and primers 3-5 were used to screen heterozygous (mZP3+/−) and homozygous (mZP3−/−) mutant mice.](image)

![Fig. 4. Light micrographs of an ovarian follicle in wild-type (A) and mZP3−/− (B) adult (~6 weeks of age) female mice. Note that in A the oocyte has a zona pellucida and is completely surrounded by follicle cells closely apposed to the oocyte. In B the oocyte does not have a zona pellucida and is not completely surrounded by follicle cells closely apposed to the oocyte. ZP, zona pellucida.](image)
coupled to the follicle. However, ultrastructural evidence suggests that the extent of follicle-cell-oocyte interaction is compromised in mZP3−/− mice (Liu et al., 1995, 1996). Perhaps, the ZP normally serves as a sort of ‘glue’ to maintain the stability of gap junctions between oocyte and follicle cell plasma membranes. In the absence of a ZP the junctions either cannot form or are very labile. It is possible that interactions between the ZP and plasma membrane, especially the oocyte’s microvillous membrane, are critical for junction formation. Further investigation of the mZP3+/− mice will be required to resolve these issues.

Many aspects of ZP assembly during oogenesis remain to be addressed experimentally. Some of these, including the extent of ZP filament assembly inside and outside the growing oocyte, already have been mentioned. The availability of homozgyous and heterozygous null mutant mice will certainly facilitate experiments on the mechanism of ZP assembly in vivo. For example, these mice can be used to introduce mutated forms of the mZP3 gene, as a transgene, into the null background and determine which regions of mZP3 polypeptide are essential for mZP2-mZP3 dimer, as well as filament formation.

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REFERENCES


