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Aldose Reductase and Macrophage Migration Inhibitory Factor Are Associated with Epididymosomes and Spermatozoa in the Bovine Epididymis[1]

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ABSTRACT

During the epididymal transit, mammalian spermatozoa acquire new surface proteins necessary for male gamete function. We have previously shown that membranous vesicles, called epididymosomes, interact with spermatozoa allowing the transfer of some proteins to sperm surface within the epididymal lumen. The protein composition of those vesicles has been investigated to document the mechanisms of protein transfer from epididymosomes to spermatozoa. Electrophoretic analysis revealed that protein composition is different from the epididymal soluble compartment as well as from similar vesicles present in the semen. Protein association with epididymosome is very strong as revealed by resistance to extraction with detergent. Matrix-assisted laser desorption ionization time-of-flight as well as immunodetection techniques have been used to identify some proteins associated to epididymosomes and spermatozoa. An aldose reductase known for its 20α-hydroxysteroid dehydrogenase activity and the cytokine (macrophage migration inhibitory factor) have been identified. These two proteins have been immunolocalized in principal cells of the epididymal epithelium, a more intense signal being detected in the distal epididymal segment as well as in the vas deferens. Database search revealed that these two proteins are characterized by the lack of a signal peptide. These results are discussed with regard to a possible apocrine mode of secretion of these proteins acquired by spermatozoa during the epididymal transit.

epididymis, gamete biology, male reproductive tract, sperm, sperm maturation

INTRODUCTION

During the epididymal transit, spermatozoa acquire new surface proteins involved in maturation of the male gamete [1]. Synthesis and secretion of these proteins by the epididymal epithelium are known to be under androgen control [2, 3]. The mechanisms of interaction of these proteins with the sperm surface have been thought to involve electrostatic interactions, thus referring these epididymal sperm proteins as coating proteins. Using different extraction procedures, it appears that many sperm surface proteins acquired during the epididymal transit behave as integral membrane proteins [2]. Some of these proteins have been proposed to be anchored to the sperm plasma membrane via a glycosylphosphatidylinositol (GPI) [4–6]. The mechanisms by which a protein from the extracellular compartment is GPI anchored to a cell plasma membrane remain to be determined [2, 7].

Using different mammalian species, we previously characterized a family of proteins sharing common features with carbonyl reductases [8, 9]. These proteins named P26h [10, 11], P34H [12], and P25b [13] in hamster, human, and bull, respectively, are involved in sperm-zona pellucida interactions. P26h and P25b have also been shown to be GPI anchored to the sperm plasma membrane during the epididymal transit [5, 14]. In the epididymal lumen, these proteins are associated with membranous vesicles called epididymosomes. Many other unidentified proteins are associated with these vesicles. Selected proteins, including P26h/P25b, are transferred from epididymosomes to define membranous domains of the spermatozoa during the epididymal transit, at least in hamster and bovine. In vitro studies have shown that this protein transfer is pH and temperature dependent and that zinc, at a concentration similar to that found in the epididymis, enhances the efficiency of transfer [15]. Prostasomes are vesicles similar to epididymosomes that have been characterized in the semen of different mammalian species. Prostasomes have been described as secretory constituents of the prostate and proposed to play key functions in sperm physiology such as protection against complement attack, modulation of the female's immune system, enhancement of sperm motility, and stabilization of the sperm plasma membrane [7, 16].

To further understand the involvement of epididymosomes in sperm maturation, this work was undertaken to identify other proteins associated to epididymosomes. Bovine was used as a model to obtain sufficient quantity of noncontaminated epididymal fluid from which epididymosomes are prepared.

MATERIALS AND METHODS

Biological Material

Bull epididymides obtained at the slaughterhouse were immediately kept on ice for transport to the laboratory. Only epididymides with swollen tubule in the distal cauda epididymidis were used in this study. Cauda epididymal fluid was recovered by retrograde flushing by applying air pressure with a syringe inserted in the scrotal portion of the vas deferens. This procedure was applied with great care to avoid contamination. Only fluids without red blood cells were used. The epididymal fluid was diluted with 150 mM NaCl and centrifuged twice at 700 × *g* for 10 min to eliminate spermatozoa followed by a centrifugation at 3000 × *g* to eliminate debris. Supernatant was ultracentrifuged at 120 000 × *g* for 2 h. The pellet containing epididymosomes was resuspended in 150 mM NaCl and resubmitted to ultracentrifugation under the same conditions. The pellet was resuspended in a small volume of 150 mM NaCl, aliquoted, and kept at −80°C until used.

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In some experiments, protein content of total cauda epididymal fluid as well as of the 120 000 × *g* ultracentrifugation supernatant was analyzed by two-dimensional gel electrophoresis. For this purpose, proteins precipitated with MeOH/$CHCl_3$ were resuspended in sample buffer.

Epididymosomes were submitted to detergent to evaluate the interaction of proteins with these membranous vesicles. In these experiments, the 120 000 × *g* pellet was resuspended in an aqueous solution of 0.1% Triton X-100. After 10 min at room temperature, epididymosomes were pelleted at 120 000 × *g* for 2 h, and proteins remaining associated to these vesicles were analyzed by two-dimensional gel electrophoresis.

Prostasomes are membranous vesicles similar to epididymosomes that have been described as a constituent of semen in different species including bovine [16]. To compare protein composition of prostasomes and epididymosomes, prostasomes were prepared from bull semen. Fresh semen samples were collected from healthy bulls at the ''Centre d'Insémination Artificielle du Québec'' and brought to the laboratory within 3 h. Semen samples diluted with PBS and prostasome preparations were chromatographed on Sephacryl S-500 [17] and processed according to the procedure used to prepare epididymosomes from cauda epididymal fluid.

Percoll Gradient Centrifugation of Cauda Epididymosomes

Freshly prepared cauda epididymosome suspensions were mixed with an isotonic Percoll solution at a final 55% Percoll concentration. This solution was centrifuged at 17 500 rpm for 30 min in a Beckman 50.2 Ti fixed angle rotor in parallel with density marker beads (Pharmacia Fine Chemicals, Baie d'Urfé, QC, Canada). Fractions were recovered from the bottom of the tube, proteins were precipitated with MeOH/$CHCl_3$, and resuspended in sample buffer for subsequent Western blot analysis.

Two-Dimensional Gel Electrophoresis and MALDI-TOF Identification of Proteins

Two-dimensional gel electrophoresis was performed as previously described [18]. Isoelectric focusing was conducted under equilibrium conditions, and the second dimension was run under denaturating conditions [19]. Gels were stained with Coomassie blue, and protein spots of interest were cut and digested with trypsin for peptide mass spectrometric analysis using matrix-assisted laser desorption ionization time-of flight technique (MALDI-TOF). Protein identification was performed by mass fingerprint in a peptide mass computer database (www.expasy.ch/tools) at our core facility service (Service Protéomique de l'Est du Québec).

SDS-PAGE and Western Blotting

Epididymosomes, prostasomes, and protein fractions separated by centrifugation on Percoll gradient as well as proteins from supernatant of 120 000 × *g* ultracentrifugation of cauda epididymal fluid were denatured in sample buffer and submitted to SDS-PAGE [20]. Electrophoretic patterns were transferred to a nitrocellulose membrane using a semidry milliblot- graphite blotter system [21]. After saturation in PBS containing 0.1% Tween 20, and 5% skim milk, the membrane was incubated in rabbit polyclonal antisera directed against macrophage migration inhibiting factor (MIF), aldose reductase, or P26h/P25b, diluted 5 μg IgG/ml, 1/2000, and 1/1500, respectively. The anti-MIF was produced against MIF purified from bovine liver and generously provided by Dr. M. Nishibori (Department of Pharmacology, Okayama University, Japan), the aldose reductase antiserum was directed against a recombinant protein produced from cDNA amplified from bovine endometrium [22], and the P26h/P25b antibodies were produced against the P26h hamster sperm protein that cross-reacts with the bovine P25b protein [13]. After washing, the membranes were incubated with a peroxidase-conjugated goat anti-rabbit IgG diluted at 1/10 000. The immune complexes were revealed using a peroxidase chemiluminescent substrate (Amersham, Buckinghamshire, UK).

Immunolocalization of Proteins

Epididymal segments and vas deferens were obtained from the slaughterhouse and rapidly fixed in 4% paraformaldehyde solution and embedded in paraffin. Paraffin sections were cleared by immersions in xylene, followed by successive baths of ethanol. Endogenous peroxidases were neutralized by immersion of sections in methanol containing 3% hydrogen peroxide for 30 min. The sections were blocked with 5% goat serum diluted in PBS to avoid unspecific antibody binding sites. After washing, the sections were incubated with either the anti-MIF or the anti-aldose reductase antibody both diluted 1/1000 in PBS containing 0.2% BSA in the case of anti-MIF or 0.5% goat serum for the anti-aldose reductase for 60 min at room temperature, and preimmune sera were used as negative controls. After washing, sections were incubated for 60 min with biotinylated goat anti-rabbit immunoglobulins and processed for staining using an ABC Vectastain kit according to the supplier's instructions (Vector Laboratories, Burlingame, CA). Tissue sections were counterstained with hematoxylin. For each antigen detection, sections of all epididymal segments were processed in parallel to allow comparison.

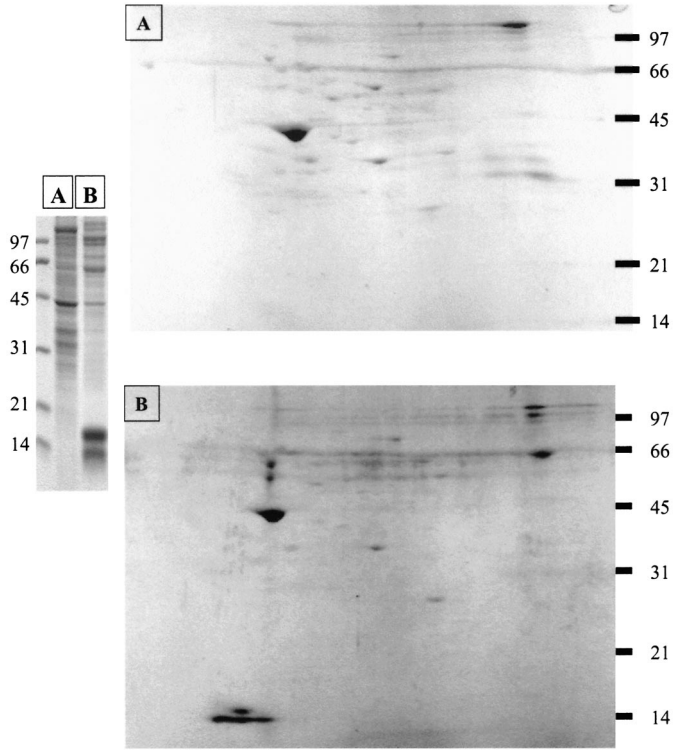

FIG. 1. Coomassie blue-stained two-dimensional gel electrophoresis of proteins associated to epididymosomes prepared from cauda epididymal fluid (**A**) and prostasomes (**B**) prepared from bull semen. Molecular weight standards (× 10^{-3}) are indicated on the right. Left panel shows corresponding one-dimensional gel electrophoresis. In this panel, molecular weight standards (× 10^{-3}) are indicated on the left.

RESULTS

Figure 1 presents electrophoretic patterns of proteins associated to epididymosomes prepared from cauda epididymal fluid (Fig. 1A) as well as prostasomes isolated from fresh bovine semen (Fig. 1B). Both one- and two-dimensional electrophoretic patterns revealed major differences in protein composition of these two types of membranous vesicles. Even though their relative abundance may be different, some protein spots appeared to be comparable in pI and size, as the major 45 kDa and >97 kDa proteins detected in both two-dimensional patterns.

Total cauda epididymal fluid (Fig. 2A: fluid containing epididymosomes) and the 120 000 × *g* supernatant (Fig. 2B: fluid without epididymosomes) showed very similar two-dimensional electrophoretic patterns. By contrast, electrophoretic pattern of proteins associated with purified epididymosomes (Fig. 2C: 120 000 × *g* pellet) showed major differences when compared with the two other protein samples. To estimate the interaction of epididymosomal proteins with these membranous vesicles, epididymosomes purified from cauda epididymidis were treated with 0.1% Triton X-100, and proteins remaining associated to epididymosomes were analyzed by two-dimensional electrophoresis. Treatment of

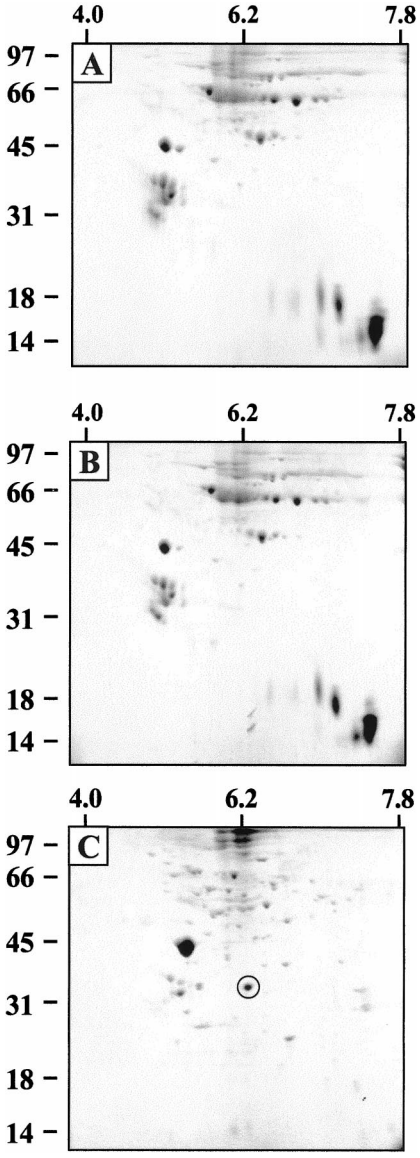

FIG. 2. Coomassie blue-stained two-dimensional gel electrophoresis of proteins associated with total cauda epididymal fluid (3000 g supernatant) (**A**), 120 000 g supernatant of cauda epididymal fluid (**B**), and epididymosomes from cauda fluid (**C**). Molecular weight standards ($\times 10^{-3}$) are indicated on the left and the pI on the top of each panel. Circle indicates protein spot submitted to MALDI-TOF analysis.

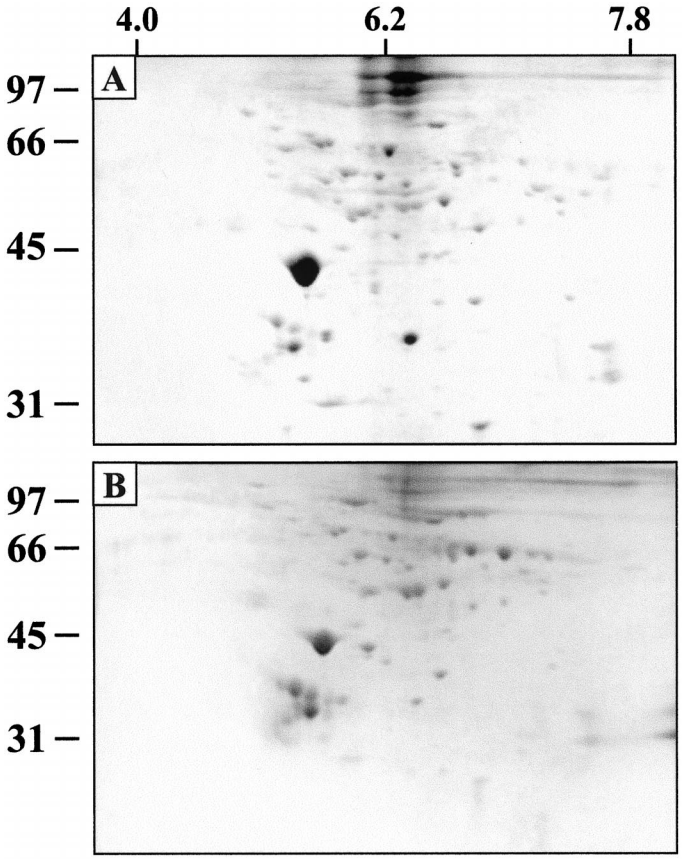

FIG. 3. Coomassie blue-stained two-dimensional gel electrophoresis of proteins associated to cauda epididymosomes previously treated with 150 mM NaCl (**A**) or 0.1% Triton X-100 (**B**). Molecular weight standards ($\times 10^{-3}$) are indicated on the left and the pI on the top of the upper panel.

epididymosomes with Triton X-100 detergent has minimal or no effect on protein composition of these vesicles (Fig. 3).

MALDI-TOF analysis was used to identify proteins associated with epididymosomes. A protein of 35 kDa and with a pI of 6.3 on a two-dimensional electrophoretic pattern of proteins from purified cauda epididymosomes was chosen on the basis of its enrichment, compared with total or soluble fraction of cauda intraluminal fluid (see Fig. 2: the protein is identified by a circle). This spot was cut out from the gel, and the masses of trypsin-generated peptide fragments were evaluated by MALDI-TOF and screened for identity in the Swiss-Prot databank. A bovine aldose reductase was identified (Swiss-Prot code: P16116, gene: AKR1B5). Twelve matching peptides were first confirmed using a PeptIdent tool using a mass tolerance of 60 ppm and one missed cleavage by the trypsin. Screening for the nonmatching peptides using FindMod increased the matching score (Table 1). To confirm the protein identity, Western blots of one-dimensional electrophoretic pattern of cauda epididymosomes were probed with an anti-aldose reductase. A single band of 35 kDa was detected on Western blots of epididymosomes prepared from caput and cauda epididymal fluid as well as on proteins extracted from spermatozoa collected in the same epididymal segments (Fig. 4).

Sequence analysis of the cDNA encoding the bovine aldose reductase shows that this protein is lacking a signal peptide. Considering that epididymosomes and associated proteins are secreted by the epididymis, this was an unexpected feature. We have previously identified other signal peptide lacking proteins transferred to the sperm surface during the epididymal transit [for review, see Ref. 23]. To investigate whether other proteins associated with epididymosomes are also characterized by the absence of a signal peptide, we searched for the presence of MIF in protein patterns of epididymosomes.

Indeed, MIF is another example of a signal peptide lacking protein secreted by the epididymal epithelium and transferred to spermatozoa during the transit along the excurrent duct in the rat [24]. Western blot analysis revealed that MIF is associated with both spermatozoa and epididymosomes collected in the caput and cauda epididymal lumen. The single band detected has an apparent Mw of 12 kDa, as expected (Fig. 4).

The association of P25b, aldose reductase, and MIF with epididymosomes was investigated using continuous Percoll gradient centrifugation. Prostasomes, membranous vesicles

TABLE 1. Peptide matching of the 36-kDa protein associated to epididymosomes identified by MALDI-TOF.*

Considered chain	Matching	Matching after modification	
Aldose reductase (20α-hydroxysteroid dehydrogenase)	901.53	1163.36	PHOS or SULF
	906.48	1993.95	GLUT
	1025.57	2012.92	CSEA or HYDR
	1083.42	2211.04	METH or NTRY
	1821.82	2239.11	ACET or TRIMETH or BROM
	2213.07	2292.16	PALM or HYDR
	2232.09	2298.17	PHOS or SULF
	2276.20	2308.15	OCTA
	2423.16	2367.25	ACET or TRIMETH
	3051.33	2433.37	METH or GERA
	3067.47	3053.46	NTRY
	3262.77	3278.62	HYDR
		3332.43	PHOS or SULF
		3348.66	PALM

* Matching peptides were evaluated using the PeptIdent tool (mass tolerance 60 ppm, monoisotopic mass, one missed cleavage by the trypsin). The hypothetical modification had been made using the FindMod tool. ACET, Acetylation; BROM, bromination); CSEA, cysteine sulfenic acid; GLUT, gluttahionylation; HYDR, hydroxylation; METH, methylation; NTRY, S-nitrosylation; OCTA, N-octa-noate; PALM, palmitoylation; PHOS, phosphorylation; SULF, sulfation; TRIMETH, trimethylation.

similar to epididymosomes characterized in semen of many mammalian species, have a buoyant density of 1.05 [25]. Epididymosome suspensions were submitted to Percoll gradient and proteins associated with each buoyant density fraction were analyzed by Western blot. All three proteins, P25b, aldose reductase, and MIF, were associated with the 1.05 density fraction (Fig. 5).

Distribution along the male reproductive tract of aldose reductase as well as of MIF has been documented by immunohistological technique. Aldose reductase was undetectable in the testis, faintly present in the basement membrane of the caput epididymal tubules, and uniformly distributed in the thickness of the epithelium bordering the lumen of the corpus and cauda epididymidis and vas deferens. The intensity of signal detection progressively increased from the corpus epididymidis to the vas deferens, in which it reached its maximum (Fig. 6). MIF was also undetectable in the testis, and a weak signal was present in the basement membrane of the caput epididymis tubule. In the corpus segment, a very weak signal at the border of the lumen could be visualized. MIF detection was very intense in all the thickness of the cauda epididymis epithelium as well as in the vas deferens (Fig. 6).

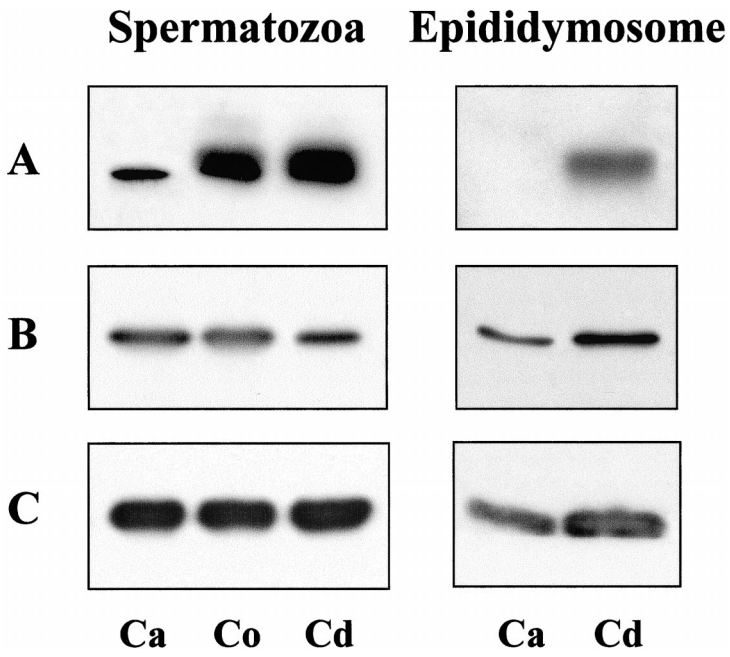

FIG. 4. Western blot immunodetection of P25b (25 kDa) (**A**), aldose reductase (36 kDa) (**B**), and MIF (12 kDa) (**C**) on spermatozoa and epididymosomes collected in the caput (Ca), corpus (Co), and cauda epididymal fluid (Cd). Only regions of the blots showing labeling are illustrated.

DISCUSSION

Two-dimensional electrophoretic patterns of proteins associated with cauda epididymosomes are very complex. The identity and function of these proteins remain to be established. Few of them are selectively transferred to the sperm surface during epididymal transit [15]. Such complexity in protein composition has also been described in prostasomes purified from seminal plasma, and these proteins may be involved in many functions in sperm physiology such as immunosuppressive, antibacterial, and anti-

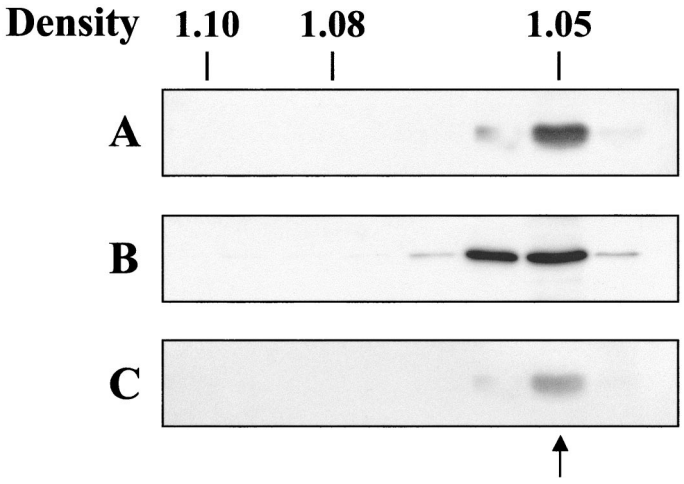

FIG. 5. Western blot immunodetection of P25b (25 kDa), aldose reductase (36 kDa), and MIF (12 kDa) along a Percoll density gradient obtained following centrifugation of a suspension of epididymosomes obtained from bull cauda epididymal fluid. Relative buoyant densities along the gradient are indicated in the upper part of the panel. Arrow indicates epididymosomes localization along the Percoll gradient.

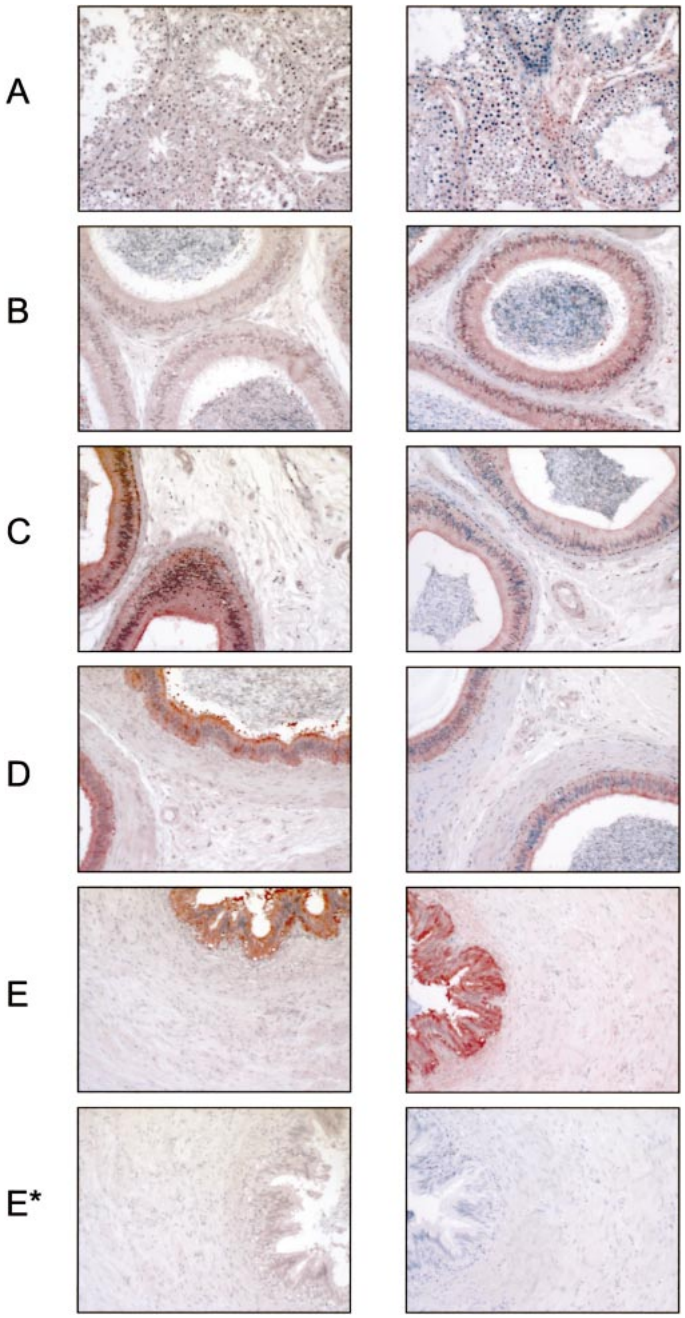

FIG. 6. Immunohistochemical localization of aldose reductase (left panel) and MIF (right panel) in bull testis (**A**), caput (**B**), corpus (**C**), cauda epididymidis (**D**) and vas deferens (**E**). E*, Negative control: vas deferens section incubated with a preimmune serum. Aldose reductase and MIF are revealed by red staining, and the sections are counterstained in blue. Magnification ×200.

oxidant activities as well as enhancement of sperm motility [for review see Ref. 7]. Except for P26h/P25b characterized by our laboratory, GPX5 [26] and ubiquitin [27] are the only identified proteins associated with epididymosomes. Although it can be expected that epididymosomes are present in semen, their contribution to the total membranous vesicles present in semen appears to be minor. Comparison of the electrophoretic pattern of proteins associated with epididymosomes with those present in total epididymal fluid and soluble epididymal proteins contained in the supernatant following epididymosomes pelleting indicated that epididymosomes contribute minimally to the total protein content of the epididymal fluid and thus to the total protein content in semen. Thus, functions of epididymosomes in sperm physiology are probably different from those played by prostasomes; epididymosomes being involved in sperm maturation, and prostasomes in postejaculatory events. Whatever the functions of epididymosome-associated proteins, their interaction with these vesicles is very strong. Detergent treatment of the epididymosomes has a minor effect on the amount of proteins associated with these vesicles. This possibly is due to composition and/or cholesterol:phospholipid ratio of epididymosomes. Epididymosomes may share common physicochemical properties with prostasomes because they both have high cholesterol content [28] and show similar buoyant density when submitted to continuous Percoll gradient centrifugation (Fig. 5).

To further document the function of epididymosomes, MALDI-TOF analysis was performed on protein spot enriched in two-dimensional electrophoretic pattern when compared with total or soluble fractions of epididymal fluid protein patterns. Using this approach, a 35-kDa protein with a pI around 6.2 was identified as a bovine aldose reductase encoded by the gene AKR1B5. The cDNA encoding this enzyme has been cloned from a bovine uterine endometrium cDNA library and revealed a theoretical size of 35 919 Da and pI of 5.76 [22]. These values are in agreement with the electrophoretic behavior of the protein spot identified as aldose reductase by MALDI-TOF analysis. The identity of the protein that is abundant in epididymosomes was confirmed by Western blotting using an antiserum raised against the recombinant protein produced using the bovine endometrial cDNA encoding AKR1B5 [22]. This enzyme possesses an aldose reductase activity, and many functions associated with this enzyme in sperm physiology can be hypothesized.

Aldose reductase is a member of the aldo-keto reductases family and can thus be involved in detoxification process of cytotoxic carbonyl compounds [29]. The epididymis is rich in steroid hormones, and their metabolites and the excurrent duct can thus be exposed to oxidative stress. Aldose reductase may protect the male gamete against carbonyl-containing metabolites. The aldose reductase can also be involved in the polyol pathway within the epididymis [30]. In this metabolic pathway, it is the rate-determining enzyme converting glucose in fructose via sorbitol. It is well known that spermatozoa use these three carbohydrates as energy sources [31, 32]. If aldose reductase remains in the intraluminal epididymal compartment as a component of epididymosomes, this enzyme probably favors the accumulation of sorbitol within the epididymal fluid. Sorbitol being poorly permeable through plasma membrane, this metabolite will accumulate in the intraluminal compartment of the epididymis, contributing to the relatively high osmotic pressure of the epididymal fluid and/or depriving sperm intracellular compartment of energy sources involving the polyol pathway metabolites. By its accumulation in the excurrent duct luminal compartment, aldose reductase activity associated with epididymosomes, via sorbitol production, could thus contribute to sperm transient immobilization within the epididymis.

It is well documented that epididymosomes can transfer some of their protein constituents to preferential sperm membrane domains [6, 15] as well as to intracellular sperm compartments [24]. If aldose reductase is transferred via epididymosome-spermatozoa interactions within the intracellular compartment of the maturing male gamete, this would result in sorbitol accumulation within the cytoplasm,

conferring a protection of epididymal spermatozoa against hypertonic conditions [33]. In fact, the 5′flanking region of AKR1B1, the human ortholog of the bovine gene (AKR1B5) encoding for aldose reductase, is characterized by an ''osmotic responsive element'' [34]. Considering that the epididymal milieu is hyperosmotic, sorbitol may be involved in sperm resistance to this intraluminal condition. Hyperosmolality, in extreme cases of bat epididymis during hibernation period, is responsible for prolongation of sperm life [35]. At moderate hyperosmolality as in the bovine epididymis, this could enhance sperm survival during epididymal transit and storage.

MIF is another protein associated with epididymosomes identified using specific antibodies. This peptide, first described as a classical T-cell cytokine [36], possesses multiple functions such as tautomerase [37] and thiol-protein oxydoreduction [38], regulation of glucocorticoid-mediated suppression of the immune response [39], and Leydig cell modulation of Sertoli cell inhibin production [40]. In the rat, MIF has also been shown to be secreted by the epididymis as a constituent of small vesicles that interact with sperm surface within the epididymal lumen. Downstream of the epididymis, MIF has been shown to be associated with sperm-dense fibers. This suggests an epididymal epithelial cell-to-sperm transfer of MIF via membranous vesicles [24]. The association of MIF with epididymosomes in the bovine epididymal lumen and spermatozoa suggest a similar phenomenon in this species. The function of MIF in sperm physiology remains, however, to be determined.

Sequence analysis of the two proteins, aldose reductase and MIF, identified in this study as constituents of epididymosomes reveal the lack of a signal peptide. P25b is another protein transferred to spermatozoa via epididymosomes during the epididymal transit. The cDNA sequences of P25b ortholog proteins, the hamster P26h [8] and P34H in humans [41], reveal that these translational products also lack a signal peptide trafficking newly synthesized protein through the ''classical'' secretory pathway. This property suggests an unusual pathway of secretion by the epididymal epithelium. The association of these proteins to vesicular structures (epididymosomes) and their lack of signal peptide suggest an apocrine mode of secretion of these proteins by the epididymal epithelium [42, 43]. This peculiar mode of secretion is particularly well documented along the male reproductive tract [44], including the epididymis [45].

Apocrine secretion involves formation of apical blebs containing selected organelles, Golgi structure, lysosomes, and secretory granules being absent. Apical blebs of principal cells of the epididymal epithelium contain small vesicular structures that are secreted in the intraluminal compartment when the apical blebs detach from the apical membrane and fragment in the extracellular milieu. Proteins secreted by this mechanism are often GPI anchored to these vesicular organelles (epididymosomes), and their sequences are characterized by the absence of a signal peptide. This is the case for aldose reductase, MIF, and probably P25b. Considering that other proteins associated with epididymosomes are transferred to sperm surface during the epididymal transit, apocrine secretion of these vesicles probably plays a major role in sperm maturation. Further work is required to understand the mechanism of interaction between epididymosomes and spermatozoa contributing to male gamete maturation within the male reproductive tract.

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