PrP<sup>C</sup>, the normal isoform of the prion component PrP<sup>Sc</sup>, is a 33–35-kDa glycoprophosphatidylinositol-anchored glycoprotein expressed in the plasma membrane of many cells and especially in the brain. The specific role of PrP<sup>C</sup> is unknown, although lately it has been shown to bind copper specifically. We show here that PrP<sup>C</sup> is present even in mature sperm cells, a polarized cell that retains only the minimal components required for DNA delivery, movement, and energy production. As opposed to PrP<sup>C</sup> in other cells, PrP in ejaculated sperm cells was truncated in its C terminus in the vicinity of residue 200. Sperm PrP, although membrane-bound, was not released by phosphatidylinositol phospholipase C as well as not localized in cholesterol-rich microdomains (rafts). Although no infertility was reported for PrP-ablated mice in normal situations, our results suggest that sperm cells originating from PrP-ablated mice were significantly more susceptible to high copper concentrations than sperm from wild type mice, allocating a protective role for PrP in specific stress situations related to copper toxicity. Since the functions performed by proteins in sperm cells are limited, these cells may constitute an ideal system to elucidate the function of PrP<sup>C</sup>.

GPI-anchored proteins, both PrP isoforms are associated with cholesterol-rich membranal microdomains, denominated rafts (14). In an effort to elucidate the function of PrP<sup>C</sup>, we decided to look for its expression in cells that perform only limited functions, such as sperm cells.

During spermiogenesis, spermatoocytes differentiate into sperm in a process in which the Golgi develops into the large acrosome and mitochondria migrate from the rest of the cytoplasm to form a tightly wrapped sheet around the upper part of the flagellum (15, 16). After the excess cell cytoplasm is pinched off, the sperm cell only carries with it an acrosome containing enzymes required for the penetration of the membranes covering the egg, as well as those other cellular components essential to provide energy for its movement. Sperm cell become independently motile following an additional maturation step that occurs during their passage through the epididymis and are considered fully mature in ejaculates (17). There is no de novo protein synthesis in mature sperm cells, and therefore changes occurring in protein patterns are due either to protein degradation or protein processing during sperm maturation.

In this work we studied the properties of PrP in sperm cells from several species during different stages of maturation. For practical reasons, we only obtained sperm ejaculates containing mature sperm cells from cattle and human, whereas sperm cells isolated from the epididymis (semi-mature) were available from cattle and rodents. Our results show that during the process of sperm maturation, PrP<sup>C</sup> changes from its regular rafts-associated, GPI-anchored structure to a unique C-terminal-truncated peptide devoid of a GPI group and, as a result of that, is not released from the cell surface by phosphatidylinositol phospholipase C (PIPLC). In addition, mature sperm PrP was not longer associated with membranal rafts. Our results also suggest that sperm cells from PrP-ablated mice were more susceptible to copper-induced toxicity than sperm cells from wt mice, although no difference in the motility or survival could be identified when comparing these cells at control conditions. Sperm cells may be the ideal system to investigate whether a protective role against copper toxicity-induced stress situations can be attributed to PrP<sup>C</sup>.

**EXPERIMENTAL PROCEDURES**

**Tissue Samples**—Human ejaculated semen samples were obtained from the laboratory for human fertility in the Hadassah University Hospital in Jerusalem. The samples received for our experiments tested in the fertility laboratory as normal regarding morphology and motility. Bovine ejaculated semen samples were received from an Israeli company for artificial insemination of cattle.

Hamster, mouse, or bovine epididymal sperm samples were obtained by mincing epididymis in saline and subsequently collecting floating cells from the saline suspension as described (18). Samples were looked upon by light microscopy to ensure that most cells are indeed differentiated sperm cells. Hamster nondifferentiated spermatocytes were obtained by a similar procedure, using testis instead of epididymes.

**Preparation of Cells for Immunoblotting**—10<sup>7</sup> N2a-C10 cells (an N2a clone expressing a chimeric mouse/hamster PrP or sperm cells reacting with αPrP mAb 3F4 (19)) were extracted in 1 ml of lysis buffer contain-

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*This work was supported by grants from the Israeli Academy of Science and the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ These authors contributed equally to this work.

§ To whom correspondence should be addressed. Fax: 972-2-6429441; E-mail: gabizon@hadassah.org.il.

The abbreviations used are: BSE, bovine spongiform encephalopathy; GPI, glycosphatidylinositol; PIPLC, phosphatidylinositol phospholipase C; wt, wild type; mAb, monoclonal antibody.
ing 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, and 1% Nonident P-40. The samples were centrifuged at 3000 rpm for 15 min at 4 °C. The supernatants were concentrated by methanol precipitation.

**Flotation Assay**—Flotation of detergent-insoluble complexes was performed as described by Naslavsky et al. (19). Briefly, 100–150 mg of cerebellum or appropriate cells were homogenized in 700 μl of an ice-cold buffer containing 150 mM NaCl, 25 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1% Triton X-100. Insoluble particles were spun down for 5 min at 2000 rpm, 4 °C, and the lysate was loaded at the bottom of Beckman Instruments TLS-55 ultracentrifuge tubes. An equal volume of ice-cold 70% Nycodenz in TNE (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA) was added and mixed with the lysate. A 8–35% Nycodenz linear step gradient in TNE was then overlaid above the lysate. The tubes were spun at 55,000 rpm for 4 h at 4 °C in a TLS-55 rotor (200,000 g).

Fractions of 180 μl each were collected from the top to the bottom of the tube and immunoblotted with the appropriate αPrP antibody.

**Copper Sensitivity Assay**—FVB or PrP<sup>C<sup>0/0<sup> epididymal sperm cells were resuspended in activation buffer as described (18). After a 10-min incubation in the activation buffer, sperm cells were separated into 3 wells, to which 0, 50, or 100 μg/ml CuCl<sub>2</sub> was added. Every 5 min the percentage of motile to nonmotile cells were established in each of the samples. 100% motile cells represent the percentage of motile cells at time zero for the sample without copper.

**RESULTS**

Fig. 1 shows anti PrP immunoblots of sperm cells extracts, either mature (human, bovine) or from the epididymis (hamster, mouse, and bovine), as compared with brain membranes of the same species. The antibodies used were 3F4 (directed against residues 108–111) for human and hamsters (20), 6H4 ("Prionics," directed against residues 144–152) for bovine samples, 1E (against residue 200 in humans and mice) (21), and R073 (polyclonal anti-PrP antisera reacting mostly against mouse and hamster PrP (22)). The molecular weight of PrP in semi-mature (epididymis) sperm cells was similar to that of the brain, whereas in mature sperm cells obtained from ejaculates, it appears as if the apparent M<sub>r</sub> of PrP was reduced to 24,000. The fact that such a large panel of polyclonal and monoclonal αPrP antisera reacted with the same bands confirms that this is indeed PrP.

To test whether the reduced molecular weight of PrP in mature sperm, as compared with epididymal sperm cells and brain samples, is due to a different N-glycosylation pattern of the PrP protein in these cells, human and hamster brain and sperm samples were deglycosylated by endoglycosidase F (Fig. 2). The deglycosylated and control samples were subsequently immunoblotted with mAb 3F4. The full-size mature PrP peptide (residues 23–231) encloses two N-glycosylation sites at residues 181 and 197 and a GPI anchor at its C-terminal (23, 24). Endoglycosidase F deglycosylation of brain PrP<sup>C</sup> resulted in a major band of an apparent M<sub>r</sub> of about 26,000, whereas faint additional bands representing either partial deglycosylation or degradation products of PrP<sup>C</sup> could also be observed (25, 26). Deglycosylation of PrP from hamster epididymis sperm cells produced a major band of the same apparent molecular weight as in brain PrP. These results show that sperm and brain PrP share a similar polypeptide backbone and probably also a similar GPI (glycophosphatidylinositol) anchor, since otherwise their deglycosylation products would not be identical in molecular weight.

As opposed to the results with brain and with semi-mature sperm cells, the apparent molecular weight of deglycosylated PrP in mature human sperm from ejaculates, as detected by immunoblotting with mAb 3F4, was of about 17,000. These results raise several interesting points. 1) The lower molecular weight of PrP in untreated mature sperm (24,000) could not be only a result of PrP deglycosylation occurring during sperm maturation, since there is a further reduction in M<sub>r</sub> of sperm PrP following endoglycosidase F treatment. 2) α PrP mAb 3F4 reacts with a site composed of residues 108–111 in the human or hamster PrP sequence; therefore it is unlikely that a 17-kDa peptide that still reacts with this antibody can be produced by proteolysis of only the N-terminal part of PrP. Indeed, during the normal degradation pathway of PrP<sup>C</sup>, a 17-kDa peptide, which does not react with mAb 3F4, is produced (14, 27). These results suggest the possibility that PrP in mature sperm is truncated in its C terminus.

To further delineate the site of such a putative C-terminal trimming, human mature sperm samples were immunoblotted with an antisemur raised against a synthetic peptide comprising residues 195–213 of the human and mouse PrP sequence (1E) and that has been shown to preferentially recognize the glutamate residue at position 200 (21). As can be seen in Fig. 3, although human brain PrP reacted readily with both the 1E antisera and the mAb 3F4, sperm PrP reacted only with mAb 3F4. That the 1E antisera can react in principle with sperm PrP can be seen from the fact that PrP from mouse epididymis samples (semi-mature sperm cells) did react with this antiserum (Fig. 1). These results show that mature sperm carries a C-terminal-truncated PrP isoform, unlike the previously characterized PrP<sup>C</sup> degradation products, that are truncated in the N-terminal part of the protein. Since bovine mature sperm PrP reacted with a PrP mAb 6H4 that is directed against residues 144–152 and, we must assume from the endoglycosidase F digestion results that at least one if not both mature sperm PrP N-glycosylation sites are occupied, it is conceivable that mature sperm PrP is cleaved between residues 181 to 200. The molec-
were immunoblotted either with mAb 3F4 or with a PrP antiserum 1E (21), which recognizes glutamate at codon 200 of the wt PrP sequence. As other GPI-anchored proteins, PrP<sup>C</sup> can be released from cell membranes by the enzyme PIPLC (29). After cleavage of the lipid group, the remaining protein is converted into a soluble peptide. We therefore tested whether PIPLC can release PrP from sperm cells. Most of the PrP is released by PIPLC from either N2a C10 cells as well as from early, pre-differentiated spermatocytes (from the testis). This was not the case for PrP from sperm cells, although, at least in semi-mature sperm cells, the molecular weight of PrP suggests the presence of the GPI group on PrP (Fig. 4). These results suggest either (i) that the GPI anchor became inaccessible to the enzyme, (ii) that the GPI anchor has been chemically modified to resist PIPLC hydrolysis, or (iii) that PrP, in addition to its GPI-membrane anchoring, is attached to the membrane by an additional mechanism. The fact that PrP in testis spermatocytes is releasable by PIPLC but that this property is lost during cell maturation suggests the appearance of an additional binding site for PrP in the membrane during cell processing. This conclusion was reinforced by the presence of the C-terminal-truncated PrP isoform in ejaculation samples, since this isoform is probably devoid altogether of the GPI anchor and, therefore, must be attached to the sperm outer membrane via a different mechanism. Interestingly, it has been shown lately that also PrP in platelets, another example of cells that do not synthesize new proteins and perform only specific functions, cannot be released from the cell membrane by PIPLC (30). As opposed to PrP, other GPI-anchored proteins are normally processed in sperm cells, and some of them are known to play a crucial role in cell maturation (31, 32).

Most GPI-anchored proteins have been shown to be targeted to special cholesterol-rich membranal microdomains denominated rafts (33). Rafts are insoluble in cold Triton X-100, and as such, proteins inserted in them can be separated from other membrane proteins as well as from soluble proteins by an assay in which the insoluble rafts will float to the top of density gradients and the soluble or detergent-solubilized proteins will remain in the lower fractions of these gradients (19). PrP is also a raft protein. In fact, since both prion proteins (PrP<sup>C</sup> and PrP<sup>S</sup>) are colocalized in those membranal microdomains, it has been speculated that the conversion of PrP<sup>C</sup> to PrP<sup>S</sup> occurs in these rafts (19, 34). Transfection of genetically engineered transmembrane PrP<sup>C</sup> into ScN2a cells (that are not targeted to rafts) has shown that the membrane localization of PrP<sup>C</sup> is essential for the conversion process, since transmembrane PrP<sup>C</sup> did not convert into PrP<sup>S</sup> (14, 35).

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When brain membranes, early spermatocytes (from testis), and semi-mature sperm cells (from epididymis) were subjected to a flotation assay, PrP in sperm, as opposed to PrP from spermatocytes and brain, could not be found in the buoyant fractions where rafts migrate (Fig. 5). It is possible that rafts are altogether absent from mature sperm cells as a result of changes in lipid membrane composition, which are known to occur during the different stages of sperm maturation (36, 37). This speculation is reinforced by the fact that ESA, a transmembrane raft protein (38), also changed its membrane association during sperm maturation. It is, however, still conceivable that this is a unique effect for a small group of sperm proteins that includes PrP.

To investigate whether PrP is exposed to the outer plasma membrane, we digested intact semi-mature hamster and mouse (full-length PrP) as well as human cells (C-terminal-truncated PrP) with a low concentration of protease K and compared the digestion pattern with sperm cells digested in the presence of detergents (not shown). The intact cell samples digested with protease K were immunoblotted with the appropriate anti-PrP antibodies. Sperm PrP was completely accessible to low protease K concentrations in all the species studied (Fig. 6A), as can be seen from the fact that after protease K digestion, no PrP could be detected with diverse antibodies. The association of sperm PrP with the cell membrane is probably extremely strong, since it is resistant to incubation with 100 mM Na<sub>2</sub>CO<sub>3</sub>, a reagent that is known to dismantle protein-protein interactions and that has been used operationally to define integral membrane proteins (Fig. 6B).
During prion disease, most of the conversion of PrP<sub>C</sub> to PrP<sub>Sc</sub>, the prion component, occurs in the brain of the infected animal and subsequently causes neurodegeneration. The question of whether sperm PrP can be converted into PrP<sub>Sc</sub> may have important implications for the possible transmission routes of prion diseases. However, as can be seen in Fig. 7, PrP in sperm cells of scrapie-infected hamsters is not more resistant to protease digestion than PrP from normal sperm. These results are consistent with the general notion that prion diseases are not transmitted sexually. Interestingly, it has been shown that male mice inoculated with prions lose their fertility before clinical signs of the disease are noticeable (39).

PrP has been shown lately to bind copper specifically. Although a specific role in copper metabolism as not yet been attributed to PrP, it has been shown that cerebellar cells from PrP-ablated mice are more susceptible to copper toxicity and oxidative stress than cerebellar cells from control mice (40). Interestingly, sperm cells are notorious for their sensitivity to high copper concentrations, as can be appreciated from the fact that the intrauterine device contraception principle is based on copper toxicity (41). We therefore tested the motility of wt FVB mouse sperm cells as compared with the motility of sperm cells of PrP<sup>0/0</sup> mice in the presence and absence of copper (PrP<sup>0/0</sup> mice contain 94% of the FVB genotype) (5). Motility of sperm cells is considered as a good criteria for cell viability (18, 41, 42). Epididymal sperm cells from wt or PrP<sup>0/0</sup> mice were placed in a buffer described previously (18). At time point zero, CuCl<sub>2</sub> was added at either 50 or 100 µg/ml to the appropriate wt or PrP<sup>0/0</sup> sperm samples, and subsequently the motile sperm cells were counted every 5 min. At both copper concentrations, sperm cells from PrP<sup>0/0</sup> mice lost their motility faster than sperm cells from wt mice; however, since 100 µg/ml CuCl<sub>2</sub> was very toxic to both types of sperm cells, the difference between 50% survival was only 5 min between wt and PrP<sup>0/0</sup> cells. At 50 µg/ml CuCl<sub>2</sub> the different in survival between both types of sperm cells was significantly larger (Fig. 9). Although no motile cells remained in the ablated samples after 30 min of incubation with 50 µg/ml CuCl<sub>2</sub>, about 50% control cells were still motile at that time point. These results were extremely reproducible. Fig. 8 represents the average of four similar experiments. In each experiment, sperm samples from 3–5 animals of each kind (wt or PrP<sup>0/0</sup>) were tested individually in triplicates. The results were not dependent of the animals ages, since copper sensitivity was higher for PrP-ablated sperm that for wt sperm in animals 6, 12, or 18 month old (not shown).

**DISCUSSION**

The finding of substantial amounts of PrP in sperm cells provides us with a new tool to be used in the quest for the elusive function of this interesting protein. As stated above, mature sperm cells retain only the minimal components required for their unique function.

In addition, the presence of this unusual PrP isoform in sperm cells (for summary see Table I) may help us elucidate both the sites in the PrP sequence that are essential for the
normal function of PrPc as well as to the molecules or cofactors required to accomplish such function. These sites in the PrP sequence seem to be different from the ones fundamental for the conversion of PrPc into PrPsc, since that process is independent of the presence of the N-terminal PrP sequence but does require a GPI anchor and a raft membrane location for both prion isoforms participating in the conversion process (14, 43).

As we have shown here, this is not the case for sperm PrP, which is devoid both of the PrP C terminus containing the GPI anchor and of its raft location, but even so, is probably as active as PrPc. The mechanism by which PrPsc is truncated during the sperm maturation process and thereafter remains attached to the sperm plasma membrane remains to be established.

PrPsc has been shown lately to bind copper specifically (12, 13, 44, 45). The binding site for copper in PrPc is believed to be in the octa-repeats present in the N-terminal section of PrP (44), which are probably intact even in mature sperm PrP. This finding suggests a possible role for PrP in copper metabolism. Copper ions serve as cofactors of many enzymes and, in particular, of energy-associated systems (46, 47). In addition, copper has also been shown to be toxic when present in excess, and therefore, fine balance of copper concentration is required for good health (41, 48, 49). The putative role of PrP in copper metabolism can vary from a copper transporter to a suppressor of copper-induced cell damage.

The possibility that PrP protects against copper damage was recently suggested from experiments showing that brain cells from PrP knock-out mice were more susceptible to copper toxicity than brains cells from wt mice (50). The results of the experiments described in Fig. 8 are also consistent with such a protective role for PrP against copper induced damage. Although no difference in motility was observed between wt mice sperm and PrP knock-out mice sperm at control conditions, the addition of copper was remarkable more toxic to PrP knock-out sperm cells than to wt sperm cells. Interestingly, a group of markers present in CSF and serum during prion disease (S100 proteins) (51) have been shown to perform as copper-binding proteins and thereby protect against copper-induced cellular damage (52), suggesting a possible compensatory effect, which may be required during the last stages of prion infection.

A putative role for PrP as a protector against specific stress situations is also consistent with the fact that the absence of PrP in ablated mice did not cause any mayor apparent damage (5). If indeed the function of PrP is to protect cells during a specific stress situation such as a toxic copper concentration, no apparent damage should occur in the absence of such an insult.

The new PrP isoform described in this manuscript may play an important role in the identification of PrPsc metabolism and function. The relative simplicity of sperm cells, as compared with nerve cells, may shorten the pathway leading to the complete elucidation of PrPsc function. Once a reliable assay is developed for the function of PrPsc, it will be possible to establish whether the loss of such function plays any role in the pathogenesis of prion diseases.
PrP in Mature Sperm

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