Sperm arylsulfatase A binds to mZP2 and mZP3 glycoproteins in a nonenzymatic manner

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Abstract

We have shown previously that sperm surface arylsulfatase A (ASA) of mouse, pig, and human is involved in sperm–egg zona pellucida (ZP) binding. By treating capacitated mouse sperm with A23187 to induce the acrosome reaction, we demonstrated by immunoblotting that ASA also existed in the acrosomal content and on the inner acrosomal membrane. Since mZP2 and mZP3 are known as sperm receptors, whereas mZP1 as a cross-linker of mZP2/mZP3, we determined whether purified ASA bound to mZP2 and mZP3 selectively. The three mZP glycoproteins were purified from solubilized ovarian ZP by size exclusion column chromatography. Immuno-dot blot analyses revealed that purified sperm ASA bound to mZP2 at the highest level followed by mZP3, whereas the binding of ASA to mZP1 was minimal. The results confirmed the physiological significance of sperm ASA in the ZP binding process. The binding of ASA to mZP2 and mZP3 was, however, not dependent on the active site pocket amino acids, Cys69, Lys123, and Lys302, which are pertinent to the capturing of an arylsulfate substrate, since ASA mutant with Ala substitution at these three residues still bound to mZP2 and mZP3. The availability of the active site pocket of ASA bound to the ZP suggested that ASA would still retain enzymatic activity, which might be important for subsequent sperm penetration through the ZP.

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Introduction

Gamete interaction that culminates in fertilization starts with specific interaction between sperm and the zona pellucida (ZP). Consistently, the ZP of all mammals consists of three to four sulfoglycoproteins; these are mZP1 (~200 kDa – dimeric form), mZP2 (120 kDa), and mZP3 (83 kDa) in mice. mZP2 and mZP3 form fibrils that are cross-linked by mZP1 (Wassarman & Litscher 2008, Litscher *et al.* 2009). Early work of Wassarman and colleagues indicate that mZP3 is the primary receptor of acrosome intact (AI) sperm, whereas mZP2 is the secondary receptor for acrosome reacted (AR) sperm, and that the glycans of both mZP3 and mZP2 are essential for sperm binding (Wassarman & Litscher 2008, Litscher *et al.* 2009). mZP3 also induces the acrosome reaction of capacitated sperm (Florman & Ducibella 2006, Wassarman & Litscher 2008). More recently, Dean and colleagues present evidence that the supramolecular structure of the ZP, regulated by intactness of mZP2, is required for AI sperm binding (Rankin *et al.* 2003,

Gahlay *et al.* 2010). Once mZP2 is cleaved at Ala166 to form mZP2_f, as occurring after fertilization (Burkart *et al.* 2012), the ZP matrix becomes refractory to sperm binding (Florman & Ducibella 2006). Accumulated evidence also reveals the participation of acrosomal matrix proteins in ZP binding, a basis for how acrosome reacting sperm remain bound to the ZP (Kim & Gerton 2003, Buffone *et al.* 2008a, 2008b). Proacrosin is an example of these acrosomal proteins, and it has affinity for both mZP2 and mZP3 (Howes *et al.* 2001). While all of these results confirm the significance of mZP3 and mZP2 in sperm binding, they argue against the exclusiveness of mZP3 and mZP2 as primary and secondary sperm receptors respectively.

In contrast to the situation on the ZP, a number of proteins on the sperm head have been described for ZP binding ability (Lyng & Shur 2007, Tanphaichitr *et al.* 2007), and these observations are explained by the possibility that these proteins are backups for one another and/or act sequentially in gamete interaction.

We have previously shown that sperm surface arylsulfatase A (ASA, EC 3.1.6.8) is involved in ZP binding in mice, pigs, and humans, and purified ASA can bind to intact or solubilized ZP directly (Rattanachaiyanont *et al.* 2001, Carmona *et al.* 2002a, Tantibhedhyangkul *et al.* 2002). Nonetheless, the identity of the ZP glycoproteins to which ASA binds was not known, and this was one specific aim of the current research study. We have postulated that ASA, as a sulfatase, interacts with sulfated sugar residues (Carmona *et al.* 2002a), which are components of the ZP glycans (Shimizu *et al.* 1983, Hirano *et al.* 1993, Noguchi & Nakano 1993, Hokke *et al.* 1994, Takasaki *et al.* 1999, Dell *et al.* 2003, Topfer-Petersen *et al.* 2008, Pang *et al.* 2011). Our computational analyses indicate that the active site pocket of ASA is the positively charged area, which is essential for capturing the sulfated substrate (Lukatela *et al.* 1998, Schenk *et al.* 2009). Cys69, which is posttranslationally modified to become formylglycine, is the active site amino acid, but Lys123 and Lys302 present in the active site pocket are essential in capturing an arylsulfate substrate into the pocket (Lukatela *et al.* 1998, Schenk *et al.* 2009). Sulfogalactosylglycerolipid (SGG, aka seminolipid, present selectively and substantially in male germ cells) and sulfogalactosylceramide (SGC, present mainly in the brain and kidney) are known natural substrates of ASA, provided that the sulfoglycolipids are presolubilized into a micelle form by saposin B (Tanphaichitr *et al.* 2003). However, the ability of purified ASA to desulfate galactose sulfate has been documented (Waheed & Van Etten 1980). Therefore, the second aim of our study was to determine whether Cys69, Lys123, and Lys302 in the active site pocket of ASA were important for ASA–ZP interaction. In addition, we wanted to verify whether ASA existed in the acrosome of mouse sperm, as documented in other mammalian sperm (Dudkiewicz 1984, Nikolajczyk & O'Rand 1992, Brandon *et al.* 1997). Information of the

ASA localization on the sperm head should shed more light to its roles during sperm binding and penetration through the ZP.

Results

Binding of sperm to the egg ZP involves AI sperm in the initial step and acrosome reacting and AR sperm in the subsequent stages. We have previously shown the presence of ASA (68 kDa) on the convex ridge surface of the mouse AI sperm head (Tantibhedhyangkul *et al.* 2002). However, in other mammals ASA has also been shown as an acrosomal enzyme (Dudkiewicz 1984, Nikolajczyk & O'Rand 1992, Brandon *et al.* 1997). Therefore, we asked whether mouse ASA was present as part of the acrosomal content (AC) and on the surface of AR sperm. A23187 was used to treat capacitated sperm to produce AR sperm along with the AC and acrosomal vesicles. Immunoblotting revealed the same anti-ASA reactive 68 kDa band in AI and AR sperm, the AC, and acrosomal vesicles (Fig. 1A). The 68 kDa molecular weight of ASA observed herein in all of the four samples is similar to that previously described for AI sperm (Tantibhedhyangkul *et al.* 2002, Wu *et al.* 2007). Among the AR sperm, AC, and acrosomal vesicles samples, ASA existed at the highest level in the AC. The amount of ASA in the acrosomal vesicles (hybrid vesicles of the sperm head plasma membrane and outer acrosomal membrane) was the least; the 68 kDa ASA band was apparent when the acrosomal vesicles sample was prepared from 10 million sperm. This was in contrast to the AC and AR sperm, where the 68 kDa ASA band was already prominent from the samples prepared from 1 million sperm. Immunofluorescence was further employed to localize ASA on AR and AI sperm. OBF13, a mAb generated against the sperm membrane protein, Izumo (Kawai *et al.* 1989, Inoue *et al.* 2005), was used as a marker of AR sperm in this ASA localization. Izumo, a transmembrane protein, is localized over the entire head

Figure 1 (A) Immunoblotting for ASA of proteins isolated from acrosome intact (AI) sperm, A23187-treated sperm with ~80% acrosome reacted (AR), acrosomal content (AC) and acrosomal vesicles (V). Proteins from 1 million AI sperm, 1 and 2 million AR sperm, from AC prepared from 1 and 2 million sperm, and from acrosomal vesicles prepared from 10 million sperm were used for gel loading. (B) Indirect immunofluorescence of ASA in AI and AR sperm. AR sperm were those that were positively labeled with OBF13 (green), a mAb against Izumo which is localized to the entire sperm head only after acrosome reaction. Note that ASA staining (red) was observed in the convex ridge of both AI (arrowhead) and AR (arrow). Blue staining from Hoechst denotes the sperm nucleus. The results shown are representative of two replicate experiments. Bar = 10 μ m.

of AR sperm and is exposed to react with OBF13 only after acrosomal exocytosis. The validity of identifying OBF13 stained sperm as AR sperm was first shown by comparing the number of AR sperm assessed by OBF13 reactivity with that obtained by Coomassie blue staining (a more widely used method; Bleil & Wassarman 1990). Supplementary Figure S1 (see section on supplementary data given at the end of this article) indicates that the numbers acquired from the two methods were the same. Figure 1B shows the presence of ASA (red staining) on the convex ridge surface of an AI sperm (arrowhead), which had negative staining with OBF13. In AR sperm (arrows), which exhibited OBF13 staining (green), ASA was also present on the convex ridge surface; it was likely that ASA was localized on the inner acrosomal membrane of AR sperm. At the present time, the possibility that ASA is also present on the outer acrosomal membrane cannot be excluded.

Since ASA has direct binding ability to the mouse ZP (Tantibhedhyangkul *et al.* 2002), we asked whether ASA could bind selectively to mZP2 and mZP3 glycoproteins, both known as sperm receptors. These two mZP glycoproteins were prepared in quantity from isolated mouse ovarian ZP. Nonreducing SDS-PAGE/silver staining of solubilized mZP glycoproteins revealed the highest abundance of mZP2 followed by mZP3 and mZP1 (Fig. 2A, left lane) in agreement with the previous report (Green 1997). Size exclusion HPLC on a Bio-Sil SEC 250 column separated solubilized mouse ZP glycoproteins into three peaks with retention times of 57.3, 62.2, and 66.8 min (Fig. 2C). SDS-PAGE with silver staining revealed these three peaks in the elution order to be mZP1 (200 kDa – dimeric form), mZP2 (120 kDa), and mZP3 (83 kDa) (Fig. 2A). The identity of the isolated mZP2 and mZP3 was further revealed by their specific reactivity to the monoclonal anti-mZP2 and anti-mZP3 antibody respectively (Fig. 2B). We further showed that the isolated mZP3, but not mZP2, was able to induce the sperm acrosome reaction, as previously described (Wassarman & Litscher 2008; Fig. 2D). The yield of mZP1, mZP2, and mZP3 was about 35, 200, and 150 ng/ovary, respectively. The weight ratio of the purified mZP2 to mZP3 (1:0.75) was similar to that previously described (1:0.7) (Green 1997), although the ratio of the obtained mZP1 to that of mZP2 (0.18:1) was lower than the described value (0.3:1). Subsequently, mZP2 and mZP3 were also purified by electroelution from the polyacrylamide gel of total solubilized ZP glycoproteins that were subjected to SDS-PAGE. The yield of electroeluted mZP3 was comparable to that obtained by HPLC, although the efficiency to prepare mZP2 by electroelution was lower than the HPLC method (see Supplementary Table S1, see section on supplementary data given at the end of this article).

To demonstrate direct affinity of ASA to mZP glycoproteins, mZP1, mZP2, and mZP3 were individually immobilized onto the nitrocellulose membrane.

Total solubilized mZP (1 µg, ~9 pmol using 110 kDa as the average molecular weight of the whole mZP glycoproteins), previously shown for ASA affinity, served as a positive control, whereas ovalbumin (1 µg) without ASA binding ability (Tantibhedhyangkul *et al.* 2002) was

Figure 2 Isolation and characterization of purified ZP glycoproteins. (A) Nonreducing SDS-PAGE of ovarian mouse ZP. The isolated zonae pellucidae were solubilized in 0.2 M sodium phosphate buffer, pH 6.8, containing 0.1% SDS by heating at 65 °C for 10 min and then subjected to SDS-PAGE followed by silver staining (total ZP lane). Two micrograms of solubilized total ZP were used for gel loading. For ZP1, ZP2, and ZP3, the amount loaded was equivalent to that purified from the solubilized total ZP of 2 µg. The apparent molecular masses of mZP1, mZP2, and mZP3 were 200, 120, and 83 kDa respectively. (B and C) Fractionation of solubilized mouse ZP by HPLC on a Bio-Sil SEC 250 size-exclusion column. The chromatogram (C) shows the separated fractions of mZP1, mZP2, and mZP3, which had distinctive electrophoretic mobility by nonreducing SDS-PAGE (ZP1, ZP2, and ZP3 lanes in A panel). The identity of the mZP2 and mZP3 fractions was further confirmed by immunoblotting (B). mZP2 (lanes 1 and 2) and mZP3 fractions (lanes 3 and 4) (0.5 µg each) were probed with both anti-ZP2 (lanes 1 and 4) and anti-ZP3 (lanes 2 and 3). (D) Ability of solubilized mouse ovarian ZP and purified mZP3 to induce the acrosome reaction. Capacitated sperm were incubated with solubilized mouse ZP, and purified mZP1, mZP2, and mZP3 at a final concentration of 8 ZP/µl or an equivalent. Sperm treated with 10 µM A23187 served as a positive control. Sperm were also treated with ovalbumin (40 ng/µl) and fetuin (40 ng/µl). Only total solubilized mZP and mZP3 could induce the acrosome reaction in a similar capacity to A23187, and the levels of AR sperm produced were highly significantly different from control untreated sperm ($P < 0.005$, as analyzed by Student's *t*-test and denoted by **). mZP1 and mZP2 and ovalbumin did not induce acrosome reaction. Fetuin slightly increased the number of AR sperm (~8% with $P < 0.05$ %) (*) as compared with the untreated control sperm sample. Data were expressed as mean $\pm$ s.d. from three replicate experiments.

used as a negative control. Fetuin, a serum glycoprotein, which is anionic due to sialylation of its carbohydrate moieties (Takasaki & Kobata 1986), was also determined for its affinity for ASA. Three micrograms of purified ASA (44 pmol, $\sim 5 \times$ mole ratio to the mZP glycoprotein dot) were used for all mZP glycoprotein dot incubation. In the first set of experiments, the proportion of the three mZP glycoproteins used for this immobilization mimicked that present in the total solubilized ZP (i.e. 0.3 μg for mZP1, 1 μg for mZP2, and 0.7 μg for mZP3; Green 1997). At this weight ratio of the three mZP glycoproteins, ASA bound to mZP2 at the highest level, followed by mZP3, whereas the binding of ASA to mZP1 was at a background level as observed with ovalbumin (Fig. 3A). The order of the ASA affinity for the three mZP glycoproteins was still observed when the three mZP glycoproteins were compared at the same weights of 0.3 and 0.7, and 1 μg . However, mZP1 also bound to ASA but at the lowest level (Fig. 3C and D). The levels of ASA binding to 0.1 μg mZP2 and mZP1 glycoproteins appeared to be minimal and similar to each other, suggesting that these amounts of mZP2 and mZP1

glycoproteins may be under the limit of detection for their ASA binding (Fig. 3C and D). The binding of ASA to mZP2 and mZP3 appeared to reach a plateau level already at 0.7 μg of the two mZP glycoproteins, suggesting that the ASA–mZP2/mZP3 interaction was specific. Further, preincubation of ASA with 10 μg of solubilized mZP glycoproteins ($5 \times$ and $10 \times$ mole ratio to mZP2 and mZP3, immobilized onto the nitrocellulose membrane respectively) abolished its affinity to both mZP glycoproteins (Fig. 3B). In addition, we showed that ASA did not bind to another anionic glycoprotein, fetuin, suggesting that ASA–ZP glycoprotein binding is a specific phenomenon (Fig. 3A).

ASA is a sulfatase with known desulfation activity for two natural sulfoglycolipids, SGG and SGC (Hanson *et al.* 2004, Schenk *et al.* 2009, Xu *et al.* 2011), each expressing galactose sulfate as their head group. Galactose sulfate has also been shown to be desulfated by purified ASA (Waheed & Van Etten 1980). Since sulfated sugar residues are part of the ZP glycans (Shimizu *et al.* 1983, Hirano *et al.* 1993, Noguchi & Nakano 1993, Hokke *et al.* 1994, Takasaki *et al.* 1999,

Figure 3 Binding of purified sperm ASA to solubilized mouse ovarian ZP and individual ZP glycoproteins. (A, B, C and D) ASA had affinity for mZP2 and mZP3. (A) One microgram of fetuin, ovalbumin, and solubilized whole mZP glycoproteins, 0.3 μg of mZP1, 1 μg of mZP2, and 0.7 μg of mZP3 were dotted onto nitrocellulose membrane. The weight ratio of mZP1, mZP2, and mZP3 used for the dotting was as described previously (Green 1997). After incubation with purified ASA (3 μg), the membrane was washed extensively to remove unbound protein and probed for the ASA binding by anti-ASA antibody and HRP-conjugated secondary antibody. (B) One microgram each of mZP, mZP2, and mZP3 was immobilized onto the nitrocellulose membrane. ASA was preincubated with 10 μg of solubilized mZP prior to the incubation with the membrane. Note that solubilized mZP saturated the ZP binding site(s) on ASA, thus preventing it from interacting with mZP, mZP2, and mZP3 immobilized onto the nitrocellulose membrane. (C) Various amounts of mZP1, mZP2, and mZP3 were immobilized onto the nitrocellulose membrane, which was then incubated with ASA. The incubation conditions and the detection of ASA–ZP binding were as described in (A). (D) Densitometric analyses of the binding of ASA to mZP1, mZP2, and mZP3. Note that mZP2 bound to ASA to the highest degree followed by mZP3. The binding of ASA to mZP1 was markedly lower. (E) Binding of ASA to mouse zona glycoproteins was inhibited by dextran sulfate (lower panel) but not by dextran (upper panel). Total mZP glycoproteins, mZP1, mZP2, mZP3, ovalbumin, and fetuin were dotted onto the nitrocellulose membrane as in (A). The conditions of ASA incubation and detection of the binding were also the same as in (A), except that the ASA incubation was performed in the presence of 1 μM of dextran sulfate or dextran. All the results were representative of two or three replicate experiments.

Figure 4 Purity of native ASA and recombinant WT, C69A, and CKK mutant ASAs. Native ASA was purified from pig sperm. All recombinant ASAs were produced in CHO-K1 cells and were directed to be secreted into the medium. Recombinant ASAs were purified as described in Materials and Methods. Medium from 'sham' transfection (using uninserted pcDNA3.1) was also collected for parallel purification (pcDNA). Left panel: SDS-PAGE/silver staining of native ASA (purified ASA), WT recombinant (rec) ASA, C69A (rec ASA with C69 substituted with Ala), CKK (rec ASA with C69, K123, and K302, all substituted with Ala), and pcDNA; right panel: corresponding immunoblotting with anti-ASA antibody. The same amount of protein after the final purification step (50 ng) was used for gel loading for all samples except for pcDNA, which was loaded based on an equivalent volume of the sample at the final step of purification. Note the purity of all rec ASAs, which appeared as a single major band on the silver stained gel.

Dell *et al.* 2003, Topfer-Petersen *et al.* 2008, Pang *et al.* 2011), it is possible that the interaction between ASA and ZP glycoproteins may be via the binding of ASA to sulfated sugars on the ZP. This prompted us to test whether dextran sulfate, a long-chain polymer of sulfated glucose, could inhibit ASA–ZP binding. Figure 3E (lower panel) reveals that binding of ASA to total solubilized mZP glycoproteins was minimal in the presence of 1 μ M dextran sulfate. However, dextran of the same concentration did not inhibit ASA–ZP interaction (Fig. 3E, upper panel). Therefore, the results suggested that sulfation of the ZP glycans may be important for the ASA–ZP interaction.

We have shown previously that the active site pocket of the three-dimensional (3D) structure of ASA is the positively charged area to which SGG and SGC and an artificial substrate, *p*-nitrocatechol sulfate (NCS), would readily dock (Schenk *et al.* 2009). In this active site pocket, Cys69, which is posttranslationally modified to be formylglycine, is the active site amino acid responsible for the removal of the sulfate group from the substrate. Lys123 and Lys302, present also in the active site pocket, are the positively charged amino acids that

are mainly responsible in capturing sulfated sugar residue of the substrate in this active site pocket (Schenk *et al.* 2009). It can be speculated that the active site pocket of ASA is important in capturing the ZP glycan via its interaction with the sulfated sugar residue of the ZP. The mutation of Cys69 to Ala prevents ASA's desulfation activity and should thus prolong the binding of the sulfated sugar to the active site pocket. In contrast, mutant ASA, with Cys69, Lys123, and Lys302 substituted with Ala (CKK), was expected to have reduced binding to ZP glycoproteins, if the active site pocket of ASA is important for ZP glycan interaction. Figure 4 (left side) shows that partially purified rec wild type (WT) ASA, and the two mutant ASAs, C69A and CKK, appeared as a major band of about 70 kDa molecular mass upon SDS-PAGE/silver staining, and they were recognized by anti-ASA antibody (Fig. 4, right side). The 'sham' purification sample from CHO-K1 cells transfected with pcDNA did not possess the ASA band. It was noted that all rec ASAs had a slightly slower mobility than native ASA purified from pig sperm (Fig. 4, left side). It was possible that the glycosylation of rec ASA in CHO-K1 cells was at a higher level, as compared with that in pig reproductive cells. As expected, rec WT ASA contained desulfation activity on NCS and its specific activity increased 7.6-fold after its purification using the Mono-Q-Sepharose resin. In contrast, the two mutant ASAs, C69A and CKK, had minimal NCS desulfation activity, slightly above the background value of the pcDNA sample (Table 1). However, the ability of rec WT and the two mutant ASAs to bind to the solubilized ZP immobilized onto nitrocellulose membrane was at similar levels (Fig. 5), a result suggesting that the active site pocket of ASA was not essential for the ZP binding.

Discussion

We have shown previously that ASA on the plasma membrane of the mouse, human, and pig sperm head is

Table 1 Desulfation activity of recombinant wild type and mutant ASAs. CHO-K1 cells transfected with full-length ASA cDNA, C69A construct, C69A/K123A/K302A (CKK) construct, and empty vector pcDNA3.1 were treated with 10 mM ammonium chloride in serum-free culture medium to induce secretion of newly synthesized ASA. The spent culture media were collected and an aliquot was used for protein quantification and NCS desulfation activity assay (medium). ASA in the remaining media was further purified using Q-sepharose resin and the total protein obtained and NCS desulfation activity was measured (after QS).

	WT	C69A	CKK	pcDNA
Medium				
Protein (mg)	3.67	5.51	6.69	6.76
Total activity (U)	17.90	2.90	2.42	1.94
Specific activity (U/mg)	4.88	0.53	0.36	0.29
After QS				
Protein (mg)	0.57	1.98	1.12	2.79
Total activity (U)	21.1	2.65	0.65	0.68
Specific activity (U/mg)	37.02	1.34	0.58	0.24

Figure 5 Binding of recombinant mutant ASAs to solubilized mouse ovarian ZP. One microgram of solubilized mZP glycoproteins or ovalbumin (labeled 'ovalbumin' in the bottom right corner panel), dotted onto the nitrocellulose membrane, was incubated with 1 µg of native purified ASA, rec WT ASA and mutant ASAs C69A, and CKK. An equivalent volume of pcDNA sample from the final purification step was also used for incubation. Note that all types of ASAs bound at a similar level to solubilized mZP, but no binding was observed with ovalbumin or when pcDNA was used in place of ASA for the incubation. The results were representative of two replicate experiments.

involved in sperm–ZP interaction and purified ASA binds to both intact and solubilized ZP (Rattanachaiyanont *et al.* 2001, Carmona *et al.* 2002a, Tantibhedhyangkul *et al.* 2002, Wu *et al.* 2007). In this report, we provided evidence that ASA was also present in the mouse AC and on the inner acrosomal membrane (Fig. 1) and it had a preferential binding ability to mZP2 and mZP3. Like ASA on the sperm plasma membrane (Weerachatanukul *et al.* 2003), ASA may be anchored onto the inner acrosomal membrane via its affinity for SGG (Carmona *et al.* 2002b), which also exists on this surface membrane (Supplementary Figure S2, see section on supplementary data given at the end of this article). Our finding on the presence of ASA as an AC component is in agreement with the previous reports in other mammalian sperm (Dudkiewicz 1984, Nikolajczyk & O'Rand 1992, Brandon *et al.* 1997). It is still unclear whether ASA in the AC binds to any ligands. Notably, ASA in the AC was of the highest quantity as compared with ASA on AR sperm and acrosomal vesicles (hybrid membranes between the sperm head plasma membrane and outer acrosomal membrane; Fig. 1A). ASA itself oligomerizes forming a dimer at neutral pH and an octamer at pH 5 (Lukatela *et al.* 1998). Since the intra-acrosomal pH is between 5.3 and 6.2 (Nakanishi *et al.* 2001), ASA in the AC should exist in the oligomeric form of a size in between a dimer and an octamer. The oligomeric forms of ASA, like those of sp56, another acrosomal protein with ZP binding ability, would likely exist as scaffolds beneficial for the interaction between acrosome reacting sperm and the ZP (Buffone *et al.* 2008a, 2008b).

Previous studies of Wassarman and colleagues have indicated that mZP3 is the primary sperm receptor, binding to AI sperm, whereas mZP2 acts as the secondary receptor, interacting with AR sperm (Bleil

et al. 1988, Wassarman & Litscher 2008). Our results indicating the binding ability of ASA, which exists on the sperm head plasma membrane, in the AC and on the inner acrosomal membrane to both mZP2 and mZP3 challenge the sharp demarcation of mZP3 and mZP2 as a receptor of AI and AR sperm respectively. In fact, more recent work indicates that both purified mZP3 and mZP2 can bind to both AI and AR sperm (Kerr *et al.* 2002). Furthermore, the supramolecular structure model of Dean *et al.* argues that sperm would interact with both mZP3 and mZP2, provided that mZP2 is intact; only after a cleavage of mZP2 by a cortical granule protease to generate mZP2_f (Burkart *et al.* 2012), sperm can no longer bind to the ZP (Gahlay *et al.* 2010). Supporting the argument that the binding of mZP2 and mZP3 to sperm is a continuous event are the findings that a number of acrosomal proteins bind to the ZP (e.g. proacrosin, sp56, zonadhesin, sp38), and proacrosin, like ASA, has the affinity for both mZP2 and mZP3. Electron microscopy immunogold labeling also indicates that mZP2 and mZP3 are localized next to each other on the ZP (El Mestrah *et al.* 2002), and both of these ZP glycoproteins have the propensity to form fibrils (Greve & Wassarman 1985, Litscher *et al.* 2008, Jimenez-Movilla & Dean 2011). The ability of sperm proteins, including ASA (this report) and proacrosin (Howes *et al.* 2001), to bind to both mZP2 and mZP3 should give enhanced interaction between sperm and the ZP. The binding ability of mZP2/mZP3 fibrils, as compared with solubilized mZP2 and mZP3, to sperm should be the matter of the future investigation.

Mammalian ZP glycans contain sulfated sugar residues, and their significance in sperm binding has been implicated (Shimizu *et al.* 1983, Oehninger *et al.* 1991, Mori *et al.* 1993, Yonezawa *et al.* 1995, Howes *et al.* 2001, Howes & Jones 2002, Moreno *et al.* 2002, Dell *et al.* 2003, Gaboriau *et al.* 2007, Lin *et al.* 2007, Topfer-Petersen *et al.* 2008, Yu *et al.* 2009, Pang *et al.* 2011). A number of sulfatases have desulfation activity on sulfated sugar residues (Hanson *et al.* 2004). Although ASA is better known for its desulfation activity on sulfoglycolipids (SGG and SGC; Schenk *et al.* 2009), the ability of ASA to desulfate galactose sulfate residues has also been documented (Waheed & Van Etten 1980). The binding of sulfated sugar residues on the ZP glycans to the active site pocket of ASA may thus be a basis of ASA–ZP interaction. Results revealing that dextran sulfate, but not dextran, could inhibit ASA–ZP2/ZP3 interaction (Fig. 3E) indicated the involvement of sulfated glycans in this interaction. However, ASA–ZP2/ZP3 interaction was still retained when the key amino acids that capture the sulfated substrate in the active pocket (i.e. Cys69, Lys123, and Lys302) were all mutated to Ala (the so-called CKK mutant ASA; Fig. 5), indicating that the ZP glycans did not bind to ASA's active site pocket. Our previous computational modeling for electrostatic potentials of ASA reveals that the active site pocket is

the highest for positive charges, although it is not well exposed. Positively charged areas are also found on certain areas on the surface of ASA's 3D structure (Schenk *et al.* 2009). Computational docking shows that SGG/SGC can also fit into a surface cleft consisting of Arg496, a positively charged amino acid, and Ile487, a hydrophobic amino acid (Supplementary Figure S3, see section on supplementary data given at the end of this article). This ASA–SGG interaction is likely a basis on how ASA from the epididymal fluid deposits onto the head convex ridge of transiting sperm without subsequent SGG hydrolysis (Carmona *et al.* 2002b). Likewise, ASA synthesized in spermatogenic cells and targeted to the acrosomal process may remain on the inner acrosomal membrane (Fig. 1B) via affinity for SGG at this R496/I487 site. ASA at neutral pH exists as a dimer and at acidic pH as a higher-ordered oligomer (Lukatela *et al.* 1998). Only one ASA molecule in the dimer or oligomer may bind to SGG on the sperm membrane, allowing the R496/I487 site on the other ASA molecule(s) for interaction with sulfated sugar residues of the ZP glycans. In addition, other surface positively charged amino acids may participate in binding to sulfated ZP glycans. In fact, we have previously shown that purified ASA can bind to chondroitin sulfate B glycosaminoglycans (disaccharide repeats of iduronic acid – N-acetylgalactosamine-4-sulfate) in a nonenzymatic manner (Wu *et al.* 2007). However, the computational docking of a long-chain glycan such as the ZP glycan to a protein is not practical and likely to be inaccurate due to the high flexibility of the glycan chain. In addition, information on the primary sequence of sulfated mZP glycans as well as higher-ordered structures of ZP glycans is not known. The latter piece of information may be relevant to ASA binding, considering that mZP1 is sulfated to the same extent as mZP2, but mZP1, in contrast to mZP2, showed only minimal binding ability to purified ASA (Fig. 3).

Since the binding of the ZP to ASA was not dependent on the active site pocket of the enzyme, ASA should still contain desulfation activity, which may be relevant during fertilization. It may desulfate SGG released as part of the acrosomal vesicles from the sperm head plasma membrane, provided that saposin B, the co-factor of ASA is available to 'solubilize' SGG from acrosomal vesicles and deliver the sulfoglycolipid into ASA's active site pocket (Ahn *et al.* 2003). This is possible, since prosaposin is present in the oviductal fluid (M Schenk, DC Santos and N Tanphaichitr, unpublished observations) and cathepsin D, which is known for its processing activity of prosaposin to saposin B (Hiraiwa *et al.* 1997), is also released as part of the AC (Erickson & Martin 1974). This SGG desulfation may be essential; acrosomal vesicles would still adhere to the ZP due to SGG affinity for the ZP (White *et al.* 2000), thus impeding sperm migration through the ZP layer. On the other hand, galactosylglycerolipid (GG), the desulfated product of SGG, has minimal affinity for the ZP (White

et al. 2000) and would thus be released into the surrounding. We are currently investigating these possibilities to gain a better understanding of ASA–ZP interaction. Regardless, our results herein showed that ASA bound preferentially to mZP2 and mZP3, the known mouse sperm receptors, and was localized on the convex ridge head surface of both AI and AR sperm as well as in the AC. All these properties implicate that sperm ASA has physiological significance in ZP binding.

Materials and Methods

Animal use

Sexually mature CF-1 female mice (6–8 weeks of age) and CD-1 male mice (~10 weeks of age) were purchased from Charles Rivers (Montreal, QC, Canada), and were housed in temperature and humidity controlled rooms with 14 h light:10 h darkness cycles. They were provided with food and water *ad libitum*. Both male and female mice were killed by cervical dislocation. The use of all mice was in accordance to the guidelines of the Canadian Council on Animal Care, as approved by the Animal Care Committee of OHRI.

Immunolocalization of ASA on AI and AR sperm

Mouse sperm were collected from the cauda epididymis and vas deferens in the KRB medium and subjected to Percoll gradient centrifugation (PGC), following our described method (Tantibhedhyangkul *et al.* 2002). PGC AI sperm were capacitated (30 min, 5% CO₂, 37 °C) in KRB–0.3% BSA, and then treated with 10 µM A23187 as previously described (Iamsaard *et al.* 2011). This treatment induced acrosome reaction in 70–80% of the total sperm population. Localization of ASA in AI and AR sperm was performed by immunofluorescence with the concurrent use of mouse mAb OBF13 IgM (recognizing the sperm membrane protein, Izumo, only in AR sperm (Kawai *et al.* 1989, Inoue *et al.* 2005), a gift from Dr Masaru Okabe, Osaka University) to differentiate AR from AI sperm. The A23187-treated sperm were washed twice with PBS by centrifugation (350 g 10 min, room temperature (RT)) and blocked with 5% de-complemented normal goat serum (prepared in PBS) for 15 min at RT. After washing with PBS by centrifugation, the sperm were incubated (30 min, RT) with 10 µg/ml rabbit polyclonal anti-ASA IgG (produced and purified for the IgG fraction as described (Wu *et al.* 2007)) plus 0.5 µg/ml OBF13 IgM in PBS. Following washing with PBS by centrifugation, the sperm were incubated (30 min, RT) with secondary antibodies, 25 µg/ml Alexa 594-conjugated goat anti-rabbit IgG and 5 µg/ml Alexa 488-conjugated goat anti-mouse IgM (Invitrogen). Sperm treated with preimmune rabbit serum IgG and normal mouse IgM in place of the primary antibodies served as negative controls. To assess cell viability during the staining process, propidium iodide (Invitrogen) was added to the cell suspension at a final concentration of 1 µg/ml 5 min before the secondary antibody incubation period ended. Hoechst 33342 (Invitrogen, at the final concentration of 0.5 µg/ml) was also added at the same time as propidium iodide to stain the sperm nucleus. The sperm were then washed twice in PBS, mounted onto a slide in 50% PBS/50% glycerol

and viewed under a Zeiss IM35 epifluorescence microscope (Carl Zeiss Canada, Toronto, ON, Canada). Fluorescent images of sperm were recorded by a Spot Junior CCD camera (Carl Zeiss Canada).

Isolation of AI capacitated sperm, AR sperm, acrosomal vesicles, and AC for immunoblotting

The preparation of AI capacitated and AR sperm was as described above. However, in the immunoblotting detection of ASA, which had a molecular mass (68 kDa) similar to BSA present in the capacitation medium, the high amount of BSA remaining in the supernatant of A23187-treated sperm could cause distortion of the ASA-reactive band, especially in the AC fraction. Therefore, the AI capacitated sperm were prewashed in KRB supplemented with 0.01% BSA prior to A23187 treatment (Iamsaard *et al.* 2011). Acrosomal vesicles and AC were prepared from the supernatant of A23187-treated sperm by ultracentrifugation as previously described (Iamsaard *et al.* 2011); acrosomal vesicles sedimented as a pellet and AC remained in the supernatant. AI and AR sperm (1 million each) and AC and acrosomal vesicles isolated from 1 million A23187-treated sperm were subjected to SDS-PAGE and immunoblotting for ASA.

Purification of native ASA and production of recombinant WT and mutant ASA

Native ASA was purified from boar sperm following the described method (Wu *et al.* 2007). The purified ASA had a specific activity of ~60 U/mg (assayed with NCS as a substrate), and appeared as a single band on a 10% polyacrylamide gel following SDS-PAGE/silver staining (see below).

Recombinant (rec) WT ASA and C69A and CKK mutant ASAs were produced in Chinese hamster ovary-K1 (CHO-K1) cells, following transient transfection of the cells with pcDNA3.1 expression vector containing WT and mutant sequences of ASA, as previously described (Schenk *et al.* 2009). Although the ASA sequence in the pcDNA3.1 plasmid was of the pig species, pig and mouse ASA sequences bear 96% conserved homology. Further, purified pig ASA binds specifically to mouse intact ZP (Tantibhedhyangkul *et al.* 2002). C69A was the mutant ASA that had the active site amino acid, Cys69, mutated to Ala. For CKK, Cys69 and the two lysine residues, Lys123 and Lys302, important for capturing the sulfate group of the substrate, were substituted with Ala. By culturing the transfected cells in medium containing 10 mM ammonium chloride for 18 h, ASA, which is a lysosomal enzyme, was secreted into the medium. The spent medium was collected for rec ASA purification, following our described method (Schenk *et al.* 2009), which involved size exclusion dialysis, Q-Sepharose resin capturing, and size exclusion Microcon YM-100 (Millipore Canada Ltd, Etobicoke, ON, Canada) centrifugation. However, in this current preparation, an additional step was included to remove transferrin (a CHO-K1 medium supplement) from rec ASA. Anti-transferrin IgG (Sigma) was coupled with Aminolink beads (Pierce, Rockford, IL, USA), following the manufacturer's instruction. The Microcon YM-100 retentate containing rec

ASA + transferrin (~100 µg) was then incubated with 30% v/v antibody-coupled Aminolink beads in a total volume of ~600 µl Tris-buffered saline (TBS: 137 mM NaCl in 20 mM Tris-HCl, pH 7.6) for 2 h at 4 °C with gentle shaking. After centrifugation (3000 g, 1 min, RT), the supernatant, containing mainly recombinant ASA, was collected. The pelleted beads were washed three times with 0.4 ml of TBS, pH 7.4, and all three washes were collected and combined with the supernatant. The combined samples were further concentrated in TBS with a Microcon YM-50. Aliquots of the final product were assayed for NCS desulfation activity and subjected to SDS-PAGE and immunoblotting.

SDS-PAGE and immunoblotting for ASA and mZP glycoproteins

Proteins were resolved by SDS-PAGE according to Laemmli (1970). For detection of ASA (native and rec ASA, AI and AR sperm, AC and acrosomal vesicles) the proteins were electrophoresed under a reducing condition in a 10% polyacrylamide gel (0.75 mm thickness). On the other hand, a nonreducing condition was used in electrophoresing mZP glycoproteins on a 7% polyacrylamide gel (1.5 mm thickness). Protein detection was by silver staining using a Bio-Rad Silver Stain kit.

Proteins separated on gel were transelektrophoresed (250 mA, 1 h) onto nitrocellulose membrane (0.45 µm; Towbin & Gordon 1984) for immunoblotting and chemiluminescence detections. For ASA, our previously described method was followed (Wu *et al.* 2007). For mZP2 and mZP3, rat monoclonal anti-mZP3 IgG and anti-mZP2 IgG antibodies were used (East *et al.* 1984, 1985). IgG fraction of rat anti-mZP3 and anti-mZP2 was purified, using a Protein G column kit from Thermo Scientific (Rockford, IL, USA), from the spent medium of hybridomas, which produced these mAbs (gifts from Dr J Dean, NIH). Nonspecific binding sites on the membrane were blocked (1 h, RT) with 5% nonfat milk in TBS containing 0.05% Tween 20 (TBST). The membrane was then incubated (1 h, RT or 4 °C, overnight) with 5 µg/ml of rat anti-mZP3 IgG or anti-mZP2 IgG, washed three times (5 min each) with TBST and then incubated with goat HRP-conjugated anti-rat IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:1000 dilution in TBST). After washing the membrane (3 × 10 min) with TBST, the protein-antibody recognition was detected using an enhanced chemiluminescence ECL western blotting detection kit (Pierce). The emitted chemiluminescence was captured by Hyperfilm ECL film in the dark; the film was developed on a Kodak M35A X-OMAT processor (Kodak Canada, Inc.).

Isolation of ovarian ZP and purification of mZP1, mZP2, and mZP3

Large numbers of ovarian ZP ($5\text{--}20 \times 10^3$) were isolated by Percoll gradient ultracentrifugation of mouse ovarian homogenates, according to the previously described method (Litscher & Wassarman 1999) with modifications. Briefly, a homogenate in triethanolamine buffer containing 1% deoxycholate was prepared from ovaries dissected from 20 to 30 mice (6–8 weeks old) and subjected to Percoll gradient ultracentrifugation. ZP (both intact and fragmented),

sedimented as a band on the upper third of the tube at 1.02 g/ml density, were aspirated through a needle into the syringe and re-subjected to Percoll gradient ultracentrifugation. ZP collected from the second ultracentrifugation round were then washed free of Percoll solution in 25 mM triethanolamine, pH 8.5, 150 mM NaCl in a 15 ml conical polypropylene tube (1500 g, 10 min, RT). The ZP pellet was transferred into a microcentrifuge tube and was washed with PBS (10 000 g, 5 min, RT). The final ZP pellet was stored in 50% glycerol in PBS at -20°C . Solubilization of the isolated ZP was done by first washing the ZP in PBS, pH 6.8, followed by resuspension and vortexing in 100 μl of 0.2 M sodium phosphate buffer, pH 6.8, containing 0.1% SDS. The ZP became solubilized by heating at 65°C for 10 min. After the tube was cooled down to RT, the insoluble material was pelleted by centrifugation (14 000 g, 1 min). Total amount of zona glycoproteins in the supernatant was determined by A280 using a weighted average of ZP1, ZP2, and ZP3 extinction coefficients and a 1:5:5 molar ratio for the three ZP glycoproteins in each ZP (Kerr *et al.* 2002). The supernatant, containing solubilized ZP, was used to purify individual ZP glycoproteins, employing two separate methods – HPLC and electroelution.

The HPLC method was as described by Litscher & Wasserman (1999). Solubilized ZP was fractionated on a size exclusion column BioSil SEC-250 (Bio-Rad) at RT and eluted with HPLC buffer (same as the ZP solubilization buffer) at a flow rate of 0.1 ml/min with online A280 monitoring. Eluted fractions, 100 μl each, were collected and measured for A280. The fractions of each protein peak were pooled, determined for protein amount by A280 using the calculated extinction coefficients for mZP1, mZP2, and mZP3 of 60 980, 74 230, and 35 010 $\text{cm}^{-1}\text{M}^{-1}$ respectively (Kerr *et al.* 2002). From the mole amounts obtained from this calculation, the weight amounts were derived using molecular weight of 100 000, 120 000, and 83 000 for mZP1, mZP2, and mZP3 respectively. The mZP fractions were then dialyzed for 24–48 h against distilled water at 4°C using a tubing with a molecular mass cutoff of 30 kDa. Finally, the samples were lyophilized and stored at -80°C prior to use.

In the electroelution method, the solubilized ZP was first resolved by SDS–PAGE as described above. The sample was applied on several lanes, with each lane containing no more than 25 μg of total ZP glycoproteins. To locate the band position of each mZP glycoprotein, 1 μg of solubilized ZP was applied to a separate lane on the same gel. Following gel electrophoresis, the lane with 1 μg of solubilized ZP was cut off the gel and stained with silver nitrate using the Bio-Rad Silver Stain kit to reveal the positions of mZP1 (200 kDa), mZP2 (120 kDa), and mZP3 (83 kDa). Corresponding regions of each ZP glycoprotein from lanes loaded with 25 μg of solubilized ZP were marked by matching with the stained gel. The marked areas were cut to produce gel slices, which were subjected to electroelution using a Centrilot Micro-Electroeluter (Millipore). The electroelution was carried out at a constant voltage (200 V) for 2 h at RT using degassed SDS-electrophoresis buffer minus SDS. The eluted proteins were washed extensively with PBS, pH 7.4, through six cycles of volume reduction–resuspension (more than tenfold volume reduction each time)

in Centricon YM-30 (Millipore). Solubilized ZP and each purified ZP glycoprotein were quantified by A280.

The purity of the purified ZP glycoproteins (by either HPLC or electroelution) was examined by silver staining and for mZP2 and mZP3, and was further validated by immunoblotting using a mAb against mZP2 and mZP3 respectively. The biological activity of total solubilized ZP and the purified ZP3 as a sperm acrosome reaction inducer was also assessed. Briefly, capacitated PGC sperm were incubated (30 min, 37°C , 5% CO_2) with six ZP equivalent amounts of solubilized ZP and each individual ZP glycoproteins per microliter of the sperm suspension. The acrosome status of the sperm was determined by Coomassie blue staining (Bleil & Wassarman 1990). Capacitated sperm untreated or treated with ovalbumin or fetuin, which were also subjected to the same electroelution as the ZP glycoproteins, served as negative controls (Carmona *et al.* 2002b), whereas sperm treated with 10 M A23187 were a positive control.

Binding of ASA to mZP glycoproteins by dot blotting

Various concentrations of solubilized mouse ZP (containing the three mZP glycoproteins) and individual mZP glycoproteins were separately spotted to the size of 0.5 cm in diameter onto nitrocellulose membrane and air dried. In certain experiments, ovalbumin and fetuin, 1 μg each, were also spotted on the same membrane. The membrane was blocked in 5% nonfat milk in TBST (1 h, RT) and incubated (1 h, RT) with purified ASA (3 μg) in 1 ml of TBST. The membrane was then washed three times (5 min each) with TBST and incubated (1 h, RT) with anti-ASA antiserum (1:1000 dilution in 5% nonfat milk). After washing three times in TBST, the membrane was incubated (1 h, RT) with HRP-conjugated goat anti-rabbit IgG (1:5000 dilution in TBST containing 5% skim milk), and processed for chemiluminescence detection as described in the Immunoblotting section and the scanned image (in TIFF format) was subjected to densitometric analysis.

To demonstrate that the binding of ASA to mZP glycoproteins immobilized onto the nitrocellulose membrane was specific, ASA was preincubated (30 min, RT) in solution with 10 μg of solubilized total mZP glycoprotein ($\sim 10\times$ mole ratio to the dotted mZP glycoproteins and mZP3 on the membrane, and $\sim 5\times$ mole ratio to the dotted mZP2) prior to co-incubation with the immobilized mZP. In alternate experiments, dextran sulfate and dextran (1 μM each) (both from Sigma and with the size of 500 kDa) were included during the co-incubation of ASA with the immobilized mZP glycoproteins. Detection of ASA–mZP glycoprotein interaction was then performed as described above.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-11-0338>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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