Synthesis and glycosylation of CD52, the major 'maturation-associated' antigen of rat spermatozoa, in the cauda epididymidis

P. Derr¹, C. H. Yeung², T. G. Cooper² and C. Kirchhoff^{1*}

¹IHF Institute for Hormone and Fertility Research at the University of Hamburg, Germany;
and ²Institute of Reproductive Medicine of the University Münster, Germany

A western and lectin blot analysis was performed of the major 'maturation-associated' antigen of rat spermatozoa, which is the rat counterpart of human CD52. In the absence of a suitable antibody, direct study of this approximately 26 kDa antigen, named previously SMemG, had been difficult. In the present study, these problems were overcome by raising a polyclonal antibody against a chemosynthetic peptide predicted from the cDNA sequence of the antigen. The antibody bound to a glycoprotein of rat cauda epididymidal tissue and spermatozoa, this glycoprotein was cleaved by phosphatidylinositol-specific phospholipase C and, after deglycosylation, was reduced to approximately 6 kDa. Northern blot analysis confirmed that the CD52 mRNA

was transcribed only post-testicularly, and antibody binding to testicular and sperm proteins of different molecular masses was shown to be nonspecific. Flow cytometry also indicated that the antigen was inserted into the sperm membrane during epididymal transit. Moreover, despite the presence of CD52 mRNA in all parts of the rat epididymis, only the 'long' mRNA molecules of the cauda region were efficiently translated and the antigen glycosylated, indicating that expression of rat CD52 is regulated on a post-transcriptional level. Lectin binding and deglycosylation studies supported the contention that there is extensive mucin-type O-glycosylation of rat CD52. In rats, there was no indication of complex N-linked carbohydrates similar to those described for human CD52.

Introduction

Post-testicular remodelling of the sperm glycocalyx occurs within the epididymis (for a review, see Yanagimachi, 1994), and a considerable number of changes seem to be due to the uptake by spermatozoa of glycoproteins from epididymal secretions (for a review, see Kirchhoff, 1998). As an alternative mechanism, it has been suggested that modifications of carbohydrates of pre-existing sperm glycoproteins from the testis occur during epididymal transit, possibly catalysed by a variety of extracellular carbohydrate-modifying enzyme activities in the epididymal fluid and on the sperm surface (for a review, see Tulsiani *et al.*, 1999). Rat CD52, previously named the 'major maturation-associated sperm membrane antigen' (Zeheb and Orr, 1984; Moore *et al.*, 1989), an approximately 26 kDa protein (Myles, 1993), and sperm membrane glycoprotein (SmemG) (Eccleston *et al.*, 1994) may be an example of such a glycoprotein. Rat CD52 was originally identified as the principal molecule labelled by surface carbohydrate-labelling experiments using intact mature spermatozoa from the cauda epididymidis (Olson and Hamilton, 1978; Jones *et al.*, 1981; Brown *et al.*, 1983; Zeheb and Orr, 1984; Hamilton *et al.*, 1986; Olson *et al.*, 1987; Moore *et al.*, 1989; Eccleston *et al.*, 1994). Furthermore,

carbohydrate labelling of rat sperm glycoproteins on western blots *in vitro* (Pera *et al.*, 1997) showed an unexpectedly high selectivity: the only molecule accessible to the periodate oxidation reaction was the counterpart of CD52. Biochemical studies revealed that > 60% of this molecule consisted of carbohydrate (Eccleston *et al.*, 1994).

Other studies have indicated that rat CD52 may undergo extracellular carbohydrate modification during epididymal transit, which explains its post-testicular disclosure on the sperm surface (for a review, see Myles, 1993). As a glycosylphosphatidyl-inositol (GPI)-anchored integral sperm membrane protein, it was initially assumed to be a pre-existing component originating at spermatogenesis and that its carbohydrate moiety underwent extensive modification only at a later stage within the epididymal duct (Hamilton *et al.*, 1986; Moore *et al.*, 1989; Eccleston *et al.*, 1994; for a review, see Myles, 1993). However, molecular cloning and *in situ* transcript hybridization proved that the CD52 mRNA is synthesized by the epididymal duct epithelium (Kirchhoff, 1994, 1996). This result indicates that uptake of the lipid-anchored antigen by spermatozoa was one of the major events occurring to sperm plasma membrane during epididymal transit (Kirchhoff and Hale, 1996). In several mammalian species, including humans, it has been shown that CD52 mRNA is produced only post-testicularly, and not by spermatogenic cells within the testis (Kirchhoff *et al.*, 1993; Krull *et al.*, 1993; Pera *et al.*, 1994; Kirchhoff, 1994, 1996). The glycopeptide is produced by the epididymal

*Correspondence
Email: kirchhoff@ihf.de

Fig. 1. Characterization of polyclonal anti-CD52 antibody specificity. (a) Putative structure of rat CD52 as predicted from its cDNA. The mature peptide sequence is arranged vertically. A 12-mer peptide sequence chosen for antibody production is boxed; putative O-glycosylation sites are shown by the open symbols; N-terminal and C-terminal signal peptides are arranged horizontally. A standard

duct epithelium and transferred to the sperm membrane with its lipid anchor intact (Yeung *et al.*, 1997).

Another important feature of the expression of epididymal CD52 is its regional specificity: maximum mRNA content is found in the distal part of the epididymis in various mammalian species (Krull *et al.*, 1993; Pera *et al.*, 1994). Furthermore, in the rat epididymis there is a region-specific variation in the length of the CD52 mRNA poly(A) tail (Pera *et al.*, 1997). Although 'short' CD52 mRNA was present in all parts of the epididymis, only the cauda epididymidis contained 'long' CD52 mRNA carrying an extended poly(A) tail. This finding indicated that regulation of the expression of CD52 in rats occurs on a post-transcriptional level; however, this contention was difficult to prove in the absence of a suitable antibody (Pera *et al.*, 1997). Moreover, carbohydrate modification of rat CD52 during epididymal transit could not be excluded (Pera *et al.*, 1997). Therefore, a reinvestigation of rat epididymal CD52 synthesis and glycosylation was undertaken using an antipeptide antiserum.

Materials and Methods

Materials, tissues and animal experiments

Chemicals were obtained from Sigma Deisenhofen, Germany, unless otherwise stated. Sprague-Dawley male rats (Charles River, 408 $\pm$ 28 g body weight) were housed under a 12 h light : 12 h dark photoperiod, and had access to rat chow (Altromin GmbH, Lage) and water *ad libitum*. Animal experiments were performed as described by Pera *et al.* (1997). Briefly, for various types of surgery animals were anaesthetized by injection of 20 mg sodium pentobarbital kg⁻¹ (Nembutal) or inhalation of halothane (Hoechst, Frankfurt/Main) and surgery was performed through a mid-line abdominal incision for bilateral castration, efferent duct ligation, and the establishment of unilateral cryptepididymis, combined with either bilateral castration or dissection of efferent ducts as described by Pera *et al.* (1997). After 2 weeks, the animals were killed by CO₂ asphyxiation and the testes and epididymides were removed immediately. Epididymides were cut into caput, corpus and cauda epididymides. Control tissues were taken from untreated animals. Tissues were shock-frozen in liquid nitrogen and stored at -80°C.

Collection of epididymal spermatozoa and fluid for protein extraction

Spermatozoa and fluid from the cauda epididymides were collected by cannulation of the proximal vas deferens

with a 27-gauge needle connected to a polyvinyl chloride (PVC) catheter and retrograde perfusion of the tubule using PBS. The PVC catheters had been pulled out over a flame such that they tapered at one end to an outer diameter small enough to fit the size of the lumen of the vas deferens, and were tied in place by a 7/0 silk ligature. Luminal contents were flushed out gradually and were drawn into a positive displacement pipette as they exuded from the cut end of the tubule at the proximal cauda epididymidis. Spermatozoa and fluid from the cauda epididymidis were collected from lobules of mid-caput tubule by a similar technique using pulled-out PVC catheters with fine tips for insertion into the caput tubule. The luminal contents collected were dispersed into 0.1 or 0.5 ml PBS (for caput and cauda epididymidis, respectively) and the diluted fluid separated from the spermatozoa by centrifugation at 4000 *g* for 1 min and subsequently at 12 000 *g* for 2 min. Sperm pellets were washed three times with PBS by centrifugation at 2000 *g* for 1 min. Sperm-free fluid and spermatozoa were stored at -80°C. Although the cannulation and perfusion method enabled collection of uncontaminated luminal fluid, the yield of spermatozoa from the caput epididymidis was very low as only a short length of caput tubule could be perfused at a time because of the build up of perfusion pressure in the highly convoluted tubule. Therefore, an alternative method was used to increase the number of spermatozoa collected from the caput epididymis. The whole capita were decapsulated and cleaned of blood vessels. Spermatozoa were released into PBS by coarse mincing and applying gentle pressure to the tubules. Tubule fragments were removed by centrifugation at 300 *g* for 10 s. Spermatozoa obtained in the supernatant were washed three times as described above. For the collection of testicular spermatozoa, efferent ducts were tied off close to the testes on both sides under halothane anaesthesia without causing damage to the vasculature. The rats were killed 20 h later by CO₂ asphyxiation and the turgid testes were removed. The fine tip of a 50 μ l glass capillary tube, made by drawing out one end over a flame, was inserted into the avascular zone of the extratesticular rete to allow spermatozoa and rete testis fluid to flow into the capillary tube.

Digestion of sperm proteins by phospholipase C

Epididymides were separated into proximal and distal parts, cut with iridectomy scissors and suspended in Dulbecco's PBS containing 0.2 mmol phenylmethanesulfonyl fluoride (PMSF) l⁻¹. Spermatozoa from the proximal part (named caput spermatozoa) and the distal part (named

phospholipase C-sensitive mammalian GPI anchor structure is proposed. (b) Specificity of antibody binding was shown by western blot analysis with antiserum of crude cauda epididymidal tissue protein extracts before (lane 1) and after (lane 2) competition with the chemosynthetic CD52 peptide. A major band is present at approximately 26 kDa, which is competed for by the peptide. (c) Phosphatidylinositol-specific phospholipase C (PI-specific PLC) digestion of the 26 kDa protein (arrow) as analysed by western blot analysis (lanes 1-3) and glycoconjugate labelling (lanes 4-6). Lanes 1 and 4: cauda epididymidal sperm protein extract; lanes 2 and 5: cauda epididymidal sperm extract incubated for 2 h at 37°C without PI-specific PLC; lanes 3 and 6: extract incubated for 2 h at 37°C with PI-specific PLC.

cauda spermatozoa) were collected and treated with phosphatidylinositol-specific phospholipase C (PI-specific PLC; Sigma; Type III) as described by Moore *et al.* (1989). Briefly, the number of spermatozoa was determined and the sample was divided into aliquots, each containing approximately 2×10^7 to 1×10^8 spermatozoa. Sperm samples were incubated with 20 U PI-specific PLC for 2 h at 37°C. Comparable sperm samples were incubated at 37°C without the enzyme as a control.

RNA extraction and northern blot analysis

RNA extraction and northern blot analysis were performed as described by Pera *et al.* (1996, 1997). Sequences of oligonucleotide primers used for the preparation of a DIG-labelled RB7/CD52 550 bp probe were 5'-AGTGGCCTGCAGACTGTCCT-3' and 5'-TTATTGTGCCCTAGCTGGGT-3'.

Western blot analysis, lectin binding and detection of immobilized glycoconjugates

A 12-mer oligopeptide (see Fig. 1) was chosen as immunogen and, after coupling to keyhole limpet haemocyanin (KLH), was used to produce a polyclonal antiserum in guinea-pigs (Pineda Antikörper Service, Berlin). The oligopeptide was used as an antigen to obtain affinity-purified antibodies. Protein extracts were prepared from pulverized epididymal tissue or washed spermatozoa as described by Pera *et al.* (1997), dispensed in equal aliquots and stored at -80°C. Methanol-chloroform (Folch) extracts of protein samples were prepared as described by Schröter *et al.* (1999). Samples of protein or Folch extract (2–10 µl), depending on the number of spermatozoa or protein content as determined with the Protein Assay Kit No.1 (Biorad, München), were loaded on to 15% (w/v) polyacrylamide Laemmli-gels, and the proteins separated and transferred to PVDF membranes (Amersham, Braunschweig) as described by Schröter *et al.* (1999). Anti-CD52 antiserum from guinea-pigs was applied at a dilution of 1:1000 and purified antibody at 1.85 mg ml⁻¹. Biotinylated *Arachis hypogaea* lectin (PNA), *Maackia amurensis* lectin (MAA), *Sambucus nigra* lectin (SNA), *Concanavalina ensiformis* lectin (Con A), *Datura stramonium* lectin (DSA) and *Phaseolus vulgaris* lectin (PHA-E) (all from Oxford GlycoSystems, Abingdon) were used at a dilution of 1 µg ml⁻¹. For carbohydrate labelling *in vitro*, membranes were processed as described by Pera *et al.* (1997). Primary binding was detected using peroxidase-coupled secondary antibodies (0.4 µg ml⁻¹). Alternatively, anti-DIG-Fab-POD (150 U ml⁻¹, Roche-Boehringer Mannheim) was used at a dilution of 1:1000 in 100 mmol Tris-Cl I⁻, pH 7.5, 150 mmol sodium chloride I⁻ and 1% (w/v) blocking reagent. Chemiluminescence film detection was carried out using the SuperSignal™ CL-HRP substrate system (Pierce Chemical Company, Rockford) according to the instructions of the supplier. Exposure was to

Fuji X-ray film. Specificity of antibody binding was shown by competition with the chemosynthetic peptide, incubating the guinea-pig antibody (1:1000 dilution) with the peptide at a final concentration of 100 µg ml⁻¹.

Immunocytochemical staining and flow cytometry of spermatozoa

The capsule of the epididymis was slit open in each region studied to expose a lobule of tubule. The lobule was washed to remove the blood, cut free and placed in a drop of medium (bicarbonate buffered, supplemented with glucose, lactate and pyruvate; see Yeung *et al.*, 1992) on a small plastic spatula. The isolated epididymal tubule segment was cut further to release the spermatozoa. After the spermatozoa were transferred and dispersed in 200 µl medium in a tube at 37°C for 2 min, a 2 µl aliquot was diluted with the medium and used for determining the percentage of motile spermatozoa. Another 10 µl aliquot was diluted with PBS and absorbance at 450 nm was measured to estimate sperm concentration. Testicular spermatozoa were collected from the rete testis as described above. Spermatozoa were washed with PBS containing 4 mg BSA ml⁻¹ and resuspended to 2×10^7 ml⁻¹. For each staining, 2.5 µl rabbit serum was added as a blocking agent to 40 µl washed spermatozoa and incubated for 10 min at room temperature before the addition of the antiserum, pre-immune or normal guinea-pig serum at a final dilution of 1:30 for a further 25 min of incubation. Spermatozoa were washed twice with 1 ml PBS-BSA by centrifugation at 450 *g* for 5 min and resuspended in 45 µl PBS-BSA before incubation with 5 µl fluorescein isothiocyanate (FITC)-conjugated rabbit anti-guinea-pig IgG (final dilution 1:100) for 30 min in the dark. After washing and resuspension in 300 µl PBS-BSA, a total of about 5000 spermatozoa was analysed for green fluorescence emission at 505–545 nm with excitation at 488 nm using the Epics XL flow cytometer (Coulter, Krefeld) with forward scatter and side scatter signals to set gatings for spermatozoa. For light microscopy, stained spermatozoa were pelleted by centrifugation at 450 *g* for 5 min and dried on to glass slides, mounted with anti-fade mounting medium and observed using a Olympus BX-40 microscope with excitation filter BP450–480 and emission filter BA515.

Desialylation and deglycosylation reactions

Chemical desialylation and β-elimination of blotted glycoproteins were performed as described by Duk *et al.* (1997). For the cleavage of putative *N*-glycans, crude protein extracts from rat cauda epididymal fluid collected as described above were denatured in 0.1 mol mercaptoethanol I⁻ and 0.5% (w/v) SDS, and digested overnight at 37°C with 5 mU *N*-glycosidase F ml⁻¹ (Roche-Boehringer Mannheim). Complete deglycosylation of proteins was carried out using the Glyco Free Deglycosylation Kit (Kit K 500, Oxford Glyko Systems Ltd, Abington) according to the instructions of the suppliers.

Results

Western blot analysis of the rat CD52 antigen

A chemosynthetic peptide was chosen as an immunogen, comprising 12 amino acid residues of the rat CD52 C-terminus (Kirchhoff, 1994, 1996; Fig. 1a). In this region of the peptide backbone, only one putative *O*-glycosylation site is predicted by the NetOglyc program (Hansen *et al.*, 1998). Database searches using BLAST (Altschul *et al.*, 1997) detected no similarities to peptides other than rat CD52. Western blot analysis was performed using protein extracts of whole cauda epididymal tissue from adult rats and revealed one prominent immunostained band of approximately 26 kDa (Fig. 1b). Specificity of the immune reaction was confirmed using the chemosynthetic oligopeptide to compete for antibody binding before western blot analysis. The immunostained band was also prominent in protein extracts of spermatozoa from rat cauda epididymidis. However, the band was not observed when intact rat spermatozoa were treated with PI-specific PLC before protein extraction and western blot analysis (Fig. 1c), supporting other reports on GPI anchoring of the antigen (Moore *et al.*, 1989; Eccleston *et al.*, 1994). Congruent results, albeit with a more intense background staining, were obtained using the *in vitro* carbohydrate labelling technique (Fig. 1c), which also detected a major PI-specific PLC-sensitive glycoconjugate of approximately 26 kDa in rat cauda spermatozoa (for a comparison, see Pera *et al.*, 1997).

The CD52-related antigen was also present in high concentrations in cauda epididymal fluid. Direct electro-

phoretic comparison of the antigen from spermatozoa from cauda epididymidis and cauda fluid revealed a small but reproducible difference in electrophoretic mobility, in which the sperm antigen migrated at a slightly slower rate (Fig. 2a). However, this difference was not observed or was even reversed when the antigen from both sources was partially purified by methanol-chloroform (Folch) extraction before gel electrophoresis and western blot analysis (Fig. 2b; for a comparison, see Schröter *et al.*, 1999), which does not support the contention that there are structural differences. It is possible that the lipid extraction destroyed hydrophobic interactions of the antigen with other membrane constituents of spermatozoa, thereby increasing its electrophoretic mobility.

Intense immunostaining of the antigen of approximately 26 kDa was observed only in protein extracts of cauda epididymal spermatozoa and cauda epididymal tissue, whereas protein extracts of testicular and caput spermatozoa as well as extracts from epididymal tissue of more proximal regions were negative (Fig. 3). Spleen protein extracts were also negative. However, occasionally, staining of an additional 15–17 kDa protein band was observed and was most prominent in sperm protein extracts. N-terminal peptide sequencing of the two major protein constituents of this band (Toplab, München; data not shown) revealed that this staining was nonspecific and ruled out any relationship with the CD52 antigen. The data indicate nonspecific crossreaction with two proteins present in spermatozoa: cytochrome C oxidase (Goto *et al.*, 1989) and testis lipid-binding protein (Pouresmaeili *et al.*, 1997).

Fig. 2. Western blot analysis using purified antibody of CD52-related antigen in crude protein extracts (a) and Folch-extracts (b) of rat spermatozoa recovered from different regions of the male genital tract and from cauda epididymal fluid. (a) Analysis of crude protein extracts. Lane 1: caput epididymal sperm proteins (Ca); lane 2: cauda epididymal sperm proteins (Cu); lane 3: proteins extracted from cauda epididymal fluid (Cef). (b) Analysis of Folch-extracted samples. Lane 4: caput epididymal sperm proteins (Ca); lanes 5, 7 and 10: cauda epididymal sperm proteins (Cu); lanes 6 and 9: molecular mass marker proteins (M); lanes 8 and 11: proteins extracted from cauda epididymal fluid (Cef).

Fig. 3. Western blot analysis of CD52 antigen in protein extracts of rat spermatozoa recovered from different regions of the male genital tract and from whole tissue samples. (a) Lane 1: cauda sperm proteins (Cu); lane 2: molecular mass marker proteins (M); lane 3: testicular sperm proteins (Te); lane 4: caput sperm proteins (Ca); lane 5: cauda sperm proteins (Cu); lane 6: caput proteins (Ca); lane 7: corpus proteins (Co); lane 8: cauda proteins (Cu); lane 9: splenic proteins (Spl). Arrow indicates a 15–17 kDa protein band that occasionally reacted with the antibody. (b) Corresponding Coomassie blue stained protein blot with similar amounts of proteins loaded in all lanes.

Post-transcriptional regulation of the expression of CD52

CD52 mRNA was confirmed to be produced only post-testicularly (Fig. 4a). However, although the mRNA appeared to be present in all parts of the rat epididymis in relatively high concentrations, staining of the glycoconjugate of approximately 26 kDa and the corresponding protein band was observed only in the cauda epididymis (Fig. 4b,c), coinciding with the occurrence of 'long' CD52 mRNA molecules. Abdominal positioning of the epididymis, the so-called cryptepididymis (Bedford, 1978; Foldes and Bedford, 1982; Pera *et al.*, 1997), resulted in a small reduction in the amounts of both 'long' cauda epididymidal CD52 mRNA and the antigen of approximately 26 kDa compared with 'normal' scrotal epididymides of sham-operated rats (Fig.

Fig. 4. Expression of CD52 in the rat epididymis after different treatments as analysed by northern blot analysis (a), western blot analysis (b,d) and *in vitro* glycoconjugate labelling (c). (a–c) Results obtained in unilateral cryptepididymal rats (scrotal and abdominal side, respectively) at 14 days after treatment; (d) results obtained from cryptepididymal rats that had also been castrated for 14 days (scrotal and abdominal side). Ca: caput epididymidis; Co: corpus epididymidis; Cu: cauda epididymidis; Nt: nucleotides; Te: testis. Similar amounts of RNA or protein, respectively, were loaded in each lane.

4b,c). The cryptepididymal condition combined with the castration of animals, which has been shown to abolish CD52 mRNA completely (Pera *et al.*, 1997), also completely abolished the corresponding cauda epididymidal antigen (Fig. 4d), thus extending and confirming the results of Pera *et al.* (1997) at the peptide level.

Immunostaining and flow cytometric measurements of rat epididymal spermatozoa

Indirect immunofluorescence revealed that most spermatozoa recovered from the cauda epididymidis (73–95%) showed strong fluorescence over their entire surface (Fig. 5). Flow cytometric analysis confirmed that in the more proximal regions, the percentage of stained spermatozoa was significantly lower (Fig. 6), although large individual variations were observed. Heterogeneity of antibody binding among immature spermatozoa was also reflected by the flow cytometric analysis, showing the distribution of two populations of spermatozoa or a broad distribution peak in contrast to a narrow peak from the cauda spermatozoa (Fig. 6). Testicular sperm preparations

consistently showed about 50% stained cells, which unlike the cauda epididymidal spermatozoa showed staining of the post-acrosomal head region in addition to some mid-piece staining (Fig. 5). In the negative control that was stained using the preimmune guinea-pig serum, testicular spermatozoa gave a more intense background staining than mature cauda epididymidal spermatozoa.

Lectin binding and deglycosylation of rat CD52

A range of lectins as well as deglycosylation reactions were used to investigate possible *N*- and *O*-glycosylation of the CD52 antigen. Strong binding of MAA in the range of the approximately 26 kDa immunostained band of untreated rat tissue and sperm protein extracts is shown (Fig. 7), whereas

Fig. 5. Indirect fluorescein isothiocyanate (FITC) immunofluorescence of freshly washed non-fixed rat spermatozoa recovered from various regions of the male genital tract. Caput epididymidal spermatozoa (a) and cauda epididymidal spermatozoa (c), both stained with the polyclonal anti-CD52 antiserum and (e) cauda epididymidal spermatozoa reacted with negative control serum. Higher magnification illustrates the difference between staining patterns obtained with testicular and cauda epididymidal spermatozoa. Testicular spermatozoa (b) incubated with antiserum showed unstained heads (arrows), but occasional staining of the post-acrosomal region in addition to some irregular mid-piece staining, whereas staining of cauda epididymidal spermatozoa (d) with the antiserum appeared strong and homogeneous over the whole sperm surface, including the entire head, mid-piece and tail. Testicular spermatozoa (f) incubated with control serum showed post-acrosomal plus mid-piece staining (arrows). Scale bars represent 20 μ m.

Fig. 6. Flow cytometry histograms of representative samples of spermatozoa recovered from the rat epididymis showing the distribution of fluorescence intensity of individual sperm cells (abscissa) stained with the polyclonal anti-CD52 antibody (right panels) or pre-immune guinea-pig serum as control (left panels). Percentages shown refer to spermatozoa within the gating (double-headed arrows), which distinguish positive staining from control staining (background). The net percentage of stained cells for each sample is obtained by subtraction of the respective control value. Note the small shift of peak intensity to the left from immature to mature spermatozoa, reflecting a decrease of unspecific sperm staining by the pre-immune serum control.

Fig. 7. Lectin binding studies of rat epididymal tissue protein extracts followed by subsequent western blot analyses of the CD52 antigen. Lane 1: binding pattern of *Arachis hypogaea* lectin (PNA) to epididymal protein extracts after desialylation; lane 2: antibody control (AC) of the same blot; lane 3: *Maackia amurensis* lectin (MAA) binding of epididymal proteins; lane 4: marker proteins (M); lane 5: *Phaseolus vulgaris* lectin (PHA-E); and lane 6: *Datura stramonium* lectin (DSA) binding of epididymal proteins; lanes 7–10: anti-CD52 antibody control (AC) of the blot (lanes 3–6).

other lectins did not bind specifically to a protein in this molecular mass range. After chemical desialylation of the protein blot (Duk *et al.*, 1997), PNA bound to a protein of this molecular mass, whereas the other lectins did not. A summary of the lectin binding results is provided (Table 1), which predict *O*-linked, α 2–3-sialylated carbohydrates and do not support the presence of large and complex *N*-linked glycans. This contention was corroborated by attempts to cleave putative *N*-linked glycans enzymatically. Protein preparations from rat cauda epididymal fluid and spermatozoa were subjected to PNGase F digestion before electrophoresis. No change in either microheterogeneity or electrophoretic mobility was observed by western blot analysis, even after overnight incubation with the enzyme (Fig. 8a; lanes 1–2). In a parallel experiment, using the human 15–25 kDa CD52 antigen (Schröter *et al.*, 1999), loss of the large and heterogeneous *N*-linked glycans was reflected by a marked loss of antigen heterogeneity combined with an increase in electrophoretic mobility (Fig. 8a; lanes 3–4), showing the effectiveness of the enzyme reaction used.

Complete deglycosylation of the rat antigen of approximately 26 kDa by β -elimination (Duk *et al.*, 1997) was attempted to remove all carbohydrate side chains, including *O*-linked glycans. After the two-step deglycosylation procedure, during subsequent western blot analysis a shift to significantly higher electrophoretic mobilities was

observed. In addition to a broad smear, an immunostained band at approximately 6 kDa was observed (Fig. 8b) which is in agreement with the calculated molecular mass of the unmodified rat CD52 peptide backbone. This finding indicated that deglycosylation of the antigen had been achieved (see also Bergeron *et al.*, 1997), although partial degradation of the peptide backbone could not be excluded.

Discussion

Rat CD52, named previously as the major 'maturation-associated' antigen, was shown to correspond to a major sperm membrane antigen with an M_r of approximately 26 000, which was produced and glycosylated only by the distal epididymis. The late occurrence of the antigen indicates a functional association with sperm storage rather than with sperm maturation. The present study confirmed and extended previous results (Pera *et al.*, 1997) that CD52 association of spermatozoa is cauda-specific. The glycopeptide was also detected in high concentrations in rat cauda fluid, and results from the present study indicate uptake by spermatozoa during epididymal transit, possibly as a GPI-anchored antigen (for a comparison, see Kirchhoff *et al.*, 1996; Yeung *et al.*, 1997). Olson *et al.* (1987) reported that rat caput spermatozoa take up a major 26 kDa protein from cauda epididymal fluid *in vitro*. Furthermore, related

Table 1. Lectin binding properties of an immunoreactive protein of approximately 26 kDa from rat cauda epididymidal tissue and spermatozoa

Lectin	Cauda epididymidal tissue protein	Cauda epididymidal sperm protein	Specificity and linkage
PNA	+	+	β -Gal, Gal β 1-3GalNAc, O-linked
MAA	+	+	Sialic acid, NeuNAc α 2-3Gal
SNA	-	-	Sialic acid, NeuNAc α 2-6Gal(NAc)
Con A	-	-	α -Man, α -Glc, α -GlcNAc, branched mannoses
DSA	-	nd	β -GlcNAc, N-acetylglucosamine repeats
PHA-E	-	nd	Gal β 1-4 GlcNAc β 1-2 Man α 1-6 $\rightarrow$

+, positive staining; -, no staining; *staining after desialylation; nd, not determined.

Fig. 8. Deglycosylation analysis of CD52 glycoprotein as enriched from cauda epididymidal fluid. (a) Western blot analysis of rat and human CD52 samples after PNGase F treatment. Lane 1: rat CD52 antigen after 24 h of incubation without PNGase F; lane 2: rat CD52 antigen after 24 h of incubation with PNGase F; lane 3: human CD52 antigen after 24 h of incubation without PNGase F; lane 4: human CD52 antigen after 24 h of incubation with PNGase F. (b) Western blot analysis of rat CD52 during chemical deglycosylation in a two-step procedure. Lane 1: untreated rat CD52 sample; lane 2: aliquot of the same sample after the first deglycosylation step; lane 3: aliquot of the same sample after the second deglycosylation step.

integral sperm membrane antigen was found in hamsters (Hoos and Olson, 1988).

GPI anchoring of rat CD52 was demonstrated by its sensitivity towards PI-specific PLC treatment, corroborating previous results obtained with this antigen (Moore *et al.*, 1989; Eccleston *et al.*, 1994). Acquisition of the highly glycosylated and lipid-anchored CD52 antigen is thus one of the few well-defined modifications that occur to the sperm membrane during epididymal passage. Sensitivity towards PI-specific PLC also implied that, in contrast to its human counterpart (Schröter *et al.*, 1999), the GPI anchor of

rat CD52 is not acylated at the 2-position of the inositol. Furthermore, there was no indication of significant structural differences between the antigen as extracted from whole cauda epididymidal tissue, fluid and spermatozoa.

Compared with other mammals (Krull *et al.*, 1993; Pera *et al.*, 1994), the rat epididymis produces relatively high amounts of CD52 mRNA in all parts of the organ (see Pera *et al.*, 1997), whereas the corresponding glycopeptide was detected only in the cauda epididymidis. The appearance of the antigen with an M_r of approximately 26 000 and the glycoconjugate band was congruent with the appearance in

the cauda epididymidis of CD52 mRNA molecules with a 'long' poly(A) tail (Pera *et al.*, 1997), the direct relationship between both 'long' mRNA and translation product emphasized further by various types of animal treatment. The polyadenylation state of mRNA has long been implicated in message translation and RNA stability, the cap and poly(A) tail synergistically stimulating the initiation of translation (for a review, see Pestova and Hellen, 1999). Evidence has also been provided for the role of a reduction in the initiation of translation in stimulating deadenylation of mRNA (Schwartz and Parker, 1999). Results from the present study confirm regulation of the expression of rat CD52 antigen at a post-transcriptional level. Although no antigen could be detected in the proximal parts of the organ, initiation of translation occurred in the cauda epididymidis and seemed to imply stabilization of the CD52 mRNA poly(A) tail.

During immunocytochemical staining, the antiserum was less specific and showed considerable crossreaction with testicular spermatozoa, although CD52 mRNA, and hence the CD52 antigen is not present in the testis (Kirchhoff, 1994, 1996; Pera *et al.*, 1997). Therefore, this staining cannot reflect a precursor relationship of CD52-related molecules originating at spermatogenesis, and must be due to nonspecific binding. The head region of immature testicular and caput epididymidal spermatozoa appeared susceptible to nonspecific binding and was also stained by negative control antisera. Some nonspecific binding to sperm proteins was occasionally observed during western blot analyses. The most prominent crossreaction appeared to be with an unrelated protein band of approximately 15–17 kDa, which did not contain any CD52-related peptide sequences. Polyclonal antiserum raised against an acidic (pI approximately 4.0) 26 kDa protein of spermatozoa from rat cauda epididymidis (Olson *et al.*, 1987) detected its antigen only over the flagellum.

Post-testicular uptake by the sperm membrane of the GPI-anchored CD52 does not rule out the possibility of carbohydrate modification within the epididymal duct lumen or on the sperm surface. Previous studies have indicated this mechanism (Hamilton *et al.*, 1986; for a review, see Myles, 1993; Eccleston *et al.*, 1994), and there is circumstantial evidence to support the plausibility of this idea. However, carbohydrate-modifying enzymes are usually intracellular enzymes that are active *in vivo* only as Golgi constituents, and their activity in male genital tract secretions has been assessed only *in vitro*. Moreover, it is not known whether the appropriate sugar donors are available from epididymal fluid. From the structural data presented by Schröter *et al.* (1999) after analysis of the N-linked glycans of human CD52, it seems unlikely that extensive extracellular enzyme action could alter CD52-bound carbohydrates *in vivo*.

Since the mature CD52 peptide sequences are so diverse among mammals (Kirchhoff, 1996; Kirchhoff and Hale, 1996), it has been suggested that they represent a scaffold for the presentation of the functionally more important

carbohydrates. To date, only the carbohydrate structures of the human counterpart, as purified from pooled ejaculates, have been studied in detail (Schröter *et al.*, 1999). Human CD52 has a single N-glycosylation site that is completely occupied and carries more than 50 different highly heterogeneous and complex glycans. O-glycosylation of human CD52 has been excluded (Schröter *et al.*, 1999). Although a highly conserved consensus site for N-glycosylation was predicted from all CD52 sequences cloned thus far, including rat CD52 (Kirchhoff and Hale, 1996), lectin-binding and deglycosylation results from the present study do not support the presence of large and complex N-linked carbohydrates of CD52 in rats. The NetOglyc program (Hansen *et al.*, 1998) and other analyses of the purified glycopeptide (Eccleston *et al.*, 1994) predict extensive mucin-type GalNAc O-glycosylation. In the molecular mass range of the immunoreactive protein band of the approximately 26 kDa, specific binding of *Maackia amurensis* lectin (MAA) and, after chemical desialylation, of *Arachis hypogaea* lectin (PNA), was observed, indicating the presence in rat CD52 of O-linked Gal β 1–3-GalNAc-containing carbohydrates which are terminally α 2–3-sialylated. Most of the N-linked glycans of CD52 in human seminal plasma are also α 2–3-sialylated (Schröter *et al.*, 1999). However, the results from the present study do not support the idea of conservation of major structurally related glycans between human and rat CD52, but indicate profound species differences in CD52-linked carbohydrates in addition to differences in their peptide sequences and GPI anchor structures.

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