Oligomerization of VIP21-caveolin in vitro is stabilized by long chain fatty acylation or cholesterol

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Abstract VIP21-caveolin is one of the components which form the cytoplasmic surface of caveolae. In vivo, this integral membrane protein is found in homo-oligomers with molecular masses of approximately 200, 400 and 600 kDa. These oligomers are also formed by the addition of cytosol to the in vitro synthesized and membrane inserted VIP21-caveolin. Here we show that long chain fatty acyl coenzyme A esters can completely substitute for cytosol in inducing 200 kDa and 400 kDa complexes, whereas 25-hydroxy-cholesterol can produce the 200 kDa oligomer. In order to understand whether acylation of VIP21-caveolin itself is a prerequisite for oligomerization, we studied a mutant protein lacking all three cysteines. When analyzed by velocity sucrose gradient centrifugation in the presence of the non-ionic detergent octylglucoside, both palmitoylated and non-palmitoylated VIP21-caveolin formed oligomers that were indistinguishable. However, only the oligomers of the non-palmitoylated protein are disrupted when analyzed by SDS-PAGE without boiling. These data suggest that the protein domains of VIP21-caveolin are the primary determinants of oligomerization, but that palmitoylation of cysteine residues can increase the stability of the oligomers.

Key words: VIP21-caveolin; Caveolae; Oligomerization; Cholesterol; Fatty acylation

1. Introduction

The plasma membrane of many mammalian cells contains non clathrin coated membrane invaginations, called caveolae or plasmalemmal vesicles (reviewed in [1]). The cytoplasmic surface of caveolae is covered by multiple filaments wrapped around the invaginated plasma membrane [2–5]. In the last few years, caveolae have been investigated intensively and several possibilities for their function have been suggested. These include: alternative endocytic pathway [3,6,7]; transcytosis [8,9]; receptor-mediated uptake of small molecules (potocytosis [10,11]); regulation of intracellular calcium concentration [12,13]; and signal transduction [14–16].

VIP21-caveolin, a protein of 21–22 kDa, is so far the best biochemical or structural marker for caveolae (for review see [17]). Antibodies against this protein decorate the caveolar filaments [5]. VIP21-caveolin is also a constituent of *trans*-Golgi network derived vesicles [18] and is found on the Golgi apparatus [19].

VIP21-caveolin is an integral membrane protein which has an unusual hairpin loop structure in the membrane, exposing regions which flank the hydrophobic segment to the cytosol [19–21]. The protein has two remarkable and peculiar properties which are possibly connected to its function: it is insoluble in non-ionic detergents such as Triton X-100 or CHAPS [15,18] and it can associate with itself to form high molecular mass homo-oligomers in vivo as well as in vitro [21,22]. The homo-oligomers can be produced from in vitro synthesized and membrane inserted VIP21-caveolin by the addition of cytosol. This process also requires ATP and GTP. The oligomers produced in vitro share several properties with those isolated from dog lung or from cultivated cells: they are of about the same size, are not solubilized by SDS at 25°C and are formed in the endoplasmic reticulum (ER) [21]. However, in contrast to the oligomers detected in vivo, they do not form the Triton insoluble, very high molecular mass complexes. The detergent insolubility might correspond to a higher degree of 'maturation' of the oligomers. This maturation may be acquired at the time the oligomers leave the ER, as they pass through the Golgi apparatus or upon their arrival at the plasma membrane.

In this report, we have tried to identify active factor(s) in cytosol that are required for the oligomerization of VIP21-caveolin. We find that the role of the cytosol is actually to stabilize the VIP21-caveolin oligomers (as judged by stability in the detergent SDS), and this property depends on palmitoylation of the protein.

2. Materials and methods

2.1. Materials

Detergents were purchased from Calbiochem (San Diego, CA, USA); reagents for SDS-PAGE from ICN (Meckenheim, Germany); palmitoyl-carnitine and coenzyme A derivatives (lauroyl-, myristoyl-, palmitoyl- and stearoyl-CoA) from Sigma (Deisenhofen, Germany). Palmitoyl-dethio-CoA was a kind gift from Dr. Theodor Wieland (MPI for Medical Research, Heidelberg, Germany). The reagents used for cDNA cloning, for in vitro transcription, translation and translocation were obtained from the sources described [23].

2.2. Cytosol extraction

Cytosol was prepared from canine brain according to the procedure used to prepare cultivated cell cytosol [21]. Before the homogenization with a Dounce apparatus, frozen canine brain was cleaned of most of its white matter.

Cytosol was fractionated using a two phase chloroform/methanol/water extraction system as described by Urbani and Simoni [24] and

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modified as follows. Briefly, 80 μl of dog brain cytosol, at a concentration of approximately 35 mg/ml protein, was extracted overnight at 4°C in a rotating shaker in 1 ml of chloroform/methanol (1/1); an equal volume of 0.04 N HCl was then added. Alternatively, the extraction was performed with the same volume of chloroform/methanol/water (1/2/0.25) and followed on the next day by the addition of half a volume of chloroform and half a volume of water. After thorough vortexing, the two phases were separated by centrifugation at 5000 rpm for 3 min in an Eppendorf centrifuge. The upper aqueous phase was removed, concentrated in a rotating evaporator and either acetone precipitated (9 volumes acetone for 1 volume aqueous solution) or evaporated to dryness. The chloroform phase was carefully removed through the protein precipitate present at the interface. Both organic phase and interface were dried down.

The lipid fraction (chloroform phase) was used directly, i.e. the post-translational assay was performed in the same tube (see below). The interface and aqueous phases were solubilized in the oligomerization buffer (HMKD buffer: 20 mM HEPES pH7.6, 1 mM MgAc, 60 mM KOAc, 0.2 mM DTT) and stored at −80°C.

2.3. Post-translational oligomerization assay

The oligomerization assay was performed after translation of VIP21-caveolin in the presence of dog pancreas microsomes was completed [21]. Samples, made up of 3 μl of translation products in 5 μl total volume, were incubated at 30°C for 1 h in the presence of HMKD buffer and of various compounds.

All coenzyme A derivatives were solubilized in HMKD. Palmitic acid was solubilized in 100% ethanol and then diluted in HMKD. Sphingomyelin and 25-hydroxy-cholesterol were solubilized in chloroform/methanol (1/1), aliquoted to the suitable concentration, and the solvent evaporated to dryness. As for the test of lipid fraction, the translation mix and buffer were added directly to the dry material and pipetted up and down to maximize the lipid 'dispersion'.

2.4. Construction and expression of VIP21-caveolin mutants

The construction and expression in human embryonic kidney 293 cells of the VIP21-caveolin mutants with all sets of one, two, or all three (Cys^-) of its cysteine residues changed to serine has already been described [20]. In the in vitro oligomerization assay, the truncated form of Cys^- (Tr Cys^-) utilizing the Met^{32} start codon was used, as done previously for the wild-type protein. For this construct, the *NcoI/XbaI* fragment was excised from the pcDNA3 vector and cloned in pSP64. The resulting plasmid encodes a form of VIP21-caveolin, in which the first 31 amino acids were deleted and the cysteine residues at positions 133, 143 and 156 were changed for serine residues. The protein bears a c-*myc* tag at the carboxy terminus.

2.5. Analysis of oligomerization products

The products generated in the post-translational assay were subjected to SDS-PAGE on a 3–16% gradient gel as previously described [21] after solubilization in the usual SDS-gel sample buffer without boiling. The possible thioester bonds were released by incubating the sample for 20 min at 37°C in the presence of 0.3 M methanolic KOH [25]. To 5 μl of the reaction mix 5 μl of 0.6 M methanolic KOH were added. The latter was prepared by dilution of a 4 M aqueous solution of KOH in absolute methanol. The control sample was incubated in the presence of 42.5% methanol alone.

Quantification of oligomerization was performed by measuring the relative radioactivity in various complexes with a phosphoimager (BAS2000, FujiX, Japan). Results were expressed as stimulation above basal activity after incubation in the presence of the HMKD buffer.

Analysis of VIP21-caveolin oligomers in transfected 293 cells was performed using cell lysates solubilized in SDS-PAGE buffer without boiling [21]. An alternate assay for the presence of VIP21-caveolin oligomers used velocity sucrose gradient centrifugation. Cells were lysed on ice in 0.5 ml 60 mM *n*-octylglucoside/25 mM MES, pH 6.5/150 mM NaCl with protease inhibitors; this lysate was overlaid onto a 4.2 ml 5–30% sucrose gradient prepared in the same cell lysis buffer, centrifuged in an SW55 rotor for 6 h at 53000 rpm (340000 × *g*), and 12 equal fractions collected from the top (plus the pellet solubilized in gel loading buffer). Samples were analyzed by SDS-PAGE (with or without boiling as indicated) and detection utilized western blotting with monoclonal anti-c-*myc* antibodies as described [20].

3. Results

3.1. Oligomerization of VIP21-caveolin is stimulated by long chain fatty acyl-CoA esters

We have recently described an in vitro oligomerization assay for VIP21-caveolin. The protein synthesis performed in the wheat germ translation system in the presence of dog pancreas microsomes is followed by a post-translational assay by incubation the samples at 30°C for 1 h. The major bands formed after addition of cytosol (but not in its absence) are a doublet of about 200 kDa and a 400 kDa complex, as detected by SDS-PAGE after treatment of samples at 25°C. We used a truncated form of VIP21-caveolin (TR VIP), which comigrates with the smaller isoform of the protein [26] and is more effective in forming oligomers [21].

Our aim was to seek the active component(s) of cytosol involved in the oligomerization of VIP21-caveolin. Previous results have shown that the VIP21-caveolin complexes isolated in vivo contained not only the protein monomers but also glycosphingolipids and cholesterol [27,28]. In our initial experiments, therefore, we subjected cytosol to delipidation by extraction with chloroform/methanol. The oligomerization activity was recovered in the pellet, which consisted mostly of proteins (not shown). To dissect this activity more precisely, the cytosol was subjected to a two phase chloroform/methanol/water extraction system. This system allows the partition of most lipids into the organic phase, of glycolipids and other amphiphilic compounds into the aqueous phase and the concentration of the precipitated proteins at the interface. As expected, no activity was found in the chloroform phase. Surprisingly, the protein containing fraction was inactive as well, whereas the aqueous phase was fully active (Fig. 1A).

It is known that the aqueous phase among other amphiphilic compounds contains CoA derivatives [29]. Indeed, using reversed phased chromatography on a Smart system (Pharmacia, Uppsala, Sweden) we detected significant amounts of acyl-CoA esters in the aqueous fraction (data not shown).

We therefore investigated whether an acylation reaction could be involved in the oligomerization of VIP21-caveolin. This was tested by a mild alkaline methanolysis of the in vitro formed complexes. Incubation in the presence of methanolic KOH led to complete disruption of all types of oligomers, while no effect of methanol alone was observed (Fig. 1B, lanes 1 and 2). This suggests that ester or thioester bonds may be required to retain the integrity of the complexes. Next, in the absence of cytosol, acyl-CoA esters were tested in the oligomerization assay, as acylating agents. Their effect was indistinguishable from the effect of cytosol (Fig. 1C). Results, expressed as the stimulation of oligomerization over the basal activity induced by HMKD buffer on the production of 400 kDa complex, are shown in Fig. 1C. The various acyl-CoA esters, bearing saturated fatty acids (lauroyl-, myristoyl-, palmitoyl-, and stearoyl-CoA) tested at a concentration of 2 μM, were equally efficient to induce the oligomer formation. No specificity linked to the length of a fatty acid was observed.

To determine that the effect of acyl-CoA esters was not simply due to their detergent-like property, we used two reagents with structures similar to palmitoyl-CoA. Both palmitoyl-carnitine (another amphiphilic compound) and palmitoyl-dethio-CoA (a non-cleavable analogue of palmitoyl-CoA) were inactive in the oligomerization assay when tested at 2 μM (Fig. 1C). Palmitoyl-dethio-CoA did not act irreversi-

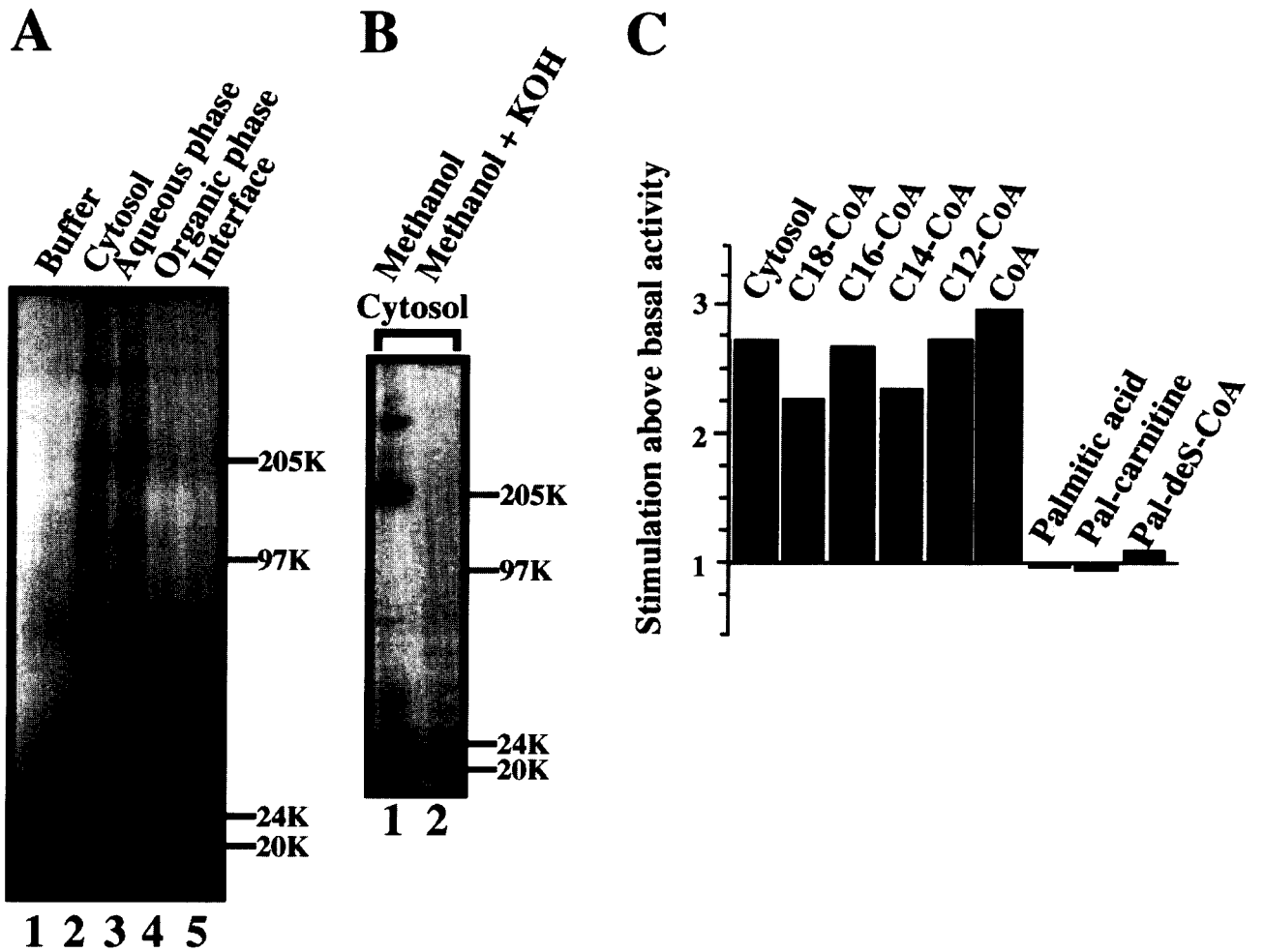

Fig. 1. Fatty acylation stimulates the in vitro oligomerization of VIP21-caveolin. A: The oligomerization assay was performed in the presence of buffer (lane 1), cytosol (lane 2) or different fractions of the chloroform/methanol extracted cytosol (aqueous phase, organic phase and interface, lanes 3–5 respectively). B: The oligomerization assay was performed in the presence of cytosol; the samples were treated either with methanol (lane 1) or with 0.3 M KOH in methanol (lane 2). C: The oligomerization assay was performed in the absence of cytosol, and in the presence of various compounds indicated at a concentration of 2 μM. The stimulation of the oligomerization above basal activity was measured in the 400 kDa complex. It is expressed as a ratio of radioactivity in 400 kDa in the presence of a tested compound to the radioactivity produced by the incubation with buffer. C18-CoA, C16-CoA, C14-CoA, C12-CoA and Pal-deS-CoA are stearoyl-, palmitoyl-, myristoyl-, lauroyl- and palmitoyl-dethio-CoA, respectively. Pal-carnitine designates palmitoyl-carnitine. Molecular mass markers here or in subsequent figures are: rabbit muscle myosin (205 K), rabbit muscle phosphorylase *b* (97 K), bovine pancreas trypsinogen (24 K) and soybean trypsin inhibitor (20 K).

bly, because 2 μM palmitoyl-CoA added together with 0.2, 0.5 and 1 μM of the non-cleavable analogue induced oligomerization (data not shown). A complete competition of 2 μM palmitoyl-CoA was achieved with 5 μM palmitoyl-dethio-CoA. Coenzyme A itself was also active in the post-translational assay, at the same concentration as palmitoyl-CoA. In contrast, palmitic acid alone could not induce the complex formation.

3.2. 25-Hydroxy-cholesterol can induce the formation of the 200 kDa complex

Next, we assessed whether other lipids could substitute for acyl-CoA in this oligomerization process. We used 25-hydroxy-cholesterol and sphingomyelin, instead of cytosol, in the post-translational assay. As shown in Fig. 2, incubation with 1 mg/ml 25-hydroxy-cholesterol led to the formation of the 200 kDa VIP21-caveolin complexes (lane 3). This process could not be inhibited by palmitoyl-dethio-CoA (lane 5). In contrast to the complexes generated in the presence of cytosol [21], the formation of the 200 kDa complexes induced by 25-hydroxy-cholesterol was not GTP dependent (Fig. 2, lanes 6 and 7). Sphingomyelin had no effect at the concentrations tested (15–150 μM, data not shown).

3.3. The oligomerization property of VIP21-caveolin is modified by cysteine residues

The data above suggest that lipid modification of a molecule involved in the oligomerization of VIP21-caveolin can influence that oligomerization process. As VIP21-caveolin was recently shown to be palmitoylated in vivo on three cysteines present at the carboxy-terminal part of the protein [20], the simplest explanation of the above findings would be that palmitoylation of VIP21-caveolin influences its oligomerization. To provide further support for this hypothesis, we

decided to use the non-acylated Cys$^-$ mutant lacking all three cysteine residues, as we have shown that this mutant is not palmitoylated [20]. For the in vitro oligomerization assay, in analogy to the TR VIP, a truncated form of the mutant (TR Cys$^-$) utilizing the Met^{32} initiation codon was produced. As shown in Fig. 3, the SDS resistant oligomerization of the cysteine deleted protein was almost completely abolished in comparison to the wild type VIP21-caveolin (cf. lanes 1 with 3, 4, 5, and 6). It was only possible to observe a very small amount of the 400 kDa oligomers (< 5%) formed upon addition of cytosol, 5 μM palmitoyl-CoA or CoA (lanes 8, 9 and 10 respectively) by overexposing the autoradiographs, and no 200 kDa oligomers were visible.

We next examined whether the requirement for cysteine residues as acylation sites for oligomerization of VIP21-caveolin also held in vivo. The wild-type VIP21-caveolin and Cys$^-$ mutants were expressed by transfection in the human embryonic kidney 293 cell line [20]. As previously reported for endogenously expressed VIP21-caveolin in MDCK cells [21], the transfected VIP21-caveolin formed SDS resistant, high molecular mass oligomers of approximately 200 and 400 kDa (Fig. 4). In striking contrast, none of these oligomers were formed by the Cys$^-$ mutant, although it was expressed at approximately equal protein levels. On extreme overexposure of the Western blot, a very small amount of 400 kDa oligomer was seen in some experiments (data not shown). Thus, the in vivo and in vitro data are in agreement and demonstrate the requirement for acylation of the cysteine residues in VIP21-caveolin for formation of SDS resistant oligomers of the protein.

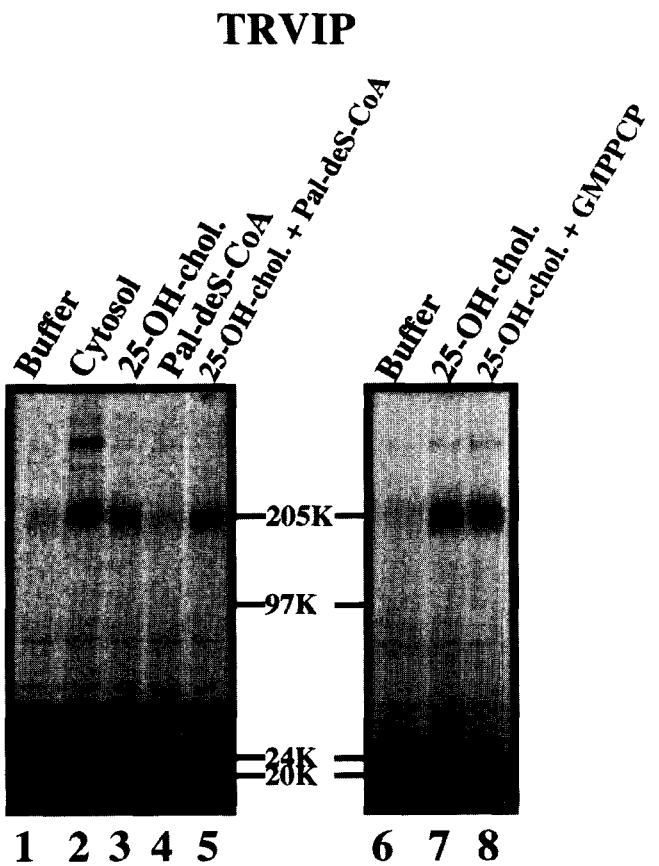

Fig. 2. 25-Hydroxy-cholesterol (25-OH-chol.) induces the formation of the 200 kDa complex. The oligomerization of TR VIP was performed in the absence of cytosol, and in the presence of the indicated compounds.

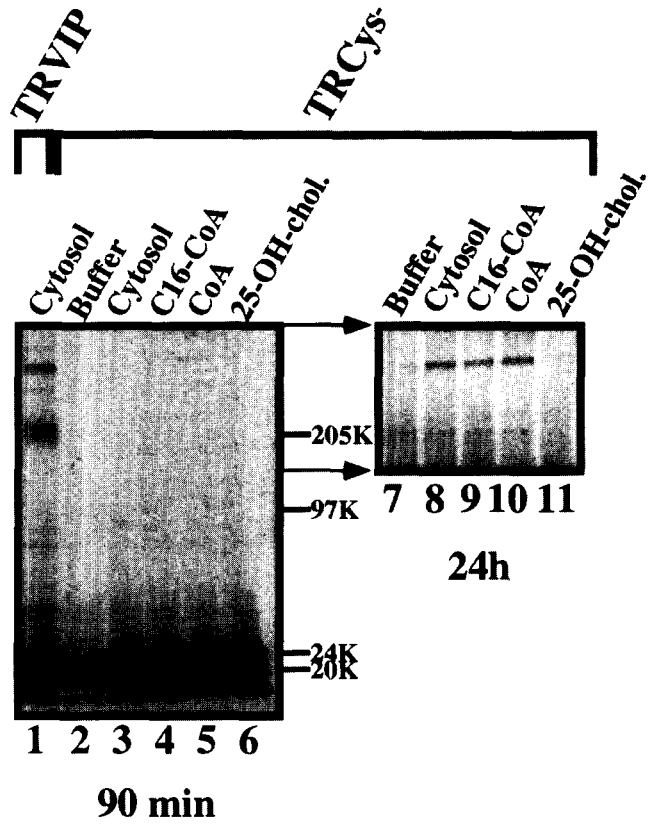

Fig. 3. SDS resistant oligomerization of VIP21-caveolin lacking all three cysteines is strongly impaired in vitro. The oligomerization of the truncated VIP21-caveolin (TR VIP, lane 1) or of the truncated mutant lacking all three cysteine residues (TR Cys$^-$, lanes 2–11) was performed in the presence of the indicated compounds. C16-CoA and CoA were used at a concentration of 5 μM. 25-hydroxy-cholesterol at 1 mg/ml. 25-OH-chol. designates 25-hydroxy-cholesterol. Lanes 7–11 show a longer exposure of lanes 2–6 (24 h and 90 min, respectively).

The three cysteine residues in VIP21-caveolin are located at positions 133, 143, and 156, and all three sites are palmitoylated [20]. To ascertain if there was any differential influence of the individual cysteine residues on the oligomerization process, we expressed VIP21-caveolin mutants that had one or two of the cysteine residues mutated to serine, and analyzed cell lysates from transfected 293 cells for the presence of VIP21-caveolin oligomers. The single cysteine mutants (Fig. 4) showed that the substitution of Cys^{133} by Ser had no apparent effect on oligomerization, that Cys^{143} was necessary for the formation of the 200 kDa oligomer (i.e. the Cys^{143} to Ser mutant had almost exclusively the 400 kDa oligomers), and Cys^{156} was necessary for the formation of the 400 kDa oligomers. The double cysteine substitution mutants showed an oligomerization pattern that matched the added effects of the two single substitutions. Thus, substitution of Cys^{133} plus one of the other cysteine residues produced a similar pattern to that of the other cysteine residue alone, and the substitution of Cys^{143} and Cys^{156} completely abolished oligomerization as assessed by SDS-PAGE (Fig. 4).

3.4. The effect of palmitoylation of VIP21-caveolin is to modify and stabilize oligomers that form independently of acylation

We next attempted to relate the above data showing an effect of palmitoylation on the oligomerization of VIP21-caveolin with other studies that indicate a clear role for peptide sequences in the oligomerization process. Specifically, we had earlier shown that complete removal of the amino-terminal cytoplasmic tail of VIP21-caveolin (amino acids 2–101) and replacement with sequences from human growth hormone led to loss of oligomerization [21]. Narrowing down the critical sequences further, we have found that deletion of amino acid residues 3–48 had no influence on oligomerization, but deletion of amino acids 46–95 completely abrogated oligomerization, as judged both by SDS-PAGE and by velocity sucrose gradient centrifugation in the presence of octylglucoside (data not shown). These results are in agreement with a recently published study [22] which showed that a fusion protein containing amino acids 61–101 of VIP21-caveolin undergoes the same oligomerization as the intact VIP21-caveolin protein as judged by velocity sucrose gradient centrifugation performed in the presence of octylglucoside. Thus, these amino acids in the membrane proximal part of the amino-terminal cytoplasmic tail appear necessary and sufficient for the oligomerization process.

The question then arises as to why the Cys$^-$ mutant of VIP21-caveolin, which contains these amino acid residues 61–101, did not appear to oligomerize by the SDS-PAGE assay (Figs. 3 and 4). To address this issue, we analyzed 293 cells transfected with the wild-type or Cys$^-$ VIP21-caveolin for the presence of oligomers, using both the SDS-PAGE assay under non-boiling conditions and by velocity sucrose gradient centrifugation in octylglucoside detergent. By the latter assay, which involves milder non-ionic detergent conditions, the wild-type and Cys$^-$ mutant VIP21-caveolin have formed the same size oligomers (Fig. 5A), both migrating to the bottom of the gradient (compare the migration to the 70 kDa protein DAF which migrates much closer to the top of the gradient). However, when comparable fractions from the gradient are analyzed by SDS-PAGE under non-boiling conditions, only the oligomers from the wild-type VIP21-caveolin are observed, whereas those from the Cys$^-$ mutant have been completely broken up by exposure to 2% SDS and do not appear in the >200 kDa region of the gel (Fig. 5B). Thus, the overall size of the oligomers (reflecting the number of molecules of VIP21-caveolin in the complex) is determined by the protein sequence (mapped to the amino-terminal cytoplasmic tail), but palmitoylation of cysteine residues in the carboxy-terminal cytoplasmic tail leads to some modification of this oligomeric structure resulting in increased stability (as assessed by the detergent SDS).

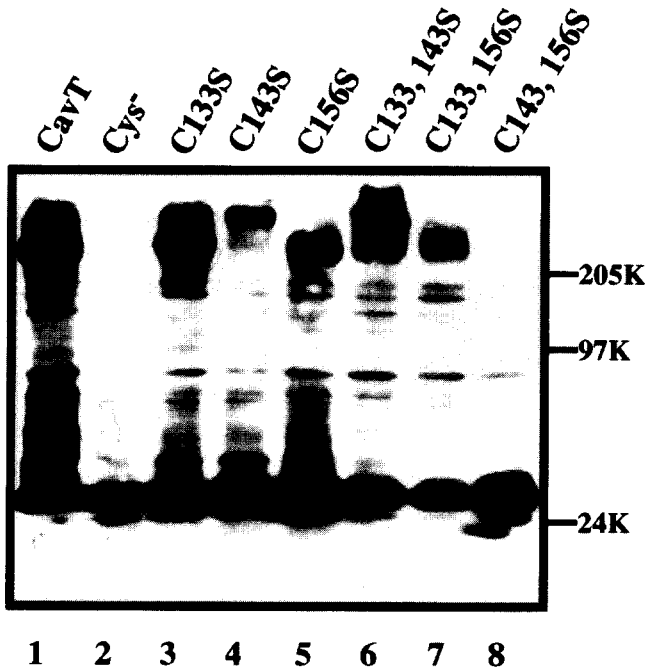

Fig. 4. SDS resistant oligomerization pattern of VIP21-caveolin determined by specific cysteine residues. The oligomerization of various VIP21-caveolin mutants containing zero, one, or two cysteine to serine substitutions was tested in transfected 293 cells. The cell lysates were analysed by SDS-PAGE separation of samples treated in SDS loading buffer at room temperature, followed by Western blot analysis for VIP21-caveolin. The various mutants are indicated above the lanes. The experiment has been repeated at least five times for each mutant, and this is a representative Western blot. On this gel there is still some 200 kDa visible in the lane C133,143S but it is relatively depleted compared to the wild type or C133S.

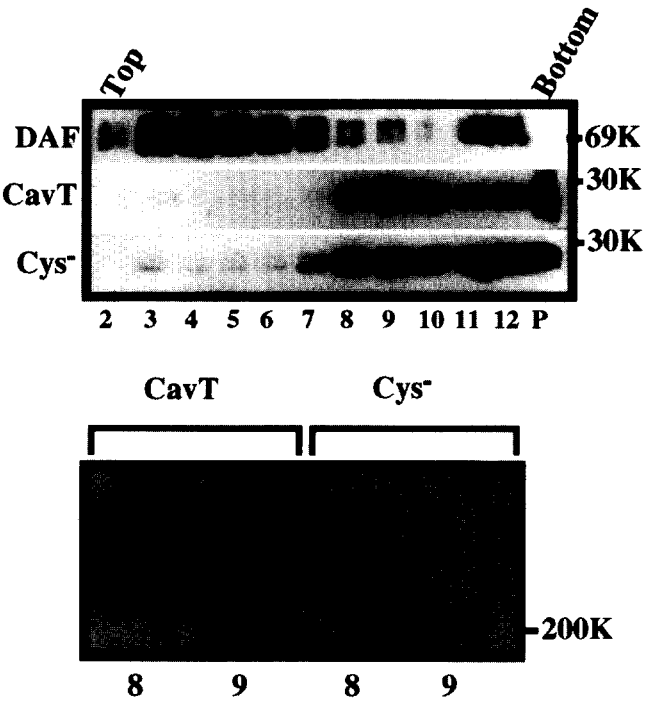

Fig. 5. VIP21-caveolin mutant lacking all three cysteine residues forms oligomers that are disrupted by SDS. The wild type VIP21-caveolin and the Cys$^-$ mutant were expressed in 293 cells. A: Octylglucoside cell lysates were subjected to velocity sucrose gradient centrifugation and the fractions from the gradient were analyzed by SDS-PAGE under boiling conditions and analyzed by Western blot for DAF (as a 70 kDa marker protein), CavT and Cys$^-$. B: Duplicate aliquots from fractions 8 and 9 from the gradient were analyzed as above except the samples were not boiled before SDS-PAGE.

4. Discussion

VIP21-caveolin is a component of the coat of caveolae [5].

By immunoelectron microscopy, antibodies to VIP21-caveolin decorate the striated filaments on the cytoplasmic face of the caveolae [5]. The demonstration of oligomerization of VIP21-caveolin [21,22] elucidates part of the structure of this caveolar coat. In our opinion, to form the caveolar coat VIP21-caveolin must acquire two properties: it should become a part of a very high molecular mass complex ($>10^4$ kDa), which contains cholesterol and glycosphingolipids and is insoluble in Triton [15,18]; this complex can resolve into high molecular mass oligomers (200–600 kDa) upon treatment with 2% SDS at room temperature [21,22]. In the present work we have investigated the contribution of different features of VIP21-caveolin, especially lipid modification, to the formation of the oligomers. The data support a complex model for oligomerization of the protein; peptide sequences in the membrane proximal region of the amino-terminal tail are necessary and sufficient for oligomerization of the protein [22], yet palmitoylation of cysteine residues in the carboxy-terminal cytoplasmic tail [20] leads to a modification of the oligomers that results in their resistance to disruption by 2% SDS at room temperature. Both in vitro and in vivo, the stabilized oligomerization requires a form of VIP21-caveolin capable of being S-acylated. Although we still can not exclude that the oligomerization occurs due to the formation of S-S bridges upon solubilization, the fact that the SDS-PAGE solubilization buffer contained 2% βME (sometimes 100 mM DTT was used) and also dependence of in vitro oligomerization on GTP or Mg^{2+} [21] speaks against unspecific oxidation of sulfhydryl groups. Fatty acyl CoA esters, as well as free CoA, produce the same effect in vitro as cytosol, and are likely to be the active components found in the cell extract. The fact that free CoA and all the tested CoA esters exhibit the same efficiency probably reflects the activity of acyl-CoA synthetases (thiokinases) which are partially associated with the ER. The dependence of the in vitro oligomerization assay on ATP shown in our previous paper [21] can be explained by this activity.

Our results give new insight into the link between caveolae (or VIP21-caveolin) and lipids, a link which had already been detected before, and consist of several observations. First, the addition of cholesterol binding drugs like filipin or nystatin changes the morphology of caveolae, making them flat [5,30]. Furthermore, the treatment of cells with cholesterol oxidase, an enzyme that produces cholestenon, leads to redistribution of VIP21-caveolin within the cell [31]. In our previous work, the 400 kDa homo-oligomer was prepared starting from Triton X-114 insoluble floating fraction [21], which is enriched in glycosphingolipids as well as in cholesterol [27,32]. Moreover, as we recently showed, the 400 kDa complex contains cholesterol and VIP21-caveolin itself seems to be a cholesterol-binding protein [28].

There are still several open questions concerning the role of palmitoylation in the structure and function of VIP21-caveolin and caveolae. We have shown that palmitoylation is not necessary for VIP21-caveolin to acquire Triton insolubility [20]. Moreover, electron microscopy studies show that the Cys^- mutant of VIP21-caveolin is localized to caveolae in transfected 293 cells, indistinguishable from the wild-type protein (R. Parton, D.M. Lublin and T.V. Kurzchalia, unpublished observations). Also, it is not clear how the palmitates modify the structure of VIP21-caveolin. All three of the cysteine residues of the protein are located in the carboxy-terminal cytoplasmic tail of VIP21-caveolin and are palmitoylated [20]. Acylation should lead to attachment of the carboxy terminus to the membrane surface and thus the flattening of this domain of the polypeptide. It is possible that with the carboxy-terminal cytoplasmic tails flattened against the membrane, the amino-terminal cytoplasmic tails can interact more tightly with each other in forming an oligomer.

Having elucidated the basic features of palmitoylation and oligomerization of VIP21-caveolin, the challenge now is to determine the role of these structural modifications in the function of VIP21-caveolin and caveolae.

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