Molecular Human Reproduction vol.4 no.11 pp. 1058–1064, 1998

Human zona pellucida glycoproteins: characterization using antibodies against recombinant non-human primate ZP1, ZP2 and ZP3

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Characterization and classification of human zona pellucida glycoproteins is essential to understand the functions of these components during fertilization. To achieve this, antibodies were raised in rabbits against recombinant non-human primate [Bonnet Monkey (*Macaca radiata*)] zona pellucida proteins, bmZP1, bmZP2 and bmZP3 expressed in *Escherichia coli*. Antibodies against the three recombinant zona proteins reacted with human zonae as revealed by indirect immunofluorescence. Such antibodies were used as specific probes to further characterize human zona pellucida glycoproteins in Western blot of heat solubilized human zonae pellucidae (hSIZP) resolved by one dimensional sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). Under non-reduced conditions human (h) hZP1, hZP2 and hZP3 resolved as 60, 100 and 53 kDa bands respectively. Under reduced conditions, dominant reactivity of hZP1, hZP2 and hZP3 was localized to 63, 65 and 58 kDa and faint reactivity to 53, 96 and 138 kDa bands respectively. In two-dimensional SDS–PAGE, hZP1 was shown to comprise two chains at 63–58 and 55–45 kDa, each consisting of multiple isomers. hZP2 was less acidic when compared with hZP1 and hZP3 and comprised a major component of 65 kDa and a minor component of ~96 kDa. The 65 kDa component displayed a higher degree of charged isomers in comparison with the 96 kDa component. hZP3 comprised a broad band in the range 68–58 kDa. These studies show conclusively that the hZP1 heavy train overlaps with hZP3 and that in previous studies, hZP2 was likely to have been misinterpreted as being hZP1. Our studies failed to distinguish two distinct species of hZP3, unlike previous reports. These studies will further help in our understanding of the nature of human zona pellucida glycoproteins.

Key words: anti-ZP antibodies/recombinant ZP1, ZP2 and ZP3/zona pellucida glycoproteins

Introduction

In mammals, the fertilization process is initiated by a species-specific recognition of spermatozoa by the extracellular glycoprotein matrix of the oocyte, the zona pellucida. The zona pellucida comprises three biochemically and immunologically distinct acidic glycoproteins, which are synthesized and secreted during oocyte growth and follicular development (Wassarman, 1988; Harris *et al*., 1994; Gupta *et al*., 1997a). These glycoproteins mediate critical steps in the fertilization process, including initial attachment of spermatozoa to the zona pellucida followed by tight binding, the induction of the acrosome reaction, and the block to polyspermy (Wassarman and Lischter, 1995). The genes corresponding to the three zona pellucida glycoproteins in several species have been cloned and sequenced, including the mouse (Kinloch *et al*., 1988; Liang *et al*., 1990; Epifano *et al*., 1995), pig (Yurewicz *et al*., 1993; Harris *et al*., 1994), rabbit (Schwoebel *et al*., 1991; Lee at al., 1993; Harris *et al*., 1994) and human (Chamberlin and Dean, 1990; Harris *et al*., 1994). Differing nomenclatures have been used for zona proteins and/or their genes in different species, and for this report we apply that of the mouse zona pellucida to denote homologues present in the zona pellucida of other species. The mouse zona pellucida genes encode predicted precursor proteins of ~80 (ZP2), 69 (ZP1) and 46 (ZP3) kDa that are processed into mature glycoproteins with apparent molecular masses of 200 (ZP1), 120 (ZP2) and 83 (ZP3) kDa on non-reducing sodium dodecyl sulphate (SDS) gels; ZP1 is a homodimer and, following reduction of disulphide bonds, migrates as a 130 kDa monomer (Bleil and Wassarman, 1980). Due to differences in post-translational modifications of the three precursor zona pellucida polypeptides, the masses of the mature glycoproteins and their relative order of migration on SDS gels vary between species. For example, biochemical studies show that the pig homologues of mouse ZP1, ZP2 and ZP3 migrate with apparent masses of 55, 90 and 55 kDa respectively, on non-reducing gels; the pig homologue of mouse ZP2 dissociates into 65 and 25 kDa fragments when separated under reducing conditions (Hedrick and Wardrip, 1987; Yurewicz *et al*., 1987). Consequently, for the zona pellucida from pig and other species, the relationship between the corresponding zona genes and the mature glycoproteins that are visualized on one and two-dimensional gels with indiscriminate protein stains, cannot be definitively established in the absence of biochemical or immunological

data. In particular, the paucity of human zona material and non-availability of purified individual human zona pellucida glycoproteins has confounded attempts to correlate the three human zona pellucida genes with the corresponding bands observed on SDS gels of human zona pellucida.

Recently, the nucleotide sequence of cDNA corresponding to Bonnet Monkey (*Macaca radiata*) ZP1 (bmZP1, Gupta *et al*., 1997b), bmZP2 (Jethanandani *et al*., 1998) and bmZP3 (Kolluri *et al*., 1995) has been described. The deduced amino acid sequence of bmZP1, bmZP2 and bmZP3 revealed 92.0, 94.2 and 93.9% sequence identity with deduced human (h) hZP1, hZP2 and hZP3 respectively. Subsequently, bmZP1 (Gupta *et al*., 1997b), bmZP2 (Jethanandani *et al*., 1998) and bmZP3 (Kaul *et al*., 1997) have been expressed in *Escherichia coli*.

In this report, we use polyclonal antibodies raised against well-characterized recombinant (r) bmZP1, bmZP2 and bmZP3 as highly specific immunological probes to identify and classify hZP1, hZP2 and hZP3 on Western blots of heat-solubilized human zonae pellucidae.

Materials and methods

Isolation and solubilization of human zonae pellucidae

Unfertilized, non-viable oocytes and non-viable, abnormal embryos used in this investigation were donated by patients from the Wayne State University IVF Program following project approval by the institutional Human Investigation Committee and signed patient consent. For use in gels, such oocytes and embryos were initially collected in P-1 medium supplemented with 10% synthetic serum substitute (Irvine Scientific, Santa Ana, CA, USA), washed in three changes of 0.002 M Tris buffer and stored at –70°C in Tris buffer in glass tubes. Additional oocytes and embryos were added until each collection tube contained ~100 in a volume of ~250 µl. Immediately prior to use, the material was thawed, heat-solubilized directly in the collection tube for 20 min in a 73°C water bath and lyophilized by speed vac.

Expression of Bonnet Monkey ZP1, ZP2 and ZP3 in E.coli

The bm*ZP1* (Gupta *et al*., 1997b), bm*ZP2* (Jethanandani *et al*., 1998) and bm*ZP3* (Kaul *et al*., 1997) excluding their signal sequences and their transmembrane-like domains following the putative furin cleavage sites were cloned in pQE-30 vector (QIAexpress; Qiagen GmbH, Hilden, Germany) downstream of the T5 promoter under the *lac* operator control and expressed as $(His)_6$ fusion proteins in *E.coli* as described previously (Gupta *et al*., 1997b; Kaul *et al*., 1997; Jethanandani *et al*., 1998). Recombinant proteins were purified using nickel–nitrilotriacetic acid (NTA) resin (Qiagen GmbH).

Generation of polyclonal antibodies against recombinant bmZP1, bmZP2 and bmZP3

Six month old male New Zealand White (NZW) rabbits (Small Experimental Animal Facility, National Institute of Immunology, New Delhi, India) were immunized intradermally at multiple sites with 200 µg of either r-bmZP1 or r-bmZP2 emulsified in complete Freund's adjuvant. The animals were boosted i.m. after 4 weeks with an equivalent amount of respective r-protein in incomplete Freund's adjuvant.

To generate the antibodies against r-bmZP3, the purified r-protein was conjugated with diphtheria toxoid (DT) using the 'one-step' glutaraldehyde coupling procedure as described previously (Kaul *et al*., 1997). Female NZW rabbits were immunized i.m. with r-bmZP3-DT conjugate (equivalent to 125 µg of the r-bmZP3) and boosted twice at monthly intervals using Squalene:Arlacel A (1:4) (Sigma Chemical Co, St. Louis, MO, USA) as an adjuvant. The primary injection had 1 mg of sodium phthalyl derivative of lipopolysaccharide (SPLPS) as an additional adjuvant. Animals were bled 15 days after the booster. Antibodies thus generated against r-bmZP1, r-bmZP2 and r-bmZP3 reacted with the respective recombinant protein in the Western blot and Bonnet Monkey zonae by indirect immunofluorescence as described previously (Gupta *et al*., 1997b; Kaul *et al*., 1997; Jethanandani *et al*., 1998).

Immunofluorescence on human oocytes

For use in immunofluorescent studies, oocytes and embryos were washed in three changes of phosphate-buffered saline (PBS), pH 7.2. Primary antisera were tested at 1:10 dilution and the anti-rabbit immunoglobulin fluorescein conjugate (Sigma Chemical Co) at a 1:200 dilution. Zona-encased oocytes/embryos were exposed to antisera for 30 min at room temperature, followed by washing in three changes of PBS. Rabbit anti-porcine ZP3 immune sera and pre-immune serum samples (1:10 dilution) from rabbits immunized with r-bmZP proteins were run as positive and negative controls respectively with all test samples. Samples were viewed using a Leitz microscope fitted for epifluorescence and with appropriate filters for fluorescein. Fluorescence was recorded on Kodak T-Max 400 film.

One and two-dimensional SDS–PAGE and immunoblotting

SDS–PAGE was performed on 12% Ready Gels (Bio-Rad Laboratories, Hercules, CA, USA) in the Laemmli (1970) buffer system. Lyophilized preparations of heat-solubilized human zona pellucida were dissolved at two zonae/µl in SDS–PAGE sample buffer (63 mM Tris–HCl, 10% glycerol, 2% SDS, 0.0025% Bromophenol Blue) in the presence or absence of reducing agent, 20 mM dithiothreitol (DTT), and heated at 98°C for 2 min prior to loading in the sample wells.

For two-dimensional isoelectric focusing (IEF)/SDS–PAGE, lyophilized preparations of heat-solubilized human zona pellucida were dissolved (10 zonae/µl) at ambient temperature in IEF sample buffer [8.3 M urea, 3% CHAPS, 1% nonidet P-40, 2% ampholine (LKB: pH 3.5–10), 0.5% SDS and 10 mM DTT] and then applied to 4% polyacrylamide IEF gels (1.0×60 mm) containing 9.0 M urea, 1.5% CHAPS, 0.5% nonidet P-40 and 2% ampholines (LKB: 0.4%, pH 4–6, 1.6% pH 3.5–10). After IEF (3142 V/h), extruded gels were kept for 10 min in equilibration buffer (62.5 mM Tris–HCl, 3% SDS, 20 mM DTT, 0.003% Bromophenol Blue) and then applied directly to the top of an 11% polyacrylamide slab gel for second dimension SDS–PAGE.

After electrophoretic transfer of human zona pellucida glycoproteins to nitrocellulose membranes, the Western blots were either stained with colloidal gold total protein stain (Bio-Rad) or blocked with Blocker™ BLOTTO (Pierce, Rockford, IL, USA) and then immunostained with antisera to recombinant Bonnet Monkey zona pellucida proteins. Membranes were incubated with 1:500 dilution of rabbit primary antiserum followed by 1:5000 horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (Ig)G secondary antibody (Amersham International plc, Little Chalfont, Bucks, UK). Immunoreactive human zona pellucida glycoproteins were visualized with an enhanced chemiluminescence substrate (Amersham) and Hyperfilm-ECL. For detection of biotinylated marker proteins, peroxidase-conjugated streptavidin (1:10 000) was added during incubation with secondary antibody.

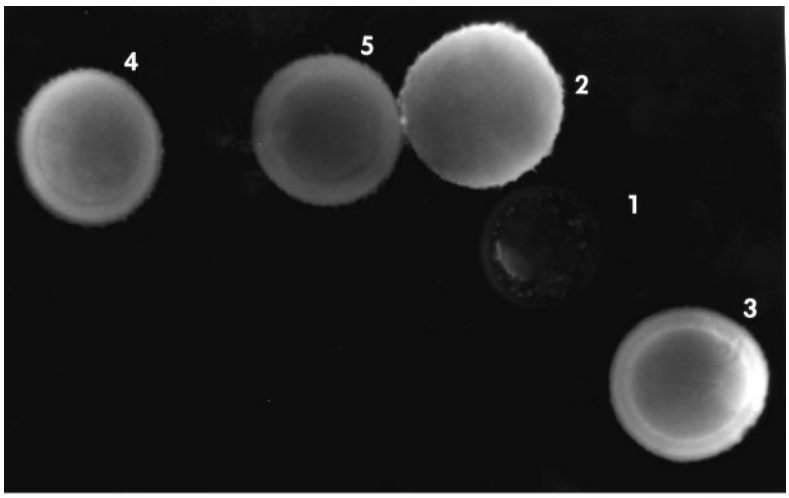

Figure 1. Micrographs showing binding of antibodies against Bonnet Monkey recombinant zona pellucida proteins to human oocytes by indirect immunofluorescence: oocytes were incubated with a 1:10 dilution of the preimmune (1) and immune serum samples of rabbits immunized with r-bmZP1 (3), r-bmZP2 (4), r-bmZP3-DT (5), and porcine ZP3 (2) followed by incubation with fluorescein isothiocyanate-labelled anti-rabbit immunoglobulins. Original magnification ×200.

Results

Reactivity of antibodies against r-bmZP1, r-bmZP2 and r-bmZP3 with native human zona pellucida

The in-vitro reactivity of the antibodies raised in rabbits against r-bmZP1, r-bmZP2 and r-bmZP3 with native human zona pellucida was analysed by indirect immunofluorescence. Antibodies against all three r-proteins showed positive fluorescence with human zona pellucida (Figure 1). No fluorescence was observed using pre-immune serum from immunized animals. Reactivity with anti-r-bmZP3 antibodies was less intense in comparison with antibodies against r-bmZP1 and r-bmZP2 though the pattern of the fluorescence of the three antibodies was comparable.

Immunoblot analysis of human SIZP resolved by 1D SDS–PAGE

Human solubilized isolated zona pellucida (SIZP; ~30 zonae per lane) was resolved by one-dimensional SDS–PAGE under non-reduced and reduced conditions, processed and probed with antibodies against r-bmZP1, r-bmZP2 and r-bmZP3. Results are shown in Figures 2 and 3 and summarized in Table I. Under non-reduced conditions, from the several bands as revealed by Aurodye staining, anti-r-bmZP1 antibodies showed specific reactivity with a 60 kDa band whereas r-bmZP2 antibodies reacted with a 100 kDa band (Figure 2). Antibodies against r-bmZP3 showed reactivity with a broad band of mean mobility of ~53 kDa. In addition to the 53 kDa band, r-bmZP3 antibodies also showed reaction with a high molecular weight band (~200 kDa).

Under reduced conditions anti-r-bmZP1 antibody reacted with a dominant band of 63 kDa in addition to a minor band of 53 kDa (Figure 3). Anti-r-bmZP2 antibodies reacted predominantly with a 65 kDa band. In addition, this immune sera demonstrated a faint reactivity with a 96 kDa band. Anti-r-bmZP3 reacted with a dominant band of 58 kDa and a minor band of 138 kDa. No immunoreactivity was observed in blots probed with preimmune sera (data not shown).

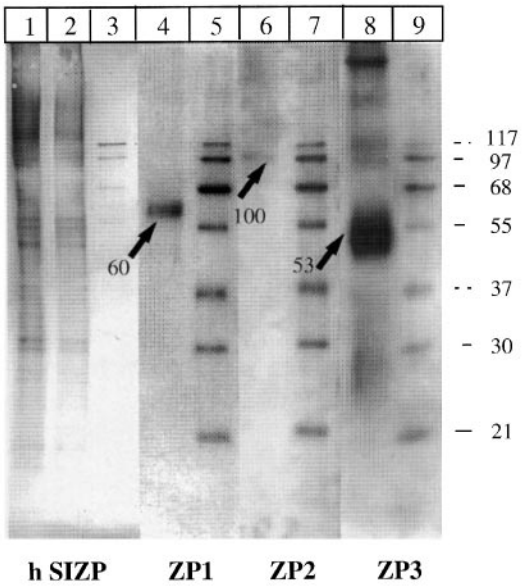

Figure 2. Reactivity in Western blot of antibodies against Bonnet Monkey recombinant zona pellucida proteins with human solubilized isolated zona pellucida (SIZP) resolved by one-dimensional sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) under non-reduced conditions. Human SIZP was resolved by SDS–PAGE under non-reduced conditions, transferred to nitrocellulose membrane and probed with either Aurodye or immune serum samples (1:500 dilution). Lane 1 (30 zonae) and lane 2 (10 zonae) were stained with Aurodye; lanes 4, 6 and 8 (30 zonae) represent reactivity with antibodies against r-bmZP1, r-bmZP2 and r-bmZP3; lane 3 shows molecular weight markers stained with Aurodye and lanes 5, 7 and 9 show biotin-labelled molecular weight markers stained with streptavidin–horseradish peroxidase conjugate.

Immunoblot analysis of human SIZP resolved by two-dimensional SDS–PAGE

To further characterize and classify human zona pellucida glycoproteins, the reactivity of antibodies developed to r-bmZP1, r-bmZP2 and r-bmZP3 was also tested in Western

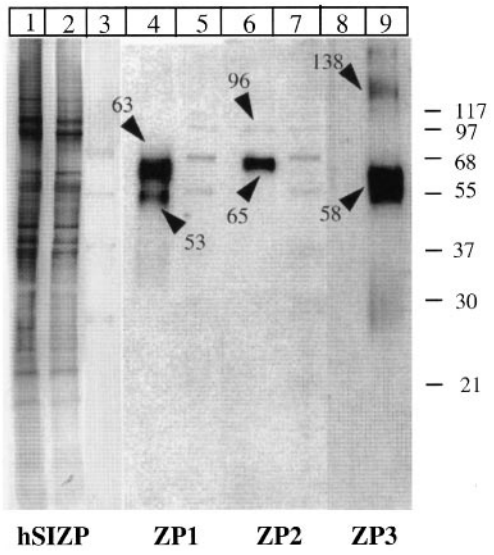

Figure 3. Reactivity in Western blot of antibodies against Bonnet Monkey recombinant zona pellucida proteins with human solubilized isolated zona pellucida (SIZP) resolved by one-dimensional sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) under reduced conditions, and probed with either Aurodye or immune serum samples (1:500 dilution). Lane 1 (30 zonae) and lane 2 (10 zonae) were stained with Aurodye; lanes 4, 6 and 9 (30 zonae) represent reactivity with antibodies against r-bmZP1, r-bmZP2 and r-bmZP3 respectively; lane 3 shows molecular weight markers stained with Aurodye; lanes 5 and 7 show biotin-labelled molecular weight markers stained with streptavidin–horseradish peroxidase conjugate. Lane 8 is an empty lane.

blots against hSIZP resolved by two-dimensional SDS–PAGE (Figure 4, Table I). Aurodye staining of two-dimensional SDS–PAGE resolved hSIZP revealed an acidic band around 66–55 kDa and two less acidic bands at 65 and 96 kDa. In addition, several oocyte/embryo associated proteins were also present. Antibodies to r-bmZP1 recognized two major chains in the acidic region, which were more intense at 63–58 kDa and less intense at 55–45 kDa (Figure 3). Both chains are comprised of multiple isomers. For both chains, the molecular weight increased slightly towards the acidic end and heavier reacting products were also located more towards the acidic end of the isomer charge train. In addition, a minor component was also observed at 80 kDa. Antibodies against r-bmZP2 recognized a major component at 65 kDa and minor component around 96 kDa. Both the components are less acidic as compared to hZP1 and hZP3. The 65 kDa component not only appears to be dominant but also showed a higher degree of charged isomers. Anti-r-bmZP3 antibodies recognized a broad band in the range of 68–58 kDa. hZP3 appears to be less acidic when compared with hZP1. Heavier reacting products were more towards the basic end of the isomer charge train.

Discussion

In the present study, we have employed antibodies developed against three well characterized r-zona proteins from non-human primates, i.e. Bonnet Monkey (r-bmZP1, r-bmZP2 and r-bmZP3) to characterize and classify human zona pellucida

Table I. Molecular weights of bands observed following Western blot analysis of human solubilized isolated zona pellucida (SIZP) using antibodies to r-bmZP1, r-bmZP2 and r-bmZP3 as probes

Zona pellucida glycoproteins	Size of bands recognized in Western blot (kDa)		
	1D SDS–PAGE		2D SDS–PAGE
	Reduced	Non-reduced	Reduced
ZP1	63 53	60	80 63–58 55–45
ZP2	96 65	100	96 65
ZP3	138 58	high MW band (~200 kDas) ~53	68–58

1D = one dimensional; 2D = two-dimensional; SDS–PAGE = sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

glycoproteins. The observed reactivity of these antibodies with native human zona pellucida is not surprising since antibodies against pig and rabbit zona pellucida have been shown previously to react with human zona pellucida (Sacco *et al*., 1981; Drell and Dunbar, 1984; Maresh and Dunbar, 1987). Furthermore, comparison of the deduced amino acid sequence of ZP1, ZP2 and ZP3 among mouse, rabbit, pig, Bonnet Monkey and human revealed highest sequence identity between Bonnet Monkey and human thus reiterating the high degree of similarity of Bonnet Monkey zona pellucida proteins to human (Kolluri *et al*., 1995; Gupta *et al*., 1997b; Jethanandani *et al*., 1998).

Zona pellucida glycoproteins have been initially classified based upon their electrophoretic mobility. From such studies, mouse zona pellucida consists of ZP1 (180–200 kDa), ZP2 (120–140 kDa) and ZP3 (83 kDa) (Bleil and Wassarman, 1980). The porcine zona pellucida (pZP) under non-reducing conditions, shows ZP1 (80–90 kDa) and ZP3 (55 kDa) and, under reducing conditions, resolves into ZP1 (82 kDa), ZP2 (65 kDa), ZP3 (55 kDa) and ZP4 (25 kDa) (Yurewicz *et al*., 1987). Porcine ZP1 (pZP1) revealed immunological cross-reactivity with ZP2 and ZP4, which do not share reactive epitopes between themselves, thereby suggesting that they are derived from reduction of disulphide bonds in ZP1.

The pattern of zona pellucida glycoproteins isolated from either a single oocyte or a pool of oocytes from species such as pig (Hedrick and Wardrip, 1987) and mouse (Bleil and Wassarman, 1980) revealed similar patterns, thereby suggesting that their composition is homogenous. Hence in this study, zonae pellucidae isolated from a pool of unfertilized oocytes and non-viable embryos are used to characterize human zona pellucida glycoproteins.

Using antibodies developed against Bonnet Monkey recombinant zona pellucida proteins as probes, we show in this study that under non-reduced conditions, hZP1 has a molecular weight of 60 kDa, hZP2 of 100 kDa and hZP3 of 53 kDa. Under non-reducing conditions where human zona pellucida glycoproteins were resolved by one-dimensional SDS–PAGE and revealed by silver staining, Bercegeay *et al*. (1995) reported that hZP1 corresponds to 96 kDa, hZP2 to 76 kDa and hZP3

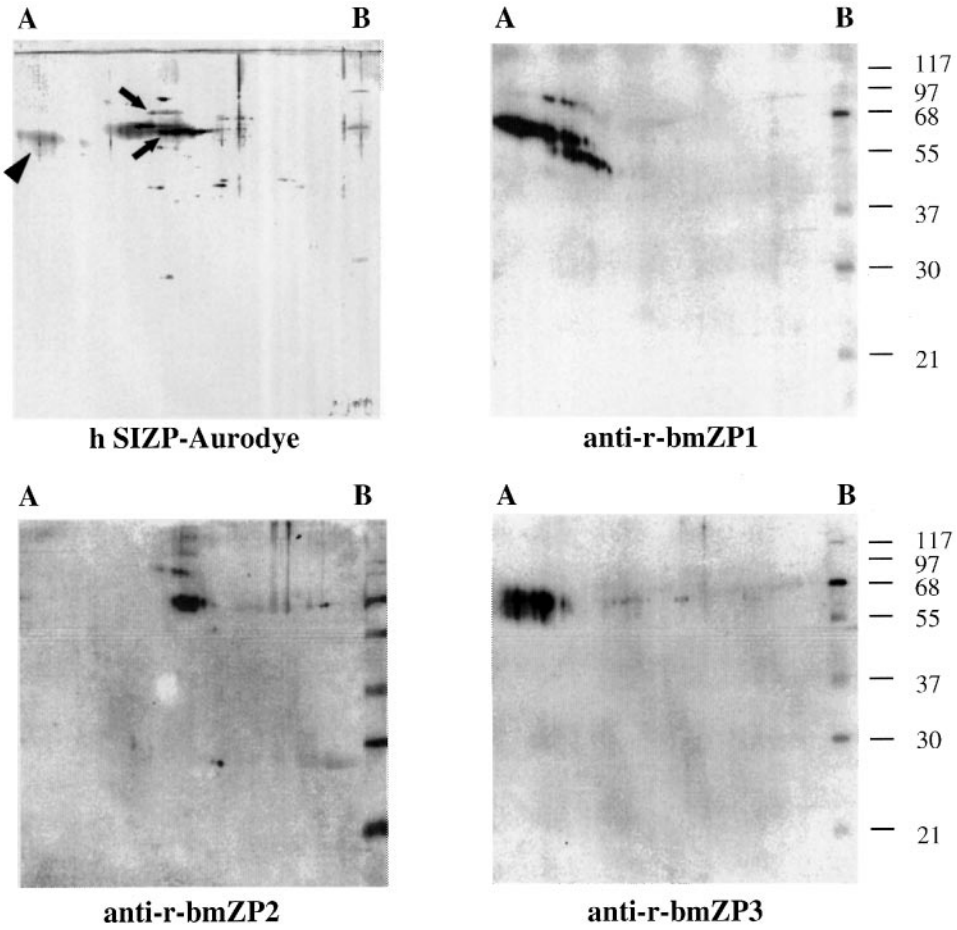

Figure 4. Reactivity in immunoblots of human solubilized isolated zona pellucida (SIZP) resolved by two-dimensional sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) with antibodies developed to Bonnet Monkey recombinant zona pellucida proteins. Human SIZP from 30 oocytes was run in an isoelectric focusing gel using a pH gradient of 3.5–10. The gel for the second dimension was run under reduced conditions using 0.1% SDS-11% PAGE. Probes were 1:500 dilutions of anti-bmZP1, anti-bmZP2 and anti-bmZP3 immune serum samples. A and B represent acidic and basic ends respectively. Molecular weight markers (kDa) are shown on the right hand side of each panel.

to 54 kDa. Using one-dimensional SDS–PAGE, non-reducing conditions and lectin blots, Moos *et al*. (1995) suggested that human zona pellucida was comprised of hZP2 (100 kDa) and two species of hZP3 [hZP3a (62–65 kDa) and hZP3b (55–60 kDa)]. Using this methodology hZP1 was not detectable. In Western blots, using one-dimensional SDS–PAGE, non-reduced conditions and antibodies against human zona pellucida, three bands of 97 kDa, 61 kDa (hZP3H) and 51 kDa (hZP3L) were observed (Shabanowitz, 1990). Under reduced conditions the 97 kDa band disappeared. It could not be determined whether the 97 kDa band represented ZP1, ZP2 or a ZP1/ZP2 complex. In the present study, using reduced conditions, hZP1 was found to consist of 63 and 53 kDa bands, hZP2 65 and 96 kDa bands and hZP3 is a broad band with mean molecular weight of 58 kDa. In addition, antibodies against r-bmZP3 reacted with a high molecular weight diffuse band of 138 kDa which may represent aggregated zona protein (Table I).

In this study, under reduced conditions, hZP1 and hZP2 are each resolved as two distinct bands. Under non-reduced conditions (in comparison with reduced conditions), hZP2 showed weak reactivity with antibodies against r-bmZP2. This was anticipated as the antibodies were generated against the denatured r-bmZP2 protein. On the other hand, it has been shown previously that antibodies generated against human zonae (native protein) reacted poorly with the zona pellucida glycoproteins resolved in SDS–PAGE under reduced conditions in comparison with non-reduced conditions (Shabanowitz, 1990).

In immunoblots of hSIZP resolved by two-dimensional SDS–PAGE, in this study, we demonstrate that hZP1 comprises two major charge trains, heavier at 63–58 kDa and lighter at 55–45 kDa (Figure 4). Our studies conclusively showed for the first time that the hZP1 heavier train is overlapping with hZP3 (68–58 kDa). Earlier investigators have described hZP1 as 92–80 kDa (Bercegeay *et al*., 1995) or 90–110 kDa (Shabanowitz and O'Rand, 1988). Our study indicates that this may not be the correct classification. The protein classified in these previous studies as hZP1 was likely hZP2. It is anticipated that hZP1 is not as heavily glycosylated as in mouse and hence hZP1 has a molecular weight in the range of 63–58 kDa. It seems probable that the classification followed

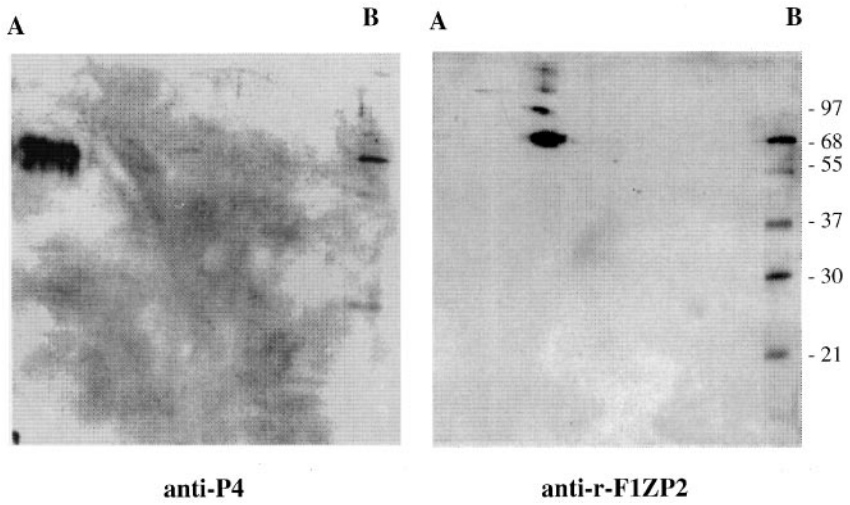

Figure 5. Reactivity in two-dimensional sodium dodecyl sulphate–polyacrylamide gel electrophoroesis (SDS–PAGE) Western blot of antibodies against bmZP3 peptide (left panel) and recombinant *N*-terminal fragment of bmZP2 (right panel) with human solubilized isolated zona pellucida (SIZP). Anti-P4 are antibodies against synthetic peptide (324–347 amino acids) corresponding to bmZP3, raised as described previously (Afzalpurkar *et al*., 1997). The *N*-terminal fragment (r-bm-F1ZP2) corresponding to 39–314 amino acid residues was expressed as polyhistidine fusion protein in *Escherichia coli* using a similar strategy to that described for the full length bmZP2. A and B represent acidic and basic ends respectively. Molecular weight markers (kDa) are shown on the right hand side.

by Shabanowitz and O'Rand (1988) and subsequently by Bercegeay *et al*., (1995) was based upon the electrophoretic mobility of mouse zona pellucida glycoproteins in SDS–PAGE and may not be correct. Further, Moos *et al*., (1995) indicated that hZP3 is more acidic in comparison with hZP1, which is contradictory to our observations, which indicate that hZP1 is slightly more acidic than hZP3.

Using methodologies such as antibodies to hZP, lectin-blots or silver staining, it has been proposed that hZP3 comprises two species (Shabanowitz, 1990; Bercegeay *et al*., 1995; Moos *et al*., 1995). In the present study, however, two distinct hZP3 trains are not discernable (Figure 4) with only a broad, single band being visible. It was not clear whether hZP3H and hZP3L were representing two distinct gene products or a result of differential post-translation modification of a single gene product (Shabanowitz, 1990). Our results clearly showed that hZP1 and hZP3 blots are partially superimposable and that hZP1 and hZP3 represent two distinct gene products.

It has been observed that the zona pellucida glycoproteins have shared amino acid sequences (Hedrick, 1996). The comparison of deduced amino acid sequence of bmZP1 revealed a sequence identity of 92.0, 25.2 and 12.3%, bmZP2, 23.5, 94.2 and 12.3% and bmZP3, 11.1, 9.9 and 93.9% with hZP1, hZP2 and hZP3 respectively. Even though deduced amino acid sequence of bmZP1, bmZP2 and bmZP3 showed high degree of amino acid sequence identity with respective human zona pellucida glycoprotein homologues, nonetheless, the lower degree of amino acid sequence identities with other human zona glycoproteins cannot be ignored. Further careful analysis revealed that none of the sequences compared showed any continuous stretch of more than five identical amino acid residues, except ZP1 and ZP2. The shared amino acid stretches reside in the *C*-terminal part of the bmZP2 (after 570 amino acids).

To rule out the possibility of artefacts due to shared amino acid residues in the Western blots, two-dimensional SDS–PAGE Western blots of hSIZP were carried out using antibodies raised against the r-bmF1ZP2 (39–314 amino acids). The results are shown in Figure 5. The observed pattern of reactivity was similar to the one observed by antibodies raised against the full length r-bmZP2. Antibodies were raised against synthetic peptide (P4) corresponding to bmZP3 (324–347 amino acids) as described earlier (Afzalpurkar *et al*., 1997). Using anti-P4 antibodies, two-dimensional SDS–PAGE Western blot of hSIZP showed similar pattern as obtained by employing antiserum against r-bmZP3 (Figures 4 and 5). These results further support the fact that the characterization of human zona pellucida glycoproteins in Western blot using antisera against r-bmZP1, r-bmZP2 and r-bmZP3 may not be influenced due to the shared amino acid sequences amongst zona pellucida glycoproteins.

In summary, antibodies raised against recombinant non-human primate zona pellucida proteins expressed in *E.coli* react with human zonae. Using these as specific immunological probes, Western blot analysis revealed that one of the dominant components of hZP1 overlaps with hZP3. Previous nomenclature based on the mobility of hZP1, in comparison with mouse zona pellucida, is likely to be incorrect as our studies demonstrate that the highest molecular weight component of hZP is hZP2 and not hZP1.

Acknowledgements

Support for this project (CSA-95–181) was provided by the Contraceptive Research and Development (CONRAD) Program, Eastern Virginia Medical School, Norfolk, Virginia, USA and the Department of Biotechnology, Government of India under a Cooperative Agreement. The views expressed by the authors do not necessarily reflect the views of the funding agencies.

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Received on April 28, 1998; accepted on August 5, 1998