T1 α /Podoplanin Shows Raft-Associated Distribution in Mouse Lung Alveolar Epithelial E10 Cells

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Key Words

T1 α • Podoplanin • Alveolar epithelial cells • Mouse lung • Lipid rafts

Abstract

Aims: T1 α /(podoplanin) is abundantly expressed in the alveolar epithelial type I cells (ATI) of rodent and human lungs. Caveolin-1 is a classical primary structural protein of plasmalemmal invaginations, so-called caveolae, which represent specialized lipid rafts, and which are particularly abundant in ATI cells. The biological functions of T1 α in the alveolar epithelium are unknown. Here we report on the characteristics of raft domains in the microplicae/microvillar protrusions of ATI cells, which contain T1 α . **Methods:** Detergent resistant membranes (DRMs) from cell lysates of the mouse epithelial ATI-like cell line E10 were prepared using different detergents followed by flotation in a sucrose gradient and tested by Western and dot blots with raft markers (caveolin-1, GM1) and nonraft markers (transferrin receptor, PDI and β -Cop). Immunocytochemistry was employed for the localization of T1 α in E10 cells and *in situ* in rat lungs. **Results:** Our biochemical results showed that the solubility or insolubility of T1 α and caveolin-1 differs in Triton X-100 and Lubrol WX, two distinct non-ionic detergents.

Caveolin-1 was insoluble in both detergents, whereas T1 α was Triton X-100 soluble but Lubrol WX insoluble. Immunofluorescence double stainings revealed that both proteins were colocalized with GM1, while caveolin-1 and T1 α were not colocalized in the plasma membrane. Cholesterol depletion modified the segregation of T1 α in Lubrol WX DRMs. Cellular processes in ultrathin sections of cultured mouse E10 cells were immunogold positive. Immunoelectron microscopy (postembedding) of rat lung tissue revealed the preferential localization of T1 α on apical microvillar protrusions of ATI cells. **Conclusion:** We conclude that T1 α and caveolin-1 are located in distinct plasma membrane microdomains, which differ in their protein-lipid interactions. The raft-associated distribution of T1 α may have an impact on a specific, not yet clarified function of this protein in the alveolar epithelium.

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Introduction

T1 α , a type I transmembrane sialomucin-like glycoprotein, is the first molecular marker, which has been cloned and sequenced and which is specific for alveolar

epithelial type I (ATI) cells of the lung [1]. The T1 α gene is identical with OTS-8 [2], and encodes a 38-40 kDa glycoprotein, known as T1 α (rat, mouse), E11 antigen (rat [3]), gp38 (mouse [4]), RTI40 (rat [5]), MEP-1 (rat [6, 7]), PA 2.26 antigen (mouse and human cell lines [8]), podoplanin [9] and aggrus (human carcinomas [10]). Known cellular localizations of T1 α are lymphatic endothelial cells (podoplanin), late osteoblasts at the transition to osteocytes [3, 11], glomerular podocytes, the salivary gland, the fetal brain, the choroid plexus and ciliary body epithelial cells [12].

Immunocytochemical analysis of adult rat lung has shown that T1 α is localized to the apical surfaces of ATI cells [1]. ATI cells are flattened squamous epithelial cells which form a long thin layer over most of the alveolar wall and which have numerous microvilli or microplicae, particularly abundant near the intercellular junctions to the neighbouring ATII cells ([13]). These protrusions, however, are not comparable with the microvilli of ATII cells, which are larger in size.

Microvilli are cell membrane specialized structures of epithelial cells that mainly function as highthroughput absorptive/secretory areas [14]. It has been well established that microvilli are particularly rich in membrane lipids characteristic to lipid rafts. The glycolipid-based rafts described above are not the only type of rafts reported to exist in microvilli. More than one type of lipid raft microdomains may be present at the same cell surface. The apical pentaspanning protein prominin resides in microvillar lipid rafts from MDCK cells that are soluble in cold Triton X-100, but insoluble in another nonionic detergent, Lubrol WX, and physically separated from "normal" rafts containing the GPI-anchored alkaline phosphatase that occupy the planar parts of the apical surface of this cell type [15]. A similar, differential sensitivity to Triton X-100 and Brij58 was recently observed for the EGF receptor [16], also suggesting the existence of lipid rafts with lipid-protein interactions, but too weak to be detected by the conventional criterion of Triton X-100 insolubility. The raft hypothesis proposes that apical proteins are selectively incorporated into lipid microdomains in the *trans* Golgi network (TGN) and then transported directly to the apical membrane [17]. Interestingly, rafts involved in the direct pathway are Lubrol WX insoluble but Triton X-100 soluble, whereas rafts involved in the indirect pathway are both Lubrol WX and Triton X-100 insoluble. In polarized hepatic cells, two types of lipid rafts seem to be operating in exocytotic trafficking of apical resident proteins [18].

The molecular interactions underlying sorting of T1 α

to the plasma membrane (apical membrane) domain and its preferential association with lipid rafts are unknown. T1 α has previously been shown with immunoelectron microscopy to be localized in cellular protrusions of osteoblastic cells and osteocytes [19].

In this study we want to analyze the localization as well as the association of T1 α with lipid rafts in the alveolar epithelial cell line E10 and consider the hypothesis that lipid rafts might contribute to a specific subcellular targeting and functional activation of T1 α . In addition, we are interested in the relationship of T1 α to caveolin-1, a structural protein of caveolae. These microdomains represent distinct, specialized lipid rafts, which are particularly abundant in ATI cells.

Materials and Methods

Cell lines and reagents

The mouse E10 lung cell line was kindly provided by M. Williams (Pulmonary Center, Boston University School of Medicine, Boston, MA, USA). This cell line was generated by Dr. A. Malkinson (Denver, CA; [20]) and has an ATI cell like phenotype e.g. expression of caveolin-1, aquaporin-5, T1 α and other ATI cell related proteins [21]. DMEM/Ham's F12 was purchased from Gibco (Invitrogen, Karlsruhe, Germany). Fetal bovine serum was obtained from HyClone Perbio Science Deutschland GmbH (Bonn, Germany). L-glutamine and trypsin/EDTA were purchased from Biochrom AG Seromed (Berlin, Germany).

Cell culture conditions

E10 cells were cultured in DMEM/Ham's F12 medium (1:1) supplemented with 10% heat inactivated fetal bovine serum and 2.5 mM L-glutamine. They were grown at 37°C in a 5% CO₂ atmosphere. Cells were seeded at a density of 3x10⁴ cells/ml and passaged continuously.

Western blot analysis

SDS-PAGE and Western blot analysis were performed as previously described [22] using the following antibodies: monoclonal hamster anti-mouse T1 α /podoplanin (Santa Cruz Biotechnology Inc., Heidelberg, Germany; dilution 1:1,000), monoclonal mouse anti-caveolin-1 (clone 2297, BD Biosciences, Pharmingen, San Jose, USA; dilution 1:500), monoclonal mouse anti-human transferrin receptor (TfR) (clone H68.4, Zymed Laboratories Inc., South San Francisco, USA; dilution 1:500), polyclonal rabbit anti-protein disulfide isomerase (PDI) (StressGen Biotechnologies Corp., Victoria, Canada; dilution 1:1,000), polyclonal rabbit anti- β -coatamer protein (β -Cop) (Oncogene Research Products, Boston, USA; dilution 1:750).

Detergent solubility

One subconfluent T75 tissue culture flask was washed twice with ice-cold phosphate-buffered saline (PBS), scraped

into 500 µl ice-cold MBS buffer (25 mM Mes, pH 6.5; 150 mM NaCl) containing 1% detergent (Triton X-100, Serva, Heidelberg, Germany; Lubrol WX, Serva, Heidelberg, Germany; Brij98, Brij58 or Brij35, Sigma-Aldrich, München, Germany) and protease inhibitors (complete EDTA-free, Roche, Prenzberg, Germany), incubated for 30 min on ice and pelleted by centrifugation (10 min, 17,000g or 1 h, 100,000g) with a Beckman F2402H rotor (Allegra™ 64R centrifuge). Afterwards the detergent soluble fraction (supernatant) was collected. To dissolve the remaining detergent insoluble fraction the pellet was resuspended in 500 µl MBS buffer containing 0.1% SDS and protease inhibitors. The homogenate was sonicated (UP100H, Dr. Hielscher GmbH, Germany) and syringed 10 times using a 26-gauge needle. Equal volumes of the detergent soluble and insoluble fractions were separated by SDS-PAGE and subjected to Western blot analysis.

Preparation of detergent insoluble membrane fractions

For floating on a sucrose density gradient, subconfluent E10 cells from 6 T75 tissue culture flasks were washed twice with ice-cold PBS and scraped into ice-cold MBS buffer containing 1% detergent and protease inhibitors, incubated for 30 min on ice and pelleted by centrifugation (10 min, 2,000g) with a Beckman F2402H rotor (Allegra™ 64R centrifuge). The pellet was resuspended in 1 ml of MBS buffer supplemented with protease inhibitors and homogenized with a 2 ml-douncer (20 strokes). Afterwards it was mixed with an equal amount of 80% sucrose in MBS buffer containing protease inhibitors and overlaid with 1.4 ml of 35% sucrose followed by a layer of 0.8 ml of 5% sucrose. The gradient was centrifuged at 200,000g (Optima™ Max Ultracentrifuge, Beckman Coulter) for 4 h at 4°C. Thirteen 300 µl fractions were collected, beginning from the top.

Detection of GM1 by cholera toxin binding (CTXB)

5 µl of each fraction were diluted with 40 µl MBS buffer. Afterwards 5 µl of each diluent were spotted onto a cellulose acetate membrane. After washing with TBS-T (137 mM NaCl, 2.7 mM KCl 20 mM Tris-HCl, 0.2% Tween20; pH 7.4) the dried membrane was incubated with blocking solution (TBS-T containing 5% non fat dry milk) for 1 h at room temperature (RT). Next, it was incubated with cholera toxin subunit B horseradish peroxidase (CTXB-HRP) (Molecular Probes, Eugene, USA; dilution 1:1,000) in blocking solution for 1 h at RT, followed by washing with TBS-T for three times. Chemiluminescent signal was generated using ECL™ Western Blotting Detection Reagents (Amersham Biosciences, Uppsala, Sweden) and detected with an Image Reader (LAS-3000 Fujifilm, Tokyo, Japan).

Saponin, cytochalasin D and ammonium sulfate treatment

For saponin treatment, subconfluent E10 cells from one T75 tissue culture flask (6 T75 flasks were used for preparation of detergent-insoluble membrane fractions) were washed twice with ice cold PBS, scraped into 500 µl PBS containing 0.2% saponin (AppliChem, Darmstadt, Germany), incubated for 40 min on ice and pelleted by centrifugation (5 min, 500g, 4°C) with a Beckman F2402H rotor (Allegra™ 64R centrifuge). The

pellet was resuspended in 500 µl ice-cold MBS buffer containing 1% Lubrol WX and protease inhibitors. After 30 min incubation on ice the homogenate was directly subjected to solubility experiments or sucrose density gradient centrifugation as previously described. To confirm that saponin itself has no influence on the solubility, control experiments were performed without Lubrol WX treatment.

For cytochalasin D treatment E10 cells were incubated for 4 h at 37°C in complete culture medium containing 1 µM cytochalasin D (Sigma-Aldrich). Afterwards detergent solubility experiments or preparation of detergent-insoluble membrane fractions were carried out. Control cells were incubated in complete culture medium containing 0.1% DMSO.

For (NH₄)₂SO₄ (ammonium sulfate) treatment detergent solubility and preparation of detergent-insoluble membrane fractions were performed as described above using MBS buffer containing 250 mM (NH₄)₂SO₄.

Coimmunoprecipitation

Coimmunoprecipitation experiments were performed as previously described [23] using polyclonal rabbit anti-caveolin-1 (clone N-20; Santa Cruz Biotechnology; Santa Cruz, CA) for immunoprecipitation.

Lipid analysis

To analyze the lipids, 200 µl of each fraction were mixed with 1.2 ml methanol/chloroform (2:1). After centrifugation (5 min, 4,000g) the supernatant was mixed with one volume acetic acid (20 mM), one volume chloroform and eight volumes aqua dest. The mixture was separated in two phases. The upper phase was carefully but not completely removed, the lower phase (lipid phase) was transferred into a new tube. The solvent was evaporated in a stream of nitrogen. Dried lipids were redissolved in 50 µl methanol/chloroform (1:2) and applied to a silica gel thin layer chromatography (TLC) plate. When the solvent front had reached 2/3 of the plate, the plate was developed with chloroform/triethylamine/ethanol/aqua dest (35:35:40:9), thereafter it was dried and developed with isohexane/ethyl acetate (5:1) until the solvent front had reached the upper edge of the plate. For lipid staining, the plate was stained with 20% sulfuric acid and heated to 200°C for several minutes. The lipid bands were compared with the standards.

Immunofluorescence

Double immunofluorescence staining was performed as previously described [24]. E10 cells grown on poly-L-lysine (Biochrom AG Seromed) coated glass cover slips were fixed with methanol/acetone (1:1). The fixed cells were incubated with monoclonal hamster anti-mouse T1α/podoplanin (Santa Cruz Biotechnology; dilution 1:50) or polyclonal rabbit anti-caveolin-1 (clone N-20, Santa Cruz Biotechnology; dilution 1:40) for 1 h at RT followed by specific anti-hamster IgG conjugated to fluoresceine isothiocyanate (FITC, dianova, Hamburg, Germany; dilution 1:200), anti-rabbit IgG conjugated to Texas Red (dianova; dilution 1:100) or anti-rabbit IgG conjugated to FITC (dianova; dilution 1:80) for 1 h at RT.

For GM1 staining, cells grown on glass cover slips were incubated for 1 h at 37°C with GM1 (200 µg/ml in medium,

Avanti Polar Lipids Inc., Alabaster, AL, USA). Unfixed cells were washed with ice cold $\text{Ca}^{2+}/\text{Mg}^{2+}$ -PBS twice and incubated for 20 min at 4°C with Alexa555-coupled CTXB (Molecular Probes; dilution 1:500 in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -PBS). For double immunofluorescence staining the cells were fixed and proceed as described before.

For immunofluorescence staining after saponin treatment, E10 cells, grown on cover slips, were incubated with 0.2% saponin in medium for 40 min at 37°C. Afterwards the cells were fixed and incubated with antibodies as described before.

Immunofluorescence labeling of the actin cytoskeleton was performed with cells fixed with methanol/acetone (1:1). After washing with PBS, TRITC conjugated phalloidin (1:1,000 in PBS, Sigma-Aldrich) was added for 1 h at RT followed by washing with PBS for three times. All immunostainings were examined with an Olympus microscope (Olympus BX60, Olympus optical Co., Ltd. Tokyo, Japan).

Immunoelectron microscopy

Cryoimmunogold electron microscopy. To determine the localization of T1 α , mouse lung E10 cells were fixed in 2% (w/v) paraformaldehyde and 0.5% (w/v) glutaraldehyde in 0.1 M phosphate buffer (PB), pH 7.4 for 2 min at 37°C and 2h at RT. The fixed samples were mixed with 10% gelatine in 0.1M PB at 37°C and incubated overnight in cold 2.3M sucrose in 0.1M PB at 4°C. Finally the probes were frozen in fluid nitrogen.

Ultrathin sections (65-70nm) were cut on an Ultracut S microtome (Leica) with a cryoattachment (Leica EM-FCS) at -110°C and collected on formvar-coated nickel grids. Then they were incubated 0.1M phosphate buffered saline (PBS), pH 7.4, containing 0.1% glycine for 10 min and washed in PBS for 10 min at RT. Before incubation (overnight at 4°C) with hamster anti-mouse T1 α antibody (undiluted supernatant), cells were incubated 2x 15 min with PBG (0.1M PBS with cold water fish skin gelatine and BSA-C, Aurion, Wageningen, Netherlands). After washings in PBG followed the incubation with goat anti-syrian hamster IgG, 12 nm gold, dilution 1:2, (dianova, Hannover, Germany). After six additional 5 min-washings in PBG, and further six 5 min-washings in PBS, incubation with 2.5 % (w/v) glutaraldehyde in PBS, further brief washes in distilled water, sections were stained by incubation with 2% methyl cellulose-3% uranyl acetate (1:9) for 10 min.

Lowicryl HM20 postembedding. Tissue pieces (about 1-2 mm³) from rat lungs (n=5) were embedded in Lowicryl HM20 and immunostained as previously described [25].

Briefly, paraformaldehyde-fixed samples were frozen in liquid nitrogen, cryosubstituted and embedded in lowicryl (through a graded series of methanol). Ultrathin sections were mounted on nickel grids, previously coated with 3% collodion. The grids were blocked with 10% normal goat serum in buffer (TBS, pH 7.6) for 45 min, followed by incubation with anti-T1 α antibody E11 (hybridoma supernatant, kind gift from Dr. A. Wetterwald; Berne, Switzerland) overnight at 4°C and for 1 h at RT. After washing with buffer (TBS containing 0.2% BSA) sections were incubated with 12 nm gold-conjugated goat anti-mouse (1:50 in TBS, pH 8.2) for 1 h. After further washing with buffer, the sections were stained with 2.5% uranylacetate in 50% ethanol and 1% lead citrate for 2 min each.

Fig. 1. Solubility of T1 α and caveolin-1 in different detergents. E10 cells were lysed in a buffer containing 1% of Triton X-100 or 1% Lubrol WX (upper part) or Brij35, 58 or 98 (lower part) to obtain soluble and insoluble fractions. These fractions were adjusted to equal volumes, and an aliquot of each (30 μ l) was analyzed by Western blot using caveolin-1 (Cav-1), or T1 α specific antibodies. Caveolin-1 was used as a marker of the lipid rafts. Representative data from three separate experiments are shown.

Results

Solubility of T1 α in various detergents

We first investigated whether T1 α is associated with cholesterol-sphingolipid rafts that are recovered as detergent-insoluble complexes upon solubilization of cells in non-ionic detergents, such as Triton X-100 at 4°C [26, 27]. E10 cells were lysed in 1% Triton X-100 at 4°C. The lysates were fractionated into supernatant (soluble fraction) and pellet (insoluble fraction) and analyzed by SDS-PAGE followed by immunoblotting for caveolin-1 and T1 α . Caveolin-1 was predominantly found in the detergent insoluble raft fraction, and T1 α was only present within the soluble fraction of the membrane (Fig. 1A). There exist two subtypes of caveolin-1 (caveolin-1 α and caveolin-1 β), which originate from an alternative initiation during translation [28].

Insolubility of a membrane protein in Triton X-100 does not exclude the possibility that it also engages in other, albeit Triton X-100-sensitive horizontal interactions. Röper et al. [15] published that Lubrol WX is a detergent for solubilization of such potential horizontal interactions possible involving T1 α . E10 cells were lysed at 4°C with 1% Lubrol WX and fractionated into supernatant and

Fig. 2. Characterization of membrane fractions prepared by Triton X-100, Lubrol WX or Brij98 from E10 cells. E10 cells were homogenized in a buffer containing 1% Triton X-100 (A) (or Lubrol WX (B) or Brij98 (C)) and subjected to sucrose density gradient centrifugation. Thirteen fractions were collected (fraction 1, top of the gradient; fraction 13, bottom of the gradient), and an aliquot of each fraction (30 μ l was resolved by SDS-PAGE and subjected to Western blot analysis with antibodies against T1 α , caveolin-1, TfR, PDI and β -Cop. As expected, caveolin-1 was enriched in fractions 2-5, representing caveolae-enriched membrane fractions. Furthermore, an aliquot of each fraction (5 μ l was blotted onto a cellulose acetate membrane and the distribution of GM1 was determined by the binding of CTXB. Representative data from three separate experiments are shown.

pellet by centrifugation (17,000g and 100,000g). Caveolin-1 was found in the Lubrol WX-insoluble fraction, as expected. Independently from the centrifugation mode, T1 α was also found in the Lubrol WX-insoluble fraction (Fig.

Fig. 3. Cholesterol depletion affects the Lubrol WX-resistant complexes containing T1 α . E10 cells were treated for 40 min at 4°C without or with 0.2% saponin, lysed in 1% Lubrol WX (30 min) and either centrifuged for 10 min at 10,000g (A) or subjected to flotation on a sucrose density gradient (B). Supernatants (S) and pellets (P) (A) and gradient fractions (B) were analysed by Western blot using antibodies against T1 α and caveolin-1. The gradient fractions one to five were also analyzed for cholesterol content by TLC (C). E10 cells grown on poly-L-lysine coated glass cover slips were incubated for 40 min at 37°C in the absence (control) or presence (saponin) of 0.2% saponin, fixed with methanol/acetone (1:1) and analyzed by immunofluorescence for T1 α (D). Bar = 10 μ m.

1A). To investigate whether the insolubility of T1 α in Lubrol WX is unique (delimited) for this detergent, we also examined others, such as Brij35, Brij58, and Brij98. The majority of T1 α was also present in the unsoluble

fractions, consistent with potential localization in lipid rafts (Fig. 1B).

T1α insolubility in Lubrol WX reflects its association with detergent-resistant complexes

We isolated lipid rafts from E10 cell membrane fractions on the basis of their insolubility in different detergents (Triton X-100, Lubrol WX, Brij98) at 4°C and their ability to float in density gradients. All fractions were analyzed with antibodies directed against caveolin-1 and T1α, the non-lipid raft-specific marker TfR, and intracellular markers β-Cop and PDI. A small quantity of T1α was detected in the low-density fractions together with caveolin-1 (Fig. 2A), but more amounts of T1α were found in the high-density fractions along with β-Cop, PDI, and TfR. In addition to Triton X-100, other nonionic detergents, such as Lubrol WX and Brij98, have been employed to isolate lipid rafts [29]. Rafts isolated by Lubrol WX (Fig. 2B) and Brij98 (Fig. 2C) presented quantitatively more T1α protein than those isolated by Triton X-100. Overall, the detection of T1α in the lipid raft fractions indicated that T1α is partially colocalized with lipid rafts.

Low-density fractions isolated by Triton X-100, Lubrol WX or Brij98 were free of Golgi apparatus or endoplasmic reticulum, as assessed by the presence of specific markers (β-Cop and PDI, respectively). The TfR was mainly found in high-density fractions, although small amounts were also present in the low-density fractions (Fig. 2B,C). This fraction is much lower than the enrichment in caveolin-1 and T1α.

The Lubrol WX resistant complexes containing T1α are sensitive to cholesterol depletion

The effect of saponin was also investigated, as it is known to sequester cholesterol, thereby destabilizing the lipid-rich microdomain structure and rendering them sensitive to solubilization by non-ionic detergents. The addition of saponin to E10 cells resulted in a redistribution of caveolin-1 and T1α from fractions 2 and 3 to the fractions 7 to 13 (T1α) or 3-13 (caveolin-1) (Fig. 3B). To analyze the cholesterol content, lipids were extracted from the low density fractions and resolved by TLC. Qualitative changes in the cholesterol content of the low density fractions after saponin treatment were found (Fig. 3C). Thus, saponin treatment resulted in a redistribution of T1α indicating that, when cholesterol was removed from the E10 cells, T1α association with the light microdomains was disrupted. Saponin treatment of E10 cells shifted T1α, caveolin-1 and cholesterol from lipid microdomains to

Fig. 4. T1α insolubility in Lubrol WX is not due to linkage to the actin cytoskeleton or other protein-protein interactions. E10 cells were incubated for 4 h at 37°C in the absence (control) or presence of 1 μM cytochalasin D (CytD) or 250 mM (NH₄)₂SO₄ followed by lysis in 1% Lubrol WX and either centrifuged for 10 min at 10,000g (A) or subjected to flotation on a sucrose density gradient (B). Supernatants (S) and pellets (P) (A) and gradient fractions (B) were analysed by Western blot with antibodies against T1α and caveolin-1. The cytochalasin D effect was controlled by immunofluorescence detection of TRITC-conjugated phalloidin. Note the complete disruption of the actin cytoskeleton (C).

Fig. 5. A-C: Single or double immunofluorescence analysis of methanol/acetone (1:1) fixed E10 cells are shown; A: T1α (FITC)/Cav-1 (Texas Red), B: T1α (FITC)/GM1 (Texas Red) and C: Cav-1(FITC)/GM1 (Texas Red).

Fig. 6. Immunoprecipitation of caveolin-1 and T1 α . Native membrane protein extracts were immunoprecipitated with anti-caveolin-1 antibody and analyzed by Western blot with caveolin-1 and T1 α antibodies. For comparison, total membrane protein lysates (lane 1), immunoprecipitate (IP-P; lane 2), and supernatant of the immunoprecipitate (IP-S; lane 3) were loaded. Lane 4 shows the immunoprecipitate of the negative control and its supernatant was loaded in lane 5. In addition, PVDF-membrane was probed with anti-caveolin-1 antibody to ensure appropriate binding of caveolin-1 protein to the antibody-agarose-complex. A representative result from three independent experiments is shown.

detergent-soluble fractions. Immunofluorescence data of normal and saponin treated E10 cells revealed a strong redistribution of T1 α from cellular processes to an ectoplasmic staining pattern (Fig. 3D).

Insolubility of T1 α in Lubrol WX is not due to interaction with cytoskeletal elements

Insolubility of a membrane protein in detergents such as Lubrol WX can be due to its association with detergent-resistant lipid rafts and/or its anchoring cytoskeletal elements.

To investigate this, we repeated the lysis of E10 cells in Lubrol WX using buffers of high ionic strength, containing 250 mM (NH₄)₂SO₄. At this concentration (NH₄)₂SO₄ disrupts the cytoskeleton in MDCK cells [30]. The proportion of T1 α recovered in the insoluble fraction was the same as in low ionic strength buffers (Fig 4A). As control that the concentration of (NH₄)₂SO₄ disrupts the cytoskeleton in E10 cells, we used b-actin, a protein, which is associated with the cytoskeleton. Pre-treatment of E10 cells with the actin disrupting drug cytochalasin D before lysis in Lubrol WX did not affect the insolubility of T1 α .

Furthermore we isolated raft-like membranes from E10 cells using Lubrol WX, which were pretreated with

Fig. 7. A Mouse lung epithelial E10 cells (ultrathin frozen sections). Immunogold labeling of cellular processes. B.C Rat lung (Lowicryl embedding). Immunogold localization of T1 α on microvilli of the pleural mesothelium (B) and on microvilli of ATI cells (C). A: Bar = 0,7mm; B,C: Bar = 2 mm.

cytochalasin D (Fig. 4B). As expected, T1 α and caveolin-1 were predominantly found in the detergent-insoluble raft fractions 2 to 4.

These observations confirmed that the insolubility of T1 α in Lubrol WX is not due to interaction with cytoskeletal elements.

The cytochalasin D effect was controlled by immunofluorescence detection of TRITC-conjugated phalloidin. After treatment with cytochalasin D E10 cells showed an alteration in cell shape and a loss of filament organization (Fig. 4B).

The detergent resistant complexes containing T1 α are distinct from those containing caveolin-1

Immunofluorescence double stainings of E10 cells revealed that both proteins are colocalized with GM1, while caveolin-1 and T1 α were not colocalized in the plasma membrane (Fig. 5).

To assess caveolin-1 interaction with T1 α , we performed coimmunoprecipitation experiments using cell lysates from E10 cells expressing caveolin-1 and T1 α (Fig. 6). Immunoprecipitation was carried out with a rabbit polyclonal antibody directed against caveolin-1. In a control experiment, the identical procedure was performed without the caveolin-1 antibody (lane 4 and 5).

As shown in Fig. 6 (lane 2), no protein of the expected size of T1 α (43 kDa) was precipitated with the caveolin-1 antibody, although both proteins can be detected in the cell lysate (lane 1). However, T1 α was found in the non-precipitated supernatant (lane 3). The specific precipitation of caveolin-1 was confirmed by Western blot analysis. T1 α was completely present in the supernatant of the cell lysates. The results of coimmunoprecipitation experiments suggest that caveolin-1 does not exist in a complex together with T1 α *in vivo*.

Immunoelectron microscopy

Immunogold labeling of ultrathin frozen sections of cultured mouse epithelial E10 cells revealed strong and selective immunoreactivity of cellular processes (Fig. 7A). In addition, for ultrastructural localization of T1 α in ATI cells *in situ* we used rat lung tissues. This was necessary, since we had no suitable antibody for the postembedding demonstration of T1 α in ATI cells in mouse tissues. Post-embedding immunoelectron microscopy of rat lung using monoclonal antibodies against T1 α (clone E11) revealed a moderate labeling of the AT I cell surface, but a strong labeling of the numerous microplacae (microvillar ridges) near the border to ATII cells (Fig. 7C). In mesothelial cells of rat lungs (used as positive control; [31]) microvilli could be seen along the apical cell membrane, which are immunogold-positive (Fig. 7B).

Discussion

T1 α is an integral membrane protein of unknown function that is expressed strongly at the apical plasma membrane of type I alveolar epithelial cells [1, 32].

Our biochemical data shows that the solubility or insolubility of T1 α and caveolin-1 differs in Triton X-100 and Lubrol WX, two distinct non-ionic detergents. Caveolin-1 is insoluble in both detergents, whereas T1 α is Triton X-100 soluble but Lubrol WX insoluble. We could show that both proteins are colocalized with GM1, a classical raft marker, while caveolin-1 and T1 α are not colocalized in the plasma membrane.

We conclude that T1 α and caveolin-1 are located in distinct plasma membrane microdomains which differ in their protein-lipid interactions. Delaunay et al. [33] compared the compositions of Lubrol and Triton DRMs. Similar to Triton DRMs, Lubrol DRMs were highly enriched with cholesterol, which is characteristic for membrane rafts. They could show that the two DRM fractions differed considerably in the ratio between lipids of the inner

Fig. 8. Localization of T1 α and caveolin-1 in the plasma membrane. Triton X-100 and Lubrol WX rafts are delivered to the apical plasma membrane (PM) either separately in two distinct transport vesicles or together in one common transport vesicle. Caveolin-1 is localized in a Triton X-100-resistant complex in caveolae and T1 α in a separate complex in microplacae of the plasma membrane.

and outer membrane leaflets. The absolute amount of the outer lipids, phosphatidylcholine (PC) and sphingolipids, was slightly greater in Lubrol than in Triton DRMs. The amount of inner lipids, especially phosphatidylethanolamine (PE), and to a lesser extent phosphatidylserine (PS), was very different. Lubrol DRMs contained more PE, and additional molecular species with long unsaturated fatty acid chains. The latter were absent in Triton DRMs. The results of Delaunay et al. [33] suggest that Triton X-100 solubilizes a part of the inner leaflet lipids of rafts, especially unsaturated PE, and partially solubilizes certain raft-associated proteins. By contrast, Lubrol WX would preserve the internal leaflet of rafts, and maintain raft association of transmembrane proteins and proteins anchored to the cytoplasmic leaflet only.

The manipulation of plasma membrane cholesterol by saponin affects the detergent-resistant complexes containing T1 α and caveolin-1 in the same manner. Saponin pretreatment decreased the buoyant density of the Lubrol WX-resistant complexes containing T1 α and caveolin-1. The microvillus-specific cell-surface staining for T1 α is also lost after extraction of plasma membrane cholesterol.

On the basis of these results we propose that there may exist multiple, distinct types of raft-like assemblies

of lipids and proteins, which can coexist within one biological membrane (Fig. 8). The coexistence in the same membrane of such distinct types of rafts with differential sensitivity to various detergents may explain some of the previously reported differences in the extent of detergent insolubility of various plasma membrane proteins.

Several other types of membrane proteins have been shown to reside in microvillar rafts. These include peripheral membrane proteins such as annexin A2 and galectin-4, as well as integral membrane proteins like the Na⁺-H⁺ exchanger 3 the epithelial sodium channel, melanotransferrin, a GPI-linked iron-receptor, prominin, and stomatin [34].

At present, several concepts of protein sorting in epithelial cells suggest that apical and basolateral proteins are segregated in the TGN and are transported separately in distinct vesicular carriers [35]. The association or nonassociation of these proteins with detergent-insoluble lipid microdomains enriched in cholesterol and sphingolipids is one of the main sorting criteria. It was shown that T1 α is clearly localized on the apical site of type I alveolar epithelial cells.

Alfalah et al. [36] have demonstrated the existence of multiple sorting pathways for apically sorted proteins based on their association or nonassociation with lipid rafts in MDCK cells. It was shown that hepatocytes transport apical cargo by two different pathways. In these cells an indirect apical pathway transports the classical Triton X-100 insoluble microdomains, and a direct route is taken by Triton X-100-soluble but Lubrol WX insoluble membrane components [18]. T1 α is a further example for an apical localized protein, which is Triton X100 soluble but

Lubrol WX insoluble.

Our study implicates the involvement of T1 α in specific not yet determined functions of microvillus-type cell protrusions of ATI cells in the lung. These protrusions can only be detected near the junction of ATI cells with ATII cells but not between neighbouring ATI cells [13]. The functional role of these microplicae near the border to ATI cells is completely unknown. We have observed such protrusions in a similar distribution also in the mouse lung (Kasper, unpublished data).

Depending from the cell type, microvilli are involved in important regulatory functions such as hyaluronan synthesis [37], cell migration [38], accumulation of enzymes and growth factor receptors as well as secretion processes [14]. For prominin, a microvillar lipid raft protein of embryonic and adult epithelial cells as well as of non-epithelial cells [39], a shedding within membrane particles has been shown [40]. T1 α was found in the bronchoalveolar lavage of injured lungs in different experimental models of lung injury [41, 42] and is regarded as biochemical marker of ATI cell injury [43]. A vesicular shedding of T1 α like prominin involving membrane particles is currently not known. The present finding that T1 α has unique biochemical properties offers a novel approach to study the role of this protein in cell biology of the alveolar epithelium.

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References

- 1 Rishi AK, Joyce-Brady M, Fisher J, Dobbs LG, Floros J, VanderSpek J, Brody JS, Williams MC: Cloning, characterization, and development expression of a rat lung alveolar type I cell gene in embryonic endodermal and neural derivatives. *Dev Biol* 1995;167:294-306.
- 2 Nose K, Saito H, Kuroki T: Isolation of a gene sequence induced later by tumor-promoting 12-O-tetradecanoylphorbol-13-acetate in mouse osteoblastic cells (MC3T3-E1) and expressed constitutively in ras-transformed cells. *Cell Growth Differ* 1990;1:511-518.
- 3 Wetterwald A, Hoffstetter W, Cecchini MG, Lanske B, Wagner C, Fleisch H, Atkinson M: Characterization and cloning of the E11 antigen, a marker expressed by rat osteoblasts and osteocytes. *Bone* 1996;18:125-132.
- 4 Farr AG, Berry ML, Kim A, Nelson AJ, Welch MP, Aruffo A: Characterization and cloning of a novel glycoprotein expressed by stromal cells in T-dependent areas of peripheral lymphoid tissues. *J Exp Med* 1992;176:1477-1482.
- 5 Vanderbilt JN, Dobbs LG: Characterization of the gene and promoter for RT140, a differentiation marker of type I alveolar epithelial cells. *Am J Respir Cell Mol Biol* 1998;19:662-671.
- 6 Kasper M, Haroske G: Alterations in the alveolar epithelium after injury leading to pulmonary fibrosis. *Histol Histopathol* 1996;11:463-483.
- 7 Tsuchiya T, Takahashi K, Takeya M, Hosokawa Y, Hattori T, Takagi K: Immunohistochemical, quantitative immunoelectron microscopic, and DNA cytofluorometric characterization of chemically induced rat malignant fibrous histiocytoma. *Am J Pathol* 1993;143:431-445.
- 8 Scholl FG, Gamallo C, Vilar inverted question mark S, Quintanilla M: Identification of PA2.26 antigen as a novel cell-surface mucin-type glycoprotein that induces plasma membrane extensions and increased motility in keratinocytes. *J Cell Sci* 1999;112:4601-4613.

- 9 Breiteneder-Geleff S, Matsui K, Soleiman A, Meraner P, Poczewski H, Kalt R, Schaffner G, Kerjaschki D: Podoplanin, novel 43-kd membrane protein of glomerular epithelial cells, is down-regulated in puromycin nephrosis. *Am J Pathol* 1997;151:1141-1152.
- 10 Kato Y, Fujita N, Kunita A, Sato S, Kaneko M, Osawa M, Tsuruo T: Molecular identification of Aggrus/T1alpha as a platelet aggregation-inducing factor expressed in colorectal tumors. *J Biol Chem* 2003;278:51599-51605.
- 11 Schulze E, Witt M, Kasper M, Lowik CW, Funk RH: Immunohistochemical investigations on the differentiation marker protein E11 in rat calvaria, calvaria cell culture and the osteoblastic cell line ROS 17/2.8. *Histochem Cell Biol* 1999;111:61-69.
- 12 Williams MC, Cao Y, Hinds A, Rishi AK, Wetterwald A: T1 alpha protein is developmentally regulated and expressed by alveolar type I cells, choroid plexus, and ciliary epithelia of adult rats. *Am J Respir Cell Mol Biol* 1996;14:577-585.
- 13 Bastacky J, Lee CY, Goerke J, Koushafar H, Yager D, Kenaga L, Speed TP, Chen Y, Clements JA: Alveolar lining layer is thin and continuous: low-temperature scanning electron microscopy of rat lung. *J Appl Physiol* 1995;79:1615-1628.
- 14 Sbarbati A, Osculati F: A new fate for old cells: brush cells and related elements. *J Anat* 2005;206:349-358.
- 15 Roper K, Corbeil D, Huttner WB: Retention of prominin in microvilli reveals distinct cholesterol-based lipid microdomains in the apical plasma membrane. *Nat Cell Biol* 2000;2:582-592.
- 16 Roepstorff K, Thomsen P, Sandvig K, van Deurs B: Sequestration of epidermal growth factor receptors in non-caveolar lipid rafts inhibits ligand binding. *J Biol Chem* 2002;277:18954-18960.
- 17 Simons K, Ikonen E: Functional rafts in cell membranes. *Nature* 1997;387:569-572.
- 18 Slimane TA, Trugnan G, Van ISC, Hoekstra D: Raft-mediated trafficking of apical resident proteins occurs in both direct and transcytotic pathways in polarized hepatic cells: role of distinct lipid microdomains. *Mol Biol Cell* 2003;14:611-624.
- 19 Schulze E, Witt M, Kasper M, Lowik CW, Funk RH: Immunohistochemical investigations on the differentiation marker protein E11 in rat calvaria, calvaria cell culture and the osteoblastic cell line ROS 17/2.8. *Histochem Cell Biol* 1999;111:61-69.
- 20 Thompson DC, Porter SE, Bauer AK, Das KC, Ou B, Dwyer-Nield L, White CW, Malkinson AM: Cytokine-induced nitric oxide formation in normal but not in neoplastic murine lung epithelial cell lines. *Am J Physiol* 1998;274:L922-932.
- 21 Kathuria H, Cao Y, Hinds A, Ramirez MI, Williams MC: ERM is expressed by alveolar epithelial cells in adult mouse lung and regulates caveolin-1 transcription in mouse lung epithelial cell lines. *J Cell Biochem* 2007;102:13-27.
- 22 Barth K, Weinhold K, Guenther A, Young MT, Schnittler H, Kasper M: Caveolin-1 influences P2X(7) receptor expression and localization in mouse lung alveolar epithelial cells. *FEBS J* 2007;274:3021-3033.
- 23 Barth K, Weinhold K, Guenther A, Linge A, Gereke M, Kasper M: Characterization of the molecular interaction between caveolin-1 and the P2X receptors 4 and 7 in E10 mouse lung alveolar epithelial cells. *Int J Biochem Cell Biol* 2008;40:2230-2239.
- 24 Barth K, Gentsch M, Blasche R, Pfuller A, Parshyna I, Koslowski R, Barth G, Kasper M: Distribution of caveolin-1 and connexin43 in normal and injured alveolar epithelial R3/1 cells. *Histochem Cell Biol* 2005;123:239-247.
- 25 Kasper M, Huber O, Grossmann H, Rudolph B, Trankner C, Muller M: Immunocytochemical distribution of E-cadherin in normal and injured lung tissue of the rat. *Histochem Cell Biol* 1995;104:383-390.
- 26 Fiedler K, Kobayashi T, Kurzchalia TV, Simons K: Glycosphingolipid-enriched, detergent-insoluble complexes in protein sorting in epithelial cells. *Biochemistry* 1993;32:6365-6373.
- 27 Melkonian KA, Chu T, Tortorella LB, Brown DA: Characterization of proteins in detergent-resistant membrane complexes from Madin-Darby canine kidney epithelial cells. *Biochemistry* 1995;34:16161-16170.
- 28 Song KS, Tang Z, Li S, Lisanti MP: Mutational analysis of the properties of caveolin-1. A novel role for the C-terminal domain in mediating homo-typic caveolin-caveolin interactions. *J Biol Chem* 1997;272:4398-4403.
- 29 Hollerer-Beitz G, Heinemann SH: Influence of detergents on the function of cloned potassium channels. *Receptors Channels* 1998;5:61-78.
- 30 Fey EG, Wan KM, Penman S: Epithelial cytoskeletal framework and nuclear matrix-intermediate filament scaffold: three-dimensional organization and protein composition. *J Cell Biol* 1984;98:1973-1984.
- 31 Kimura N, Kimura I: Podoplanin as a marker for mesothelioma. *Pathol Int* 2005;55:83-86.
- 32 Dobbs LG, Williams MC, Gonzalez R: Monoclonal antibodies specific to apical surfaces of rat alveolar type I cells bind to surfaces of cultured, but not freshly isolated, type II cells. *Biochim Biophys Acta* 1988;970:146-156.
- 33 Delaunay JL, Breton M, Trugnan G, Maurice M: Differential solubilization of inner plasma membrane leaflet components by Lubrol WX and Triton X-100. *Biochim Biophys Acta* 2008;1778:105-112.
- 34 Danielsen EM, Hansen GH: Lipid raft organization and function in brush borders of epithelial cells. *Mol Membr Biol* 2006;23:71-79.
- 35 Keller P, Toomre D, Diaz E, White J, Simons K: Multicolour imaging of post-Golgi sorting and trafficking in live cells. *Nat Cell Biol* 2001;3:140-149.
- 36 Alfalah M, Wetzel G, Fischer I, Busche R, Sterchi EE, Zimmer KP, Sallmann HP, Naim HY: A novel type of detergent-resistant membranes may contribute to an early protein sorting event in epithelial cells. *J Biol Chem* 2005;280:42636-42643.
- 37 Kultti A, Rilla K, Tiihonen R, Spicer AP, Tammi RH, Tammi MI: Hyaluronan synthesis induces microvillus-like cell surface protrusions. *J Biol Chem* 2006;281:15821-15828.
- 38 Barreiro O, Vicente-Manzanares M, Urzainqui A, Yanez-Mo M, Sanchez-Madrid F: Interactive protrusive structures during leukocyte adhesion and transendothelial migration. *Front Biosci* 2004;9:1849-1863.
- 39 Corbeil D, Roper K, Fargeas CA, Joester A, Huttner WB: Prominin: a story of cholesterol, plasma membrane protrusions and human pathology. *Traffic* 2001;2:82-91.
- 40 Huttner HB, Janich P, Kohrmann M, Jaszi J, Siebzehnruhl F, Blumcke I, Suttrop M, Gahr M, Kuhnt D, Nimsky C, Krex D, Schackert G, Lowenbruck K, Reichmann H, Juttler E, Hacke W, Schellinger PD, Schwab S, Wilsch-Brauninger M, Marzocco AM, Corbeil D: The stem cell marker prominin-1/CD133 on membrane particles in human cerebrospinal fluid offers novel approaches for studying central nervous system disease. *Stem Cells* 2008;26:698-705.
- 41 McElroy MC, Pittet JF, Hashimoto S, Allen L, Wiener-Kronish JP, Dobbs LG: A type I cell-specific protein is a biochemical marker of epithelial injury in a rat model of pneumonia. *Am J Physiol* 1995;268:L181-186.
- 42 Koslowski R, Dobbs LG, Wenzel KW, Schuh D, Muller M, Kasper M: Loss of immunoreactivity for RTI40, a type I cell-specific protein in the alveolar epithelium of rat lungs with bleomycin-induced fibrosis. *Eur Respir J* 1998;12:1397-1403.
- 43 McElroy MC, Kasper M: The use of alveolar epithelial type I cell-selective markers to investigate lung injury and repair. *Eur Respir J* 2004;24:664-673.