Identification, Proteomic Profiling, and Origin of Ram Epididymal Fluid Exosome-Like Vesicles

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Identification, Proteomic Profiling, and Origin of Ram Epididymal Fluid Exosome-Like Vesicles[1]

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ABSTRACT

Small membranous vesicles, between 25- and 75-nm diameter, were collected by high-speed centrifugation from the ram cauda epididymal fluid and were found to be normal constituents of this fluid and of the seminal plasma. The SDS-PAGE protein pattern of these vesicles was specific and very different from that of the caudal fluid, seminal plasma, sperm extract, and cytoplasmic droplets. After two-dimensional electrophoresis separation and mass spectrometry analysis, several proteins were identified and grouped into i) membrane-linked enzymes, such as dipeptidyl peptidase IV (DPP-IV), neprilysin (NEP), phosphodiesterase-I (E-NPP3), and protein G-beta; ii) vesicle-associated proteins, such as lactadherin (MFEG8-PAS6/7) and vacuolar ATPase; iii) several cytoskeleton-associated proteins, such as actin, ezrin and annexin; and iv) metabolic enzymes. The presence of some of these proteins as well as several different hydrophobic proteins secreted by the epididymis was further confirmed by immunoblotting. These markers showed that the majority of the vesicles originated from the cauda epididymal region. The physical and biochemical characteristics of these vesicles suggest they are the equivalent of the exosomes secreted by several cell types and epithelium. The main membrane-linked proteins of the vesicles were not retrieved in the extract from cauda or ejaculated sperm, suggesting that these vesicles did not fuse with sperm in vivo.

epididymis, epididymosomes, exosomes, gamete biology, male reproductive tract, mass spectrometry, protein, proteomic, sperm, spermatozoa, sperm maturation

INTRODUCTION

Mammalian sperm collected from the testis are immobile and unable to bind to and then fertilize the oocyte. They acquire these properties gradually while they travel through the epididymal tubule. During this transit, the sperm lose their cytoplasmic droplets and their plasma membrane undergoes profound changes, including addition, removal, or transformation of proteins and lipids [1–3]. These gradual surface modifications occur in response to variations in the luminal fluid composition and are thought to be responsible for their final maturation [4].

One of the most striking changes is the transfer to the sperm membrane of very hydrophobic proteins found in the epididymal fluid and of proteins that normally bear glycosyl phosphatidyl inositol (GPI) tails [5–7]. Because such proteins normally exist in a membrane environment and are not free in the fluid, it has been suggested that a transport system, for example, vesicles (named epididymosomes) or lipid micelles, might exist in the epididymis [2, 3, 8]. The presence of membrane vesicles in seminal plasma of human and mammals (named prostasomes) has been well documented, and it has been suggested that these vesicles are derived mainly from the accessory glands, such as the prostate or the seminal vesicles (for reviews, see [8, 9]).

We have recently provided evidence that such vesicles exist in the cauda epididymal fluid of rams [10], and in vitro experiments have shown that such vesicles, which are also present in the bovine cauda epididymis and seminal plasma, can transfer some proteins to the sperm in zinc- and pH-dependent manners (for reviews, see [8, 9]).

In this study, we definitively establish that an intraluminal system of membrane vesicles occurs naturally in the ram cauda epididymis. We have isolated, purified, and biochemically characterized these vesicles from the epididymal fluid and the seminal plasma, and we defined biochemical markers that help us to determine their origin. Their relationship to the vesicles present in the seminal plasma and to exosome vesicles secreted by other types of epithelium [11] is discussed, as well as their role in the transport and transfer of proteins to the sperm membrane.

MATERIALS AND METHODS

Fluids and Sperm Collection

Experiments on animals were conducted according to the International Guiding Principles for Biomedical Research Involving Animals as promulgated by the Society for the Study of Reproduction. Testis and epididymis were obtained from adult Ile de France and Romanov rams (at least 10 different animals). Testicular fluid was obtained after puncture of the rete testis. Epididymal fluids were collected by microperfusion with PBS from the 10 different zones (0–9) defined in the three main regions (caput, corpus, cauda) and the caudal fluid (CEP) by retroperfusion of zones 8 and 9 from the deferent duct (see Fig. 1). Ejaculates were obtained from five intact adult animals or two vasectomized animals using an artificial vagina.

The spermatozoa were separated from the fluid or seminal plasma by two successive centrifugations (15 min, 15 000 × *g*, 4°C). Where indicated, the sperm from different zones were diluted in PBS and layered on a discontinuous percoll gradient (40% and 90%) and centrifuged at 3000 × *g* for 10 min at 4°C. The sperm pellets were carefully collected, diluted with PBS, and centrifuged again to remove the percoll. Under both conditions (directly washed or after percoll gradient), the last pellet was directly extracted with an equal volume of nonreducing Laemmli sample buffer and centrifuged again; the supernatant was boiled after addition of beta-mercaptoethanol (2%) and is referred to as the sperm extract.

Cytoplasmic droplets were purified from the top layer of the discontinuous percoll gradient from the sperm samples of zones 8 and 9. This

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suspension was diluted in PBS, centrifuged at 15 000 × *g*, and the pellet resuspended and loaded on a 10–90% percoll gradient. The top white band separated after centrifugation was recovered. The purity of the droplets was assessed by microscopy after two successive washes in PBS (15 000 × *g*, 10 min, 4°C) and also by electron microscopy. This preparation was highly enriched in droplets, with very few contaminating sperm.

Each experiment was repeated at least twice with samples from two different animals. The most representative results are shown.

Vesicle Preparation

The 15 000 × *g* supernatant from the caudal fluid was ultracentrifuged at 48 000 × *g* (JA18; Beckman, Villepinte, France) or 100 000 × *g* (SW21; Beckman) for 2 h at 4°C (no major differences in the protein pattern of vesicles were observed between the two procedures by SDS-PAGE; see also [10]). After the centrifugation, a yellowish pellet was found at the bottom of the tube. This pellet was suspended in PBS and submitted to a second high-speed centrifugation. The final pellet gave a cloudy, refracting solution in PBS, which was divided into aliquots to be processed for electron microscopy or prepared for one- and two-dimensional gel separation.

Electron Microscopy

The washed pellet was fixed at room temperature with 2.5% glutaraldehyde in 0.175 M cacodylate at pH 7.2. After 24 h, the pellet was post-fixed with osmium tetroxide (2% final) and potassium ferricyanide (1.5%), washed with deionized water, and dehydrated with ethanol before embedding in epoxy-propan glycidether 100. The fixed pellet was cut with an ultramicrotome (80–90 nm). Contrast was enhanced by uranyl-acetate and lead citrate. Samples were observed on a Philips CM 10 microscope (60 KV). Measurement of the vesicle diameters and of the thickness of the vesicle membranes was performed either manually or using digital imaging system (Soft Imaging System, Munster, Germany). Both techniques gave very similar results.

Gel Electrophoresis

SDS 6–16% gradient polyacrylamide gels were used for protein separation, and iso-electrofocalization was performed as previously described [12]. The gels were Coomassie blue-stained before spot cutting and mass-spectrometry analysis or silver stained when better visualization of the protein spots was needed. For protein quantification, the Coomassie blue-stained gels were scanned and the total protein content of each lane analyzed using the 1D-Elite software package (Amersham-Pharmacia Biotech AB, Uppsala, Sweden).

Western Blotting

Semidry transfer of proteins was performed over 2 h at 0.8 mA/cm^2. The western blots were blocked with TBS-Tween 20 (0.5%, w/v) and, depending on the antibodies used, supplemented with lyophilized low-fat milk (5% [w/v]), gelatin (0.5% [w/v]), or BSA (1% [w/v]).

The first antibodies were either rabbit polyclonals or mouse monoclonals. The anti-HE1, anti-RABP, anti-PGDS, and anti-acrosin are rabbit polyclonal antibodies obtained in our laboratory against the ram epididymal cholesterol transfer protein (CTP/HE1/NP-C2), retinoic acid binding protein (E-RABP), prostaglandin D2 synthase (PGDS) [13], and sperm acrosin [14], respectively. The anti-lactadherin (P47/PAS-6/7/MFG-E8) rabbit polyclonal antibody was a gift from Dr. J.T. Rasmussen [15, 16]. The anti-phosphodiesterase I rabbit polyclonal (B10; nucleotide pyrophosphatase 3; E-NPP3) was a gift of Dr. M. Maurice [17]. Anti-CD10 (neprilysin/CALLA/neutral endopeptidase) and anti-protein G-beta (against a peptide mapping at the carboxy-terminus of human G-beta 1 and recognizing G-beta 1, 2, 3, and 4) were rabbit polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-actin was a monoclonal antibody (Sigma, Saint Quentin Fallavier, France, and Prolabo, Fontenay-sous-Bois, France).

Membranes were incubated with the antisera under mild agitation overnight at 4°C or for 2 h at 37°C. The second antibody was a goat anti-rabbit or a goat anti-mouse antibody conjugated with peroxidase (dilution 1:5000). The peroxidase was revealed with chemoluminescent substrates and the images recorded on film or digitized with a cooled CCD camera. No reaction was observed with the secondary antibodies alone or the preimmune sera when available (for anti-CTP/HE1, -PGDS, -ERABP, and -acrosin).

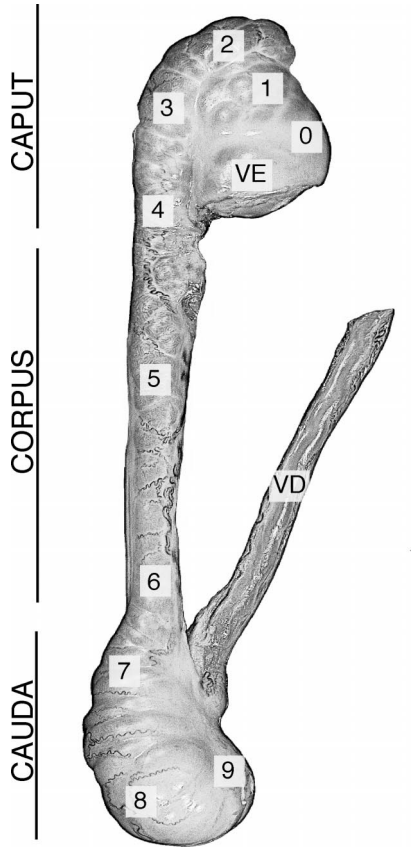

FIG. 1. Photograph of an epididymis from adult ram with the different zones of microperfusion indicated. Caput, corpus, and cauda indicate the gross morphological regions. (Real size, 15 cm.)

Mass Spectrometry

The identification of the major protein spots from two-dimensional gels was obtained by mass spectrometry. Coomassie blue-stained spots were obtained from at least two different bidimensional gels from two different separately treated preparations. The spots were cut into small blocks with a sterile scalpel blade. The blocks were rinsed, then reduced and alkylated with iodoacetamide, and incubated overnight at 37°C in a microtube with 12.5 ng/μl trypsin (sequencing grade; Roche, Meillan, France) in 25 mM NH_4HCO_3 as previously described [18]. The tryptic fragments were extracted, dried, reconstituted with 0.1% formic acid, and sonicated for 10 min.

Tryptic peptides were analyzed either directly by MALDI (M@LDI-L/R; Waters Micromass, Manchester, UK) or sequenced by liquid chromatography coupled to tandem Mass Spectrometry (nano-LC-MS/MS) (Q-TOF-Global equipped with a nano-ESI source; Waters Micromass) in automatic mode. The peptides were loaded on a C18 column (Atlantis dC18, 3 μm × 75 μm × 150 mm, Nano Ease; Waters Micromass) and eluted with 5–60% linear gradient at a flow rate of 180 nl/min for 30 min (buffer A, water:acetonitrile 98:2 [v/v] 0.1% formic acid; and buffer B, water: acetonitrile 20:80 [v/v], 0.1% formic acid). The peptide masses and sequences obtained were either matched automatically to proteins in a non-redundant database (NCBI) using the Mascot program (http://www.matrixscience.com) or de novo sequenced using the ProteinLynx Global Server program (Waters Micromass) and blasted manually against the current databases.

Chemicals

All reagents were of the best available grade (Sigma). Low molecular weight standards for electrophoresis were from Amersham-Pharmacia Biotech AB and Biorad (Ivry sur Seine, France).

RESULTS

Electron Microscopy of the Vesicle Pellet

The white-yellowish pellet obtained after ultracentrifugation of the cauda epididymal fluid was analyzed mor-

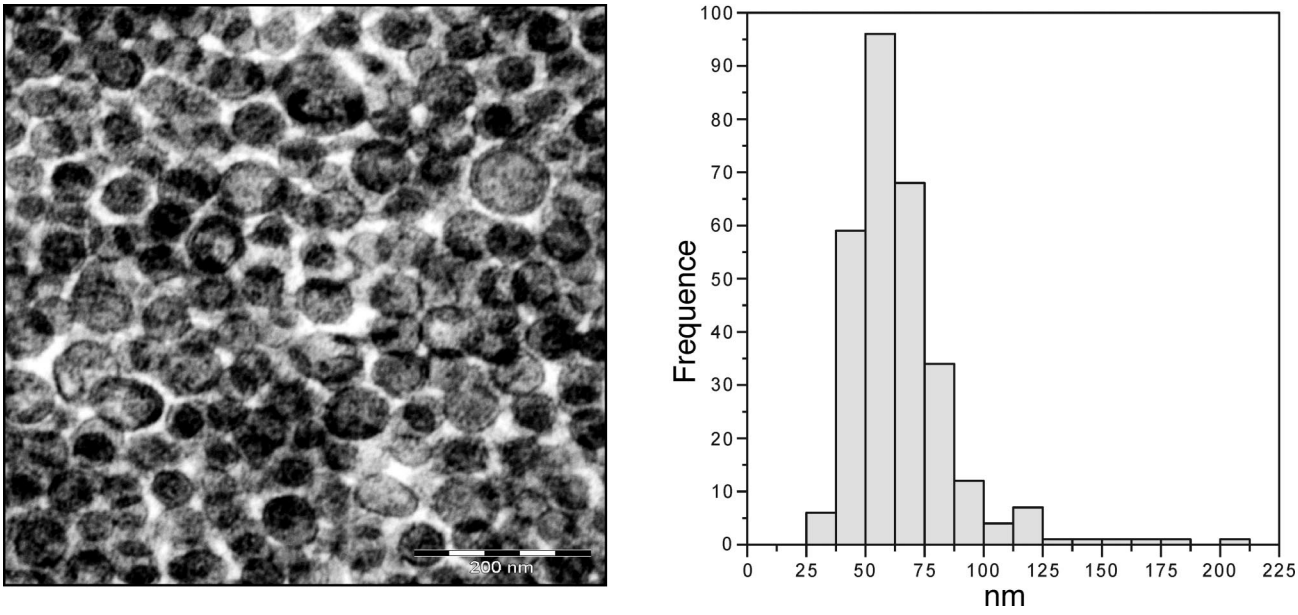

FIG. 2. Electron micrograph and analysis of the high-speed pellet from the cauda epididymal fluid. Electron microscropy of the high-speed pellet showed small vesicles with a typical bilayer membrane. The diameters of the vesicles from two different preparations (10 different microscopic fields, 292 vesicles) were measured to establish the size distribution of these vesicles.

phologically by electron microscopy (Fig. 2). This pellet was composed of vesicles with a distribution size between 25- and 75-nm diameter (64 ± 22 nm; mean ± SD, n = 292). Some of these vesicles contained electron-dense material and a large majority showed a typical membrane bilayer of an average size of 7.9 ± 1.4 nm (mean ± SD, n = 200), suggesting a homogeneous population.

Characterization of the Vesicle Proteins by 1-D SDS-PAGE

The proteins from these vesicles were analyzed by SDS-PAGE (Fig. 3A). The protein pattern obtained was very specific and different from that of the cauda fluid, particularly bands at 140, 110, and 45 kDa that were strongly enriched in the pellet. To estimate the protein quantity represented by these vesicles, the pellet was resuspended to concentrate the vesicles from the starting caudal fluid 20, 10, 5, 2.5, and 1 times (1 = no concentration). The total protein quantity of the different lanes analyzed by a digital system indicated that these proteins were less than 3% of the total protein of the caudal fluid. This value was similar to that obtained by the Bradford assay using BSA as the standard and was also demonstrated by the lack of difference in the pattern of proteins of the cauda fluid before (CEF) or after ultracentrifugation (CEF-hs).

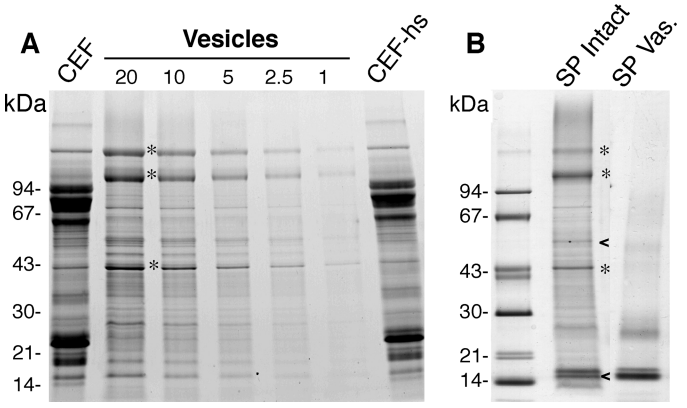

FIG. 3. Protein pattern of the vesicles. **A**) SDS-PAGE comparison of the proteins from caudal fluid (CEF), caudal fluid after high-speed centrifugation (CEF-hs), and from epididymal vesicles (vesicles). The same volume of CEF and CEF-hs were loaded while vesicles were resuspended in the initial volume of fluid (1) or concentrated 2.5, 5, 10, or 20 times. **B**) Protein separation of the vesicle pellet obtained from the seminal plasma (SP Intact) of an intact and a vasectomized (SP Vas.) ram. The same volume of 10-fold concentrated pellet was loaded per lane. 6–16% SDS-PAGE; Coomassie blue-stained gel.

Small Membraneous Vesicles Are Normal Constituents of the Epididymal Fluid

To ascertain that these vesicles had a physiological significance and were not artifacts induced by the collection method, we compared the pellet obtained with the seminal fluid of normal and vasectomized rams collected 3 mo after the surgery (no sperm could be observed in the ejaculate at this time). After high-speed centrifugation, seminal plasma from both types of animals yielded a pellet, but the pellet obtained from the vasectomized rams was much smaller than from the controls. After separation by SDS-PAGE (Fig. 3B), the protein pattern of vesicles obtained from the vasectomized animal was very different from that of the normal ram and that of the cauda epididymis. All the characteristic proteins of these latter pellets were absent from the pellet of the vasectomized animals. In contrast, the pattern obtained from the epididymal vesicles and the seminal plasma vesicles from the normal animals differed only by two protein bands at 14 and 55 kDa.

Identification of the Vesicle Proteins by Two-Dimensional SDS-PAGE, Mass Spectrometry, and Western Blotting

The vesicle proteins from the caudal fluid (Fig. 4) and from the seminal plasma (not shown) were separated by two-dimensional gel electrophoresis to allow mass spectrometry identification. Both preparations gave identical protein profiles except for two series of spots at about 14

TABLE 1. Names, accession numbers, size, sequence of the peptides obtained by LC-MS-MS, and number of peptides and percentage of the protein coverage obtained by Maldi for each protein described in Figure 3.

Spots	Identity	Accession numbers for matched sequences	Theoretical MW (Da)	LC-MS-MS	Maldi	
					Peptides	Coverage (%)
DPP-IV	Dipeptidyl peptidase IV	DPP4_BOVIN, P81425, AAL67836, DPP4_FELCA, BAA92344, Q9N217, DPP4_HUMAN, P27487, CDHU26, CAA43118, AAA52308, Q866G2	88314	IISNEEGYK TYTLADYLK RTYTLADYLK KLDFIHLHGTK QYILFEYNYVK QHIEISTTGWVGR LAYVWNNDIYVK WEYYDSVYTER LGTFEVEDQIEATR FWYQMILPPHFDK + Ox (M)* HSYTASYDIYDLNK HSYTASYDIYDLNKR FRPAEPHFTSDGNSFYK LNWATYLASTENIIVASFDGR GAWEVIGIEALTSDYLYYISNEYK QVEYLLIHGTADDNVHFQQSAQISK IAIWGWSYGGYVTSMVLGSGSGVFK	36	40.1
NEP	Neprilysin	NEP_RAT, P07861 HYRTN, NEP_MOUSE, Q61391, AAA37386, NEP_HUMAN, CAA30157, HYHUN, AAA51915, NEP_RABIT, P08049, CAA28950, HYRBN, O93394	85741	FIMDLVSSLSR HVVEDLIAQIR ALYGTTSETATWR KALYGTTSETATWR DGDLVDWWTQQSANNFK EVFIQTLDDLTWMDAETK + Ox (M)* VDKDEWISGAAVVNAFYSSGR EVFIQTLDDLTWMDAETKK + Ox (M)* NFNKDGDLVDWWTQQSANNFK SLNYGGIGMVIGHEITHGFDDNGR	21	25
E-NPP3	Ectonucleotide pyrophosphatase/ phosphodiesterase-3	NPP3_RAT, 6114760, BAA06333, AAB61535, AAB61536, Q99K07	99008	DVELLTGLDFYQEK AEYLQTWSTLLPNINK VRDVELLTGLDFYQEK		
mannosidase	Beta mannosidase	Q9TTG6, A55881, AAC48665	98524	TSAVAPFVWLDVGSIPGR LVSGEAIVAIPELNIQQR FVSEYGYQSWPSFSTLEK TVELVEEPIQNSPGLSFYFK		
Lactotransf.	Lactotransferrin precursor	Q29477, JC2323	77357	ANEGLTWNSLK LRPVAAEIYGTEK SPQTHYYAVAVVK DLLFKDSALGFVR IPSKVDSALYLGSR ETTVFENLPEKADR SVDGKENLIWELLR YLGTEYVTAIANLKK GEADALSLDGGYIYTAGK	9	15.3
Ezrin	Ezrin/Moezin	EZRI_BOVIN, I45889, P31976, P2602, Q8HZQ5, Q8VHK3, Q9DCI1 Q80ZT8, Q9YGW6	68586	APDFVFYAPR EVWYFGLQYVDNK SQEQLATELAEYTAK IAQDLEMYGINYFEIK FYPEDVAEELIQDITQK VTTMDAELEFAIQPNTTGK AKFYPEDVAEELIQDITQK QAADQIKSQEQLATELAEYTAK	36	54.8

TABLE 1. *Continued.*

Spots	Identity	Accession numbers for matched sequences	Theoretical MW (Da)	LC-MS-MS	Maldi	
					Peptides	Coverage (%)
NAP22	NAP22/BASP1	BASP_BOVIN, AAC67307, NP_006308	22866	ETPAATEAPSSTPK ASEAEAAEPAAPTKDDK AQAPAAPADEVKPAETPAANSDQTVAVKE		
HSC71	Heat shock cognate 71 kDa protein	P19120			10	18.4
V-ATPase	Vacuolar ATP synthase catalytic subunit A	P50516			19	37.1
hPAST1	Testilin-hPAST1	O14611			20	39
OSA	Serum albumin precursor	P14639			8	13.5
Pyr Kin	Pyruvate kinase	P14786			7	20
Tubulin	Tubulin	P053217			7	21.5
Carn1	MARC 4BOV/ Carnosinase 1	15376943, BAA91840, Q8WY59, Q9NW02, Q9NUV1, CAD56843	43805	MLAAYLYEVSQLKD EGGSIPVTLTFQEATGK YPSLSLHGIEGAFSGSGAK NVMLLPVGSADDGAHSQNEK NVMLLPVGSADDGAHSQNEKLNR + Ox (M)*	9	24
Crea kin	Creatine kinase	P05124			14	48
Enolase	Alpha-enolase	ENOA_BOVIN, AAD33073, Q9DDH4, ENO_XENLA, Q8AVT0, ENOA_TRASC, AAD41645, Q9DDG7, Q9XSJ4, Q9DDH1	47116	IGAEVYHNLK EALELLKNAIGK GNPTVEVDLFTAK LAQSNGWGVMVSHR + Ox (M)* AAVPSGASTGIYEALELR DATNVGDEGGFAPNILENK LAMQEFMILPVGAENFR FTASAGIQVVGDDLTVTNPK YG(K/Q)DATNVGDEGGFAPNILENK DYPVVSIEDPFDQDDWEAWQK SFIRDYPVVSIEDPFDQDDWEAWQK	20	55.9
Ald-dh	Aldehyde dehydrogenase	DHA1_SHEEP 1BXSA S14752 P51977, DHA1_HUMAN, DEHUE1, AAA35518, AAP36480 AAC51652, AAB60268, S02302, DHA1_MOUSE, Q811J0, JQ1004, AAB32754, Q9PWJ3, Q9W617, Q9YGY2	54098	VAFTGSTEVGK FPVFNPATEEK LLLATMEAMNGGK KFPVFNPATEEK EEIFGPVQQIMK TIPMDGNFFTYTR IFINNEWHSSVSGK ELGEYGFHEYTEVK IAKEEIFGPVQQIMK IFINNEWHSSVSGKK GYFIQPTVFSDVTDDMR ANNTFYGLSAGIFTNDIDK YVLGNPLTPGVSQGPQIDKEQYEK	16	38.8
Citr-dh	Isocitrate dehydrogenase	O88844			12	29.5
Actin	Actin	P53463, IC0FA, 1DGAA, ATBOB, ATBOG, ATHUG, ATRTC, ACTE_STRPU, AAA49639, AAA64871, AAA51580, CAA31455, CAA26486, CAA27396, CAA74015, JS0190, O93400, Q8WVW5	41579	AVFPSIVGR AGFAGDDAPR GYSFTTTAER EITALAPSTMK + Ox (M)* QEYDESGPSIVHR SYELPDGQVITIGNER VAPEEHPVLLTEAPLNPK DLYANTVLSGGTTMYPGIADR + Ox (M)* KDLYANTVLSGGTTMYPGIADR + Ox (M)* TTGIVMDSGDGVTHTVPIYEGYALPHAILR+Ox (M)*	16	44.1

TABLE 1. *Continued.*

Spots	Identity	Accession numbers for matched sequences	Theoretical MW (Da)	LC-MS-MS	Maldi	
					Peptides	Coverage (%)
Sorb-dh	Sorbitol dehydrogenase (L-iditol 2-dehydrogenase)	P07846			9	22
G-beta	Transducin beta chain 1 (protein G beta)	P04901 1A0RB 1GG2B RGHUB1 Q9QWG8 CAA26875 AAC72249	37236	LIIWDSYTTNK KACADATLSQITNNIDPVGR LIIWDSYTTNKVHAIPLR	9	28.2
Ald Red	Aldose reductase	P80276			8	25.7
annexin	Annexin II	P04272			20	49.4
PAS 6/7	Lactadherin precursor. (MFG-E8/PAS6/7)	Q95114			7	14.3
NCC	Nuclear Chloride Channel	AF109197, AAD26137, CAB46078		LFMVLWLK NSNPALNDNLEK GVTFNVTTVDTKR LAALNPESNTAGLDIFAK FSAYIKNSNPALNDNLEK VLDNYLTSPLPEEVDETSAEDEGVSQR	6	24
Glut-S-transf	Glutathione S transferase μ5	P48774, Q9Z1B2, BAB22327, Q9N0V4, Q9TSM4, O97117	25618	(L/I)TQSNAILR SMVLGYWDIR PMILGYWDIR MAPVLGYWDIR MLLEFTDTSYEEK LLLEYTDTNYEER LDLDFPNLPYLMDGK LGLDFPNLPYLIDGTHK LTFVDFLTYDVLDQNR	5	31.7
PEBP	Phosphoethanolamine binding protein	P13696, PEBP_HUMAN, PEBP_MACFA, 1A44, S00056, S18358	20714	LYTLVLTDPDAPSR YVWLVYEQEGPLK NRPTSITWDGLDPGK LYTLVLTDPDAPSRK YREWHHFLVVNMK GNNISSGTVLSDYVGSGPPK WSGPLSLQEVDERPQHPLQVK	11	79
SOD	Superoxide dismutase	P09670			4/5	32.4/37.1

*Ox (M), oxidized methionion.

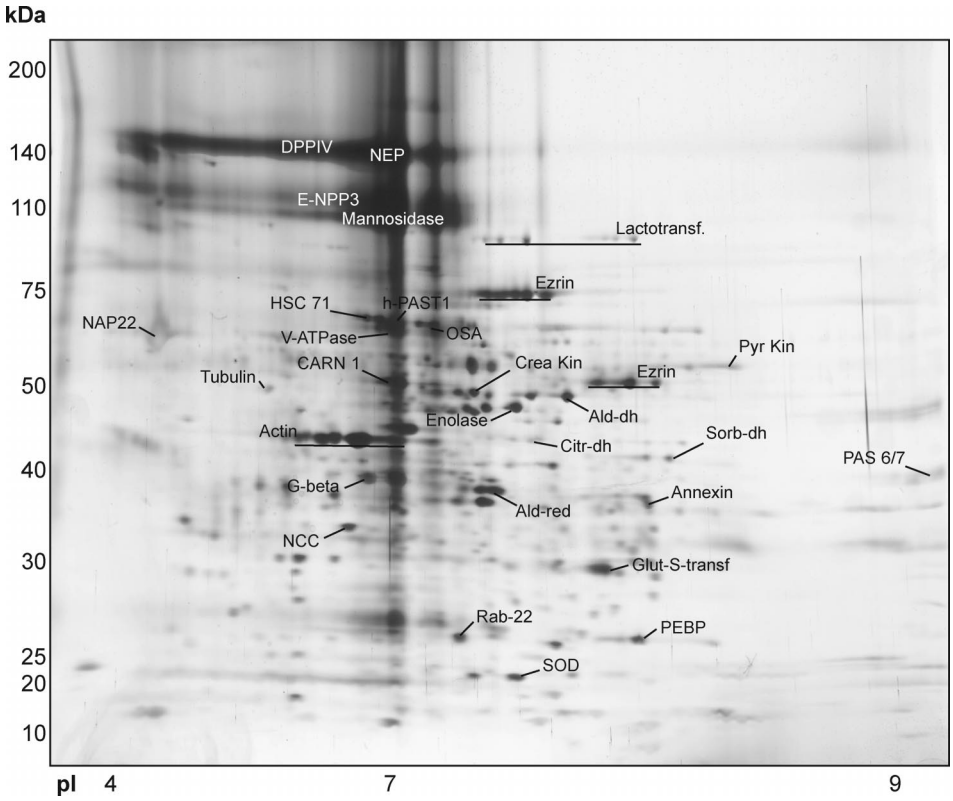

FIG. 4. Two-dimensional gel electrophoresis of the vesicle proteins. The vesicles were treated and separated by two-dimensional gel electrophoresis under denaturing conditions. The spots were cut and processed by mass spectrometry to identify the proteins (see also Table 1). 6–16% SDS-PAGE; silver-stained gel.

and 55 kDa present only in the seminal plasma (see also Fig. 3B). The major protein spots from the vesicles observed by Coomassie-blue staining (Fig. 4) were processed for identification by mass spectrometry. Two different approaches were used: Maldi-TOF, which allowed comparison of the tryptic pattern of a protein with the theoretical tryptic pattern in databases; and nano-HPLC-MS-MS, which allowed sequencing of specific tryptic fragments and search for homology in data bases (see Tables 1 and 2).

Fragments matching several proteins were obtained in the major spots at about 140 and 120 kDa, ie: dipeptidyl peptidase IV (DPPIV) and neprilysin (NEP) (140 kDa), and phosphodiesterase-I (E-NPP3) and beta-mannosidase (120 kDa). These proteins were also found when the 140 kDa and 120 kDa bands from a one-dimensional gel were processed in the same way. Because of their abundance and the vertical smear they produced in the gel, dipeptidyl peptidase IV and neprilysin signatures were found in a large number of other spots with lower molecular weights. The cytoskeleton protein actin (45 kDa) was also one of the major proteins of the vesicles; this protein was separated as a series of at least four different spots composed of alpha- and beta-actin isoforms. The actin-associated protein Ezrin/Cytovillin was found in two major trains of spots: one at 75–80 kDa, which is its normal molecular weight, and one at 50 kDa, which could represent a degradation product of this protein. Tubulin and annexin II were two other cell-membrane cytoskeleton components identified. Several other membrane-linked proteins were identified such as lactadherin-PAS6/7 protein, chaperone heat shock protein-71 (HSC71), vacuolar ATPase (V-ATPase), protein G-beta (G-beta) and NAP22/BASP1. One protein expressed in testis (HPAST1-testilin-EHD1) at the germ cell level was also identified. The metabolic enzymes creatine kinase (CreaKin), pyruvate kinase (PyrKin), glutamate carboxypeptidase-like-1 (named carnosinase 1; CARN1), alpha-enolase (Enolase), aldehyde and isocitrate dehydrogenase (Ald-dh and Sorb-dh, respectively) and aldose reductase (Ald-red) were also present. Varying amounts of lactotransferin and serum albumin (OSA) were also found depending upon the preparation variable. Because these proteins were present in the caudal fluid, they may be adsorbed to the vesicle membrane. More information on these proteins is available in Table 2.

Western blots were performed using specific antibodies to confirm the identities of some of the major vesicle proteins (Fig. 5). Immunoreactions with neprilysin, ENPP3, protein G-beta, lactadherin-PAS6/7and actin were observed in raw cauda epididymal fluid (CEF) and purified vesicles (Fig. 5), but their intensity levels decreased significantly after removal of the vesicles from the caudal fluid by centrifugation (Fig. 5, CEF-hs). This result thus confirmed that these proteins are mainly associated with the vesicles.

Presence of Epididymal Hydrophobic Proteins Within the Vesicles

To investigate whether hydrophobic proteins cosediment with the vesicles, we used a similar immunoblotting approach (Fig. 5). The first protein sought was the 17-kDa hydrophobic protein that has previously been described in rams [5]. This protein is secreted exclusively by the epithelium of the cauda region (zones 7–9) and transferred to the sperm membrane. The Western blot showed that this protein was linked to the vesicles but that a large part also remained in the fluid after centrifugation.

Because PEBP (phosphatidyl-ethanolamine binding protein) was found by mass spectrometry to be associated with the vesicles, we analyzed whether other proteins that bind and transport hydrophobic compounds in epididymal fluid were also present. One isoform of the retinol acid binding protein (E-RABP) was found to be present in the vesicles

as well as prostaglandin D2. In contrast, the cholesterol transport protein (HE1/NP-C2/CTP) was not found to be associated with the vesicles.

Epididymal Distribution of the Vesicles

Using these immunological tools, the origin of the vesicles along the epididymis was investigated. We probed the epididymal fluids from different zones with antibodies against the proteins linked to the vesicles, i.e., phosphodiesterase E-NPP3, neprylisin, lactadherin-PAS 6/7, and protein G-beta (Fig. 6A). We found that E-NPP3, NEP, and the 35-kDa G-beta protein were restricted to the cauda epididymal fluid (zones 6/7 to 9). We observed that one form (30 kDa) of PAS6/7-lactadherin was mainly associated with the caudal fluid and another (50–55 kDa) was present throughout the epididymis.

The Western blot probed with the anti-actin antibody (Fig. 6B, control) showed the presence of this protein in the fluid throughout the epididymis. Because this might suggest that some of the vesicles were transported from the upper epididymal tract, we submitted these different fluids to high-speed centrifugation. After this treatment, actin was slightly decreased in the high-speed supernatant from zone 7 (Fig. 6B, high speed) as compare with the control (Fig. 6B, control) and was completely removed from the fluid in zones 8 and 9. It was also in these zones that actin was retrieved associated with the pellet (see Fig. 5).

We therefore analyzed the behavior of other vesicle proteins. We observed that the amounts of E-NNP3, neprilysin, lactadherin-PAS 6/7, 35-kDa G-beta in zone 7 were strongly decreased after high-speed centrifugation (Fig. 6C), confirming that these proteins are associated with the vesicles as soon as they are present in the fluid.

Origin(s) of the Vesicles

Because we have previously observed that 5–10% of sperm died during epididymal transit [14] and because sperm lose their cytoplamic droplet during the corpus transit, we checked whether some of the vesicles were derived from the sperm cells or their droplets. For this, we used different antibodies to investigate whether the vesicle proteins were also present on the sperm and/or the cytoplasmic droplet.

Sperm obtained from the caput region (zone 2) that retain their cytoplasmic droplets; from the corpus region (zone 6), where sperm with or without droplets coexist; from the cauda epididymis (zones 8–9), where sperm have lost their droplets were washed by percoll gradient centrifugation before extraction. This also purified intact cytoplasmic droplets released in the caudal fluid. As a control, an equal volume of fluid from the cauda containing both the sperm and the droplets was centrifuged and washed only with PBS.

The PBS- and percoll-washed cauda sperm, the cytoplasmic droplets, the caudal fluid and the vesicles pellet were run on SDS-PAGE (Fig 7A). Almost no difference was observed in the protein pattern between the two caudal sperm preparations (first and second lanes) and the sperm extracts from zones 2 and 6 (not shown). The cytoplasmic droplet protein pattern was very different from those of the sperm extracts, the caudal fluid, and the vesicles. We have previously shown that acrosin could be released in the fluid by dying sperm [14], and thus, if some of the vesicles were derived from the acrosomal membranes of these damaged sperm, some of the acrosin should remain associated with

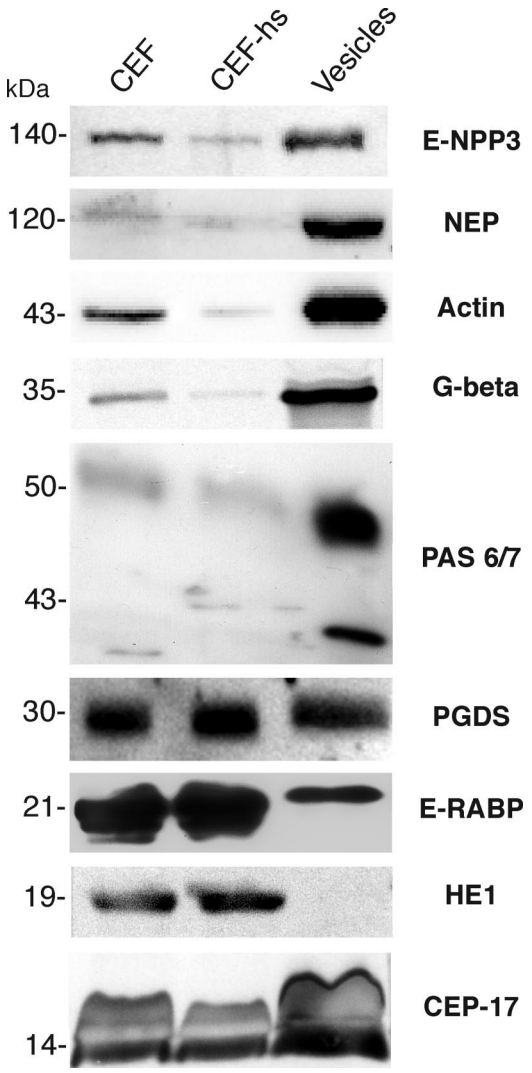

FIG. 5. Immunological identification of some of the vesicle proteins. Caudal fluid (CEF), caudal fluid after high-speed centrifugation (CEF-hS), and epididymal vesicles (10 times concentrated) were separated on a 6–16% SDS-PAGE and transferred to nitrocellulose and probed with specific antibodies to confirm the identity of some of the proteins obtained by mass spectrometry. Similar Western blots were probed with antibodies directed against hydrophobic or lipophilic proteins known to be in the ram cauda epididymal fluid and/or on sperm membrane.

it. With our ram anti-acrosin antibody, three different acrosin forms were observed in the sperm extracts between 43 and 50 kDa and a 43-kDa band was slightly visible in the caudal fluid (Fig. 7B). No reactive bands were found in cytoplasmic droplets or in the vesicle preparation, suggesting that the vesicles did not derive from the degraded acrosomal sperm membranes and indicating also that the cytoplasmic droplet preparation was not contaminated with sperm.

The previous samples and sperm extracts from zones 2 and 6 were probed with the different antibodies reacting with the vesicle proteins (Fig. 8). The anti-phosphodiesterase and anti-neprilysin antibodies showed reactive bands in the cauda fluid containing the vesicles, but no reaction was obtained with the cauda sperm extract or cytoplasmic droplet. The neprilysin antibody reacted with the caput sperm extract but not with corpus and cauda sperm extracts, but the neprilysin form observed in the fluid showed a lower molecular weight (less than 120 kDa; see Fig. 8, compare

TABLE 2. Properties of identified proteins.

Identity with comments
Dipeptidylpeptidase 4—DPP4 (also known as CD26)
DPP4 is a cell-surface glycoprotein with protease activity that removes post-proline N-terminal dipeptides from peptides of up to 100 amino acids (for review [19, 20]). This protein also interacts with numerous proteins such as adenosine deamisase or chemokine receptors. This enzyme has been reported to be involved in cancer progression and in immunology. It is of note that ablation of the *Dpp4* gene has no effect on mouse reproduction [20]. DPP4 is present on prostasomes and exosomes from enterocytes.
Membrane metallo endopeptidase—MME (also known as Neprilysin, NEP, CD10)
Neprilysin is known as the common acute lymphoblastic leukemia antigen (CALLA) [21]. This protein is an integral membrane zinc metalloendopeptidase that degrades peptides of up to 40 amino acids such as enkephalins and substance P. The MME family comprises several different members. Neprilysin is mainly expressed in brain, kidney, and testis, and one member of the MME family, NEPII or SEP/NL1, is expressed predominantly in the testis at the level of round spermatids [22–24]. This form could be the high-molecular weight immuno-reactive compound found on sperm extract from the caput epididymis. The relationship between this protein (which exists under membrane and soluble forms) and the 120-kDa immunoreactive protein from the cauda fluid remains to be established. MME has also been described in the human prostate epithelium and found in prostasomes [25]. Targeted disruption of the MME locus in mice showed that this enzyme protects against induced endotoxin lethality and acts on the arterial blood pressure, but no effect on reproduction has been reported [26, 27].
Ectonucleotide pyrophosphatase/phosphodiesterase 3 (PDNP3, ENPP3)
Phosphodiesterase (ENPP3) is an ectonucleotide pyrophosphatase/phosphodiesterase of a multigene family (for review [28]). ENPP3 is a transmembrane type II metallo-enzyme present on the apical surface of polarized cells. These enzymes have broad substrate specificity (ATP, ADP, AMP, etc.) and are involved in various physiological processes (bone mineralization, nucleotide recycling, tumor invasion, etc.). In humans, *Enpp3* mRNA has been found in the prostate; in mice, one family member, NPP1/PC-1, has been immunolocalized in the epididymis with a regional expression restricted to the corpus-cauda [29].
Beta mannosidase
Beta-mannosidase (Beta-galactosidase/beta-glucuronidase) is a lysosomal glycosidase described in sperm and in epididymal fluid from different species [30]. In humans, the epididymis is the main source of this enzyme and its activity has been used as a biological marker for obstructive azoospermia [31]. In the bull and rat this enzyme is highly present in the cauda epididymis and has been found associated with vesicles in the rat [32, 33]. The role of this enzyme in reproduction is not known, but transient association with sperm has been reported, suggesting a role in the modification of sperm protein sugars [34].
Brain abundant, membrane attached signal protein 1—BASP1 (also known as NAP22 and CAP23)
BASP1 is very similar to the chicken brain protein CAP23 and is part of a protein family (BASP1 family) that contains PEST sequences characteristic of short-lived proteins [35]. BASP1 is abundant in nerve endings but is also present in high amounts in nonnervous tissues: testis, kidney, and lymphoid organs (spleen, thymus). BASP1 is a 22- to 25-kDa protein that shows a slow migrating behavior in SDS-PAGE due to its N-terminal myristoylation, which explains is appearance at more than 50 kDa in our study. The exact role of these proteins is not known. This protein is found in prostasomes.
Heat shock protein 8—HSPA8 (also known as HSC71)
One member of the heat shock protein family HSPA8 was found expressed in the testis at the level of the sperm germ cell, where it is involved in spermatogenesis [36]. This protein remains in mature sperm and may have a role in sperm-egg binding. In the ram, we observed HSPA8 in sperm extract (unpublished results). The mRNA and the protein have also been found in other tissues, including the epididymis, although at a lower level than in the testis [37]. This protein is found in prostasomes and exosomes.
ATPase, H+ transporting, lysosomal 70 kDa, V1 subunit A. (ATP6V1A, vacuolar ATPase)
Both V-ATPase and CliC have been immunolocalized in epididymal epithelium in mice where they are involved in maintaining a slightly acidic pH of the caudal fluid. The chloride channel (CliC) is found in acidic intracellular vesicles and functions in association with the vacuolar ATPase [38, 39].
EH-domain containing 1—EHD1
EHD1 is a 62-kDa protein highly expressed in testis and particularly in germ cells [40]. EHD1 is a member of a recently characterized family of four genes in human. These proteins show multiple conserved domains that include a nucleotide-binding consensus site at the N-terminus, a bipartite nuclear localization signal, and a protein-binding domain with an EF-hand calcium-binding motif at the C-terminus. These proteins may play a role in endocytosis, and vesicle trafficking and signaling by interacting with other proteins [41].
Carnosine dipeptidase 1 (metallopeptidase M20 family)—CNDP1
Two carnosinases have been described: one is expressed as a brain-specific enzyme (CNDP1) and the second (CNDP2) is expressed in different tissues, including testis and kidney [42]. These enzymes degrade carnosine (beta-alanyl-histidine) and homo-carnosine (gamma-aminobutyric acid-L-histidine) that are dipeptides with neuroprotective and neurotransmitter functions in the brain and are abundant in muscle and certain peripheral tissues. These peptides possess strong and specific antioxidant and metal-binding properties (for review see [43]). CNDP1 is very specific for histidine and thus seems a true carnosinase while CNDP2 corresponds to a nonspecific cytosolic dipeptidase. In human, the enzyme deficiency leads to neurological defect and mental retardation.
Creatine kinase (Muscle [CKM] and Brain [CKB] isoforms)
Creatine kinase has been reported in sperm from different species where it may participate in energy transport from mitochondria to flagellar dynein-ATPase. This enzyme is also found in the cytoplasmic droplet The level of this enzyme in sperm has been related to sperm malformation and poor fertility [44, 45]. This enzyme is found in prostasomes.
Guanine nucleotide binding protein (G protein), beta polypeptide 1 (transducin beta chain 1, protein G beta)
Protein G is involved in sperm capacitation and acrosomal reaction, and different forms of protein G are involved in different cell exocytose mechanisms. Several isoforms of protein G have been immunolocalized on sperm; a 35- to 37-kDa form was observed in human sperm by Western blot [46, 47]. The immunoreactive doublet observed in sperm at 20 kDa is striking because no protein G beta of this molecular size has been reported. This doublet could be a degradation product of the 35- to 37-kDa protein.
Aldo-keto reductase family 1, member B1 (aldose reductase)
Aldose reductase has recently been found in cauda epididymal vesicles from the bull. This enzyme is immunolocalized mainly in the basal epithelium of the corpus and cauda regions of the epididymis in the bull [48]. This enzyme is found in prostasomes.
Milk fat globule-EGF factor 8 protein (MFGE8)
MFGE8/PAS67/P47 is a protein of about 50 kDa expressed in the testis and the mammary gland [15]. This protein is one of the major proteins associated with the milk-fat globule. In mice, it has been suggested that the principal cell of the initial segment secretes the protein and this protein has also been immunolocalized on sperm membrane of different species [15, 16, 49, 50]. The sperm protein is apparently processed during sperm maturation and capacitation [51]. The protein binds to integrins and zona pellucida proteins and is one of the candidates to mediate the sperm-egg binding [49–52]. Recently it was shown that the sperm's ability to recognize and bind with the zona pellucida is impaired by antibody against the protein or by the recombinant protein, and some of the null male mice for this protein were infertile [50]. We found that the main difference between the cauda and the ejaculated vesicles was 50- to 60-kDa MFGE8. This protein is found in prostasomes.

TABLE 2. *Continued.*

Identity with comments
Glutathione S-transferase μ5 (GSTM5)
Glutathione S-transferase has been described in the epididymis of different species [4]. Different forms of glutathione S-transferase are present in the testis and in the epididymal epithelium and may protect the sperm membrane from lipid peroxidation during long-term storage in the cauda epididymis. Some of the enzyme isoforms seem to be secreted via an unconventional route through apocrine bleb secretion, and are found within 20-nm-diameter vesicles contained in these membrane blebs [53]. This protein is found in prostasomes.
Prostatic Binding Protein (PBP), Phosphoethanolamine-binding protein (PEBP)
PBP is a ubiquitous protein, and two different genes encoding two different proteins during spermatogenesis have been described in mouse germ cells [54]. PBP is also expressed in the epididymis, mainly in the caput-corpus junction, and has been found in epididymal fluid and associated with large vesicles [55]. The role of this protein in the transport/transfer of phosphatidyl-ethanolamine from the epididymal fluid to the sperm is uncertain, but this protein has a tendency to bind electrostatically to negatively charged domains of membranes [56]. This protein is found in prostasomes.
Superoxide dismutase (SOD3)
A specific form of superoxide dismutase, highly expressed in cauda epididymis, has been shown to be secreted in the luminal fluid [57]. This enzyme could also be involved in sperm protection against lipid peroxidation.

lanes Z2 and CEF). Moreover, this neprilysin fluid form appeared only in zones 6–7, suggesting that it was a different form than the sperm neprilysin. The anti-actin antibody reacted with the sperm extracts but the decrease in the reaction observed in the percoll-washed sperm and the substantial reaction with the cytoplasmic droplets indicated that actin was mainly present in these later (Fig. 8). As expected, the total fluid was reactive because actin was present in the vesicles.

The anti-G beta antibody recognized different bands on sperm extracts, the most reactive at 43 and 35 kDa and a strong doublet at about 20 kDa. The 43-kDa band showed varying intensity depending on the samples, and a less reactive band at 55 kDa could also be observed. The 35-kDa band was decreased on the percoll-washed sperm extract but was highly enriched in cytoplasmic droplets while the other bands remained at the same level (Fig. 7). This 35-kDa band was also found in the CEF and in the vesicles. Anti-lactadherin-PAS6/7 reacted slightly with the 50-kDa and 35-kDa bands in sperm, and a 43-kDa band was also

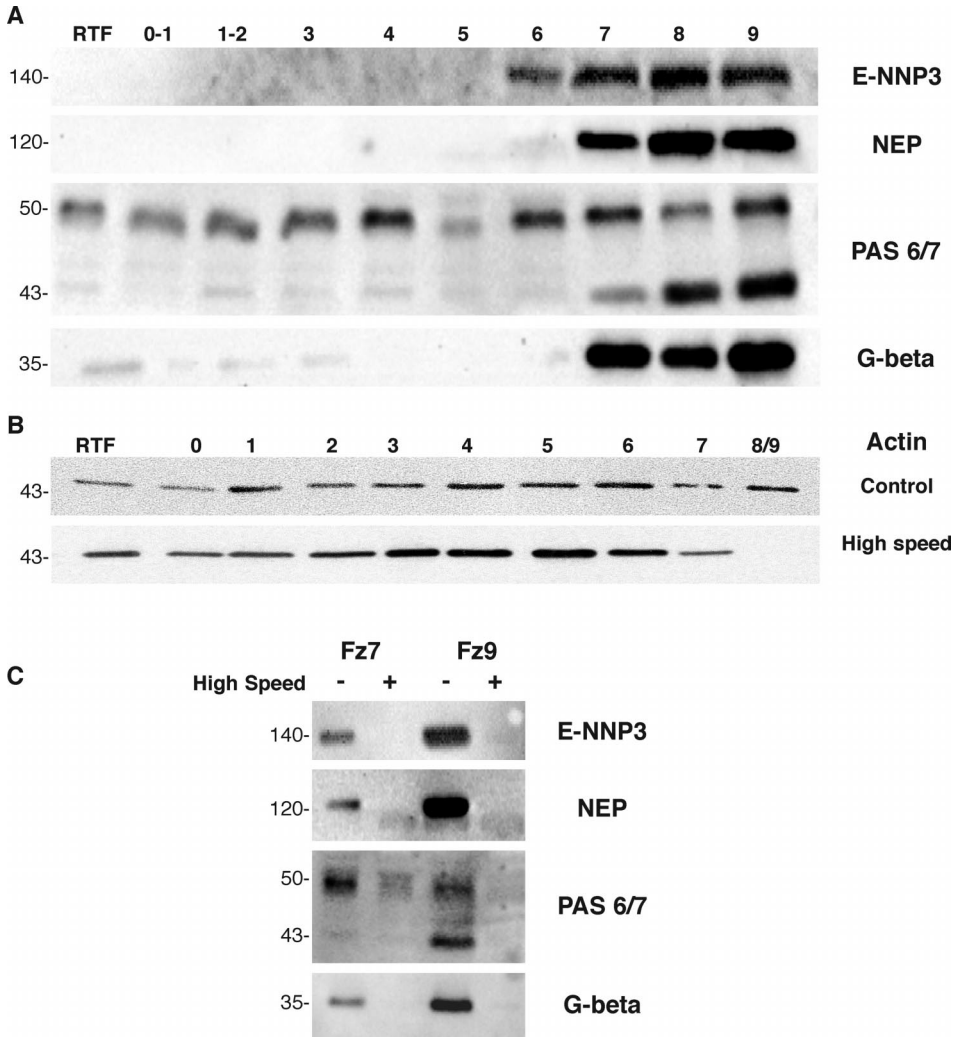

FIG. 6. Regional distribution of the vesicle in the epididymal fluid. **A**) Equivalent amounts of protein from the rete testis and the different epididymal zones were run on 6–16% SDS-PAGE and transferred to nitrocellulose and probed with antibodies reactive against vesicle proteins. **B**) An equivalent Western blot, actin was present throughout the epididymal fluid (control); high-speed centrifugation (high speed) decreased the quantity of actin in zone 7 and removed it from the fluid in zones 8/9. **C**) The fluid of zones 7 and 9 were probed with antibodies reactive against vesicle proteins before (−) or after (+) high-speed centrifugation. After this treatment, the immunoreactive proteins were significantly reduced or removed, indicating they are linked to vesicles.

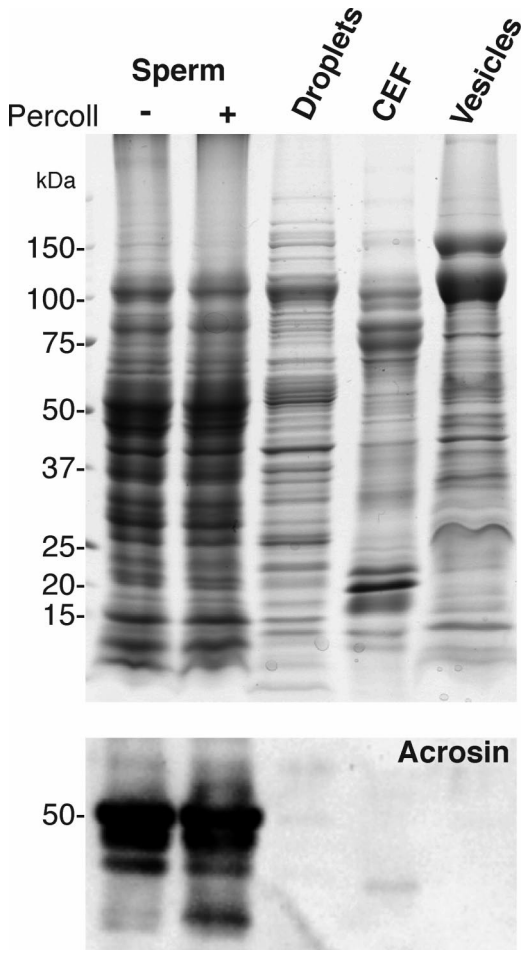

FIG. 7. Origin of the protein vesicles. Equivalent amounts of epididymal cauda sperm before (−) and after percoll wash (+), cytoplasmic droplets, cauda epididymal fluid (CEF), and purified vesicles were separated by 6–16% SDS-PAGE. After Coomassie blue staining, except for sperm before and after percoll washing, a very different pattern of proteins was observed for each type of sample. An equivalent gel was transferred to nitrocellulose and probed with a ram anti-acrosin antibody. Sperm were strongly reactive and a slight reaction was observed in the cauda fluid.

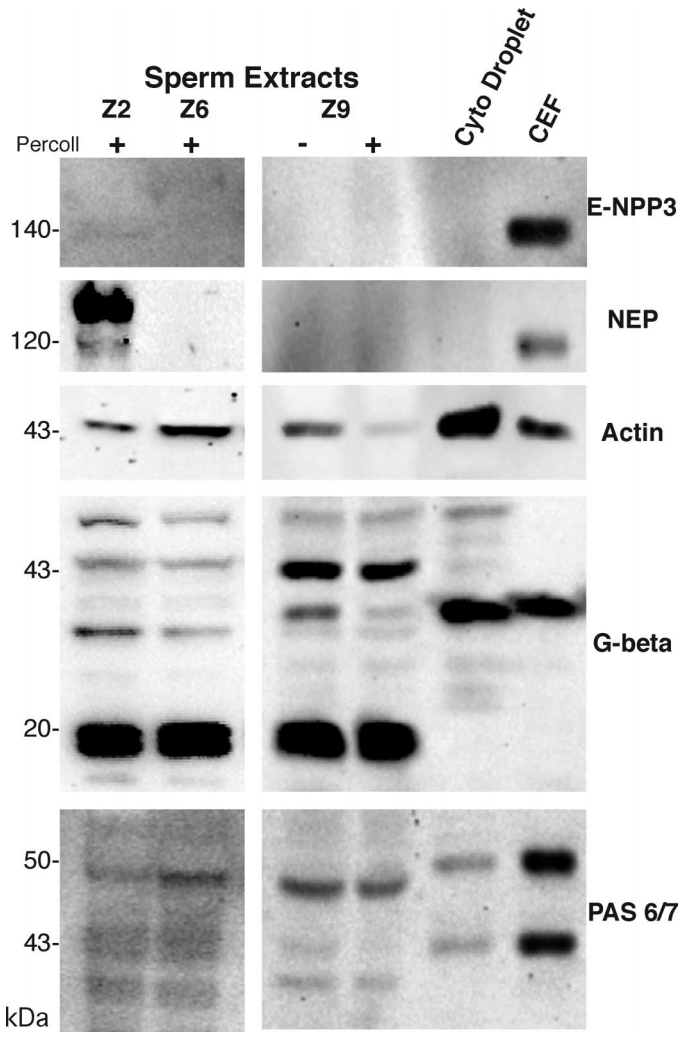

FIG. 8. Common proteins between sperm and vesicles. Equivalent amounts of epididymal percoll-washed sperm from zones 2 and 6, cauda sperm before (−) and after percoll washing (+), purified cytoplasmic droplets, and cauda epididymal fluid (CEF) were separated, transferred, and probed with antibodies reactive to vesicle proteins. The major proteins ENPP3 and neprilysin were not found on sperm from zones 6 and 9 nor on cytoplasmic droplets, while actin, G-beta, and the 43-kDa PAS6/7 were mainly associated with cytoplasmic droplets but not with cauda epididymal percoll-washed sperm.

visible in PBS-washed sperm but decreased after washing with Percoll. An equivalent 43-kDa band and the 50-kDa band were retrieved in cytoplasmic droplets and in the caudal fluid (Fig. 8). Similar results were obtained when ejaculated sperm extracts were used instead of cauda epididymal sperm extracts (not shown).

All these findings indicated that none of the immunoreactive proteins found in the vesicles were present on mature sperm extracts and that only three of the five antibodies cross-reacted between the cytoplasmic droplets and the vesicles, suggesting no direct relationships between the sperm and the vesicles.

DISCUSSION

Our results demonstrated clearly that membranous vesicles are normal constituents of the cauda epididymal fluid and that, although a small number of vesicles originate from the other accessory glands, this fluid is the main source of the vesicles retrieved in the ram seminal plasma. This is demonstrated by the very similar one-dimensional and two-dimensional SDS-Page protein profiles and the protein compositions found for the epididymal and seminal plasma vesicles of normal rams and the absence of these vesicles in the seminal plasma of vasectomized rams.

Several groups have suggested that small membranous vesicles could be formed by apocrine or bleb secretions from the testis, efferent ducts, and the epididymal epithelium [53, 58, 59]. It has also previously been shown that the seminal plasma of different mammals, including the humans, contains neurosecretory-like vesicles, and some findings have indicated that these vesicles originated from the prostate and seminal vesicles [60, 62]. The epididymal membrane vesicles described here are quite homogeneous in size and delimited by a single bilayer membrane. Although their mean diameter is smaller than the average diameter reported for seminal plasma vesicles, it can be concluded that the seminal-fluid vesicles are composed of a mixture of vesicles originating from the different accessory glands, to which the epididymis largely contributes (see also [63]). Moreover, almost all proteins identified in ram epididymal vesicles have also been found in a recent proteomic profiling study of human seminal plasma vesicles

[26], and one protein, aldose reductase, has also been described in bull epididymal vesicles [27] (see also Table 2). This suggests that, as in the ram, a large proportion of the vesicles found in seminal plasma in humans (and certainly in bulls) are derived from the epididymis. Alternatively, these different types of vesicles may be shed from the cell membrane by a very similar mechanism, including the same membrane domains containing the same proteins.

Both the epididymal and seminal plasma vesicles also show some physical and biochemical similarities to vesicles secreted by different types of epithelia and cells called exosomes [11]. They are in the same size range, they are not retained by 0.22-μm filtration, and they can be purified by size exclusion on gel filtration. Indeed, the ram cauda fluid size-exclusion peak obtained by gel filtration was found to have a similar protein pattern as the vesicle pellet, and this peak was considerably decreased when the fluid was centrifuged at high speed before separation (unpublished data). All this suggests that epididymal and seminal plasma vesicles may be members of an exosome-vesicle family. This hypothesis is also supported by the fact that the different proteome of the exosomes published to date also contain similar proteins to epididymal and seminal plasma vesicles (for review, see [11, 64]). It has been suggested that exosomal vesicles are secreted from cells upon fusion of multivesicular endosomes with the cell membrane by an unknown mechanism [64]. However, we still do not have enough information on epididymal vesicles to know whether they are a true homogeneous population of exosomes. For example, no V-ATPase subunits (a marker of intracellular acidic vesicles) were found in exosomes, while one is present in the epididymal vesicles. Alternatively, this is in agreement with the suggestion that the composition of exosomes may vary in function with the tissue from which they are derived [11, 64].

Several types of membrane secretion have been described in the efferent ducts and epididymis, including bleb secretions, which involved a large surface of the apical membrane and then form larger vesicles in the fluid. Destruction of these large vesicles could give rise to smaller vesicles similar to exosomes. Other possible origins include the confluence of smaller vesicles coming from the upper tract (which may explain the actin results we have obtained). However, our findings that these vesicles have a specific protein composition, which is different from the mature sperm and to a large extent from cytoplasmic droplets, and contain proteins that are only present in the cauda region strongly suggest that they are mainly the result of a specific secretion from this region.

Different roles have been suggested for seminal plasma vesicles (prostasomes), like immunosuppression, oxidative protection, and bacterial defense (for reviews, see [8, 9, 63, 65]). Similar properties have been also suggested for exosomes, and these diverse roles may reflect the different biological activity of the composing proteins (see Table 2). Both seminal plasma and epididymal vesicles have been suggested to be involved in sperm membrane maturation by transferring proteins, particularly hydrophobic proteins, to the cell under very specific conditions (acidic pH, presence of zinc [48, 66]). Indeed, these vesicles are rich in GPI anchored proteins such as CDw52, CD55, CD59, prion protein, and p25b, and it has been shown that, in vitro, the vesicles can fuse with sperm [8, 61, 65, 67, 68]. Immunoblotting revealed that several new hydrophobic and lipophilic compounds were bound with the epididymal vesicles. We observed the presence of the very hydrophobic protein 17 kDa secreted by the cauda epididymis [5] and of PGDS secreted in the caput epididymis [13]. One form of E-RABP is also associated with the vesicles, although several different isoforms are secreted in the epididymis [4]. The 17-kDa protein has been immunolocalized on cauda sperm flagellum, but PGDS and E-RABP were not found in the sperm membrane ([13] and unpublished results). We have also observed that the main prion protein isoform, a GPI-anchored protein present on epididymal exosomes, was not transferred to the cauda or ejaculated sperm membrane [10]. Exchange of other seminal plasma vesicle membrane-linked proteins have also been described, particularly for dipeptidyl peptidase IV [68]; transfer has been suggested after ejaculation in horse [52, 68, 69].

However, our data demonstrated that none of the epididymosomes major membrane-bound proteins was present in vivo on cauda epididymal or ejaculated sperm extracts. Thus, if these vesicles are involved in sperm membrane transformations, it is via very subtle exchange mechanisms that remain to be explained but not by a vesicle-fusion mechanism. Moreover, the epididymal exososomes represent only a small percent of the total cauda protein, and the most hydrophobic proteins were mainly present in the caudal fluid bulk phase under a soluble form. It is possible that these soluble proteins exist in micelles or complexes, which could explain the mechanism of their transfer to the cell membrane. Alternatively, these vesicles could play a role in the female genital tract, where their immunosuppressive and anti-oxidant properties might protect the sperm from degradation.

In conclusion, we have clearly demonstrated that vesicles with a specific protein content exist in the caudal fluid and form the majority of the vesicles retrieved in the seminal plasma of the ram. Using different markers, we have also provided evidence that the majority of these vesicles are mainly secreted in the cauda/corpus regions of the epididymis and accumulated in the caudal fluid. These vesicles, which are similar to the previously described vesicles from the seminal plasma, are physically and biochemically related to exosomes. They represented only a small fraction of the caudal-fluid proteins and do not contain all the hydrophobic compounds of this fluid. We found no evidence for the exchange of the major proteins between vesicles and sperm within the epididymis or during ejaculation. The role of these vesicles in male reproductive physiology requires further investigations.

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