Acyl and alkyl chain length of GPI-anchors is critical for raft association in vitro

Jürgen Benting[a,b,1], Anton Rietveld[a,b,2], Iris Ansorge[c], Kai Simons[a,b,*]

[a] *European Molecular Biology Laboratory (EMBL), Cell Biology and Biophysics Programme, Postfach 102209, Meyerhofstrasse 1, 69117 Heidelberg, Germany*
[b] *Max Planck Institute for Molecular Cell Biology and Genetics, 01307 Dresden, Germany*
[c] *Zentrum für Molekulare Biologie, Universität Heidelberg, Im Neuenheimer Feld 282, 69120 Heidelberg, Germany*

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Abstract **We determined the acyl and alkyl chain composition of GPI-anchors isolated from MDCK and Fischer rat thyroid (FRT) cells. Both cell lines synthesize GPI-anchors containing C16/C18 or C18/C18 saturated acyl and alkyl chains. The GPI-anchored placental alkaline phosphatase (PLAP) expressed in both cells is raft-associated and PLAP purified from FRT cells is raft-associated in vitro when reconstituted into liposomes containing raft lipids. In contrast, the GPI-anchored variant surface glycoprotein from *Trypanosoma brucei* which contains C14 acyl and alkyl chains shows no significant raft association after reconstitution in vitro. These data indicate that the acyl and alkyl chain composition of GPI-anchors determines raft association.**

Key words: GPI-anchor; Fatty acid; Lipid raft

1. Introduction

Glycosyl-phosphatidylinositol (GPI) -anchored proteins are poorly solubilized by non-ionic detergents like Triton X-100 (TX-100) [1,2]. The TX-100-insoluble membrane fractions are enriched in cholesterol (Chol), sphingomyelin (Sm) and glycosphingolipids which are thought to be detergent-insoluble because they form liquid-ordered (l_o) domains [3–5], also called lipid rafts [6]. As compared to a liquid-disordered phase, lipid rafts are characterized by a higher degree of acyl chain order [7,8]. Association with lipid rafts is involved in intracellular protein sorting [6,9]. Most GPI-anchored proteins are sorted to the apical surface of polarized cells and they are raft-associated probably because their lipid-anchors contain long and saturated acyl and alkyl chains, facilitating their partitioning into l_o domains [5]. As an exception from this behavior, the GPI-anchored protein gD1-DAF, comprising the ectodomain of the glycoprotein D1 (gD1) of the herpes simplex virus and the GPI-anchor signal of the decay acceleration factor (DAF), is not raft-associated in Fischer rat thyroid (FRT) cells [10]. Why this protein fails to associate with rafts is not known. We have analyzed this issue by comparing the acyl and alkyl chain content of GPI-anchors in MDCK and FRT cells to find out whether this is the reason for the difference in raft association. In addition, we analyzed the detergent-insolubility of GPI-anchored proteins in vitro and found that the fatty acid chain length seems to influence the raft association of GPI-anchored proteins.

*Corresponding author. Fax: (49) (6221) 387 512.
E-mail: simons@embl-heidelberg.de

[1] Present address: Hoechst Schering AgrEvo GmbH, H872N, Forschung Biochemie, 65926 Frankfurt am Mainz, Germany.
[2] Present address: Unilever Research Vlaardingen, P.O. Box 114, 3130 AC Vlaardingen, The Netherlands.

Abbreviations: Chol, cholesterol; ESI-MS, electrospray ionization mass spectrometry; OG, *N*-octyl-β-D-glyco-pyranoside; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PLAP, placental alkaline phosphatase; Sm, sphingomyelin; Gal-Cer, *O*-β-D-galactosyl(1→1)ceramide; TX-100, Triton X-100; VSG, variant surface glycoprotein; VSV-PLAP, VSV-G epitope-tagged PLAP

2. Material and methods

2.1. Cell lines and cell culture

A MDCK cell line stably expressing placental alkaline phosphatase (PLAP) was obtained from D. Brown [11] and was grown in MEM (Gibco BRL) as described [12]. FRT cells obtained from C. Zurzolo were grown in Coon's modified F12 medium (Biochrom) as described [13].

Bloodstream forms of *Trypanosoma brucei* strain 427 of genotype *TETR BLE* [14] were grown in HMI-9 medium [15] supplemented with 20% FCS.

2.2. Antibodies

A rabbit anti-PLAP antibody was from Dako. The rabbit anti-variant surface glycoprotein (VSG) 222 antibody was described previously [14].

2.3. Expression constructs and transfection

A cDNA construct encoding N-terminally VSV-G epitope-tagged PLAP (VSV-PLAP) with the signal sequence of lactase-phlorizin-hydrolase in pcDNA-3 was kindly provided by Dr T. Harder (Basel Insitute for Immunology, Switzerland). FRT cells were transfected with pcDNA-3/VSV-PLAP using calcium-phosphate precipitation as described [16].

2.4. Purification of surface proteins

Subconfluent MDCK and FRT cells were surface-biotinylated twice for 20 min with 0.5 mg/ml sulfo-NHS-LC-biotin (Pierce) in phosphate-buffered saline (PBS) on ice. After quenching with 10 mM glycine in PBS for 30 min, cells were scraped and lysed in 40 ml 1% deoxycholate and 1% TX-100 in 10 mM Tris-HCl pH 8, 150 mM NaCl, 1 mM EDTA (TNE) with CLAP (chymostatin, leupeptin, antipain and pepstatin A, 25 μg/ml final each) at room temperature. The lysate was passed 10 times through a G22 needle and cleared by centrifugation (30 min at 15000 rpm in a Sorvall SS-34 rotor, 4°C). Biotinylated proteins were bound to monomeric streptavidin (Pierce) for 4 h, followed by extensive washes in PBS containing 0.05% TX-100. After washes with 10 bed-volumes PBS containing 30 mM *N*-octyl-β-D-glyco-pyranoside (OG), bound proteins were eluted with 2 mM biotin in PBS containing 30 mM OG in 0.5 ml fractions.

2.5×10^9 bloodstream forms of *T. brucei* were resuspended in 10 ml lysis buffer (25 mM Tris-HCl pH 7.6, 2 mM EDTA, 1 mM Zn-acetate and 10 μM leupeptin, 2 μM E64, 1 μM pepstatin, 0.1 mg/ml pefabloc (Boehringer Mannheim)) and passed 10 times through a G27 needle.

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The lysate was centrifuged for 30 min at 38 000 × *g* and the resulting membrane pellet was solubilized in lysis buffer containing 1% TX-100. After sonification, the lysate was centrifuged as above and the supernatant was loaded on a DE52 column equilibrated with 10 mM Tris-HCl pH 8.0, washed with 100 mM NaCl, 0.05% TX-100, 10 mM Tris-HCl pH 8.0 and once in the same buffer containing 30 mM OG instead of TX-100. VSG 222 was eluted with 500 mM NaCl, 30 mM OG, 10 mM Tris-HCl pH 8.0.

2.5. Release of phosphatidylinositol (PI) from GPI-anchors and nanoelectrospray tandem mass spectrometry

Detergents and salts were removed from the purified surface protein by dialysis against water. Residual free lipids were extracted six times with 1.5 ml water-saturated 1-butanol. The aqueous phase was lyophilized and acid deamination was done in 60 μl 0.3 M sodium acetate buffer pH 4.0 and 30 μl freshly prepared 1 M sodium nitrite for 3 h at 37°C. The released PI was extracted three times with 100 μl water-saturated 1-butanol. The butanol fractions were pooled, dried and subjected to Bligh and Dyer solvent partitioning to remove salts [17]. The chloroform phases were dried and each sample was dissolved in 50 μl methanol/chloroform 2:1. GPI-derived PI species were identified on a Micromass Quattro II electrospray ionization mass spectrometer (ESI-MS). Analysis was done at 30°C with a capillary voltage of 0.8–1.2 kV in the negative ion mode. Parent scan analysis was performed by selection of a *m/z* 241 collision product (phosphoinositol-H_2O).

2.6. Reconstitution of GPI proteins in lipid vesicles and TX-100 extraction

Bovine liver phosphatidylcholine (PC), phosphatidylethanolamine (PE), brain Sm and *O*-β-D-galactosyl(1→1)ceramide (Gal-Cer) were from Avanti Polar Lipids. According to the supplier, Gal-Cer consisted of cerasine (non-hydroxylated) and phrenosin (hydroxylated) of which both approximately 50% have C24:0 and C24:1 fatty acids. The fatty acids of the Sm, PC and PE used are primarily long and suitable to support the formation of l_o domains in the presence of Chol [5]. Chol was from Sigma. Thirty μg of the purified total surface proteins was added to 750 nmol of lipids in a total volume of 800 μl containing 40 μmol OG. The mixture with a final molar protein to lipid ratio of 1:1250 was dialyzed against 5000 volumes of PBS at 4°C. Subsequently, the vesicles were collected and detergent-extracted for 30 min in a total volume of 400 μl in 25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA with 1% TX-100 on ice. After centrifugation for 30 min at 45 000 rpm in a TLA-45 rotor at 4°C without brake, the supernatant was collected carefully and precipitated in 10% TCA. Presence of PLAP and VSG 222 in the supernatant and the pellet was analyzed on Western blots. Detergent extractions of cells were performed as described [12].

3. Results

3.1. GPI-anchors contain long and saturated acyl and alkyl chains

Total cell surface proteins were purified after surface biotinylation (see Section 2) from subconfluent MDCK and FRT cells expressing PLAP or VSV-PLAP, respectively (Fig. 1, lane 7 and 8). The bands corresponding to PLAP from MDCK cells and to VSV-PLAP from FRT cells, identified by Western blotting, are marked with a triangle or an asterisk, respectively, showing that GPI-anchored proteins were present in these fractions. The analysis of the PI moiety released from GPI-anchored proteins revealed that the major GPI-derived PI species in both FRT and MDCK cells has 18:0/18:0 acyl and alkyl chains (Fig. 2). A 16:0/18:0 species occurs in FRT cells which is almost absent from MDCK cells. Both species had one ether-bonded and one ester-bonded fatty acid which is considered typical for GPI-anchors [18]. Other PI species estimated to comprise less than 10 mol% were plasmenyl 18:0/18:1 PI as well as diacyl 16:0/18:0 and diacyl 18:0/18:0 PI. Taken together, the acyl and alkyl chains found

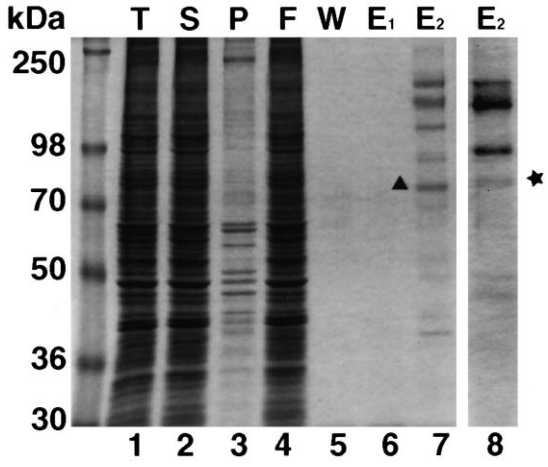

Fig. 1. Purification of surface proteins. Protein patterns of total MDCK cells (lane 1, T), of total solubilized and insoluble proteins after lysis in deoxycholate and TX-100 (lane 2, S and 3, P), of the flow-through of the streptavidin column (lane 4, F) and the wash with 30 mM OG (lane 5, W). Bound proteins were eluted with biotin in 0.5 ml fractions (E). Eluted proteins derived from MDCK (lane 7) and FRT (lane 8) cells were first present in fraction 2 (E2). The purification was performed five times with comparable results. PLAP from MDCK cells and VSV-PLAP from FRT cells are marked with a triangle or an asterisk, respectively; 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Coomassie staining.

in GPI-anchors of MDCK and FRT cells are long and almost exclusively saturated. Next, we analyzed the raft association of PLAP in FRT and MDCK cells by detergent extraction followed by floatation in an Optiprep gradient (Fig. 3). As already shown [2], PLAP expressed in MDCK cells is raft-associated as indicated by its floatation to low density (30–5% Optiprep interface, Fig. 3). Likewise, PLAP expressed in FRT cells is also raft-associated. In summary, we conclude that the GPI-anchors in MDCK and FRT cells are structurally similar and that PLAP expressed in FRT cells is raft-associated in vivo.

3.2. Chain length influences raft association in vitro

Next, we analyzed the raft association of a GPI-anchored protein with shorter acyl and alkyl chains than found in FRT cells. The PI of the GPI-anchored VSG from the protozoan parasite *T. brucei* contains two myristoyl (14:0) acyl and alkyl chains [19,20], the shortest tails of GPI found so far. VSG from *T. brucei* and PLAP purified from FRT cells was reconstituted into vesicles of different lipid compositions. Since total cell surface proteins were reconstituted, we used low protein to lipid ratios to minimize possible unspecific interactions of the reconstituted proteins. Furthermore, we assumed that the biotinylation of the protein moiety of a GPI-anchored protein does not affect its lipid interaction via its lipid-anchor. The raft association of PLAP and VSG was analyzed by TX-100 extraction of the proteoliposomes and sedimentation of the insoluble material. As a typical non-raft bilayer, pure bovine liver PC and PE vesicles were used. Raft lipid-containing vesicles consisted of Chol, SM and Gal-Cer in addition to PC and PE [3]. More than 90% of the initial amount of VSG and PLAP was incorporated into the vesicles, regardless of the lipid composition (data not shown), indicating the presence of GPI-anchors in the proteins. TX-100 extraction of the reconstituted vesicles showed that both proteins could be solubi-

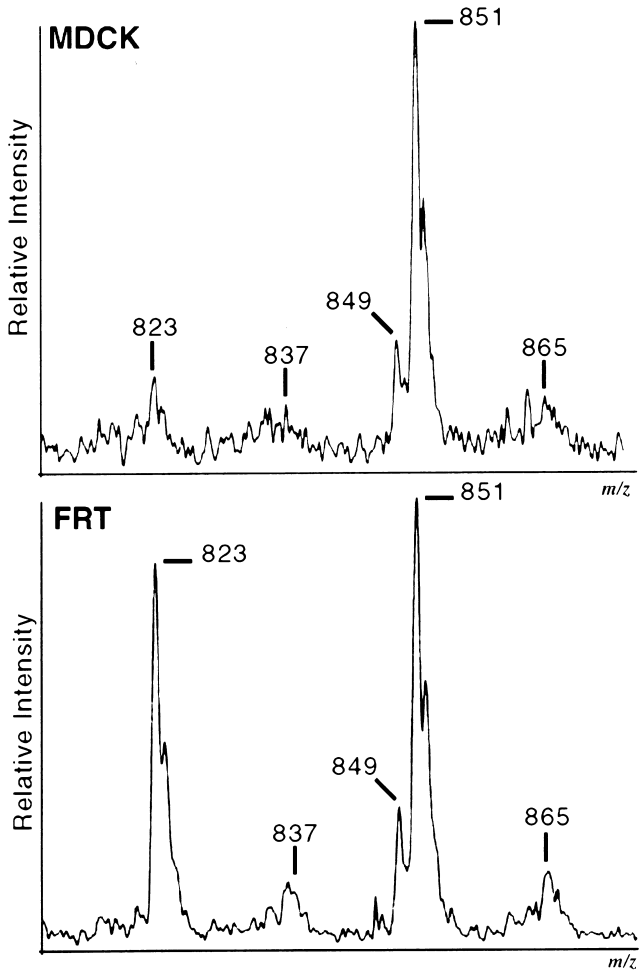

Fig. 2. ESI-MS spectra of PI species released from MDCK and FRT cells. The indicated molecular masses correspond to 1-alkyl-2-acyl-*sn*-glycero-3-phosphoinositol with C16:0/18:0 (*m/z* 823), C18:0/18:1 (*m/z* 849), C18:0/18:0 (*m/z* 851) fatty acids and 1,2-diacyl-*sn*-glycero-3-phosphoinositol with C16:0/18:0 (*m/z* 837) and C18:0/18:0 (*m/z* 865) fatty acids.

lized from PC/PE vesicles (Fig. 4). In contrast to this finding, PLAP from FRT cells was 90% resistant to TX-100 extraction when reconstituted into vesicles containing 30% Chol, 5% SM and 5% Gal-Cer (Fig. 4). Strikingly, approximately 50% of the VSG reconstituted into vesicles of the same lipid composition

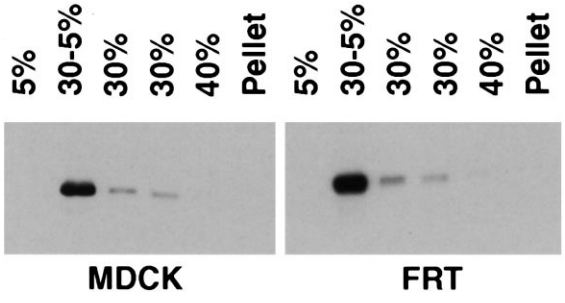

Fig. 3. Floatation of PLAP after TX-100 extraction of MDCK and FRT cells. MDCK and FRT cells were extracted with 1% TX-100 for 30 min on ice. Subsequently, the detergent-insoluble membranes were floated in an Optiprep step gradient. The presence of PLAP in the fractions collected from the gradient was analyzed by Western blotting. The results were reproduced twice.

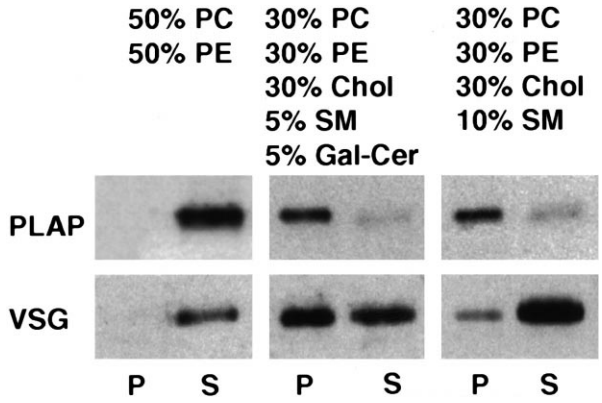

Fig. 4. Detergent extraction of VSG and VSV-PLAP reconstituted into vesicles of different lipid composition. The vesicles were extracted with TX-100 on ice and detergent-insoluble membranes were sedimented at $100\,000 \times g$. The presence of the proteins in the supernatant or the pellet was analyzed by Western blotting. Each reconstitution and detergent extraction was repeated and reproduced once.

was solubilized (Fig. 4). This shows that the VSG partitions into l_o domains to a significantly lower extent as compared to PLAP.

Gal-Cer has been shown experimentally to be poorly solubilized from liposomes by TX-100 [3]. It is possible that VSG and PLAP are insoluble due to an aggregation with Gal-Cer rather than a partitioning into l_o domains. We therefore tested vesicles composed of PC/PE/Chol/SM (3:3:3:1) which are individually soluble in TX-100 [3]. However, when present in a bilayer, they are partially in a detergent-resistant phase. As demonstrated in Fig. 4, the detergent-resistant fraction of VSG is reduced to approximately 10% in the absence of Gal-Cer, whereas PLAP is still approximately 90% insoluble in this lipid mixture. This shows that detergent-resistant membrane domains form in vesicles which lack Gal-Cer but do contain physiological levels of Chol and Sm.

4. Discussion

The acyl and alkyl chains present in the GPI-anchors of MDCK and FRT cells, as determined here for the first time, were both long (C18/C18 and C16/18) and saturated. Moreover, we did not find differences in raft association of PLAP expressed in MDCK and FRT cells in vivo. Thus, structural differences between the acyl/alkyl chains of GPI-anchors of MDCK and FRT cells do not provide an explanation for the detergent-solubility of gD1-DAF in FRT cells. Recent evidence from our lab has shown that raft association mediated by GPI-anchors is not sufficient for preferential apical targeting and that *N*-glycans are required for apical sorting [12]. Thus, it is possible that the basolateral sorting of gD1-DAF in FRT cells [10,13] is due to basolateral signals in the ectodomain of the protein or due to an interaction with a basolaterally sorted protein. Obviously, the mechanisms involved in GPI protein sorting in FRT cells require further scrutiny.

We determined the PI content of the total mixture of GPI-anchored proteins, not of purified PLAP. However, the overexpressed PLAP in MDCK and FRT cells represented approximately 5–10% of the total purified surface proteins. We

therefore assume that our structural data partially reflect the actual PI content of PLAP.

Previous studies have shown that many raft-associated proteins are palmitoylated [21,22]. Cytoplasmic proteins carrying two saturated hydrocarbon chains, e.g. myristate (C14) and at least one palmitate (C16), become raft-associated [23]. For instance, the src-family of kinases and also a GFP carrying both of these modifications associate with lipid rafts from the cytoplasmic side [24,25]. From these data, one can conclude that at least one saturated C16 fatty acid is needed for raft association, whereas one C14 fatty acid is not sufficient. We found that the C14 GPI-anchored VSG is susceptible to TX-100 extraction when reconstituted into raft lipid-containing vesicles, whereas the C16/C18 GPI-anchored PLAP from FRT cells is largely resistant to TX-100 extraction. This observation confirms the prediction that the fatty acid chain length of GPI-anchors is also a determinant for raft association in the exoplasmic leaflet [5].

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References

[1] Low, M. (1989) Biochim. Biophys. Acta 988, 427–454.
[2] Brown, D.A. and Rose, J.K. (1992) Cell 68, 533–544.
[3] Schroeder, R., London, E. and Brown, D. (1994) Proc. Natl. Acad. Sci. USA 91, 12130–12134.
[4] Ahmed, S.N., Brown, D.A. and London, E. (1997) Biochemistry 36, 10944–10953.
[5] Schroeder, R.J., Ahmed, S.N., Zhu, Y., London, E. and Brown, D.A. (1998) J. Biol. Chem. 273, 1150–1157.
[6] Simons, K. and Ikonen, E. (1997) Nature 387, 569–572.
[7] Rietveld, A. and Simons, K. (1998) Biochim. Biophys. Acta 1376, 467–479.
[8] Brown, D.A. and London, E. (1998) Annu. Rev. Cell Dev. Biol. 14, 111–136.
[9] Keller, P. and Simons, K. (1998) J. Cell Biol. 140, 1357–1367.
[10] Zurzolo, C., van 't Hof, W., van Meer, G. and Rodriguez-Boulan, E. (1994) Braz. J. Med. Biol. Res. 27, 317–322.
[11] Brown, D.A., Crise, B. and Rose, J.K. (1989) Science 245, 1499–1501.
[12] Benting, J.H., Rietveld, G.A. and Simons, K. (1999) J. Cell Biol. 146, 313–320.
[13] Zurzolo, C., Lisanti, M.P., Caras, I.W., Nitsch, L. and Rodriguez, B.E. (1993) J. Cell Biol. 121, 1031–1039.
[14] Ansorge, I., Steverding, D., Melville, S., Hartmann, C. and Clayton, C. (1999) Mol. Biochem. Parasitol. 101, 81–93.
[15] Hirumi, H. and Hirumi, K. (1989) J. Parasitol. 75, 985–989.
[16] Zurzolo, C.R., Gentile, R., Mascia, A., Garbi, C., Polistina, C., Aloj, L., Avvedimento, V.E. and Nitsch, L. (1991) J. Cell Sci. 98, 65–73.
[17] Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911–917.
[18] McConville, M.J. and Ferguson, M.A.J. (1993) Biochem. J. 294, 305–324.
[19] Ferguson, M.A.J., Homans, S.W., Dwek, R.A. and Rademacher, T.W. (1988) Science 239, 753–759.
[20] Masterson, W.J., Raper, J., Doering, T.L., Hart, G.W. and Englund, P.T. (1990) Cell 62, 73–80.
[21] Arni, S., Keilbaugh, S.A., Ostermeyer, A.G. and Brown, D.A. (1998) J. Biol. Chem. 273, 28478–28485.
[22] Melkonian, K.A., Ostermeyer, A.G., Chen, J.Z., Roth, M.G. and Brown, D.A. (1999) J. Biol. Chem. 274, 3910–3917.
[23] Rodgers, W., Crise, B. and Rose, J.K. (1994) Mol. Cell. Biol. 14, 5384–5391.
[24] Wolven, A., Okamura, H., Rosenblatt, Y. and Resh, M.D. (1997) Mol. Biol. Cell 8, 1159–1173.
[25] Galbiati, F., Volonté, D., Meani, D., Milligan, G., Lublin, D.M., Lisanti, M.P. and Parenti, M. (1999) J. Biol. Chem. 274, 5843–5850.