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Comparison Between Epididymosomes Collected in the Intraluminal Compartment of the Bovine Caput and Cauda Epididymidis[1]

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ABSTRACT

During their transit along the epididymidis, mammalian spermatozoa acquire new proteins involved in the acquisition of male gamete fertilizing ability. We previously described membranous vesicles called epididymosomes, which are secreted in an apocrine manner by the epididymal epithelium. Some selected proteins associated with epididymosomes are transferred to spermatozoa during epididymal transit. The present study compared epididymosomes collected from caput epididymal fluid with vesicles from the cauda epididymidis in the bull. Two-dimensional gel electrophoresis revealed major differences in protein composition of epididymosomes isolated from the caput and cauda epididymidis. LC-QToF analysis of major protein spots as well as Western blot analysis confirmed the differences in proteins associated with these two populations of epididymosomes. Biotinylated proteins associated with caput and cauda epididymosomes also revealed differences. When incubated with caput epididymal spermatozoa, epididymosomes prepared from these two segments transferred different protein patterns. By contrast, cauda epididymosomes transferred the same pattern of proteins to spermatozoa from the caput and cauda epididymidis. Transfer of biotinylated proteins from cauda epididymosomes to caput spermatozoa decreased in a dose-dependent manner when biotinylated epididymosomes were diluted with unbiotinylated vesicles. Caput epididymosomes added in excess were unable to inhibit transfer of biotinylated proteins from cauda epididymosomes to caput spermatozoa. Following transfer of biotinylated proteins from cauda epididymosomes to caput spermatozoa, addition of unbiotinylated cauda epididymosomes was unable to displace already transferred biotinylated proteins. These results established that epididymosomes from caput and cauda epididymidis have different protein composition and interact differently with maturing spermatozoa.

apocrine secretion, bovine spermatozoa, epididymis, epididymosomes, gamete biology, male reproductive tract, sperm, sperm maturation

INTRODUCTION

Leaving the testis, the mammalian spermatozoon has to transit along the epididymidis in order to acquire its fertilizing ability and its forward motility [1]. This maturation process depends on epididymal principal cell protein synthesis and secretion, which are under androgenic control [2]. Thus, within the epididymidis, spermatozoa will encounter intraluminal fluid of a different protein composition that will interact with the sperm surface [3]. Some epididymal secreted proteins are known to be added to sperm and to be essential for maturation [4–6]. Some of these proteins are glycosylphosphatidyl-inositol (GPI) anchored to the sperm plasma membrane [7–10], some behave as integral membrane proteins [4, 5], and others are incorporated into the intracellular structures of spermatozoa [11–13]. These imply unusual mechanisms of transfer of extracellular proteins to the sperm subcellular compartments or to specific plasma membrane domains.

The epididymal epithelium undergoes apocrine secretion at its apical pole [14]. This secretion pathway involves formation of apical cytoplasmic blebs that will detach into the intraluminal compartment. The apical blebs disintegrate, liberating their content, including small membranous vesicles named epididymosomes [14–16]. These vesicles of 50–500 nm in diameter are rich in cholesterol and show complex electrophoretic patterns of associated proteins [17–19]. These have been described in an increasing number of mammalian species, including human [13], mouse [20, 21], horse [22], sheep [23–25], chimpanzee [26], hamster [7, 27], and bull [28–30]. We have shown that selected proteins of epididymosomes are transferred to spermatozoa [28, 29]. Some of these proteins are GPI anchored [7, 28] and have been shown to be essential for the acquisition of sperm-fertilizing ability [7, 19, 27], whereas others have been proposed to modulate the motility of maturing spermatozoa [13, 31, 32]. The present study describes the differences between epididymosomes collected in two different segments of the bovine epididymis and investigates whether these vesicles collected in different epididymal segments interact differently with epididymal spermatozoa. The results are discussed with regard to the function of epididymosomes in sperm maturation.

MATERIALS AND METHODS

Sampling of Epididymal Intraluminal Fluids

Bovine reproductive tissues were obtained at the slaughterhouse immediately after killing. Testicles were brought on ice to the laboratory within 3 hours. Only epididymides with distal cauda epididymal tubules swollen by the presence of spermatozoa were used in this study.

Intraluminal fluid from the cauda epididymis was obtained by retrograde flushing by applying air pressure with a syringe in the scrotal segment of the vas deferens. The caput epididymis was dissected to avoid blood contamination. A few tubules were neatly cut with a razor blade, and fluid was recovered by applying pressure to the proximal portion of the dissected caput epididymis.

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By repeating this operation approximately 10 times on each caput epididymidis, 50–100 μl of intraluminal fluid was collected per caput epididymis. These procedures were applied with great care to avoid blood or tissue contamination. Considering that these experiments require uncontaminated intraluminal fluid to prepare epididymosomes and that the diameter of corpus epididymis tubule is too small to be flushed, this segment was not investigated in this study.

Preparation of Epididymal Spermatozoa, Epididymosomes, and Prostasomes

Epididymal fluids were diluted with 0.15 M NaCl, and spermatozoa were pelletted at 700 × *g* for 5 min and washed several times with isotonic NaCl. In preliminary experiments, different media with different Na:K ratios have been tried for spermatozoa-epididymosome co-incubations (data not shown). The same results were obtained, and this is the reason a simple isotonic solution was used to prepare the epididymosomes. Spermatozoa were kept at room temperature for further use. Supernatants were centrifuged once at 3000 × *g* to remove remaining debris and were ultracentrifuged at 120 000 × *g* for 2 h at 4°C. The pellets were resuspended in 0.15 M NaCl and were ultracentrifuged again at 120 000 × *g*. To avoid tissue contamination of caput epididymosomes, these suspensions were submitted to chromatography on a Sephacryl S-500 HR (Pharmacia) between the two ultracentrifugations at 120 000 × *g* [33]. The pellets containing isolated caput or cauda epididymosomes were resuspended in small volumes of NaCl, aliquoted, and frozen at −80°C until used.

Prostasomes are membranous vesicles similar to epididymosomes that are present in seminal plasma. In humans they are thought to be secreted by the prostate [17, 34]; their tissue origin has not been described in bovines. Prostasomes from freshly ejaculated bovine semen obtained from the artificial insemination center (CIAQ, St-Hyacinthe, QC, Canada) were prepared in a manner similar to that described for epididymosomes.

Biotinylation of Proteins Associated with Spermatozoa and Epididymosomes

Freshly prepared caput or cauda epididymosomes were suspended in 250 μl PBS, pH 7.4, containing 0.7 mg sulfo-NHS-LC-biotin (Pierce, Brockville, ON, Canada). These mixtures were kept at room temperature for 30 min and then at 4°C for 8–10 h. The biotinylated epididymosomes were diluted with 15 vol of 0.15 M NaCl and were ultracentrifuged at 120 000 × *g* for 2 h. The pellets were resuspended in 0.15 M NaCl, aliquoted, and kept at −80°C until use. Freshly collected caput and cauda spermatozoa were biotinylated as previously described [29].

Incubation of Spermatozoa with Biotinylated Epididymosomes

First, epididymosomes were co-incubated with epididymal spermatozoa to document the electrophoretic pattern of transferred proteins. Caput or cauda spermatozoa were resuspended at a concentration of 30×10^6 per 150 μl in 0.15 M NaCl, 10 mM MES-PIPES at pH 6.5, containing biotinylated caput or cauda epididymosomes for 3 h. A pH of 6.5 was previously shown to be optimum for in vitro protein transfer from epididymosomes to spermatozoa [29].

In competition experiments, caput spermatozoa were incubated with biotinylated cauda epididymosomes in the conditions described above, except that 30-, 40-, or 74-fold excess (based on protein concentrations) of unbiotinylated cauda epididymosomes was added to dilute biotinylated epididymosomes. Competition assays also were performed by adding unbiotinylated caput epididymosomes or prostasomes to the mixture containing caput spermatozoa and biotinylated cauda epididymosomes. Displacement experiments also were performed by adding 30-fold excess of unbiotinylated cauda epididymosomes after 150 min of co-incubation of caput spermatozoa with biotinylated cauda epididymosomes. At different times after mixing caput spermatozoa with biotinylated cauda epididymosomes, aliquots of sperm suspension were collected to determine the quantity of proteins of epididymosomes transferred to spermatozoa. This also was performed 150 min after adding unbiotinylated epididymosomes to the mixture.

SDS-PAGE and Immunodetection

Biotinylated epididymosomes or spermatozoa co-incubated with epididymosomes were extracted with 0.3% vol/vol Triton X-100 for 15 min at room temperature and were pelletted by centrifugation. Proteins in supernatants were precipitated with MeOH/$CHCl_3$ [35]. Proteins were dissolved in Laemmli sample buffer (2% SDS, 2% β-mercaptoethanol, 50 mM Tris, pH 6.8). After SDS-PAGE [36], proteins were transferred onto nitrocellulose membrane. Electrophoreses were performed using proteins from 4×10^6 spermatozoa or using the same quantities of epididymosomes or prostasomes based on protein concentrations as determined by Coomassie blue staining technique (BioRad protein assay, Hercules, CA). After transfer, nitrocellulose membranes were stained with Ponceau Red to verify uniformity of transfer. Membranes were blocked for 1 h in PBS-0.1% Tween-20 containing 5% defatted milk. In order to visualize biotinylated proteins, the membranes were incubated with neutravidin-conjugated horseradish peroxidase (Pierce, Brockville, ON, Canada) for 80 min in 2% skim milk prepared in PBS-Tween. They were revealed using a chemiluminescent peroxidase substrate (Roche Diagnostics, Laval, QC, Canada). Quantities of biotinylated proteins associated with spermatozoa were determined by densitometry using a gel documentation system (Alpha Innotech, San Leandro, CA). Determinations were made on a constant number of spermatozoa during the linear phase of the peroxidase enzymatic activity [29].

Some Western blots were performed to detect P25b protein, macrophage migration inhibitory factor (MIF), AKR1B1 (aldose reductase), heat shock 70-kDa protein 5 (HSPA5), and seminal plasma protein PDC-109. The rabbit antisera used for these purposes were from our laboratory (anti-P25b; Parent et al. [37]), a gift from Dr. M.A. Fortier; (anti-AKR1B1) [38], from D.R. Nishibori; (anti-MIF), and from Dr. P. Manjunath [39]. HSPA5, also known as GRP78, was detected using a commercial monoclonal antibody (BD Transduction Lab, Mississauga, ON, Canada). The immune complexes were revealed using a goat anti-rabbit IgG or a goat anti-mouse IgG coupled with horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA).

Two-Dimensional Gel Electrophoresis and LC-QToF Identification

Proteins associated with caput and cauda epididymosomes were submitted to two-dimensional gel electrophoresis [40]. Isoelectric focusing was conducted under equilibrium, and separation in the second dimension was performed under denaturing conditions. Protein electrophoretic patterns were stained with Coomassie blue, and major protein spots were cut out and digested with trypsin for peptide mass spectrometric analysis (LC-QToF). Identifications were performed by mass fingerprint using a peptide mass computer database (http://www.expasy.ch/tools) at Genome Canada Core Facilities (Montreal, QC, Canada). Protein identification was based on individual ion score >100 and a minimum of two peptides matched. For some proteins, identification was confirmed by Western blotting using specific antisera.

Statistical Analysis

Statistical analyses were performed by analysis of variance. Results were compared by Student *t*-test. Differences were considered to be significant at $P < 0.05$.

RESULTS

Comparison Between Epididymosomes from the Caput and Cauda Epididymal Fluids

Both one- and two-dimensional gel electrophoretic protein patterns of epididymosomes collected in the caput and cauda epididymidis showed major differences. The two-dimensional electrophoretic patterns revealed proteins unique to one of the epididymosome populations, others were present in both protein patterns, and others varied in relative intensity (Fig. 1).

LC-QToF analyses of selected proteins were performed. These data revealed that the endoplasmin precursor (HSP90B1, also known as GRP94), reticulocalbin 1, nucleobindin 2 precursor, tumor-associated calcium signal transducer 1, and testis-expressed sequence 101 were detectable in caput epididymosomes. Cauda epididymosomes were characterized by the presence of regucalcin. Some proteins were common to epididymosomes from the proximal and distal epididymis, such as clusterin, HSPA5, chaperonin-containing TCP-1 subunit 2, actin beta, and aldose reductase (Table 1).

Western blots were used to investigate the presence of proteins previously shown to be associated with bovine epididymosomes. The same quantities of protein from caput

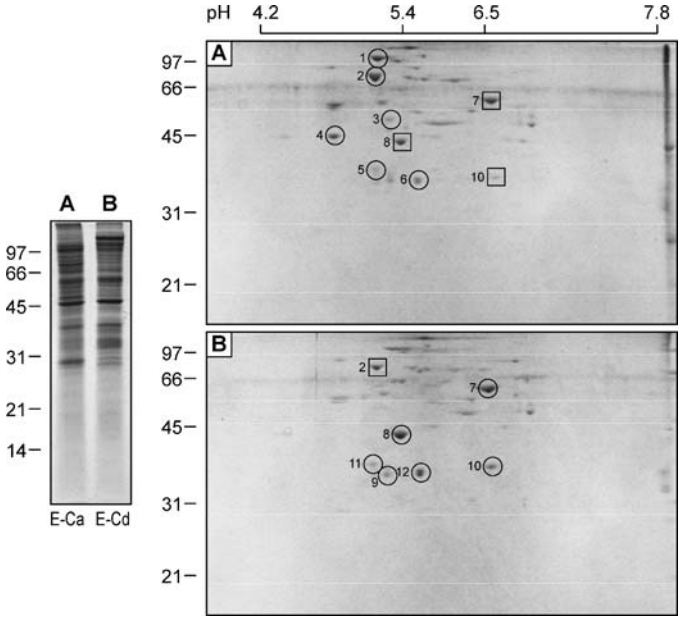

FIG. 1. Coomassie blue-stained two-dimensional electrophoretogram of proteins associated with epididymosomes prepared from caput (**A**) and cauda (**B**) epididymal fluids. Circles indicate protein spots analyzed by LC-QToF. Squares indicate proteins corresponding to the ones identified by circles on the corresponding gel but not analyzed by LC-QToF. Numbers identifying protein spots correspond to the proteins listed in Table 1. MW standards are indicated on the left. Left panel shows corresponding one-dimensional gel electrophoresis. In this panel, MW standards are indicated on the left.

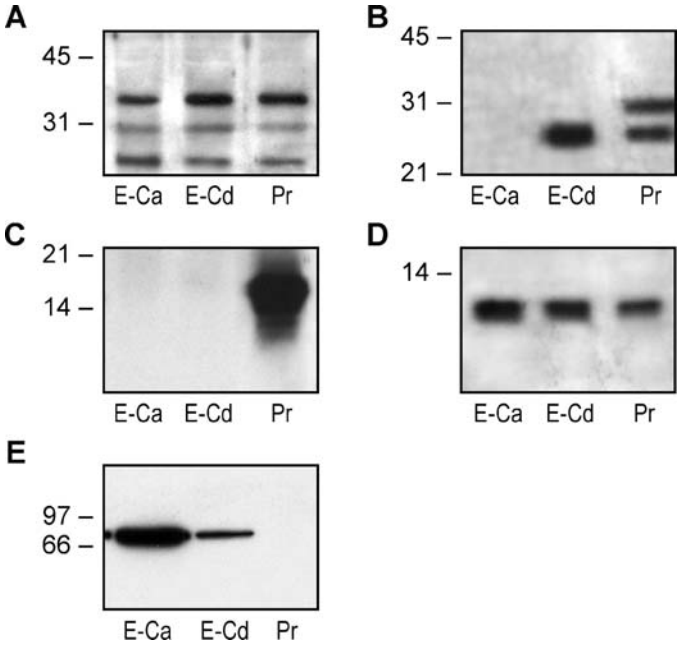

FIG. 2. Western blot immunodetection of AKR1B1 (**A**), P25b (**B**), seminal plasma protein PDC-109 (**C**), MIF (**D**), and HSPA5 (**E**) on epididymosomes collected in the caput (E-Ca) and cauda (E-Cd) epididymal fluids, and prostasomes (Pr). Only regions of the blots showing labeling are illustrated. MW standards are indicated on the left.

and cauda epididymosomes and from prostasomes prepared from semen were analyzed. AKR1B1 and MIF were detected at similar levels in the three preparations. P25b, a zona pellucida binding protein, was present in both cauda epididymosomes and prostasomes. Two forms of P25b were detected in the membranous vesicles prepared from semen. Seminal plasma protein PDC-109 was associated only with prostasomes. HSPA5 was associated with caput epididymosomes and was detectable at a lower level in epididymosomes from the cauda epididymal fluid (Fig. 2).

When epididymosomes are biotinylated, surface proteins are probed predominantly. Detection of electrophoretic patterns of biotinylated proteins also showed major differences between epididymosomes from the caput and cauda epididymal fluids (Fig. 3).

TABLE 1. List of proteins associated with epididymosomes identified by LC-QToF.

Spot no.	Protein identification†	NCBInr accession no.
1	Endoplasmin precursor (HSP90B1)	gi 27807263
2	Heat shock 70kDa protein 5 (HSPA5)	gi 76630567
3	Nucleobindin 2	gi 76635537
3	Unknown	gi 74268141
4	Reticulocalbin 1	gi 76672694
4	Keratin, type 1 cytoskeletal 10	gi 479
5, 6, 11, 12	Clusterin	gi 27806907
5, 6	Tumor-associated calcium signal transducer 1	gi 74354587
5	Testis expressed sequence 101	gi 76687188
7	Chaperonin containing TCP-1, subunit 2 beta	gi 61808645
8*	Actin, beta	gi 28189611
9	Regucalcin	gi 27806809
10*	Aldose reductase (AKR1B1)	gi 162652

* Analyzed by MALDI-ToF.
† HUGO gene nomenclature committee (http://www.gene.ucl.ac.uk/nomenclature/) and UniProt (http://www.pir.uniprot.org).

Interactions Between Spermatozoa and Epididymosomes from the Caput and Cauda Fluids

As shown previously [29], only selected proteins are transferred from cauda epididymosomes to caput spermatozoa when these two suspensions are co-incubated in vitro (Fig. 3).

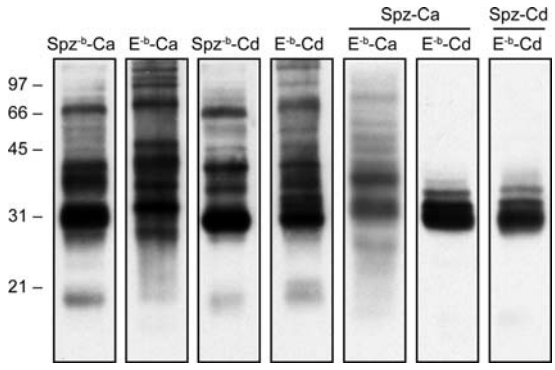

FIG. 3. Western blot analysis of biotinylated ($^{-b}$) proteins of spermatozoa (Spz^{-b}) and epididymosomes (E^{-b}) prepared from caput (-Ca) and cauda (-Cd) epididymal fluids. Also illustrated is Western blot detection of biotinylated proteins from caput (E^{-b}-Ca) or cauda (E^{-b}-Cd) epididymosomes transferred to unbiotinylated caput (Spz-Ca) or cauda (Spz-Cd) spermatozoa. MW standards are indicated on the left.

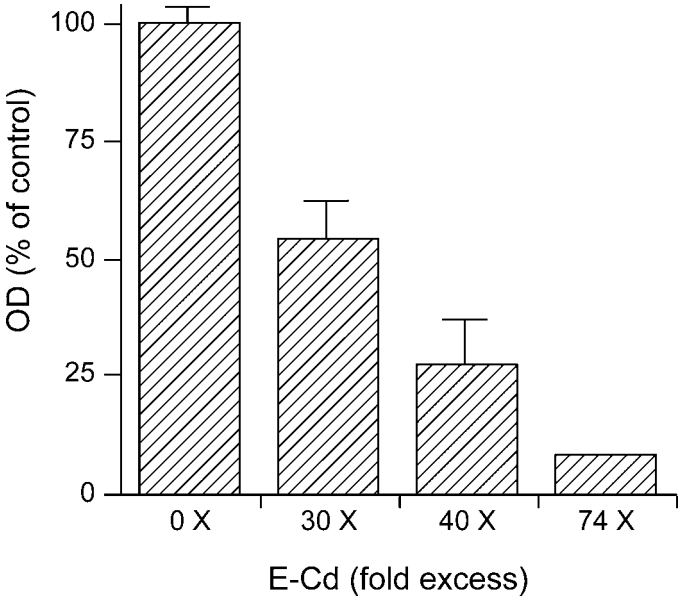

FIG. 4. Densitometric determination of biotinylated proteins of epididymosomes from the cauda epididymis (E-Cd) transferred to caput spermatozoa in presence of 30-, 40-, or 74-fold excess of unbiolinylated E-Cd. Quantities of transferred proteins are measured as arbitrary optical density (OD) units and are expressed as a percentage of control consisting in co-incubation in absence of unbiotinylated E-Cd (0X). Vertical bars express SD of at least three independent experiments.

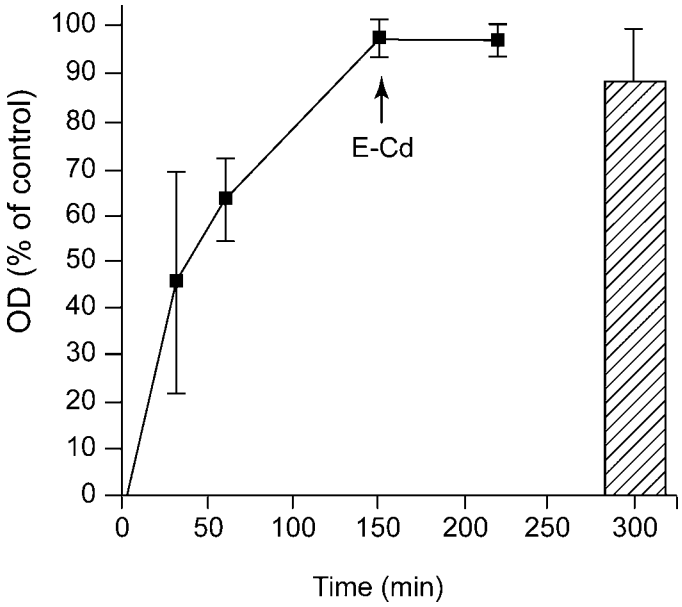

FIG. 6. Densitometric determination of biotinylated proteins of epididymosomes from the cauda epididymis transferred to caput spermatozoa as a function of time of co-incubation. Following 150 min of co-incubation, 30-fold excess of unbiotinylated epididymosomes from cauda fluid was added and quantities of transferred biotinylated proteins were determined 150 min later (hatched bar). The quantity of transferred proteins associated with a constant number of spermatozoa is expressed as arbitrary optical density (OD) units. Vertical bars express SD of five independent experiments.

The pattern of proteins transferred from cauda epididymosomes to caput spermatozoa is the same as for those transferred to cauda spermatozoa. Electrophoretic patterns of biotinylated proteins from caput epididymosomes showed major differences from those characterizing cauda epididymosomes. There also were major differences between the electrophoretic patterns of proteins from epididymosomes and the biotinylated proteins of intact spermatozoa collected in the same segments of the epididymis. The electrophoretic pattern of biotinylated proteins from caput epididymosomes transferred to caput spermatozoa was very different from that of cauda epididymosomes transferred to the same spermatozoa (Fig. 3). Thus, it is the epididymal origin of epididymosomes, not the maturational status of the male gamete, that dictates which proteins are transferred to sperm.

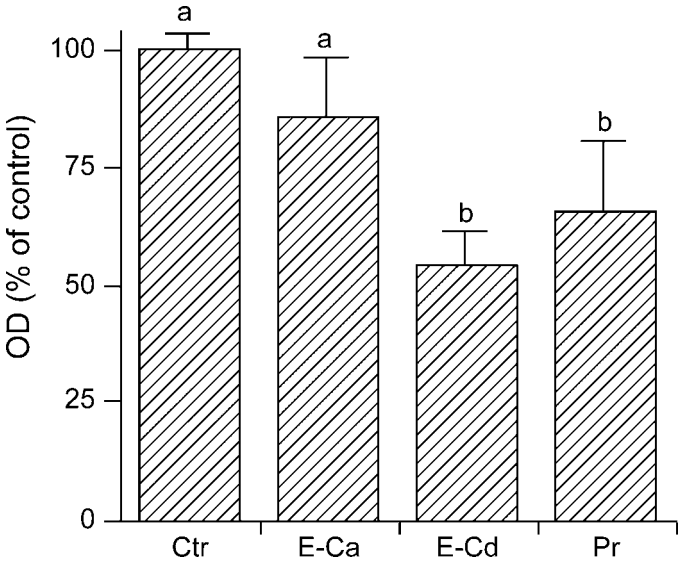

FIG. 5. Densitometric determination of biotinylated proteins of epididymosomes from the cauda epididymis (Ctr) transferred to caput spermatozoa in presence of unbiotinylated epididymosomes from caput (E-Ca) or cauda (E-Cd) epididymis or in presence of prostasomes (Pr). Quantities of transferred proteins are measured as arbitrary optical density (OD) units and are expressed as a percentage of control consisting in co-incubation in absence of unbiotinylated membranous vesicles (Ctr). Vertical bars express SD of seven independent experiments. Different letters (a, b) indicate statistical differences at $P < 0.05$.

At pH 6.5, the amount of biotinylated proteins transferred from cauda epididymosomes to caput spermatozoa reached a plateau after 2.5 h [29]. During this co-incubation period, if biotinylated cauda epididymosomes were diluted with unbiotinylated epididymosomes from the same epididymal region, the amount of biotinylated proteins transferred to the same number of caput spermatozoa decreased. The decrease was proportional to the dilution factor, reaching less than 10% of transferred protein when biotinylated proteins were diluted by a 74-fold factor (Fig. 4). Although unbiotinylated cauda epididymosomes competed with protein transfer when caput spermatozoa were co-incubated with biotinylated epididymosomes collected in the cauda epididymidis, unbiotinylated caput epididymosomes had no effect on this in vitro protein transfer experiment. Prostasomes had an inhibitory effect on this protein transfer, but they were not as efficient as unbiotinylated cauda epididymosomes (Fig. 5).

The transfer of protein from cauda epididymosomes to caput spermatozoa was time dependent and reached a plateau after 2.5 h of co-incubation in vitro (Fig. 6). The addition of an excess of unbiotinylated cauda epididymosomes after this period of in vitro co-incubation of biotinylated cauda epididymosomes with caput spermatozoa had no effect on the quantity of transferred protein to spermatozoa (Fig. 6).

DISCUSSION

Proteome analyses of epididymal fluid have been reported in different species [41–43], including humans [43].Whereas the protein composition from one epididymal segment to the other shows minor differences in humans, the proteome shows great variability along the excurrent duct in all other species studied. This also is true for epididymosomes, as shown by the protein electrophoretic patterns that vary according to the epididymal segment. These protein patterns are different from the soluble fraction of epididymal fluids [30]. This is in agreement with the proposed function of epididymosomes, which is the transfer of new proteins to the maturing spermatozoa [19].

Whereas many proteins were found to be unique to caput epididymosomes, the majority of proteins found associated to cauda epididymosomes are also present in epididymosomes of the caput epididymis. The functions of these newly identified proteins in sperm maturation remain to be defined. It is noticeable that some of them are calcium binding proteins (reticulocalbin 1 and nucleobindin 2 precursor) or are involved in calcium signaling (regucalcin), and that three chaperone proteins are associated with epididymosomes: HSP90B1 (also known as GRP94), HSPA5 (GRP78), and chaperonin-containing TCP-1 subunit 2. HSPA5 has been proposed as being involved in sperm attachment to oviductal epithelial cells in bovines [44], whereas HSP90B1 has been proposed as being involved in the formation of a functional zona pellucida receptor complex [45]. The association of HSP90B1 with epididymosomes is particularly interesting when one considers that P25b, a protein acquired by spermatozoa during the epididymal transit and involved in zona pellucida binding [46], is one of the proteins of epididymosomes transferred to spermatozoa in bovines [28]. If these proteins are transferred to spermatozoa, their proposed functions are in agreement with what we know about epididymal sperm maturation [4, 6].

The fact that many proteins associated with the caput epididymosomes are not detectable in the electrophoretic pattern of cauda epididymosomes suggests that the epididymosomes from the proximal epididymis represent a minor population of membranous vesicles present in the cauda epididymal fluid. Thus, the transiting spermatozoa interact with different populations of epididymosomes during maturation. This is particularly true if one considers P25b, which is undetectable in the Western blot of caput epididymosomes, as being associated with cauda epididymosomes (Fig. 2B). Other proteins are detectable in epididymosomes collected both in caput and cauda epididymis (Fig. 2, A and D). Following ejaculation, cauda epididymosomes are mixed with seminal plasma containing prostasomes, membranous vesicles secreted by accessory sex glands. At the ultrastructural level, epididymosomes and prostasomes are very similar [17, 34, 47]. Considering that AKR1B1, P25b, and MIF are associated with these membranous vesicles in the cauda epididymis and in semen, epididymosomes from the cauda may contribute significantly to the vesicles present in semen (Fig. 2). In semen, what is known to be prostasomes is a mixed population of vesicles secreted by the prostate and, in a smaller proportion, vesicles originating from the cauda epididymis [13]. Thus, during epididymal transit and following ejaculation spermatozoa interact with different populations of membranous vesicles that are able to transfer specific proteins to the male gamete [19].

When co-incubated in vitro, cauda epididymosomes transferred only selected proteins to the caput epididymal spermatozoa. Considering the differences in protein composition of epididymosomes of different segments of the excurrent duct, we can expect that epididymosomes will transfer different proteins to spermatozoa depending on their origin along the epididymis. In fact, caput spermatozoa will acquire different proteins if incubated with epididymosomes from the caput or the cauda epididymis. Cauda epididymosomes will, on the other hand, transfer the same protein patterns, even though they are incubated with spermatozoa from different epididymal segments. Thus, we can hypothesize that proteins intended to be transferred to spermatozoa are segregated to particular membrane subdomains of epididymosomes that are more efficient in interacting with spermatozoa.

The transfer of selected proteins from cauda epididymosomes to caput spermatozoa shows a certain level of specificity. In fact, when biotinylated epididymosomes are diluted with epididymosomes from the same epididymal segment, the quantity of biotinylated proteins transferred decreases. Caput epididymosomes are unable to compete with cauda epididymosomes in similar co-incubation experiments. Thus, even though both populations of epididymosomes are able to transfer proteins to spermatozoa, they do not interact in the same way with the male gamete. Epididymosomes from caput and cauda epididymis do not transfer the same proteins to the maturing spermatozoa. This supports the idea that the population of these membranous vesicles varies along the epididymis. The membrane subdomains or cell compartments of spermatozoa acquiring new proteins may vary according to the type of epididymosomes with which they are interacting. Prostasomes are able to compete with cauda epididymosomes in co-incubation experiments, probably due to the fact that this mixed population of membranous vesicles contains epididymosomes from the cauda segment of the epididymidis. Once transferred to sperm, proteins from epididymosomes are not displaced by co-incubation with unlabeled epididymosomes. Proteins that originate in the epididymis and are added to the sperm during maturation were first thought to be surface coated proteins [5]. Our data show that these proteins transferred by epididymosomes are more tightly bound to spermatozoa than coating proteins retained by electrostatic interactions. In fact, some of these proteins are GPI anchored to both epididymosomes and to spermatozoa following transfer [7, 8, 28]. This is the reason they behave as integral membrane proteins [5]. Other proteins from the epididymis, such as MIF, are transferred to an intracellular sperm compartment, which explains why they cannot be displaced in in vitro competition assays (Fig. 6) [11–13].

In conclusion, during the maturation in the epididymis, spermatozoa interact with different populations of small membranous vesicles called epididymosomes, which modify the male gamete in a sequential manner. Further work is needed to understand how newly acquired proteins are transferred from these epididymosomes to spermatozoa.

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