Caveolin-1 and MAL are located on prostasomes secreted by the prostate cancer PC-3 cell line

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Summary
MAL, BENE and MAL2 are raft-associated integral membrane proteins of the MAL family of proteins involved in membrane trafficking processes. We show here that the human prostate carcinoma PC-3 cell line expresses the transcripts for the three proteins simultaneously. MAL, BENE and MAL2 co-fractionated with caveolin-1 in the raft fraction of PC-3 cells, and immunofluorescence analysis showed colocalization of these proteins with caveolin-1 in a multivesicular intracellular compartment. Markers of the Golgi apparatus, early and recycling endosomes and lipid droplets were excluded from this compartment. Prostate epithelial cells contain vesicular organelles enriched in raft components named prostasomes that are secreted in the prostate fluid. Interestingly, the prostasome fraction isolated from the culture supernatant of PC-3 cells consisted mainly of 30-130 nm cup-shaped vesicles that were positive for MAL, caveolin-1 and CD59, a glycosylphosphatidylinositol-anchored protein previously found in prostasomes. CD63, an integral membrane protein found in multivesicular bodies/lysosomes and secretory granules was also found in PC-3 cell-derived prostasomes. Prostasome secretion was not inhibited by brefeldin A, a compound that blocks the conventional secretory pathway. However, wortmannin, an inhibitor of phosphatidylinositol-3 kinase, reduced the secretion of prostasomes in PC-3 cells. Our results suggest that MAL family proteins are associated with caveolin-1 in a multivesicular compartment that may be involved in prostasomal secretion in PC-3 cells.

Key words: MAL family, Caveolin-1, PC-3 cells, Prostasomes, Secretion

Introduction
Compartmentalization of biological membranes in glycolipid-and cholesterol-enriched microdomains or rafts appears to be a general feature of biological membranes from mammals (Simons and Ikonen, 1997) to yeast (Bagnat et al., 2000). Recruitment of specific proteins into rafts was initially proposed to explain the segregation and subsequent transport of newly synthesized proteins from the Golgi apparatus to the apical surface in polarized epithelial cells (Simons and Wandinger-Ness, 1990). More recently, rafts have been involved in a general mechanism for intracellular protein transport and in signalling processes (Simons and Ikonen, 1997).

Raft organisation for transport or signalling processes requires specialized protein machinery, of which the MAL (Pérez et al., 1997) and caveolin (Razani et al., 2002) families are the best characterized elements. In particular, the MAL protein was the first identified member of the integral protein machinery involved in the raft-mediated traffic to the apical surface in polarized epithelial MDCK cells (Puertollano et al., 1999; Cheong et al., 1999). The MAL protein family also includes BENE, a membrane protein that seems to be involved in cholesterol transport in endothelial cells (de Marco et al., 2001), and MAL2, an essential protein of the basolateral-to-apical transcytotic pathway in hepatoma HepG2 cells (de Marco et al., 2002). Caveolin-1 (cav-1), a multifunctional raft-associated membrane protein, is the founding member of the caveolin family (Liu et al., 2002). Cav-1 is mainly found in plasma membrane invaginations named caveolae and at the Golgi region, and forms a scaffold onto which many classes of signalling molecules are recruited to generate pre-assembled signalling complexes. Interestingly, recent evidence indicates that in certain cell lines cav-1 is preferentially targeted to other cellular destinations such as the cytosol in skeletal muscle cells and keratinocytes, mitochondria in airway epithelial cells or the lumen of secretory vesicles in exocrine and endocrine cells (Liu et al., 1999; Li et al., 2001). Curiously, in some of these locations cav-1 behaves as a soluble protein (Liu et al., 1999; Li et al., 2001). Besides cav-1, the caveolin family consists of cav-2, a protein also implicated in caveolae formation, and cav-3, a protein involved in T-tubule formation in muscle cells.

Expression of individual members of the MAL and caveolin families of proteins is cell-type specific. The question remains as to whether combinatorial expression of different members of these families in the same cell, probably with the participation of other proteins, might result in novel subcellular localizations for these proteins or in the generation of cell-type specialized compartments. In this study, we described the simultaneous expression of MAL, BENE, MAL2 and cav-1 in human prostate carcinoma PC-3 cells. The four proteins were present in rafts and colocalized in an apparently multivesicular compartment, which also contained the glycosylphosphatidylinositol (GPI)-anchored protein CD59. Interestingly, cav-1, MAL and CD59 were also found in
prostasomes secreted by PC-3 cells. Several biological functions have been assigned to prostasomes, i.e. the enhancement of spermatozoa motility, the stabilization of the spermatozoa membrane and the delay of the acrosomal reaction, the immunomodulation of the local environment, and the inhibition of viral activity (Ronquist, 1999; Kravets et al., 2000). As cav-1 stimulates cell survival and contributes to metastasis in prostate carcinoma (Tahir et al., 2001), cav-1 secretion may be a novel function of prostasomes with relevance for prostate tumor progression.

Materials and Methods

Materials
The rabbit polyclonal antibodies to cav-1 and the mouse monoclonal antibody (mAb) to giantin were from BD Biosciences (San Jose, CA, USA). The anti-human MAL, BENE and MAL2 mAbs have been described previously (Martín-Belmonte et al., 1998; de Marco et al., 2001; de Marco et al., 2002). The mouse mAb to CD59 was kindly provided by Dr V. Horejsi (Institute of Molecular Genetics, Prague, Czech Republic). The anti-CD63 mAb was from Developmental Studies Hybridoma bank (Iowa City, IA, USA). Polyclonal rabbit anti-calnexin antibodies were from Stressgen (San Diego, CA, USA). The mouse hybridoma producing mAb 9E10 to the human c-Myc epitope was purchased from the American Type Culture Collection. The mouse mAb to GFP was from Medical and Biological Laboratories (Nagoya, Japan). The anti-EFA1 mAb was from BD Biosciences (San Jose, CA, USA). The anti-transferrin receptor mAb was from Zymed Laboratories (San South Francisco, CA, USA). Peroxidase-conjugated secondary antibodies were supplied by Pierce (Rockford, IL, USA). Alexa488 and Alexa594-conjugated secondary antibodies were purchased from Molecular Probes (Eugene, OR, USA). Brefeldin A (BFA), wortmannin and Nile red were obtained from Sigma-Aldrich (St Louis, MO, USA).

Cell culture conditions and transfections
The androgen-independent human prostate cancer cell line PC-3 was purchased from the American Type Culture Collection. Cells were grown in a 1:1 mixture of Ham’s F12K medium and Dulbecco’s modified Eagle’s medium supplemented with 7% fetal bovine serum (Sigma-Aldrich), 50 units/ml penicillin and 50 μg/ml streptomycin at 37°C in an atmosphere of 5% CO₂. Transfection of PC-3 cells with plasmids expressing c-myc-tagged BENE (de Marco et al., 2001) (BENE-myc) or with green fluorescent protein-tagged MAL (MAL-GFP) (Martín-Belmonte et al., 2003) was carried out by electroporation using the Electo Cell Manipulator 600 equipment. Selection of stable transfectants was carried out by treatment with G418 sulfate (Gibco). Drug-resistant cells were selected, screened by immunofluorescence analysis with the anti-human c-myc mAb 9E10 to detect BENE-myc or directly to detect MAL-GFP, and the clones that proved to be positive were maintained in drug-free medium.

Northern blot analysis
Approximately 20 μg total RNA from the indicated cell lines were denatured in 50% formamide and 2.2 M formaldehyde at 65°C, subjected to electrophoresis and transferred to nylon membranes. RNA samples were hybridized under standard conditions to [32P]-labeled MAL, BENE and MAL2 cDNAs. As a loading control, blots were hybridized to a 0.6 kb HindIII/HindIII cDNA fragment from the 3’ untranslated region of human β-actin mRNA. Final blot washing conditions were 0.2 SSC/0.1% SDS (1×SSC=0.15 M sodium citrate, pH 7.0) at 65°C.

Prostasome isolation
Prostasomes were isolated from the medium of PC-3 or PC-3 MAL-GFP cells incubated without serum for 20-24 hours. Cell death, as assayed by trypan blue staining, was not observed under these conditions. The medium was centrifuged to remove cell debris first at 400 g for 5 minutes and then at 10,000 g for 30 minutes. Prostasomes were then sedimented by ultracentrifugation at 100,000 g for 2 hours (Wang et al., 2001) in a SW40 rotor, washed once in phosphate-buffered saline (PBS) and then sedimented again by ultracentrifugation at 100,000 g for 2 hours. Finally, the prostasomes (500 μl from the bottom of the tube) were collected and centrifuged in a TLA100.1 rotor at 100,000 g for 2 hours. Prostasome pellets isolated from one or two 100-mm diameter tissue culture dishes were analyzed by SDS-PAGE and western blotting. In control experiments, prostasomes were subjected to flotation by centrifugation in a sucrose density gradient. For this, prostasome pellets collected as previously described were resuspended in 5 ml of 2.6 M sucrose, 20 mM Tris-HCl pH 7.4 and
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Floated into an overlaid linear sucrose density gradient (2.0-0.25 M sucrose, 20 mM Tris-HCl pH 7.4) at 190,000 g for 16 hours in a Beckman SW40 rotor as previously described (Wubbolts et al., 2003). Fractions (1 ml) were collected from the bottom of the tube and the density of the fractions was determined by refractometry. The fractions were diluted with 2.5 ml of PBS, and then centrifuged first in a TLA 100.3 rotor for 1 hour at 230,000 g and then in a TLA 100.1 rotor under the same conditions. Finally, pellets containing concentrated prostasomes were analyzed by SDS-PAGE and western blotting.

Detergent extraction procedures and isolation of insoluble membranes

Lipid rafts were prepared essentially as described (Brown and Rose, 1992). PC-3 cells grown to confluency in 100-mm dishes were rinsed with PBS and lysed for 20 minutes in 0.5 ml 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100 at 4°C. The lysate was homogenized by passing the sample through a 22-gauge needle, brought to 40% sucrose (w/w) in a final volume of 4 ml, and placed at the bottom of an 8-ml 5-30% linear sucrose density gradient made in the same buffer without Triton X-100. Gradients were centrifuged for 18 hours at 39,000 rpm at 4°C in a Beckman SW40 rotor. Fractions of 1 ml were harvested from the bottom of the tube and aliquots were then subjected to SDS-PAGE under reducing conditions and transferred to Immobilon-P membranes (Millipore) for western blot analyses.

Western blotting

Samples subjected to SDS-PAGE were transferred to Immobilon-P membranes. The membranes were then blocked with 5% non-fat dry milk and 0.05% Tween 20 in PBS and incubated with the indicated primary antibody. The membranes were then extensively washed with 0.05% Tween 20 in PBS, and then incubated with secondary antibodies coupled to horseradish peroxidase. Finally, the membranes were washed and developed using an ECL western blotting kit (Amersham Biosciences). Quantitative analyses were performed with a computing densitometer.

Immunofluorescence microscopy

Cells grown on coverslips were fixed in formalin solution for 15 minutes at room temperature, permeabilized with 0.2% Triton X-100 for 5 minutes, and incubated with 3% bovine serum albumin in PBS for 30 minutes. Cells were then incubated with the indicated primary antibodies for 30 minutes, rinsed, and incubated with the corresponding secondary antibodies coupled to Alexa488 or Alexa594. After extensive washing, the coverslips were mounted on slides. Images were obtained using a Bio-Rad Laboratories Radiance 2000 confocal laser microscope. Controls to assess labeling specificity included incubations with control primary antibodies or omission of the primary antibodies. Lipid droplets were stained by mounting coverslips in Fluoromount containing the lipophilic dye Nile red (1:1000 dilution from a saturated solution in acetone) as previously described (Pol et al., 2001).

Electron microscopy

PC-3 cells were fixed with 4% paraformaldehyde and 2% glutaraldehyde in 0.1 M Na-cacodylate buffer, pH 7.4. After careful washing, the cells were scraped off the plate. After post-fixing, cells were infiltrated with Epo. Thin sections were cut, stained and examined. Isolated prostasomes were loaded on formvar-carbon coated grids and incubated with anti-CD59 or anti-CD63 mAbs followed by gold-conjugated secondary antibodies. When anti-GFP was used to detect MAL-GFP, prostasomes were previously permeabilized with saponin. Finally, the grids were analyzed by whole-mount immunoelectron microscopy.

Results

Prostate cancer PC-3 cells coexpress MAL, BENE and MAL2 transcripts

To identify a model cell line simultaneously expressing MAL,
BENE and MAL2, we carried out northern blot analysis using a panel of human cell lines and the canine MDCK cell line (Fig. 1A). Individual expression of MAL, BENE or MAL2 transcripts was found in Jurkat and HPB-ALL T cell lines, A498 cells, HeLa cells, endothelial-like ECV304 cells and hepatoma HepG2 cells (Fig. 1A). Expression of only MAL and MAL2 was found in epithelial renal MDCK cells, whereas intestinal epithelial Caco-2 cells showed expression of BENE and MAL2 (Fig. 1A). Finally, the epithelial prostate PC-3 cell line was the only cell line that simultaneously coexpressed transcripts for the genes encoding MAL, MAL2 and BENE (Fig. 1A), and was therefore chosen for further experimentation.

In agreement with previous analyses of individual members of the MAL family (Martín-Belmonte et al., 1998; Millán and Alonso, 1998; de Marco et al., 2001), MAL, MAL2 and BENE were selectively detected in the raft fraction of PC-3 cells (Fig. 1B). Control experiments showed that raft fractions from PC-3 cells also contained the raft-associated protein cav-1 and excluded the ER-associated protein calnexin (Fig. 1B). Finally, as our current antibodies to MAL and BENE are not suitable for immunofluorescence, we prepared PC-3 cells stably expressing MAL tagged with GFP (MAL-GFP) or BENE tagged with c-myc (BENE-myc). As shown in Fig. 1C, the presence of the tags did not interfere with the ability of these proteins to reside in rafts.

Subcellular distribution of MAL, MAL2 and BENE in PC-3 cells

The intracellular distribution of MAL, MAL2 and BENE was studied by confocal immunofluorescence microscopy in PC-3 cells stably expressing MAL-GFP or BENE-myc. As shown in Fig. 2, MAL (Fig. 2A), MAL2 (Fig. 2D) and BENE (Fig. 2G) were found in apparently distinct vesicular structures that were often concentrated close to the lateral plasma membrane. Dual labelling of cav-1 and either MAL, MAL2 or BENE showed that cav-1 had a similar distribution (Fig. 2E,H) and colocalized with MAL (Fig. 2C), MAL2 (Fig. 2F) and BENE (Fig. 2I). In addition, as shown in Fig. 3A-D, MAL proteins colocalized between them in PC-3 cells. Cav-1 and MAL proteins were also detected at varying degrees at the plasma membrane. Therefore, in some cells the overlap between cav-1 and MAL family members does not appear complete in the selected confocal plane. The structures containing cav-1 detected by immunofluorescence microscopy were also detected by phase-contrast microscopy (Fig. 3E,F). Similar structures were also stained with MAL (Fig. 3G,H).

Interestingly, electron microscopy examination of Epon sections revealed the presence of multivesicular structures in PC-3 cells (Fig. 3I). The raft-associated GPI-anchored protein CD59 showed a high level of colocalization with cav-1 (Fig. 4A-C) and with MAL (data not shown). In order to investigate the nature of the compartment labelled with cav-1 and MAL family members, several markers of cellular compartments were used. As shown in Fig. 4D-F, in some cells intracellular cav-1 colocalized with the transferrin receptor, a marker of recycling endosomes. To investigate the potential colocalization of transferrin receptor with cav-1, we analyzed the effect of Brefeldin A (BFA), a compound known to induce tubulation of the compartment labelled with transferrin receptor (Lippincott-Schwartz et al., 1991), on the distribution of these two proteins. In the presence of BFA, the endosomal compartment labelled with the transferrin receptor was tubulated, unlike the compartments labelled with cav-1 (Fig. 4G-I) or MAL-GFP (data not shown). This experiment suggested that cav-1 and MAL label a compartment that is close to the transferrin receptor-containing compartment. Cav-1 was also excluded from early endosomes labelled with EEA1 (Fig. 5A-C), and from lysosomes labelled with CD63 (Fig. 5D-F), also known as lysosome-associated membrane protein 3 or lysosome integral membrane protein 1 (Kobayashi et al., 2000), and with lamp-1 (data not shown). Furthermore, cav-1 labelling did not colocalize with the Golgi apparatus, as determined by double staining of cav-1 with the Golgi marker giantin (Fig. 5G-I). Finally, cav-1

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**Fig. 3.** PC-3 cells stably expressing BENE-myc or MAL-GFP. Cells were fixed and stained with MAL (A), MAL2 (B,D) and BENE (C) and analyzed with a confocal laser microscope. PC-3 cells stained with cav-1 were analyzed by immunofluorescence microscopy (E) and by phase-contrast microscopy (F). PC-3 cells stably expressing MAL-GFP were analyzed by immunofluorescence microscopy (G) and by phase-contrast microscopy (H). (I) Electron micrograph of PC-3 cells embedded in Epon. Cells in A-D have been outlined for better visualization. Bars A-H, 10 μm; I, 1 μm.
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was not found in lipid droplets stained with Nile red (Fig. 5J-L) even in the presence of BFA (data not shown), a compound that has been shown to cause the accumulation of cav-1 in the ER and the redistribution of cav-1 to lipid droplets (Fujimoto et al., 2001; Ostermeyer et al., 2001).

Characterization of prostasomes released by PC-3 cells

Prostasomes are vesicular organelles secreted by prostate epithelial cells (Ronquist and Brody, 1985; Ronquist, 1999; Kravets et al., 2000). Interestingly, the prostasomal membrane has a very high cholesterol/phospholipid ratio and lots of sphingomyelin (Arvidson et al., 1989). As cholesterol and sphingomyelin are molecules normally found in rafts, the possibility existed that proteins from the MAL and caveolin families were found in prostasomes released from PC-3 cells. Prostasomes have routinely been isolated from human seminal plasma (Carlsson et al., 2003), but granules with strong similarities to seminal prostasomes have been isolated from cytospins of several human prostate cancer cell lines in vitro, including the PC-3 cell line (Nilsen et al., 1999). In order to investigate whether prostasome-like vesicles are secreted by PC-3 cells, serum-free culture supernatants incubated for 20-24 hours with PC-3 cells were centrifuged at 100,000 g for 2 hours (Wang et al., 2001). As shown in Fig. 6A, whole-mount electron microscopy of the pellet fraction revealed the presence of cup-shaped vesicles that were mainly 30-130 nm in diameter. A recent proteomic analysis revealed the presence of 139 proteins in human prostasomes (Utleg et al., 2003), but none of these proteins appears to be specific to prostasomes. In order to investigate whether PC-3-secreted vesicles share common characteristics with seminal prostasomes, we investigated the presence of the membrane attack complex inhibitory protein CD59, a GPI-anchored protein previously demonstrated in prostasomes from semen (Rooney et al., 1993). Electron microscopy analyses of whole-mounts of the vesicles immunolabeled with anti-CD59 (Fig. 6A) and western blotting (Fig. 6B) revealed the presence of CD59 in the vesicles released by PC-3 cells. Furthermore, lysosomal proteins have also previously been found in seminal prostasomes (Aumuller et al., 1997), and we also found the tetraspanin CD63 in vesicles secreted from PC-3 cells (Fig. 6A,B). Control western blot experiments showed that calnexin, an integral membrane protein in the endoplasmic reticulum, was not present in the vesicles (data not shown). These experiments suggest that the vesicles sedimented from the culture supernatant of PC-3 cells resemble true seminal prostasomes and they will therefore be referred to as prostasomes.

Cav-1 and MAL are present in prostasomes secreted by PC-3 cells

As prostasomal membranes are enriched in raft molecules (Arvidson et al., 1989), the prostasome fraction isolated from the tissue culture medium of PC-3 cells was analyzed by western blotting with antibodies against cav-1. As shown in Fig. 6C, cav-1 was found in PC-3 cell-derived prostasomes. Approximately, 0.5-1.0% of the total amount of cav-1 was collected in the prostasomes released by PC-3 cells to serum-free culture media over ~22 hours. Prostasomal cav-1 migrated in SDS-PAGE similar to cav-1 from MDCK cells and ECV304 cells (data not shown). The presence of the raft-associated protein MAL in PC-3 cell-derived prostasomes was investigated. MAL-GFP was detected by western blot (Fig. 6C)
and by electron microscopy analyses (Fig. 6D) in prostasomes isolated from PC-3 cells stably transfected with MAL-GFP. Finally, PC-3 cell-derived prostasomes were floated on a linear sucrose density gradient to confirm that these proteins were associated with a vesicular membrane fraction. As shown in Fig. 6E, the total amount of cav-1 floated at a density of 1.11 g/ml, similar to CD59 in seminal prostasomes floated in Nycodenz gradients (Rooney et al., 1996). In agreement with previous results by these authors, a fraction of CD59 was not associated with prostasomes (Rooney et al., 1996). These flotation experiments indicate that cav-1 found in the sedimented prostasome fraction (Fig. 6C) is associated with prostasomes and not with potential aggregates that could sediment and contaminate this fraction.

Characterization of prostasome secretion in PC-3 cells

Our previous experiments indicate that cav-1 and MAL are released in association with prostasomes in PC-3 cells (Fig. 6). It is not clear so far how prostasomes are secreted, but it has been suggested that secretory granules containing prostasomes could be secreted either by fusion with the plasma membrane or by the formation of bleb-like protrusions in the plasma membrane (Rooney et al., 1996). In agreement with previous results by these authors, a fraction of cav-1 in prostasomes isolated from the culture supernatant of PC-3 cells treated with BFA (2.5 μg/ml), an inhibitor of the constitutive exocytic pathway via the endoplasmic reticulum and the Golgi apparatus (Miller et al., 1992), was measured. As expected, control immunofluorescence microscopy experiments showed that the Golgi apparatus morphology was altered in the presence of BFA (data not shown). Western blot analysis showed that BFA did not reduce the amount of cav-1 (Fig. 7A) or MAL-GFP (Fig. 7B) in prostasomes, thus suggesting that prostasomes are not constitutively released by the conventional secretory pathway. Control experiments indicated that the treatment with BFA did not significantly affect the intracellular amount of cav-1 or MAL-GFP in cell lysates and the number of cells (data not shown).

The endocytic pathway has recently been involved in an alternative secretory pathway (for review see Denzer et al., 2000; Stoorvogel et al., 2002; Théry et al., 2002; Blott and Griffiths, 2002; Raposo et al., 2002). Lysosome-related organelles (LROs) are cell-type specific organelles with several characteristics of lysosomes/late endosomes/multivesicular bodies that secrete their content to the extracellular milieu when they fuse with the plasma membrane (Denzer et al., 2000;
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Stoorvogel et al., 2002; Théry et al., 2002; Blott and Griffiths, 2002; Raposo et al., 2002). As prostasome-containing granules morphologically resemble multivesicular bodies and contain lysosomal-associated proteins (Aumuller et al., 1997), the possibility that prostasomes were secreted by an endocytic secretory pathway was explored. It has been shown that wortmannin, an inhibitor of phosphatidylinositol-3 kinase (PI3K), inhibits the formation of multivesicular bodies/late endosomes (Fernandez-Borja et al., 1999) and reduces the secretion of LRO in Daudi cells (Clayton et al., 2001). As shown in Fig. 7C, treatment of PC-3 cells with wortmannin (100 nM) reduced the amount of cav-1 released in prostasomes by 30%. The secretion of MAL-GFP in prostasomes was also reduced by wortmannin (Fig. 7D). Similar to BFA, treatment with wortmannin did not affect the intracellular amount of cav-1 and MAL-GFP in cell lysates or the number of cells (data not shown).

Furthermore, after one hour of wortmannin treatment, the morphology of the cav-1 labelled compartment was severely affected (Fig. 7E). Control experiments showed that endosomes labelled with Rab5 were also altered (Fig. 7E). These results support the hypothesis that prostasomes are secreted from endocytic compartments. When higher concentrations (2.5-10 μM) of wortmannin were used, cav-1 secretion was increased. This effect might be due to additional effects of the drug.

Discussion

In this study, we show that MAL, MAL2 and BENE are found simultaneously in the human prostate cancer cell line PC-3. These raft-associated proteins were concentrated in an intracellular compartment with multivesicular bodies/late endosomes (Fernandez-Borja et al., 1999) and reduces the secretion of LRO in Daudi cells (Clayton et al., 2001). As shown in Fig. 7C, treatment of PC-3 cells with wortmannin (100 nM) reduced the amount of cav-1 released in prostasomes by 30%. The secretion of MAL-GFP in prostasomes was also reduced by wortmannin (Fig. 7D). Similar to BFA, treatment with wortmannin did not affect the intracellular amount of cav-1 and MAL-GFP in cell lysates or the number of cells (data not shown).

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strongly suggest that cav-1 and MAL exploit the multivesicular compartment for storage and release. Furthermore, our results suggest that the intracellular organelles, where cav-1 and MAL family proteins are concentrated in PC-3 cells, are prostasomes. Prostasomes are vesicles/granules secreted to the prostatic fluid by prostate epithelial cells that intracellularly appear in the interior of large (~1 μm diameter) storage vesicles (Shalen et al., 2002). In agreement with this idea, we found that the GPI-anchored protein CD59, a protein previously found in prostasomes (Rooney et al., 1993), colocalized intracellularly with cav-1 and MAL, and it was found in the vesicles released by PC-3 cells. The lysosomal protein CD63 was also found in secreted prostasomes, although this protein did not colocalize intracellularly with cav-1. This result can be explained if the amount of CD63 is much lower in the intracellular prostasomic compartment than in lysosomes. Furthermore, vesicles derived from PC-3 cells contain the raft-proteins MAL and cav-1, and it has previously been shown that the prostasome membrane is enriched in raft molecules (Arvidson et al., 1989). This is the first time that prostasome-like vesicles have been isolated from culture supernatants of PC-3 cells, although prostasomes have previously been isolated from cytopsins of these cells (Nilsson et al., 1999).

The mechanism of prostasome secretion is not well understood, but our experiments suggest that prostasomes are not secreted by the conventional secretory pathway via the endoplasmic reticulum and the Golgi apparatus. Interestingly, many different cell lines such as red blood cells, mast cells, epithelial cells, reticulocytes and dendritic cells secrete small vesicles termed exosomes (Denzer et al., 2000; Stoovogel et al., 2002; Théry et al., 2002; Blott and Griffiths, 2002; Raposo et al., 2002). Exosomes, like prostasomes, are enriched in raft molecules (de Gassart et al., 2003; Wubbolts et al., 2003), are found in the interior of cell-specific organelles with characteristics of lysosomes/late endosomes/multivesicular bodies and are secreted when LROs fuse with the plasma membrane (Denzer et al., 2000; Stoovogel et al., 2002; Théry et al., 2002; Blott and Griffiths, 2002; Raposo et al., 2002). Interestingly, secretory vacuoles filled with prostasomes of the human prostate morphologically resemble multivesicular bodies/lysosomes and contain lysosomal proteins (Aumuller et al., 1997). Therefore, it is possible that the prostasome-containing granules found in prostate cells are in fact cell-specific LROs and that prostasomes are secreted when these LROs fuse with the plasma membrane. In agreement with this hypothesis, we show that, similar to exosome secretion in Daudi cells (Clayton et al., 2001), prostasome secretion is inhibited by wortmannin in PC-3 cells.

In normal prostate epithelial cells, the MAL family members MAL and MAL2 are expressed in a granular compartment at the apical side of the cell as demonstrated by immunohistochemical analysis (Marazuela et al., 2003; Marazuela et al., 2004). Cav-1 expression is undetectable in normal prostate epithelium, but it is upregulated in prostate cancer and an elevated expression of cav-1 has been correlated with prostate cancer progression (Yang et al., 1998; Yang et al., 1999). Interestingly, it has recently been shown that cav-1 is secreted by androgen-insensitive prostate cancer cells such as the PC-3 cell line, and that secreted cav-1 stimulates cell survival and contributes to metastasis in prostate cancer cells (Tahir et al., 2001). The mechanism of cav-1 secretion in prostate cancer cells has not been defined. However, the finding that BFA did not inhibit the secretion of cav-1 suggests that cav-1 is secreted via a Golgi-independent pathway (Wu et al., 2002; Wu and Terrian, 2002). Furthermore, as cav-1 might redistribute to lipid droplets in the presence of BFA (Fujimoto et al., 2001; Ostermeyer et al., 2001), it was suggested that lipid droplets could be involved in cav-1 secretion (Wu et al., 2002; Wu and Terrian, 2002). Our experiments show that, at least in the prostate cancer cell line PC-3, cav-1 is not found in lipid droplets. Furthermore, we found that cav-1 is released on prostasomes in PC-3 cells and that prostasomes appear to be secreted from an endocytic compartment. These findings reveal information that might be important for understanding prostate cancer progression.

The fact that the prostatic cell line PC-3 expresses MAL, MAL2 and BENE endogenously has allowed us to test whether the coexpression of different members of the MAL family in the same cell could result in novel subcellular localizations. In agreement with this idea, the raft-associated MAL proteins in PC-3 cells localized to an intracellular compartment with multivesicular morphology that was identified as a specific compartment of prostatic cells called prostasomes. Cav-1 was also found in the same compartment. Further work is required to investigate the potential role of MAL protein members in the organization and/or function of prostasomes in prostate cancer cells.

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References


Cav-1 and MAL in prostosomes

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