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Zona pellucida glycoprotein mZP3 bioactivity is not dependent on the extent of glycosylation of its polypeptide or on sulfation and sialylation of its oligosaccharides

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SUMMARY

During fertilization in mice, free-swimming sperm bind to mZP3, one of three egg zona pellucida glycoproteins. Sperm recognize and bind to specific serine/threonine-linked (O-linked) oligosaccharides located at the mZP3 combining site for sperm. Shortly after binding to mZP3, sperm undergo the acrosome reaction, a form of cellular exocytosis. Here, we examined the influence of extent of glycosylation, sulfation, and sialylation of mZP3 (M_r ~65,000-100,000) on its bioactivity; i.e. its ability to inhibit binding of sperm to eggs and to induce the acrosome reaction in vitro. Low (av. M_r ~70,000), medium (av. M_r ~82,000), and high (av. M_r ~94,000) M_r fractions of mZP3 were purified and shown to vary in extent of asparagine-linked (N-linked) glycosylation. All three size-fractions exhibited bioactivity, suggesting that the ability of mZP3 to inhibit binding of sperm to eggs is not related to the extent of glycosylation of its polypeptide (M_r ~44,000). Digestion of mZP3 by neuraminidase decreased its average M_r from ~83,000 to ~77,000 and increased its average pI from ~4.7 to ~6.0, but did not significantly affect mZP3 bioactivity. Terminal sialic acid largely accounts for the glycoprotein's acidic nature, but is not an essential element of the mZP3 combining site for sperm. Experiments with stably transfected embryonal carcinoma (EC) cells that secrete bioactive EC-mZP3 revealed that, of the sulfate present, ~70-75% was located on N-linked and ~25-30% on O-linked oligosaccharides. EC-mZP3 devoid of sulfate inhibited binding of sperm to eggs and induced the acrosome reaction to the same extent as sulfated EC-mZP3. These results suggest that sulfation of EC-mZP3 oligosaccharides is not essential for bioactivity. Overall, these findings contrast with those reported for certain other glycoproteins involved in cellular adhesion that require sulfate and/or sialic acid for bioactivity.

Key words: mZP3 glycoprotein, Sulfation, Sialylation, Glycosylation, Bioactivity

INTRODUCTION

Mammalian eggs are surrounded by a thick extracellular coat, called the zona pellucida (ZP) (Dietl, 1989; Yanagimachi, 1994; Wassarman, 1988, 1993). In general, the ZP is composed of three relatively acidic glycoproteins, called ZP1-3, that are synthesized and assembled into a filamentous network around the egg during oogenesis (Wassarman, 1988, 1990, 1993; Wassarman and Mortillo, 1991). During fertilization in mice, free-swimming sperm recognize and bind to specific serine/threonine-linked (O-linked) oligosaccharides located on the carboxy-terminal half of the mZP3 polypeptide (Wassarman et al., 1985; Wassarman, 1987, 1988, 1990, 1995; Wassarman and Litscher, 1995). Therefore, gamete adhesion during fertilization in mice, and in a number of other mammalian species (Wassarman, 1991; Litscher and Wassarman, 1993), is carbohydrate-mediated.

Not a great deal is known about the structure of mZP3 O-linked oligosaccharides that are recognized by sperm. They have an average apparent M_r of ~3,900 based on gel-exclusion chromatography (Florman and Wassarman, 1985; Bleil and Wassarman, 1988; Miller et al., 1992), but in view of the aberrant behavior of O-linked oligosaccharides under these conditions (Kobata, 1994; E. S. Litscher and P. M. Wassarman, unpublished results), it is likely that they consist of fewer sugar residues than initially thought. Furthermore, while the monosaccharide at the nonreducing-end of the oligosaccharide is essential for bioactivity, there is disagreement as to whether the monosaccharide is galactose (Bleil and Wassarman, 1988; Litscher et al., 1995) or *N*-acetylglucosamine (Shur and Hall, 1982; Miller et al., 1992).

Carbohydrate-mediated cellular adhesion can often involve more than one correctly displayed monosaccharide and/or other constituent, such as sulfate and sialic acid (Karlsson, 1991; Lis and Sharon, 1993; Sharon and Lis, 1993; Opdenakker et al., 1993; Varki, 1994). For example, extensive studies (Rosen, 1993; Varki, 1994; Hemmerich et al., 1994; Rosen and Bertozzi, 1994) of the interaction between leukocyte L-selectin and its endothelial ligand, called GlyCAM-1 (Lasky et al., 1992), have revealed that sialylation, sulfation, and extent of glycosylation

of GlyCAM-1 are essential factors in the interaction (Rosen et al., 1989; Imai et al., 1991, 1992, 1993; Berg et al., 1992; Foxall et al., 1992; Brandley et al., 1993; Hemmerich et al., 1994; Bertozzi et al., 1995). GlyCAM-1 is a mucin-like molecule that carries heterogeneous O-linked oligosaccharides that are clustered and variably modified with sulfate, sialic acid, and fucose (Lasky et al., 1992; Imai and Rosen, 1993).

Since it has been pointed out that GlyCAM-1 and mZP3 have certain features in common (Lasky et al., 1992), we examined the potential contribution of sialylation, sulfation, and extent of glycosylation of mZP3 to its bioactivity. We conclude from the results that mZP3 oligosaccharides are sialylated and sulfated and that the polypeptide is heterogeneously glycosylated. However, these three factors are not directly involved in binding of sperm to mZP3 O-linked oligosaccharides.

MATERIALS AND METHODS

Collection and culture of gametes and embryos

Sperm, ovulated eggs, and fertilized eggs were obtained from randomly bred, Swiss albino mice (CD-1; Charles River Breeding Labs) and cultured in vitro, essentially as previously described (Florman and Wassarman, 1985; Bleil and Wassarman, 1980, 1986).

Purification and size-fractionation of mZP3

mZP3 was purified by HPLC using ZP isolated by fractionation of ovarian homogenates on Percoll gradients, essentially as previously described (Moller et al., 1990). In some cases, purified mZP3 was radioiodinated using Na[^{125}I] (carrier-free, ~15.4 mCi/μg I; Amersham) at ~0.5 mCi/2.5 μg mZP3, essentially as previously described (Rosiere and Wassarman, 1990). mZP3 concentration was estimated spectrophotometrically and by gamma-counting. To obtain size-fractions of mZP3, purified ^{125}I-mZP3 (unlabeled mZP3:radiolabeled mZP3, 10:1 by weight) was subjected to SDS-PAGE followed by autoradiography of the wet gel, the region containing mZP3 was excised and sliced into three fractions based on average M_r (high, ~94,000; medium, ~82,000; low, ~70,000), and each fraction was electroeluted from the gel slice (Centrilutor; Amicon), concentrated (Centricon-30; Amicon), and dialyzed extensively, first against 8 M urea and then against distilled water. In some cases, the mZP3 size-fractions were digested with 0.5 units N-glycanase (NGase; E.C. 3.5.1.52 and 3.2.2.18; Genzyme) in 20 mM sodium phosphate, pH 7.5 (2 μl enzyme plus 8 μl buffer), for 48 hours at 37°C to remove N-linked oligosaccharides. Digestions were terminated by addition of 40 μl distilled water and boiling the sample for 5 minutes prior to SDS-PAGE.

Production and purification of EC-mZP3

A stably transfected mouse embryonal carcinoma (EC; F9) cell line that expresses mZP3 (EC-mZP3) was cultured as previously described (Kinloch et al., 1991). To radiolabel EC-mZP3 with [^{35}S]sodium sulfate or [^{35}S]methionine, EC cells were cultured in low sulfate, serum-free medium (90% sulfate-free MEM, 10% D-MEM, 5 μg/ml transferrin, 1 μg/ml insulin; Gibco-BRL) in the presence of 200 μCi [^{35}S]sodium sulfate (carrier-free, ~43 Ci/mg S; ICN Biomedicals) or [^{35}S]methionine (~1,130 Ci/mmole; ICN Biomedicals). To obtain sulfate-free EC-mZP3, 10 mM or 20 mM sodium chlorate was present in the culture medium (Baeuerle and Huttner, 1986). Radiolabeled EC-mZP3 was recovered from the medium and partially purified by HPLC (Bio-Sil SEC-250; Bio-Rad), as previously described (Kinloch et al., 1991). In some cases, radiolabeled EC-mZP3 was immunoprecipitated by standard procedures (Ausubel et al., 1989) using an antiserum directed against mZP3 (Ra-mZP3; Kinloch et al., 1991). Immunoprecipitated EC-mZP3 was separated from IgG by HPLC and was dialyzed extensively, first against 8 M urea and then against distilled water prior to subjecting samples to bioactivity assays and SDS-PAGE.

Chemical and enzymatic deglycosylation of EC-mZP3

Asparagine-linked (N-linked) oligosaccharides were removed from EC-mZP3 using a Protein de-N-Glycosylation Kit (Oxford GlycoSystems) according to the protocol supplied by the manufacturer. O-linked oligosaccharides were removed from EC-mZP3 by incubating the glycoprotein overnight at 37°C in the presence of 7.5 mM NaOH (so-called, β-elimination reaction), essentially as previously described (Florman and Wassarman, 1985). Complete deglycosylation of EC-mZP3 was carried out using trifluoromethanesulfonic acid (TFMS; GlycoFree Chemical Deglycosylation Kit, Oxford GlycoSystems) according to the protocol supplied by the manufacturer. In each case, the treated sample was brought to 600 μl, BSA was added (150 μg/ml, final concentration), and EC-mZP3 was precipitated with trichloroacetic acid (TCA, 20%; 1 hour on ice). The sample was centrifuged and the pellet was washed 3-times with ice-cold 5% TCA and 5-times with ethyl ether. The pellet was dissolved in electrophoresis sample buffer and then subjected to SDS-PAGE.

Desialylation of mZP3

Terminal sialic acid was removed from ^{125}I-labeled mZP3 (unlabeled mZP3:labeled mZP3, 10:1 by weight) by digestion with 3.3 units/ml neuraminidase (NEURase; *Arthrobacter ureafaciens;* E.C. 3.2.1.18; Oxford GlycoSystems) in 100 mM sodium acetate, pH 5.0 (2 μl enyzme plus 8 μl buffer), for 48 hours at 37°C. The enzyme was inactivated by addition of 40 μl distilled water and boiling for 5 minutes, and the sample was then dialyzed extensively against distilled water prior to analysis by two-dimensional SDS-PAGE and measurement of bioactivity. ^{125}I-labeled mZP3 digested with NEURase was subjected to high-resolution two-dimensional electrophoresis (O'Farrell, 1975) and autoradiography.

Bioactivity assays

mZP3 and EC-mZP3 were tested for their ability to prevent free-swimming sperm from binding to ovulated eggs in vitro ('competition assay'), essentially according to the procedure described previously (Bleil and Wassarman, 1980, 1986; Florman and Wassarman, 1985; Moller et al., 1990). Briefly, capacitated sperm were incubated with mZP3 or EC-mZP3 in a 20 μl drop, under oil, at 37°C for 15 minutes, at which time 12 ovulated eggs and 2-3 two-cell embryos were added to the drop and the incubation was continued an additional 40-45 minutes. Eggs and embryos were then washed by mouth-pipetting, fixed in 2% formaldehyde/PBS/PVP in M199-M (1:1 by volume), and the number of sperm bound per egg in the largest plane of focus was determined by dark-field phase microscopy.

In some cases, mZP3 was tested for its ability to induce sperm to undergo the acrosome reaction in vitro, essentially according to the procedure described previously (Moller et al., 1990). Briefly, 50 μl capacitated sperm (10^6 sperm/ml) were incubated with an equal volume of test sample for 1 hour at 37°C in a humidified atmosphere of 5% CO_2 in air. The sperm were then fixed with 4% paraformaldehyde, washed with 100 mM ammonium acetate, pH 9.0, placed on gelatin-coated slides, washed with water, then methanol, then water (5 minutes each), stained with 0.04% Coomassie Blue G-250 in 3.5% perchloric acid, and the status of the acrosome assessed by light microscopy.

RESULTS

Effect of extent of mZP3 glycosylation on bioactivity

mZP3 consists of a M_r ~44,000 polypeptide (402 amino acids) that is glycosylated to different extents (Salzmann et al., 1983;

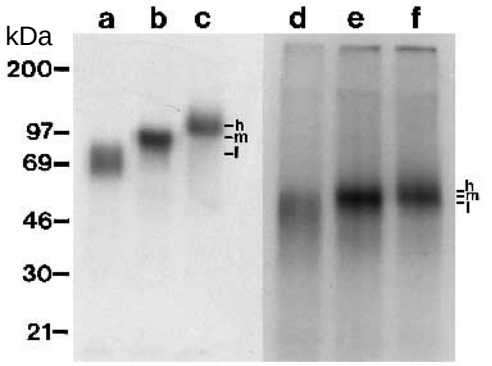

Fig. 1. SDS-PAGE analysis of mZP3 size-classes before and after treatment with NGase. Lanes a-c, autoradiograph of the three molecular mass fractions (l, m, and h, respectively) of mZP3 (see Materials and Methods). Lanes d-f, autoradiograph of the three molecular mass fractions (l, m, and h, respectively) of mZP3 following treatment with NGase to remove N-linked oligosaccharides (see Materials and Methods). Note that the resulting bands are broad and ~55,000 M_r. Gels were calibrated with prestained molecular mass standards ranging from 14,300 to 200,000 M_r.

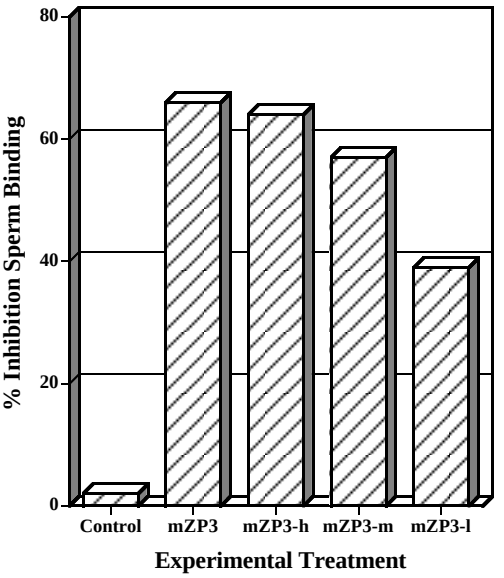

Fig. 2. Effect of mZP3 size-classes on binding of sperm to eggs in vitro. Shown are results of competition binding assays carried out with sperm exposed to intact mZP3 or to mZP3 size-classes h (av. M_r 94,000), m (av. M_r 82,000), and l (av. M_r 70,000) prior to incubation with unfertilized eggs and two-cell embryos, as described in Materials and Methods. Each experimental treatment was carried out at ~60 nM, ~120 nM, and ~240 nM; for example, mZP3 size-class h inhibited binding of sperm to eggs by 28%, 64%, and 85% over this range of glycoprotein concentrations. Shown are results of experiments carried out at ~120 nM with all three size-classes of fractionated mZP3.

Kinloch et al., 1988; Ringuette et al., 1988; Wassarman, 1988). For example, mZP3 can have either 3 or 4 complex-type, N-linked oligosaccharides and an undetermined number of O-linked oligosaccharides attached to its polypeptide (Salzmann et al., 1983). Consequently, mZP3 is very heterogeneous with respect to M_r, ranging from ~65,000 to ~100,000, as determined by migration on SDS-PAGE. It should be noted that these are all apparent M_r, since it is well known that glycoproteins and glycopeptides migrate anomolously on SDS-PAGE due, in part, to a low charge-to-mass ratio for the molecules (Leach et al., 1980).

To determine whether or not the extent of glycosylation of mZP3 polypeptide influences its sperm receptor activity, the purified glycoprotein was fractionated by SDS-PAGE according to size. Three mZP3 fractions, called high (h; M_r ~88,000-100,000), medium (m; M_r ~77,000-87,000), and low (l; M_r ~65,000-76,000) M_r, were obtained by electroelution of glycoprotein from gel slices following SDS-PAGE (Fig. 1) and were assayed for their ability to inhibit binding of sperm to eggs in vitro. As seen in Fig. 2, while fraction l appeared to be slightly less effective than h and m, all three fractions inhibited binding of sperm to eggs in the concentration range ~60-240 nM. These results suggest that there is no correlation between the extent of glycosylation of mZP3 polypeptide and its ability to inhibit binding of sperm to eggs.

To determine how much of the size heterogeneity of mZP3 is attributable to its N-linked oligosaccharides, fractions h, m, and l were digested with N-glycanase (NGase). This endoglycosidase catalyzes the hydrolysis of N-linked oligosaccharides (high-mannose, complex, and hybrid types) at the β-aspartyl-glycosylamine bond between the innermost GlcNAc and the asparagine residue (Elder and Alexander, 1982; Tarentino et al., 1985; Tarentino and Plummer, 1994). Removal of N-linked oligosaccharides decreased the average M_r of all three fractions to ~54,000-56,000 (i.e. a M_r decrease of ~10,000 to ~45,000) (Fig. 1), with fractions h and l undergoing the largest and smallest change, respectively. The NGase treated fractions continued to migrate as relatively broad bands consistent with heterogeneous O-linked glycosylation of the M_r ~44,000 polypeptide. These results suggest that the size heterogeneity of mZP3 is attributable primarily to the glycoprotein's complex-type N-linked oligosaccharides. It should be noted that excision of N-linked oligosaccharides from purified mZP3 by NGase and inhibition of N-linked glycosylation of nascent mZP3 by tunicamycin (Roller and Wassarman, 1983) result in production of a heterogeneous, ~55,000 M_r species. Furthermore, previous studies have demonstrated that excision of N-linked oligosaccharides from mZP3 has no detectable effect on bioactivity (Florman and Wassarman, 1985; Litscher and Wassarman, 1996a).

Effect of desialylation of mZP3 on bioactivity

To examine the possible involvement of sialic acid in sperm receptor activity, mZP3 was digested with NEURase from *Arthrobacter ureafaciens*, an enzyme specific for a variety of nonreducing terminal sialic acids in α2-3/6/8 linkages to various monosaccharides (Uchida et al., 1979; Reuter and Schauer, 1994). Such treatment resulted in a small decrease in the average M_r of mZP3, from ~83,000 to ~77,000, and a substantial increase in its average pI, from ~4.7 (range ~4.2-5.2) to ~6.0 (range ~5.3-6.7) (Fig. 3). These changes suggest that sialic acid was removed, but mZP3 polypeptide (~44,000 M_r) remained intact during the incubation. Despite these changes, mZP3 treated with NEURase continued to inhibit binding of

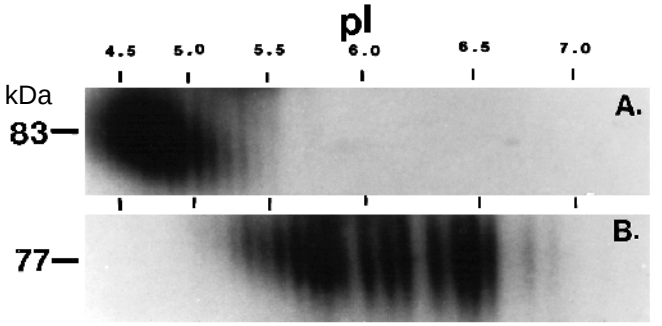

Fig. 3. High-resolution two-dimensional gel electrophoreis of mZP3 before and after treatment with NEURase. ^{125}I-labeled mZP3 was subjected to high-resolution two-dimensional gel electrophoresis before (A.) and after (B.) digestion by NEURase and then to autoradiography, as described in Materials and Methods. The isoelectric point (pI) is shown in the first dimension and the M_r in the second dimension of the gel.

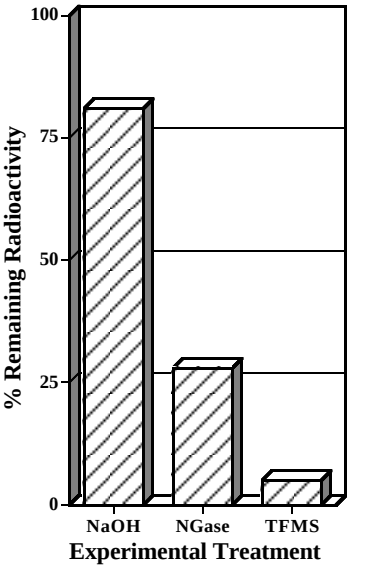

Fig. 4. Analysis of EC-mZP3 sulfation. Shown is the percentage [^{35}S]sulfate remaining with TCA precipitated EC-mZP3 following treatment with 7.5 mM NaOH, NGase, or TFMS, as described in Materials and Methods. Each bar represents the average value (% remaining radioactivity) of three individual experiments.

sperm to eggs in vitro. At a concentration of ~120 nM, untreated mZP3 and NEURase-treated mZP3 inhibited binding of sperm to eggs by 72±15% and 68±7%, respectively (n=4). Furthermore, NEURase-treated mZP3 induced sperm to undergo the acrosome reaction in vitro to the same extent as untreated mZP3 (data not shown). These results suggest that terminal sialic acid is not an essential constituent of the mZP3 combining site for sperm.

Effect of EC-mZP3 sulfation on bioactivity

Previously, we reported that EC-mZP3, secreted by EC cells stably transfected with the mZP3 gene fused to the mouse *pgk-1* promoter, inhibited binding of sperm to eggs in vitro (Kinloch et al., 1991, 1995). To evaluate the possible role of sulfate in bioactivity, transfected EC cells were cultured in low sulfate-medium containing [^{35}S]sodium sulfate or [^{35}S]methionine, culture medium was collected, and secreted EC-mZP3 was immunoprecipitated using a polyclonal antibody directed against mZP3 (Ra-mZP3). Analysis of an aliquot of the immunoprecipitated glycoprotein by SDS-PAGE and autoradiography revealed that EC-mZP3 was radiolabeled by either [^{35}S]sodium sulfate or [^{35}S]methionine. To determine whether the radiolabel was associated with EC-mZP3 oligosaccharides and/or polypeptide, aliquots of the immunoprecipitated glycoprotein were treated with N-glycanase to remove N-linked oligosaccharides, with mild alkali to remove O-linked oligosaccharides (β-elimination reaction), or with TFMS to remove both N- and O-linked oligosaccharides. Analyses of the amount of radioactivity remaining with EC-mZP3 after such treatments revealed that ~70-75% and ~25-30% of the incorporated [^{35}S]sulfate was associated with N-linked and O-linked oligosaccharides, respectively; less than 5% of the radiolabel was associated with polypeptide (Fig. 4). These results demonstrate that the oligosaccharides of bioactive EC-mZP3 are sulfated.

To prepare EC-mZP3 devoid of sulfate, transfected EC cells were cultured in the presence of sodium chlorate, a potent inhibitor of sulfation (Baeuerle and Huttner, 1986), and either [^{35}S]methionine or [^{35}S]sodium sulfate. As seen in Fig. 5, sodium chlorate (10 mM and 20 mM) completely inhibited sulfation of EC-mZP3 (lanes g and h), but did not significantly

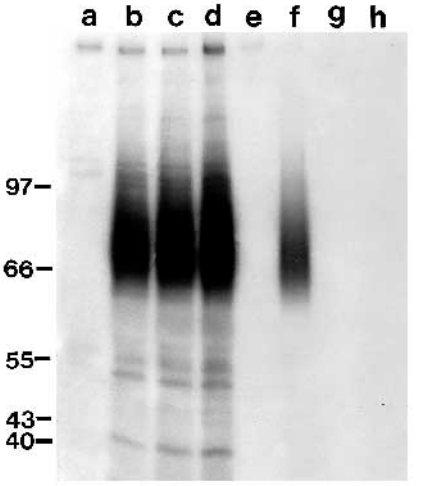

Fig. 5. Effect of sodium chlorate on sulfation and secretion of EC-mZP3. Shown are untransfected EC cells (lanes a and e) and EC cells stably transfected with the mZP3 gene (lanes b-d and f-h) cultured in low sulfate, serum-free medium in the presence of either [^{35}S]methionine (lanes a-d) or [^{35}S]sodium sulfate (lanes e-h). In some cases, 10 mM (lanes c and g) or 20 mM (lanes d and h) sodium chlorate was present in the medium. Cells were cultured for 24 hours, secreted EC-mZP3 was immunoprecipitated from the medium with Ra-mZP3, and samples were subjected to SDS-PAGE and autoradiography.

affect its synthesis and secretion into the culture medium (lanes c and d). Sulfate-free EC-mZP3 was immunoprecipitated from the medium using Ra-mZP3, partially purified by HPLC, and assayed for bioactivity. At a concentration of ~120 nM, sulfate-free EC-mZP3, sulfated EC-mZP3, and egg mZP3 inhibited binding of sperm to eggs in vitro by 60±20%, 57±10%, and 60±8%, respectively (n=3; Fig. 6). No significant difference was detected for sulfate-free and sulfated EC-mZP3 in these assays. Furthermore, at a concentration of ~120 nM, sulfate-free EC-mZP3 was just as effective as sulfated EC-mZP3 in inducing sperm to undergo the acrosome reaction in vitro (~2.4-fold induction of the acrosome reaction, as compared to

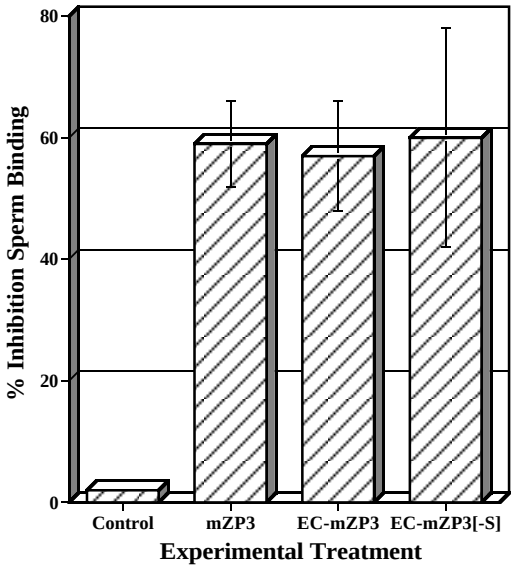

Fig. 6. Effect of sulfation of EC-mZP3 on its sperm receptor activity. Shown are results of competition binding assays carried out with sperm exposed to ~120 nM mZP3, EC-mZP3, or EC-mZP3[−S]; the latter is EC-mZP3 lacking sulfate that was synthesized in the presence of sodium chlorate (see Materials and Methods). Each bar represents the average value (% inhibition sperm binding) ± s.d. of three individual experiments.

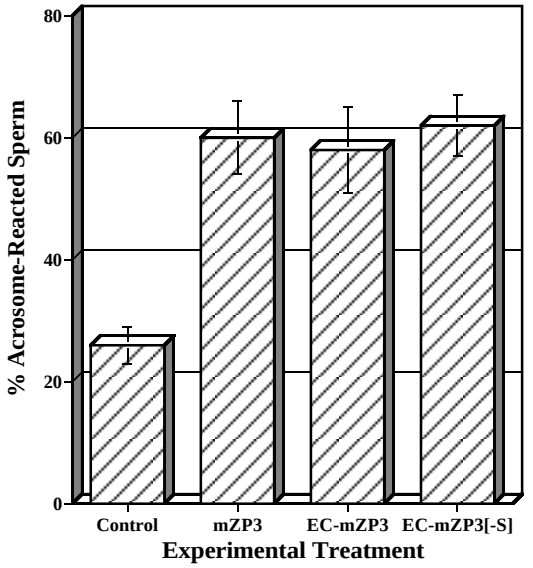

Fig. 7. Effect of sulfation of EC-mZP3 on its acrosome reaction-inducing activity. Shown are results of acrosome reaction assays carried out with sperm exposed to medium alone ('control') or to ~120 nM mZP3, EC-mZP3, or EC-mZP3[-S]; the latter is EC-mZP3 lacking sulfate that was synthesized in the presence of sodium chlorate (see Materials and Methods). Each bar represents the average value (% acrosome-reacted sperm) ± s.d. of three individual experiments (~200 sperm/experiment).

background levels) (n=3; Fig. 7). These results suggest that, in terms of bioactivity, sulfate is not an essential constituent of the EC-mZP3 combining site for sperm.

DISCUSSION

Several lines of evidence suggest that mouse sperm recognize and bind to mZP3 O-linked oligosaccharides, rather than to mZP3 N-linked oligosaccharides or polypeptide (Wassarman, 1990; Wassarman and Litscher, 1995). Relatively little is known about the structure of these O-linked oligosaccharides (see Introduction) that are located on the carboxy-terminal third of the polypeptide encoded by mZP3 gene exon-7 (Litscher et al., 1995; Rosiere and Wassarman, 1990; Kinloch et al., 1995; Litscher and Wassarman, 1996a). Here, we addressed the possibility that sialic acid and/or sulfate are essential elements of the mZP3 combining site for sperm and that the extent of glycosylation of mZP3 polypeptide also influences bioactivity.

Although mZP3 exhibits considerable M_r heterogeneity, primarily due to different extents of N-linked glycosylation (Fig.1), apparently all three size classes of mZP3 are bioactive (Fig. 2). This is unlike the case of GlyCAM-1 where only the largest size class of the ligand (~30% of total GlyCAM-1) is recognized by L-selectin (Imai and Rosen, 1993). It can be concluded that all three M_r classes of mZP3 possess O-linked oligosaccharides required for bioactivity at their combining site for sperm, but are very heterogeneous with respect to N-linked glycosylation. The results obtained with mZP3 are not unexpected since the extent of glycosylation of ZP3 polypeptides from different mammalian species varies considerably, while the polypeptides themselves are very similar to each another (Wassarman, 1993; Wassarman and Litscher, 1995). For example, the primary structures of mZP3 and hamster ZP3 (hZP3) polypeptides (M_rs ~44,000) are ~82% identical; however, the average M_r of mZP3 and hZP3 is ~83,000 and ~56,000, respectively, due to different extents of N-linked glycosylation (Moller et al., 1990; Kinloch et al., 1991; Wassarman, 1993; Wassarman and Litscher, 1995). On the other hand, despite very different extents of N-linked glycosylation, purified mZP3 binds to hamster sperm and purified hZP3 binds to mouse sperm (Moller et al., 1990; Litscher and Wassarman, 1996b). This is consistent with the fact that mouse sperm *(Mus musculus)* bind to ovulated hamster eggs *(Mesocricetus auratus)* and hamster sperm bind to ovulated mouse eggs (Schmell and Gulyas, 1980; Cherr et al., 1986; Moller et al., 1990).

Sulfation is a common post-translational modification of both membrane and secreted glycoproteins that takes place in the *trans*-Golgi (Huttner, 1988; Kato and Spiro, 1989; Skelton et al., 1991). There is considerable evidence to suggest that sulfation may be important for recognition phenomena mediated by carbohydrates present on glycoproteins (Fiete et al., 1991; Roche et al., 1991; Baenziger et al., 1992; Liau et al., 1992; Imai et al., 1993; Pittmann et al., 1994; Bansal and Pfeiffer, 1994). In the context of fertilization, sulfate has been implicated in gamete adhesion and induction of the acrosome reaction in echinoderms (DeAngelis and Glabe, 1990; Ward and Kopf, 1993; Foltz and Lennarz, 1993) and in sperm-egg interactions in mammals (Jones, 1991; Urch and Patel, 1991; LoLeggio et al., 1994).

There is previous evidence to suggest that mZP3, as well as ZP3 from other mammalian species, possess sulfated oligosaccharides (Shimizu et al., 1983; Yurewicz et al., 1991; Noguchi and Nakano, 1993; Hokke et al., 1993). For example, structural

analysis (^{1}H NMR) of porcine zona pellucida glycoproteins revealed the presence of three sulfated and sialylated O-linked oligosaccharides of low molecular mass (Hokke et al., 1993) and anion-exchange HPLC analysis of mouse ZP3 N-linked oligosaccharides also revealed the presence of significant levels of sulfate (Noguchi and Nakano, 1993). Results of experiments described here indicate that EC-mZP3, secreted by EC cells stably transfected with the mZP3 gene placed under the control of a constitutive promoter (Kinloch et al., 1991), is also a sulfated glycoprotein. Removal of both N-linked and O-linked oligosaccharides from [^{35}S]sulfate-labeled EC-mZP3 results in loss of virtually all radiolabel from the glycoprotein (Fig. 4), strongly suggesting that the oligosaccharides, but not the polypeptide, are sulfated.

Chlorate affects sulfation of nascent glycoproteins by inhibiting ATP-sulfurylase, an enzyme involved in the synthesis of the high energy sulfate donor 3′-phosphoadenyl 5′-phosphosulfate (PAPS) (Baeuerle and Huttner, 1986). While some studies suggest that inhibition of sulfation by chlorate can affect secretion of glycoproteins (Cardelli et al., 1994; Beinfeld, 1994), others indicate that chlorate does not have a significant effect on either synthesis or secretion of glycoproteins (Bernstein and Copans, 1992; Liau et al., 1992; Imai and Rosen, 1993; Pittman et al., 1994; Mintz et al., 1994). Here, we found that chlorate (10-20 mM) was, indeed, a potent inhibitor of sulfation of EC-mZP3, but did not measureably affect either the synthesis or secretion of EC-mZP3 (Fig. 5). Furthermore, sulfate-free EC-mZP3 proved to be just as effective as sulfated EC-mZP3 at inhibiting binding of sperm to eggs (Fig. 6) and inducing sperm to undergo the acrosome reaction (Fig. 7). It can be concluded from these experiments that, although sulfate is present, it is not an essential constituent of the EC-mZP3 combining site for sperm.

Sialic acid is frequently found at the nonreducing-end of oligosaccharides present on glycoproteins and, in many instances, serves as an essential ligand in carbohydrate-mediated cellular adhesion (Zeng and Gabius, 1992; Varki, 1992; Norgard et al., 1993; Roth, 1993; Pilatte et al., 1993; Powell et al., 1993; Kelm et al., 1994). For example, sialylation of GlyCAM-1 is required for L-selectin binding (Rosen et al., 1989; Imai et al., 1991; Berg et al., 1992; Foxall et al., 1992). Treatment of GlyCAM-1 with NEURase from either *Arthrobacter ureafaciens* or Newcastle Disease virus (selective for sialic acid α2-3 linkage; Paulson et al., 1979) significantly decreases L-selectin binding activity (Imai et al., 1992). On the other hand, we found that, while much of the acidic nature of mZP3 is attributable to sialic acid (Fig. 3), removal of sialic acid does not significantly affect mZP3 bioactivity. This is consistent with results of previous experiments with gp55, a proteolytic fragment of mZP3, whose sperm receptor and acrosome reaction-inducing activities are not affected by NEURase treatment (Rosiere and Wassarman, 1990; Litscher and Wassarman, 1996a).

Overall, these observations strongly suggest that neither sulfate nor sialic acid present on mZP3 oligosaccharides is directly involved in sperm receptor or acrosome reaction-inducing activity. This conclusion is consistent with results of recent experiments demonstrating that certain synthetic oligosaccharide constructs of defined structure, at relatively low concentrations (~10 μM), inhibit binding of mouse sperm to eggs in vitro (Litscher et al., 1995). The inhibitory oligosaccharide constructs lack both sulfate and sialic acid, but carry galactose in either α- or β-linkage at their nonreducing-end. Such findings support the proposal that bioactive mZP3 O-linked oligosaccharides possess galactose at their nonreducing-end and that the sugar is essential for sperm receptor activity in vitro (Bleil and Wassarman, 1988; Wassarman, 1989). On the other hand, it should be noted that mice reproduce normally following disruption of their α-1,3-galactosyltransferase gene by targeted mutagenesis in embryonic stem (ES) cells (Thall et al., 1995). At first glance, this result indicates that a terminal α-galactose on mZP3 oligosaccharides is not required for fertilization to occur in vivo. Unfortunately, in this study, mZP3 was not purified from the null mutation mice and assayed for receptor activity in vitro. Furthermore, the bioactive mZP3 O-linked oligosaccharides were not analyzed to determine the nature of the sugar at the nonreducing-end. In mice lacking α-1,3-galactosyltransferase, it is possible that changes in mZP3 O-linked oligosaccharide structure could occur, beyond elimination of terminal α-galactose residues, without compromising fertilization in vivo. Further structural studies of O-linked oligosaccharides on mZP3 purified from α-galactosyltransferase homozygous null mutation mice should resolve some of these issues.

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