Polysialic acid is present in mammalian semen as a posttranslational modification of NCAM and the polysialyltransferase ST8SiaII

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Running title: Polysialylated form of ST8SiaII and NCAM in mammalian semen

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Keywords: Polysialic acid; mammalian sperm; Polysialyltransferase, ST8SiaII, NCAM

Background: Polysialylated glycoproteins play an important role during numerous biological processes.

Results: Polysialylated ST8SiaII and NCAM are components of mammalian semen and are partially associated to spermatozoa.

Conclusion: Polysialic acid represents a further glyco-motif in mammalian ejaculates, known to influence the immune system.

Significance: Administration of polysialic acid during insemination might be useful to increase the number of spermatozoa escaping the female immune system.

SUMMARY

Fertilization in animals is a complex sequence of several biochemical events beginning with the insemination into the female reproductive tract and, finally, leading to embryogenesis. Studies by Kitajima and co-workers demonstrated the presence of polysialic acid (polySia) on sea urchin sperms. Based on these results, we became interested in the potential involvement of sialic acid polymers in mammalian fertilization. Therefore, we isolated human sperms and performed analyses including Western blotting and mild DMB-HPLC that revealed the presence of α2,8-linked polySia chains. Further analysis by a glyco-proteomics approach leads to the identification of two polySia-carriers. Interestingly, besides NCAM also the polysialyltransferase ST8SiaII has been found to be a target for polysialylation. Further analysis of testis and epididymis tissue sections demonstrated that only epithelial cells of the caput were polySia positive. During the epididymal transit polySia-carriers were partially integrated into the sperm membrane of the postacrosomal region. Since polySia is known to counteract histone- as well as neutrophil extracellular trap (NET)-mediated cytotoxicity against host cells, which plays a role after insemination, we propose that polySia in semen represents a cytoprotective element to increase the number of vital sperms.

In vertebrates, the highly negatively charged carbohydrate polysialic acid (polySia) is known to influence e.g. the regulation of cell-cell contact and repulsion (1). In mammals, polySia consists of α2,8-linked N-acetylneuraminic acid (Neu5Ac) residues and chain length of these polymers can exceed 60 sialic acid residues (2-6). While most extracellular glycoproteins are modified by monosialyl residues, polysialylation is restricted to a small number of N- and O-glycosylated
proteins. The best characterized target for polysialylation is the neural cell adhesion molecule NCAM (7). Here, glycans at the fifth and sixth N-glycosylation site can be posttranslationally modified with sialic acid polymers (8-11). The modification of NCAM with polySia creates a bulky and highly negatively charged moiety, which leads to an inhibition of the homophilic trans as well as cis interaction between NCAM molecules modulating the adhesive properties of eukaryotic cells (12-17).

More recently, four additional polysialylated glycoproteins have been identified: (I) a not in detail specified α-subunit of a voltage-gated sodium channel in adult rat brain (18), (II) a soluble form of CD36 in murine and human milk (19), (III) neuropilin-2 on human mature dendritic cells (mDC) (20) as well as (IV) the synaptic cell adhesion molecule SynCAM1 in postnatal murine brain (21).

In mammals, the generation of polySia depends on the presence of the α2,8-polysialyltransferases ST8SiaII and ST8SiaIV. Deletion of both enzymes in mice leads to a mortal phenotype since polySia is involved in the development of several essential organs like the brain, heart, kidney, pancreas and the respiratory tract (22-25). Interestingly, both polysialyltransferases are able to polysialylate their N-glycans in cell culture experiments (26-28). However, such an autopolysialylation has so far not reported in vivo.

PolySia is not only expressed in vertebrates but also in other clades (29) like sea urchins (30). In contrast to vertebrates, however, also α2,9- and α2,5O-glycolyl-linked polySia exist in addition to the α2,8-linked form. Kitajima and co-workers could show that polySia mediates important functions of sea urchin sperms. For instance, α2,9-linked polySia is suggested to influence the motility of sea urchin sperms (31) and α2,5O-glycolyl-linked polySia is discussed to potentiate the acrosome reaction (30). The function of α2,8-linked polySia has not been studied to date.

Since benefiting organs are often independently developed in different species during evolution (e.g. eye of vertebrates and sepia) we investigated, in the present study, mammalian semen for the existence of polysialylated glycoproteins. Our data reveal the presence of polysialylated ST8SiaII besides polysialylated NCAM in mammalian semen. Thus, polySia carriers may influence processes localized in the female reproductive tract.

**EXPERIMENTAL PROCEDURES**

*Materials* — NCAM-specific monoclonal antibody (mAb) 123C3 (32,33) and polySia-specific mAb 735 (33) as well as inactive and active endoneuraminidase (endoN) were purified as described previously (34,35). MAbs against human ST8SiaII and ST8SiaIV were purchased from Sigma-Aldrich (Taufkirchen, Germany).

*Separation of vital sperms* — For enrichment of vital human sperms a swim-up procedure was applied. For this purpose, 1 mL native ejaculate was stacked under 5 mL TALP medium (2 mM CaCl₂, 3.1 mM KCl, 0.4 mM MgCl₂, 100 mM NaCl, 25 mM NaHCO₃, 0.3 mM NaH₂PO₄, 1mM sodium pyruvate, 10 mM HEPES, 21.6 mM sodium lactate, 20% fetal bovine serum (v/v)). After incubation at 37°C and 5% CO₂ for 60 min, 3 mL of the supernatant of each well were isolated and centrifuged for 10 minutes at 700 g. Enriched mobile sperms were used for further analyses.

*Protein extraction and isolation of polysialylated proteins* — Whole ejaculate was diluted in water and homogenized. Precipitation of proteins was performed by adding four parts acetone of -20°C per part sample. After incubation at -20°C over night, protein pellet was isolated by centrifugation and lyophilized to dryness.

Alternatively, polysialylated proteins were isolated from enriched vital sperm by affinity precipitation. To this end, samples were homogenized in lysis buffer (50 mM Tris/HCl (pH 8.0), 5 mM EDTA, 150 mM NaCl, 1% TritonX-100 (w/v), 0.5 % sodium deoxycholate (w/v), 1 mM PMSF, 1 mM Aprotinin and 1 mM Leupeptin) on ice. Homogenized samples were incubated for 2 hours at 4°C on a shaker and centrifuged for 1 hour at 4°C. For affinity precipitation, inactivated endoN was coupled to tosylactivated magnetic dynabeads® M-280 (Invitrogen, Darmstadt, Germany) according to the manufacturer’s instructions and incubated with the supernatant over night at 4°C on a shaker. Subsequently, beads were washed twice each with 1 mL washing buffer 1 (20 mM
Tris/HCl (pH 8.0), 150 mM NaCl, 0.5% Triton-X 100 (w/v)) and washing buffer 2 (20 mM Tris/HCl (pH 8.0), 150 mM NaCl). Polysialylated proteins were disconnected from magnetic beads with elution buffer (100 mM triethylamine, 150 mM NaCl) and dried down in a vacuum concentrator.

HPLC analysis of sialic acid polymers – For detection of internal, α2-8-linked sialic acid residues, the C7/C9 method described by Sato et al. (36) was applied. After oxidation, reduction and fluorescence labeling resulting DMB-derivatives were analyzed on a Superspher 100 C-18 column (250 × 40 mm, Merck-Hitachi, Darmstadt, Germany) at 40°C using a Merck-Hitachi HPLC system (37). Mobile phases methanol / acetonitrile / water / trifluoroacetic acid (TFA) (4:4:92:0.1) (M1) and methanol / acetonitrile / water / TFA (45:45:10:0.1) (M2) were used for separation of DMB-labeled sialic acids. A linear gradient was applied from 0% to 20% M2 in 35 min at a flow rate of 0.3 mL/min.

The degree of polymerization (DP) of polySia chains was analyzed by DMB-HPLC analysis (38,39). To this end, purified polySia-carriers were dissolved in 80 µl DMB reaction buffer, and incubated for 24 h at 4°C. The reaction was stopped by adding 20 µl 1 mM NaOH, and released polySia chains were separated on a DNAPac PA-100 column (Dionex, Idstein, Germany) by HPLC (37). MilliQ water (E1) and 1 M NaNO3 (E2) were used as eluents at a flow rate of 1 mL/min. Elution was performed by the following gradient: T0 min = 0% E2; T5 min = 1% E2; T15 min = 10% E2; and T60 min = 50% E2.

SDS-PAGE and Western Blotting – Total protein lysates as well as purified polysialylated proteins were separated by 10% SDS-PAGE under reducing conditions and, subsequently, transferred onto a PVDF membrane. Binding of anti-polySia, anti-NCAM as well as anti-ST8SiaII and ST8SiaIV mAbs was detected by use of horseradish peroxidise-conjugated secondary antibodies (Dako, Hamburg, Germany) and chemiluminescence SuperSignal kit (Thermo Fisher, Kehl, Germany). Prior to SDS-PAGE, a part of the samples were treated with endoN or peptide-N-glycosidase F (PNGase F). For the release of N-glycans immunoprecipitates were resuspended in 40 mM DTT, 0.5% SDS and boiled for 10 min. After adjusting the sample buffer to the final concentration of 50 mM sodium phosphate, pH 7.5, 40 mM DTT, 0.5% SDS (w/v), and 1% NP-40 (v/v), precipitates were incubated with PNGaseF (50 U/ml) (Roche Applied Science, Mannheim, Germany) for 16 h at 37°C. EndoN digest (1 µg/ml) was performed in lysis buffer for 16 h at 37°C.

Glycoproteomics approach – Eluted polysialylated proteins were separated by electrophoresis on 10% ready-to-use SDS gels (Bio-Rad, Munich, Germany), followed by in-gel digestion with trypsin (Promega, Mannheim, Germany) as described in detail previously (21). Extracted peptides were separated on a C18 column (PepMap, 3 µm, 75 µm×100 mm, Dionex) using an Ultimate nanoLC system (Dionex) with a linear gradient from 10% acetonitrile/0.1% formic acid to 60% acetonitrile/0.1% formic acid. Peptides were directly spotted by a Probot (Dionex) onto a matrix-assisted laser desorption / ionization time-of-flight (MALDI-TOF) steel target (Bruker Daltonics, Bremen, Germany) and mixed with an equal volume of 2,5-dihydrobenzoic acid (DHB) matrix (7.5 mg DHB/ml, 1% phosphoric acid, 50% acetonitrile). Peptide mass fingerprints of tryptic digests were generated by MALDI-TOF-MS using an Ultraflex I TOF/TOF mass spectrometer (Bruker Daltonics). MS and MS/MS spectra were acquired in positive reflector mode using FlexControl 2.4 software, and analyzed by the FlexAnalysis software 3.0 (both Bruker Daltonics). External calibration of mass spectra was carried out using peptide calibration standard for MS (Bruker-Daltonics), and annotations of fragment ions in the MS/MS mode was performed according to (40).

Immunohistochemistry / Immunofluorescence – Paraffin embedded testis and epididymis tissue (formalin fixed) sections were cut into 5 µm serial sections. After rehydration in xylen and a following ascending ethanol series, sections were incubated with blocking solution for 5 min, followed by incubation with the primary antibody mAb 735 (10 µg/ml PBS containing 2% (w/v) bovine serum albumin (BSA)) overnight at 4°C. As negative control for the polySia-staining, before incubation with mAb 735 tissue sections were pretreated with endoN (3 µg/ml in PBS containing 0.1% BSA).
overnight at 37°C. For staining the Envision⁺ System HRP Kit (Dako) was used. The stained sections were counterstained with hemalaun (Roth, Karlsruhe, Germany).

For immunofluorescence, sperms were washed with PBS/0.1% BSA pH 7.4 and centrifuged at 700 × g by discarding the supernatant. Purified sperms were fixed in PBS pH 7.4 containing 2% formaldehyde (v/v) for 30 minutes at 22°C. After fixation, sperms were washed with PBS/0.1% BSA. For negative control of the polySia staining as well as the staining against NCAM and ST8SiaII sperms were pre-treated with endoN (3 µg/ml in PBS/0.1% BSA) overnight at 37°C. Primary antibodies were incubated overnight at 4 °C. For the visualization of the acrosome biotinylated peanut agglutinin (PNA) (20 µg/ml in PBS/0.1% BSA) (Vector Laboratories Burlingame, CA) was used. FITC (fluoresceinisothiocyanate) or Rhodamin (Dianova, Hamburg, Germany) conjugated secondary antibodies against mouse IgG and biotin were used for visualization of the primary antibodies. All images were taken with a Leica DMR Microscope and processed by MetaMorph® Microscopy Automation & Image Analysis Software ©2012 Molecular Devices, LLC.

RESULTS

Detection of polySia in human semen. For the detection of internal sialic acid residues in human semen proteins thereof were subjected to C7/C9 analysis after acetone precipitation. To this end, all terminal sialic acid molecules were transformed into C7 residues by periodate oxidation. Due to the α2,8-linkage, internal sialic acid residues are protected against oxidation because no vicinal hydroxyl groups exist, which are necessary for oxidation. In a last step, all sialic acid residues were released under acidic conditions and labeled with DMB. Resulting C9-Neu5Ac-DMB were separated by RP-HPLC (Figure 1A). The detection of C9-Neu5Ac-DMB after periodate oxidation indicated the presence of internal α2,8-linked sialic acid residues linked to proteins of human semen. However, the analysis of di-, tri- and oligomers of Neu5Ac might also lead to comparable chromatograms. Hence, detection of internal sialic acid residues is not an unambiguous proof for the presence of polySia.

For this reason, the chain length was analyzed in more detail. Therefore, polySia chains were cleaved off the glycans under mild acidic conditions and tagged directly with DMB at the reducing end. Fluorescently labeled chains were separated by anion exchange chromatography according to the DP (3,41,42). As shown in figure 1B, obtained chromatographic profiles show that polySia chains can comprise more than 40 sialic acid residues.

In addition to the chemical detection of polySia, we performed a dot blot analysis with a mAb against α2,8-linked polySia (Figure 1C). The protein fraction of human ejaculate revealed a strong polySia signal, whereas the immune staining was abolished after the degradation of polySia by endoN. Taken together, the chemical and immunological analyses of human semen disclosed the existence of α2,8-linked polySia.
chains, which can reach a length of more than 40 sialic acid residues.

ST8SiaII and NCAM are polysialylated. For identification of the polysialylated proteins rat epididymis was used storing maturated spermatozoa. At first polySia-carriers were purified from epididymal lysates using magnetic beads, which were coated with enzymatically inactive endoN. EndoN contains an extended binding site for polySia and targeted mutation of active site residues gives rise to an inactive form that works as an efficient polySia-specific lectin (35,43). Purified polySia-carriers were subjected to Western blotting against polySia. As shown in Figure 2A polySia-immunostaining displayed a typical diffuse band for polysialylated proteins in a region between 100 - 200 kDa. To generate peptide mass fingerprints of the polysialylated glycoproteins a SDS-PAGE was performed and two gel slices in the area of the immune signal against polySia were used for tryptic in gel digest. Resulting peptides were extracted, separated by RP-nanoLC and directly spotted onto a MALDI-TOF-MS carrier for MS(MS)-analysis. Resulting mass spectra were utilized for data base search leading to the identification of ST8SiaII and NCAM (Figure 2B). The findings were confirmed by additional fragmentation analyses of selected peptides (Suppl. 1).

After MS/(MS) based identification of ST8SiaII and NCAM in rat epididymal tissue, their presence in human semen was approved by Western blotting. Purification of the polySia-carriers with magnetic endoN-beads and immunostaining against ST8SiaII as well as NCAM were employed. Whereas the polysialylated forms of both glycoproteins were hardly (NCAM) or not (ST8SiaII) detectable, the underlying proteins NCAM-140 and ST8SiaII could be visualized after removal of polySia by endoN treatment (Figure 3). The poor visibility of the polysialylated forms of NCAM and ST8SiaII can be explained by the bulky and highly negative properties of polySia. The antibody-protein binding might be similarly inhibited as the NCAM-NCAM interaction.

PolySia carriers are detectable in the postacrosomal region of mammalian sperms. Formalin fixed human, bovine and rat sperms were used for the visualization of polySia. The immunostaining displayed a polySia positive area on the surface of the head (Figure 5A and B). However, more than 90% of all sperms were polySia negative in all analyzed species. When sperms were pretreated with endoN, no polySia positive sperms were detectable. Immunostaining with mAb against NCAM and ST8SiaII using human sperms provided comparable results (Figure 5C and D). For the detection of the protein backbone of both polySia carriers a degradation of polySia with endoN was necessary.

For a more detailed localization of the polySia-carriers the acrosome was visualized using the lectin PNA demonstrating that the polysialylated proteins were located in the postacrosomal area of the head as exemplary shown for human and bovine sperms (Figure 5E).
5E). In the case of human sperms, polysialylated proteins were primarily present on a part of the postacrosomal area close to the tail. In contrast to human sperms, the complete bovine postacrosomal area was polySia positive. Comparable results were obtained with rat sperms (data not shown).

**Epithelial cells of the epididymis express polysialylated proteins.** Spermatogenesis takes place in the testis resulting in the generation and release of morphologically differentiated spermatids (44). However, these are neither motile nor capable to fertilize. Therefore, maturation of sperms in the epididymis is essential, which comes along, amongst others, with a change of the cell surface distribution of glycoconjugates. Consequently, it is possible that polySia is present on the surface of all sperms when they leave the testis and that during the maturation in the epididymis the sialic acid polymers are degraded. A second possibility is that polysialylated NCAM and ST8SiaII reach the postacrosomal region during the transfer through the epididymis. Many essential proteins are integrated into the plasma membrane of sperms during the epididymal transit, which are generated and released in so-called epididymosomes by epithelial cells (45). To address this point, paraffin embedded tissue sections of testis as well as epididymis from mice were examined for polySia. In tubuli of the testis no specific immune signal against polySia occurred including spermatogonia, spermatocytes, spermatids and sertoli cells (Figure 6A). In contrast, a vesicular staining against polySia was visible in epithelial cells of epididymis (Figure 6B). The polySia expressing epithelial cells were located in the caput of the epididymis. Comparable results were obtained when rat and roebuck epididymis were analyzed (data not shown). Furthermore, gene transcripts of polysialyltransferases ST8SiaII and ST8SiaIV were analyzed by RT-PCR demonstrating expression of ST8SiaIV in addition to the targets for polysialylation ST8SiaII and NCAM in the epididymis (Suppl. 2). The signal intensity of ST8SiaIV was comparable with the signal intensity of NCAM. However, expression level of ST8SiaII was lower than the obtained signal for ST8SiaIV and NCAM gene transcripts.

**DISCUSSION**

In adult mammals the carbohydrate polymer polySia is mainly expressed in neuronal tissue (1,22-24). However, more and more reports associate polysialylated glycoconjugates also with other physiological systems (20,46-49). Since different polySia species contribute to the sperm motility and the acrosome reaction of sea urchin sperms (30), we investigated human ejaculates for the presence of polysialylated proteins. Thereby, α2,8-linked polySia could be detected in human semen attached to two different glycoproteins. One of these polySia-carriers was NCAM. Surprisingly, the second identified target for polysialylation was the polysialyltransferase ST8SiaII. More than sixteen years ago, autopolyosialylation of polysialyltransferases was first observed in vitro and later also in cell culture experiments (26,28,50,51). The observed autopolyosialylation occurred only in the absence of NCAM and was never observed in vivo so far. Consequently, polysialylation of ST8SiaII and ST8SiaIV was often declared as a biochemical phenomenon, which takes place only under artificial conditions. However, by identifying ST8SiaII as polySia carrier in mammalian semen, we now demonstrate for the first time that polysialylation of polysialyltransferases occurs also in vivo. Besides polysialylated NCAM and ST8SiaII no further polysialylated glycoproteins could be identified although. Nevertheless, a proteomics approach can never completely assure that all proteins were identified.

In agreement with in cellulo as well as in vitro experiments concerning the polysialylated glycosylation sites of ST8SiaII, we detected polySia chains on N-glycans of glycosylation site 5 in vivo (52). In addition to this glycosylation site, N-glycans of the third glycosylation were discussed to be a target for autopolyosialylation. However, we never observed a peptide signal corresponding to de-N-glycosylated glycosylation site 3.

Interestingly, polysialylated NCAM and ST8SiaII were present in the postacrosomal region of mammalian sperms. To determine the origin of polySia, tissue sections of testis and epididymis were investigated demonstrating that polySia was expressed by epithelial cells of the caput. It is well known, that epithelial cells in all parts of the epididymis release so-called
epididymosomes containing a number of different glycoproteins, which are integrated into the membrane of sperms during maturation (53-56). Epididymosomes contain, e.g., proteins, which are necessary to prevent a killing of sperms by the innate and adaptive immune system of females. Many studies indicated that insemination leads to a recruitment of immune cells (57-59). Mammalian seminal plasma and sperms contain different components to counteract this activation of the female immune system. For example, N-glycans in semen containing the Lewis$^x$ and Lewis$^y$ epitope are discussed to inhibit the innate and adaptive immune system of women (60). Intriguingly, α$_{2,8}$-linked polySia influences also the immune system. For example, tissue macrophages express siglec-11 that is capable to bind α$_{2,8}$-linked polySia (61). In this way polySia suppresses the immune response of tissue macrophages in the brain (microglia) after lipopolysaccharide (LPS) stimulation (62). Thus, secreted and partially integrated polySia-carriers may also contribute to the immune suppression.

The main population of invading immune cells after insemination consists of neutrophils. One mechanism of neutrophils against invading microorganisms is the formation of neutrophil extracellular traps (NET). Thereby, neutrophils sacrifice themselves and histone containing DNA forms together with anti-microbial enzymes and peptides a meshwork to capture and kill pathogens (63,64). After insemination neutrophils generate NET and sperms entangle in this network of DNA and histones (65). DNase of the seminal plasma, however, degrades DNA and spermatozoa can escape from this system. Interestingly, high levels of DNase in seminal plasma have been associated with higher fertility in bovine systems (66,67). The most cytotoxic entities of NET for endogenous cells, however, are the extracellular histones (68). Consequently, sperms and epithelial cells would be negatively influenced by exaggerated NET formation and the formation of nucleosomes after degradation of DNA. Recently, we could show that α$_{2,8}$-linked polySia of bacteria and humans can diminish the cytotoxicity of extracellular histones as well as nucleosomes and, therefore, may represent a cytoprotective component (68-70). Thus, polySia attached to NCAM and ST8SiaII as a content of mammalian ejaculates may act in the same manner in the female reproductive tract increasing the number of surviving sperms as illustrated in Figure 7. In terms of declining semen quality in men (71,72) administration of polySia during insemination could represent a useful strategy to increase number of sperms, which are able to escape the innate immune system of women.

In summary, we were able to show that polysialylated ST8SiaII as well as NCAM are partially integrated in the postacrosomal region of a subfraction of sperms. Based on previous findings, these polySia carriers may represent a cytoprotective and/or immune modulatory element of mammalian ejaculates. However, the precise role of polySia and, particularly, the reason for the polysialylation of ST8SiaII in addition to NCAM needs to be further investigated.
REFERENCES


The abbreviations used are: DMB, 1,2-diamino-4,5-methylenedioxybenzene; endoN, endoneuraminidase; mAb, monoclonal antibody; LPS, lipopolysaccharide; Neu5Ac, N-acetylneuraminic acid; NCAM, neural cell adhesion molecule; NET, neutrophil extracellular traps; peptide-N-glycosidase F, PNGase F; polySia, polysialic acid; SynCAM, synaptic cell adhesion molecule.

Acknowledgments – We thank Christian Galuska for critical remarks on the manuscript and Sandra Frank for the preparation of figures as well as proof reading. In addition, we acknowledge Christina Ulm and Caroline Feuerstacke to train the involved medical doctoral candidates and Werner Mink as well as Siegfried Kühnhardt for expert technical assistance. This work was supported by a grant of the Deutsche Forschungsgemeinschaft to SPG (GA 1755/1-1).
**Fig. 1. Detection of polySia in human semen.** (A) Terminal and internal sialic acid residues of the protein fraction of human semen were visualized by the C7/C9 method. After subsequent metaperiodate oxidation, reduction, hydrolysis and DMB labeling C7-Neu5Ac-DMB and C9-Neu5Ac-DMB were separated by RP-HPLC. Internal sialic acid residues resulted in the detection of C9-Neu5Ac-DMB, whereas terminal ones are detected as C7-Neu5Ac-DMB. (B) For characterization of the polySia chain length, protein fraction of whole ejaculate was subjected to the mild DMB method. DMB labeled sialic acid polymers were separated by anion exchange chromatography according to the chain length. Respective numbers of sialic acid residues are given for single peaks on top of the profiles. (C) Proteins were transferred to a PVDF membrane and polySia was visualized with the mAb 735. For negative control polySia was degraded with endoN.

**Fig. 2. Identification of ST8SiaII and NCAM as polySia carriers.** (A) Polysialylated proteins were purified from rat epididymis lysates using magnetic beads, which were coated with inactive endoN and separated by SDS-PAGE. Two gel slices (see labeling) were cut for tryptic in gel digest. After reduction, carboxymethylation and treatment with trypsin resulting peptides were extracted, separated by nano-LC and directly spotted onto a MALDI-TOF-MS target for MS analysis. Database search (mascot) revealed NCAM and ST8SiaII as polySia carriers with significant scores (inset). Identified peptides of NCAM and ST8SiaII are printed in bold. Signals representing tryptic peptides of trypsin, keratin, actin, myosin, and spectrin were detected in consequence of sample preparation and were not labeled.

**Fig. 3. Detection of polysialylated NCAM and ST8SiaII using human sperms.** For Western blotting against (A) NCAM and (B) ST8SiaII polysialylated proteins were purified from mobile human sperm lysates using magnetic beads, which were coated with inactive endoN. Western blot analyses were performed with or without previous endoN treatment. Apparent molecular masses of standard proteins are indicated in kDa.

**Fig. 4. Determination of the polysialylated N-glycosylation site of ST8SiaII.** (A) Purified polySia-carriers of rat epididymis were separated by SDS-PAGE. Western blot analysis was performed using anti-polySia mAb 735 with or without previous endoN or PNGaseF treatment. Apparent molecular masses of standard proteins are indicated in kDa. (B) MALDI-TOF mass spectra of precipitated polysialylated glycopeptides before (+) or after (-) PNGaseF treatment and nanoLC separation. Monoisotopic masses of the pseudomolecular ions [M+H]+ are given. (C) Fragmentation analysis of the de-N-glycosylated peptide by MALDI-TOF-MS/MS. Sequence-specific ions are labeled according to previous studies (44, 45). The conversion of Asn to Asp is illustrated in bold. (D) Assignment of a polySia chain to individual N-glycosylation sites of ST8SiaII.

**Fig. 5. Localization of polySia, NCAM and ST8SiaII on sperms.** (A) Major areas of a sperm. (B) Formalin fixed human, bovine and rat sperms were stained with an antibody against polySia. Human sperms were used to locate (C) NCAM and (D) ST8SiaII. (E) Acrosomes were visualized with PNA.
in addition to NCAM and polySia positive areas using respective antibodies. Staining of nuclei (blue fluorescence) was achieved by DAPI.

**Fig. 6.** Immunohistological localization of polySia in testicular and epididymal tissue. (A) Paraffin embedded serial (A) testis and (B) epididymis sections (caput) of mice were stained with a mAb against polySia. For negative control tissue sectioned were pretreated with endoN to degrade polySia. Scale bars = 20 µm.

**Fig. 7.** Proposed model for a putative cytoprotective function of polySia attached to ST8SiaII and NCAM. (A) After insemination neutrophils are activated and NET formation is induced. (B) DNase of semen degrades DNA and (C) cytotoxic nucleosomes are formed. (D) PolySia chains of NCAM and ST8SiaII localized on sperms as well as epididymosomes bind nucleosomes counteracting nucleosomes mediated cytotoxicity.
Figure 1

A

C9-Neu5Ac-DMB

C7-Neu5Ac-DMB

Time [min]

B

Fluorescence

Fluorescence

Time [min]

C

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- Figure 1A shows a chromatogram with peaks labeled C9-Neu5Ac-DMB and C7-Neu5Ac-DMB.
- Figure 1B illustrates the fluorescence over time, with inset figures highlighting specific time points.
- Figure 1C presents a table with columns labeled - and +, indicating the effect of EndoN.
Figure 2

A

WB: anti-polySia

- 170 kDa
- 130 kDa
- 72 kDa
- 55 kDa

B

NCAM

ST8Sia II

Intensity

m/z

800 1300 1800 2300 2800

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Figure 3

A  WB: anti-NCAM

-  +  endoN

- 250 kDa
- 130 kDa
- 95 kDa
- 72 kDa
- 55 kDa

B  WB: anti-ST8SiaII

-  +  endoN

- 250 kDa
- 130 kDa
- 95 kDa
- 72 kDa
- 55 kDa
Figure 4
Figure 5
Figure 6

A  Testis
   - EndoN
   + EndoN

B  Epididymis (caput)
   - EndoN
   + EndoN

- PolySia
- Epithelial cells
- Lumen
Figure 7

A: NET formation after insemination

B: DNase of semen

C: Formation of nucleosomes

D: Compensation of nucleosome cytotoxicity

Epididymosome and sperm associated polysialylated NCAM and ST8SiaII

Binding of nucleosomes
Polysialic acid is present in mammalian semen as a posttranslational modification of NCAM and the polysialyltransferase ST8SiaII

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J. Biol. Chem. published online May 13, 2013

Access the most updated version of this article at doi: 10.1074/jbc.M113.451112

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