Thioacylation is required for targeting G-protein subunit G_{α_1} to detergent-insoluble caveolin-containing membrane domains

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α -Subunits of heterotrimeric G_i -like proteins (α_i , α_o and α_z) associate with the cytoplasmic leaflet of the plasma membrane by means of N-terminally linked myristic acid and palmitic acid. An additional role for palmitate has been recently suggested by the observation that fusion with the palmitoylated N-terminus of α_{11} relocalizes cytosolic green-fluorescent-protein reporter to low buoyancy, Triton-insoluble membrane domains (TIFF; Triton-insoluble floating fraction), enriched with caveolin-1 [Galbiati, Volonté, Meani, Milligan, Lublin, Lisanti and Parenti (1999) *J. Biol. Chem.* **274**, 5843–5850]. Here we show that, upon transient expression in transfected COS-7 cells, myristoylated and palmitoylated α_o (α_o wt, where wt is wild-type) is exclusively found in TIFF, from where non-palmitoylated α_o wt and α_o C3S

(Cys³ → Ser) mutant are excluded. Moreover, α_o fused to N-terminally truncated human vasopressin V_2 receptor (V_2 TR- α_o), lacking myristate and palmitate, still localizes at the plasma membrane by means of first transmembrane helix of V_2 R, but is excluded from TIFF. Likewise, α_o C3S does not partition into TIFF, even when its membrane avidity is enhanced by co-expression of $\beta\gamma$ -subunits. Thus membrane association, in the absence of added palmitate, is not sufficient to confer partitioning of α_o within TIFF, suggesting that palmitoylation is a signal for membrane compartmentalization of dually acylated α -subunits

Key words: fatty acylation, caveolae, palmitoylation, myristoylation.

INTRODUCTION

The α -subunits of G_i -like subfamily of heterotrimeric G-proteins (α_i , α_o and α_z) are tethered to the cytoplasmic leaflet of the plasma membrane primarily via their dually lipid-modified N-terminus, carrying myristic acid and palmitic acid at Gly² and Cys³ respectively (for reviews, see [1–3]). In addition, when G-proteins are inactive, complexes of α -subunits with membrane-bound $\beta\gamma$ dimers act co-operatively with covalent lipid modifications to increase membrane avidity [4].

The attachment of the C₁₄ myristic acid to the N-terminal glycine residue of proteins occurs before completion of synthesis, whereas palmitic acid is added post-translationally to membrane-anchored polypeptides [5]. Therefore, the whole docking process of α -subunits to the membrane bilayer can be viewed as a complex multi-step mechanism where myristoylation provides nascent α -subunits with a first membrane tether. However, the linkage of myristate does not confer a sufficient membrane avidity for proper α -subunit anchorage to the membrane, as indicated by the substantial solubility of myristoylated, but palmitoylation-defective, α -protein mutants, upon subcellular fractionation [6–8]. Thus the subsequent thioesterification of C₁₆ palmitate to adjacent Cys³ is necessary to stabilize the interaction between α -subunits and the lipid bilayer.

As a further increase in the level of complexity, recent data support the view that α -subunits are not homogeneously distributed throughout the plasma membrane, but enriched within

Triton-insoluble low-buoyancy domains rich in sphingomyelin, glycosphingolipids and cholesterol [hereafter termed ‘rafts’ or Triton-insoluble floating fraction (TIFF)] that may or may not contain caveolins [9–12]. Here a number of lipid-modified signalling polypeptides, including G-protein α -subunits, Src-like non-receptor tyrosine kinases, Ras-like small GTPases and endothelial nitric oxide synthase, are clustered [13,14].

This suggests that lipid modifications, besides anchoring polypeptides to cellular membranes, play a role in their partitioning into rafts, where signalling partners and ancillary regulatory proteins can assemble. In this context, a specific role of palmitoylation versus myristoylation as a signal for targeting to rafts has been purported for Src-like non-receptor tyrosine kinases [15,16], endothelial nitric oxide synthase [17] and, more recently, for α -subunits [18–20].

Subcellular-fractionation experiments have shown that recombinant wild-type α_{11} (α_{11} wt) is partially localized within low-buoyancy caveolin-enriched fractions, separated by ultracentrifugation on discontinuous sucrose gradients from Triton X-100 extracts of transfected COS-7 cells [18]. However, the recovery of myristoylated, but palmitoylation-defective Cys³ → Ser mutant of α_{11} (α_{11} C3S) in caveolin-containing fractions was lower, and non-acylated Gly² → Ala (α_{11} G2A) mutant and double (α_{11} G2A/C3S) mutant were totally excluded from these fractions [18]. This has been taken as a suggestion that N-terminal

Abbreviations used: CMV, cytomegalovirus; DMEM, Dulbecco's modified Eagle's medium; Endo H, endoglycosidase H; ER, endoplasmic reticulum; α_o , α -subunit of the regulatory G_o -protein; GFP, green fluorescent protein; MBS, Mes-buffered saline; PNGase F, peptide:N-glycosidase F; PPO, 2,5-diphenyloxazole; TBS, Tris-buffered saline; TBS-T, TBS/Tween 20; TI, Triton-insoluble; TIFF, Triton-insoluble floating fraction; TS, Triton-soluble; V_2 R, human vasopressin V_2 receptor; V_2 TR- α_o , myc-tagged fusion protein consisting of the 1–71-amino-acid sequence of V_2 R and the full-length α_o ; wt, wild-type; TIU, trypsin-inhibitory unit(s); PBA, PBS + 1% BSA.

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modification with fatty acids, most notably with palmitic acid, is necessary to confer on α_{11} an association to caveolin-containing T1FF. This hypothesis is supported by subsequent studies [19] showing that fusion with doubly acylated N-terminal 32 amino acids of α_{11} was sufficient to relocalize cytosolic green fluorescent protein (GFP) to caveolin-enriched subdomains of the plasma membrane *in vivo*, as demonstrated by co-fractionation and co-immunoprecipitation with caveolin-1. When dual acylation of this 32-amino-acid domain was blocked by specific point mutation (G2A), the resulting GFP fusion protein was totally soluble, whereas the myristoylated, but non-palmitoylated C3S chimaera only partially partitioned into caveolin-containing fractions. Furthermore, both non-acylated GFP fusions (G2A and C3S) no longer co-immunoprecipitated with caveolin-1. Taken together, these results indicate that lipid modification of the N-terminal region of α_{11} is essential for targeting to its correct subcellular destination and interaction with caveolin-1.

To further substantiate the potential role of lipid modifications as a specific targeting signal to T1FF membranes, the present study was undertaken to examine the cellular fate of the α_{01} -subunit, carrying the same lipidation motif as α_{11} , whose membrane binding in transfected COS-7 cells was not provided by fatty acylation, but by fusion to the extracellular domain and first membrane-spanning helix of human vasopressin V_2 receptor (V_2R) or co-expression with $\beta\gamma$ -subunits.

MATERIALS AND METHODS

Materials

The N-terminal *myc*-tagged V_2R cDNA [21] was generously given by Dr C. Barberis (CNRS-INSERM, Montpellier, France). wt and C3S mutant of rat α_{01} have previously been described [6]. The pcDNA3 expression plasmids encoding for $G\beta_1$ and $G\gamma_2$ subunits [22] and the rabbit anti- α_0 polyclonal antiserum (ON1; [23]) were kindly given by Professor Graeme Milligan, Department of Biochemistry, University of Glasgow, Glasgow, Scotland, U.K. The characteristics of an antiserum (1028) generated against a trpE fusion protein with the 93 C-terminal amino acids of α_{01} are detailed in [6]. The monoclonal anti-*myc* antibody (9E10) and the polyclonal caveolin-1 antibody (N20) were purchased from Santa Cruz (Santa Cruz, CA, U.S.A.). [9,10- 3H]Palmitic acid (sp. radioactivity 40–60 Ci/mmol), [9,10- 3H]myristic acid (sp. radioactivity 40–60 Ci/mmol) and L-[^{35}S]methionine/L-[^{35}S]cysteine (PRO-MIX[®]) were from Amersham Pharmacia Biotech. All other biochemicals used were of the highest purity available and obtained from regular commercial sources.

Construction of V_2TR - α_0 fusion protein

The *myc*-tagged version of the V_2R was used in the generation of the construct described below; for convenience, the original nucleotide and amino acid numbering of the published untagged V_2R is maintained [21].

The V_2R truncation (V_2TR) was engineered by oligonucleotide-mediated site-directed mutagenesis (Sculptor kit; Amersham Pharmacia Biotech); a naturally occurring mutation originally described in a family affected by nephrogenic diabetes insipidus [24] was reproduced by introducing a Gly $\rightarrow$ Ala substitution of nucleotide at position 284 of the published sequence [21]; this resulted in a stop codon at residue 71 of the native protein. To generate the fusion protein, the cDNA encoding the first 71 amino acids of the V_2R (plus N-terminal *myc*-tag extension) was placed at the 5' end of the full-length rat α_{01} sequence (amino acids 1–354). To do so, the N-terminal

portion (240 bp) of the open reading frame of α_{01} DNA was amplified by PCR using the following oligonucleotides: sense (5'-GCAGGAGGTACCGGCCGGCAGGACACTGGGGATGTACTCTGAGCGCA-3'; *KpnI* and *NaeI* restriction sites are underlined) and antisense (5'-CAATGGCTGCCAGAGACTGGATGGTGT-3'; *PfI*MI site is underlined). The PCR-amplified fragment was digested with *KpnI* and *PfI*MI and ligated to the C-terminal portion of α_{01} DNA inserted in the pUC19 vector (New England BioLabs, Beverly, MA, U.S.A.). Subsequently the complete open reading frame of α_{01} (including the spacing sequence generated by PCR amplification at the 3' end) was excised with *NaeI* and *XbaI* and ligated to the N-terminal portion (228 bp) of V_2R cloned in pUC19 vector. The final fusion construct (Figure 2 below), was then subcloned into the *EcoRI* site of the cytomegalovirus-driven pcDNA3 eukaryotic expression vector (Invitrogen, Groningen, The Netherlands).

Cell culture and transient transfection

COS-7 cells were cultured in DMEM (Dulbecco's modified Eagle medium) supplemented with 10% (v/v) fetal-bovine serum, 2 mM L-glutamine, 100 mg/ml streptomycin and 100 units/ml penicillin. Cells (50–70% confluent) were transfected using the LIPOFECTAMINE[®] reagent (Gibco BRL) following manufacturer's instructions and routinely analysed 48 h post-transfection.

Immunoblot analysis

Cellular proteins were resolved by traditional Laemmli discontinuous SDS/PAGE system using 12.5 or 10% (w/v) acrylamide [25]. After semi-dry transfer to nitrocellulose membranes, blots were incubated overnight at 4 °C in Tris-buffered saline/Tween 20 [TBS-T; 20 mM Tris/HCl (pH 7.5)/150 mM NaCl/0.2% Tween 20], containing 5% powdered skimmed milk. After five 5-min washes with TBS-T, membranes were incubated for 3 h with primary antibodies diluted in TBS-T/milk and for 1.5 h with horseradish peroxidase-conjugated goat anti-mouse/rabbit IgG (Pierce). Proteins were detected using the SuperSignal[®] chemiluminescent substrate (Pierce, Rockford, IL, U.S.A.).

For cell fractionation, cells were harvested and resuspended in 0.5 ml of hypotonic buffer {5 mM Tris/HCl (pH 7.5)/1 mM MgCl₂/1 mM EGTA/0.1 mM EDTA/protease inhibitors [0.067 trypsin-inhibitory unit (TIU)/ml aprotinin (Sigma)+1 mM PMSF]}. Cell suspensions were incubated on ice for 30 min, freeze-thawed and homogenized with a Teflon/glass homogenizer. Following a low-speed centrifugation to remove unbroken cells and the nuclear pellet, samples were centrifuged for 30 min at 200 000 *g* at 4 °C in a Beckman TL100 centrifuge. The pellets (particulate fraction, P) and the acetone-precipitated supernatants (soluble fraction, S), were separated by SDS/PAGE and analysed by Western blotting.

Metabolic labelling and immunoprecipitation

Metabolic labelling was carried out on COS-7 cells, plated on 10 cm Petri dishes, mock-transfected or transfected with the various constructs 48 h before exposure to the radioactive precursors. Cells were labelled for 4 h with [3H]palmitic acid (200 μ Ci/ml) or [3H]myristic acid (200 μ Ci/ml) in DMEM supplemented with dialysed 5% (v/v) fetal-bovine serum, 5 mM sodium pyruvate, antibiotics and L-glutamine. Labelling with 100 μ Ci/ml [^{35}S]methionine/[^{35}S]cysteine was carried out as a pulse of 2 h in methionine/cysteine-free DMEM supplemented with 5% dialysed fetal-bovine serum, antibiotics and L-glutamine, and a subsequent overnight chase incubation in complete

medium. After washing with PBS, cells were lysed in 0.2 ml of 1% (w/v) SDS containing 0.067 TIU/ml aprotinin and 1 mM PMSF (TIU stands for trypsin-inhibitory units). After breakage of DNA by repeated pipetting and boiling for 4 min, 0.8 ml of the following mixture (Mix I) was added to each sample: 1.25% (w/v) Triton X-100, 190 mM NaCl, 6 mM EDTA, 50 mM Tris/HCl, pH 7.5, and protease inhibitors as indicated above. After centrifugation at 13000 *g* for 10 min at 4 °C, 10 μ l of anti-*myc* monoclonal antibody was added to each supernatant and the samples incubated overnight at 4 °C. A 25 μ l portion of a 1:1 suspension of Protein A-Sepharose CL-4B beads (Amersham Pharmacia Biotech, Cologno Monzese, Italy) in Mix II (4 parts of Mix I plus 1 part of 1% SDS) were added to each sample and incubated for 4–5 h at 4 °C with continuous rotation. Immunocomplexes were washed three times for 15 min at 4 °C with Mix II (1 ml) and once with 50 mM Tris/HCl, pH 6.8, and boiled for 3 min in Laemmli sample buffer containing 20 mM dithiothreitol, or as outlined below for endoglycosidase digestions. Samples were separated by SDS/10%-PAGE and the gels treated with 2,5-diphenyloxazole (PPO) for fluorography [26]. To maximize sensitivity we used a gel-miniaturization procedure which involved soaking the PPO-impregnated gels after water washing in 50% (w/v) poly(ethylene glycol) 3000 (Sigma) at 70 °C for 15 min before drying [27]. Dried gels were exposed on Kodak X-Omat AR-5 films at –70 °C.

Treatment with glycosidases

Endoglycosidase H (Endo H)

Immunocomplexes were boiled for 5 min in 0.15% (w/v) SDS containing 1% (v/v) β -mercaptoethanol. After sedimentation of the beads, the supernatants were supplemented with 5 vol. of 0.1 M sodium citrate buffer, pH 5.5, and PMSF (1 mM final concentration) and incubated overnight with 4 m-units of Endo H (Roche Molecular Biochemicals, Monza, Italy).

Peptide:N-glycosidase F (PNGase F)

Samples were denatured as for Endo H digestions, but the SDS-containing supernatants were diluted in a mixture containing (final concentrations) sodium phosphate buffer, pH 7.2 (50 mM), EDTA (20 mM), n-octyl glucoside (1%, w/v) and PMSF (1 mM). Samples were incubated overnight with 4 units of PNGase F (Roche Molecular Biochemicals).

Fluorescence microscopy

Transfected COS-7 cells grown on glass coverslips were incubated for 1 h at room temperature with primary antibody in PBS/ Ca^{2+} / Mg^{2+} , containing 0.5% (w/v) BSA, prior to fixation for 10 min with 4% (w/v) paraformaldehyde. After washing thoroughly with 20 mM sodium phosphate buffer, pH 7.4, containing 500 mM NaCl (high-salt buffer), samples were incubated for 1 h with Texas Red-conjugated secondary antibody in 20 mM sodium phosphate buffer, pH 7.4, containing 450 mM NaCl and 0.1% gelatin, sequentially washed with high-salt (see above) and low-salt (10 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl) buffers. After mounting with 90% glycerol/10% PBS, slides were observed under a Zeiss Axioplan fluorescence microscope.

Flow-cytometric analysis

For cell-surface detection, transfected cultures were trypsin-treated, washed in PBS and incubated in PBA (PBS + 1% BSA)

containing 1 μ g/ml of anti-*myc* antibody for 30 min. After washing with PBA, cells were incubated for 30 min in PBA containing FITC-conjugated anti-mouse antibody. Cells, washed in PBS, were then measured by FACScan flow cytometer equipped with an argon laser (BD Biosciences, Heidelberg, Germany). At least 10000 cells were collected and evaluated for fluorescence content using Lysys II software (Becton Dickinson).

Preparation of caveolin-enriched TIFF

Routinely, four 100-mm-diameter Petri dishes of confluent COS-7 cells were scraped into 2 ml of Mes-buffered saline [MBS; 25 mM Mes (pH 6.5)/0.15 M NaCl] containing 1% (v/v) Triton X-100. Homogenization was carried out with 10 strokes of a loose-fitting Dounce homogenizer. The homogenate was adjusted to 40% sucrose by the addition of 2 ml of 80% sucrose prepared in MBS and placed at the bottom of an ultracentrifuge tube. Two 4 ml layers of 30 and 5% sucrose in MBS were overlaid above the homogenate and centrifuged at 39000 rev./min for 16–20 h in an SW41 rotor (Beckman Coulter, Cassina De'Pecchi, Italy). A light-scattering band confined at the boundary between 5 and 30% sucrose was observed that contained caveolin-1 but excluded most of other cellular proteins. From the top of each gradient, 1 ml gradient fractions were collected to yield a total of 12 fractions. For Western blotting, gradient fractions were trichloroacetic acid-precipitated, and the protein contents assayed with the BCA (bicinchoninic acid) reagent (Pierce). After normalization, proteins were separated by SDS/PAGE and blotted on to nitrocellulose. Otherwise, gradient fractions were immunoprecipitated as described above.

RESULTS

The palmitoylated form of recombinant α_0 is selectively associated with TIFF in transfected COS-7 cells

Cholesterol and sphingolipids can form a liquid-ordered phase that is resistant to solubilization by non-ionic detergents, such as Triton X-100, at low temperatures, and float to low buoyancy fractions on sucrose density gradients [28]. Therefore the insolubility of proteins, including G-protein α -subunits, in cold Triton has been often interpreted, with due caution (see [29] for a discussion), as an indication of their possible association with TIFF domains.

To define the contribution of myristoylation and palmitoylation to the association with TIFF of dually acylated α -subunits, we first compared the differential partitioning into cold Triton extracts of α_0 wt and α_0 C3S mutant, whose known palmitoylation site was mutated by replacement of Cys³ by Ser. Transiently transfected COS-7 cells, metabolically labelled for 4 h with [³H]myristate or [³H]palmitate 48 h post-transfection, were subjected to sequential solubilization first with 1% (v/v) Triton X-100 at 4 °C (Triton-soluble extract, TS), and secondly with 60 mM n-octyl glucoside (Triton-insoluble extract, TI). The latter detergent, structurally resembling glycosphingolipid, acts by displacing similar endogenous lipid components that are concentrated in TI extract. Figure 1(A) shows the amount of radioactive fatty acids incorporated in each of two polypeptides immunoprecipitated from TS and TI fractions with a specific anti- α_0 antiserum (1028). After labelling with [³H]myristate, α_0 wt was almost equally distributed between TS and TI fractions, whereas palmitoylation-defective α_0 C3S partitioned differently, being substantially more concentrated in TS. This suggests that the specific attachment of palmitate confers on α_0 an increased avidity for TIFF. Labelling of α_0 wt with [³H]palmitate confirmed this assumption, by showing that radiolabelled protein could

Figure 1 Palmitoylation-dependent partitioning of α_o into caveolin-1 containing TIFF of transfected COS-7 cells

(A) COS-7 cells, transfected with α_o wt and α_o C3S, were metabolically labelled for 4 h with [3 H]palmitate (3 H-pal) or [3 H]myristate (3 H-myr) and subjected to sequential extraction with 1% (v/v) Triton X-100 (TS) and 60 mM n-octyl glucoside (TI) at 4 °C. Cell extracts were immunoprecipitated with anti- α_o polyclonal antiserum (1028) and Protein A–Sepharose beads, and immunoprecipitates were subjected to SDS/12.5%–PAGE and fluorography. (B) Western-blot analysis with anti- α_o antiserum (ON1) of TS and TI extracts prior to immunoprecipitation. (C) COS-7 cells were transfected with α_o wt and labelled for 4 h with [3 H]palmitate. After solubilization in cold Triton, cell lysates were separated by ultracentrifugation on discontinuous sucrose density gradients. Fractions, 1 ml each, collected from the top of the gradients, were immunoprecipitated with 1028 antiserum, and subjected to SDS/PAGE, fluorography and gel miniaturization to detect the presence of [3 H]palmitoylated α_o . The corresponding gradient distribution of caveolin-1 ('Cav-1'), as determined by Western blotting, is shown below. Fractions 1–4 are the 5%-sucrose layer, and fractions 5–8 are the 30%-sucrose layer. Fractions 9–12, containing 40% sucrose, represent the 'loading zone' of these bottom-loaded flotation gradients and contain the bulk of cellular membrane and cytosolic proteins, as described in [19].

almost exclusively be detected in the TI fraction. The differential partitioning of α_o wt was not due to differences in the absolute protein content between fractions, as shown by Western-blotting analysis of the corresponding cell extracts before immunoprecipitation (Figure 1B).

These results were further supported by the analysis of the distribution of [3 H]palmitoylated α_o wt on bottom-loaded sucrose density gradients, a well-defined procedure that combines extraction by Triton X-100 at 4 °C and flotation, to separate low-density Triton-insoluble complexes from the bulk of cellular membranes and cytosolic proteins. Thus Triton extracts of COS-7 cells, transfected with α_o wt and labelled with [3 H]palmitate as above, were fractionated on flotation sucrose gradients. The bulk of cellular proteins, including soluble polypeptides, was concentrated in the bottom, high-density fractions (40% sucrose layer of the gradient; fractions 9–12; results not shown), whereas caveolin-1, used here as a marker protein for TIFF, was exclusively detected by Western immunoblotting in the fractions at the boundary between 5 and 30% sucrose layers of the

Figure 2 Schematic illustration of V_2R , α_o and the V_2TR - α_o fusion protein used in the present study

The N-terminal myc-tagged 1–71 amino acids of V_2R were fused to the N-terminus of α_o to generate the V_2TR - α_o chimaera. Amino acid residues of first V_2R intracellular loop, maintained in the chimaera, are shown by black boxes. The putative site for N-glycosylation at Asn²² (N) in the extracellular tails of V_2R and V_2TR - α_o , as well as the N-terminally myristoylated glycine (G) and adjacent palmitoylated cysteine (C) residues of α_o , are indicated by open boxes. The two putative palmitoylated cysteine residues in C-terminal cytoplasmic tail of V_2R are shown in black boxes.

gradient (fractions 4 and 5) (Figure 1C). Immunoprecipitation of each fraction with 1028 anti- α_o antiserum showed that [3 H]-palmitoylated α_o wt co-localized with immunoblotted caveolin-1 in low-buoyancy fractions (Figure 1C).

Overall, these results show that myristoylated and palmitoylated α_o wt partitions within caveolin-containing TIFF, whereas myristoylated, but palmitoylation-defective, α_o wt and α_o C3S are completely excluded from TIFF, thus suggesting the notion that, uniquely depending upon the presence of palmitate, α_o may change its membrane partitioning.

Fusion with N-terminal V_2R truncation localized nonacylated α_o wt at the plasma membrane of transfected COS-7 cells without association to TIFF

The differential membrane partitioning of α_o according to the presence of palmitate led us to consider the possibility that palmitoylation may serve not only for membrane binding, but

Figure 3 Expression of V_2R and $V_2TR-\alpha_o$ chimaera in COS-7 cells

COS-7 were transiently transfected with the different constructs using the LIPOFECTAMINE[®] reagent. At 48 h post-transfection, cells were harvested and lysed in Laemmli sample buffer. Lysates were separated by SDS/10%-PAGE immunoblotted with polyclonal anti- α_o (ON1) and/or monoclonal anti-*myc* antibodies.

also for compartmentalization, in TIFF. To verify this hypothesis, we questioned whether α_o , provided with a different membrane-anchoring mechanism than fatty acylation, could similarly be enriched in TIFF. To this purpose we fused to the 5' end of full-length α_o a sequence known to be sufficient for both membrane targeting/insertion and correct orientation of V_2R , comprising its N-terminus, the first transmembrane helix, and first cytoplasmic loop [30]. The features of the resulting fusion protein ($V_2TR-\alpha_o$), carrying a *myc*-epitope at the extreme N-terminus of V_2R moiety, are detailed in Figure 2.

The cDNAs encoding V_2R and the $V_2TR-\alpha_o$ fusion protein were individually transiently transfected into COS-7 cells and their level of expression analysed by Western blotting of whole cell lysates using anti-*myc* and/or anti- α_o antibodies. Figure 3 shows that, 48 h post-transfection, the cells produced comparable levels of the recombinant proteins. As previously reported [31] the V_2R blot did not yield a single band. A predominant broad band between 45 and 50 kDa and a weaker band at about 70 kDa were detected by the *myc* monoclonal antibody, the latter perhaps representing a dimeric form of the V_2R [31]. Likewise, blotting of the $V_2TR-\alpha_o$ chimaera with the same antibody showed a major broad band between 53 and 56 kDa (finely resolved as a doublet by the α_o antiserum) and a further band at about 60 kDa (Figure 3).

As a result of the fusion, the α_o moiety loses the N-terminal glycine residue that is the site of attachment of myristate to α_o wt. To verify that the adjacent cysteine residue of $V_2TR-\alpha_o$ is still palmitoylated, we determined the incorporation of [³H]palmitate into the fusion protein by metabolic radiolabelling of transfected COS-7 cells, followed by immunoprecipitation of cell lysate with the anti-*myc* antibody. As Figure 4 shows, the $V_2TR-\alpha_o$ chimaera was not palmitoylated, whereas V_2R , used here as a positive control, successfully incorporated the C_{16} fatty acid, as previously reported [32].

The extracellular domain of tr $V_2R-\alpha_o$ carries the putative N-glycosylation site at Asn²² (see Figure 2). Thus successful glycosylation of the chimaera could serve as a marker to verify translocation into the endoplasmic-reticulum (ER) lumen, where

Figure 4 Incorporation of [³H]palmitate in V_2R but not in $V_2TR-\alpha_o$ fusion protein

Mock and transfected COS-7 cells were metabolically labelled for 4 h with [³H]palmitic acid. Cell lysates were immunoprecipitated with monoclonal anti-*myc* antibody and analysed by SDS/PAGE and fluorography. To enhance signal detection, fluorographed gels were miniaturized as described in the Materials and methods section.

Figure 5 N-glycosylation of V_2R and $V_2TR-\alpha_o$ fusion protein

Mock and transfected COS-7 cells, pulse-labelled for 2 h with L-[³⁵S]methionine/L-[³⁵S]cysteine and chased overnight, were immunoprecipitated with anti-*myc* monoclonal antibody and Protein A-Sepharose beads. Immunoprecipitates were subjected to overnight incubation at 37 °C with or without specific endoglycosidases (PNGase F, Endo H), followed by separation by SDS/PAGE, fluorography, and gel miniaturization.

this post-translational modification occurs. To achieve this objective, COS-7 cells transfected with $V_2TR-\alpha_o$, and V_2R , were pulse-labelled for 2 h with L-[³⁵S]methionine/L-[³⁵S]cysteine and chased overnight. Lysates immunoprecipitated with anti-*myc* antibody were treated with Endo H or PNGase F enzymes, and analysed by SDS/PAGE and fluorography. Again, a predominant broad band between 45–50 kDa was immunoprecipitated from cells expressing the V_2R , whose size was reduced to 40 kDa by treatment with PNGase F, and proved to be resistant to Endo H treatment (Figure 5). These data, in agreement with [31], can tentatively identify the 45–50 kDa band as the mature intact receptor, and the 40 kDa band as the deglycosylated, and/or non-glycosylated, mature receptor. Likewise, the $V_2TR-\alpha_o$ fusion was immunoprecipitated as a major band of about 55 kDa that was sensitive to PNGase F and resistant to Endo H (Figure 5). From these results we can conclude that, similarly to the wt

Figure 6 Subcellular distribution of V_2R and $V_2TR-\alpha_0$ fusion protein in transfected COS-7 cells

Postnuclear supernatants of transfected COS-7 cells were fractionated by ultracentrifugation at 200 000 *g* into soluble (S) and particulate (P) fractions. Partitioning of proteins between fractions was determined by SDS/PAGE of trichloroacetic acid precipitates, followed by immunoblotting with anti-*myc* (A) and anti- α_0 (B) antibodies.

V_2R , the chimaera is N-glycosylated and hence must have been translocated into the ER lumen, and fully processed through the Golgi network.

The subcellular localization of each of $V_2TR-\alpha_0$ and V_2R proteins was next examined by fractionating post-nuclear COS-7 cell homogenates into cytosolic (S) and membrane (P) fractions by ultracentrifugation at 200 000 *g*. Western immunoblotting analysis of each fraction with anti-*myc* (Figure 6A), and anti- α_0 (Figure 6B) antibodies revealed that the $V_2TR-\alpha_0$ chimaera was primarily concentrated in the particulate fraction, comparably with V_2R (Figure 6A) and α_0 wt (Figure 10A, below). It is noteworthy that the particulate fraction in this assay contains total cell membranes, including plasma and intracellular membranes. Thus changes in the distribution among various cell membrane compartments will not be appreciated unless morphological analysis of transfected cells is performed.

To investigate in greater detail the intracellular localization of the various proteins, COS-7 cells expressing V_2R or $V_2TR-\alpha_0$ were examined by indirect immunofluorescence after staining with anti-*myc* antibody prior to fixation. The micrographs presented in Figure 7 illustrate that the fluorescence patterns associated with V_2R and $V_2TR-\alpha_0$ fusion protein were similarly intense at the cell edges, indicating that both polypeptides gained a plasma-membrane localization and a correct topology, as the

Figure 7 Immunofluorescence detection of V_2R and $V_2TR-\alpha_0$ fusion protein in transfected COS-7 cells

Transfected cells individually expressing each of the constructs were stained with the monoclonal anti-*myc* antibody prior to fixation with 4% (w/v) paraformaldehyde. Indirect immunofluorescence was revealed under optical microscopy.

Figure 8 Flow-cytometric analysis of surface expression of V_2R and $V_2TR-\alpha_0$ fusion protein in transfected COS-7 cells

Cells were stained with anti-*myc* antibody and FITC-conjugated anti-mouse antibody. The fluorescence associated with mock-transfected cells was identical with that of the blank.

N-terminal *myc* epitope was accessible to antibody binding without permeabilization. Mock-transfected COS-7 cells showed no detectable fluorescence (results not shown). To further evaluate the surface expression of V_2R and $V_2TR-\alpha_0$ in a more quantitative manner, we carried out flow cytometry using anti-*myc* antibody on non-permeabilized cultures. As reported in Figure 8, fluorescence intensities resulting from V_2R - and $V_2TR-\alpha_0$ -expressing cells were identical, thus indicating that both polypeptides were targeted to the cell surface with comparable efficiency. Again, mock-transfected cells were undistinguishable from the blank.

Figure 9 Triton extractability and buoyant density of recombinant $V_2TR-\alpha_o$ expressed in transfected COS-7 cells

Two experimental approaches were employed to assess the association of $V_2TR-\alpha_o$ with TIFF. In (A) the content of $V_2TR-\alpha_o$ was assayed by Western blotting with anti-myc monoclonal antibody in the TS and TI extracts of transfected COS-7 cells, as detailed in the legend to Figure 1. In (B) COS-7 cells transfected with fusion protein were Triton-extracted and subjected to ultracentrifugation on flotation gradients (see Figure 1). Equal volumes of each fraction were immunoblotted with anti-myc antibody to reveal the distribution of $V_2TR-\alpha_o$.

These results clearly demonstrate that fusion with the N-terminal truncation of V_2R can efficiently substitute for fatty acylation to specify a plasma membrane localization for α_o . In spite of its membrane targeting, no association of non-acylated $V_2TR-\alpha_o$ to TIFF was assessed either by partitioning into TS and TI fractions, or by flotation on sucrose density gradients. The chimera was exclusively concentrated in the TS fraction (Figure 9A) and distributed at the bottom of the gradient (Figure 9B), i.e. in those fractions from where caveolin-1 is excluded (see Figure 1D). This observation strengthens the notion that lipid modifications are required to confer α_o partitioning within TIFF.

Co-expression with $\beta\gamma$ -subunits increases membrane avidity of non-palmitoylated α_o mutant without influencing partitioning within caveolin-containing TIFF

Degtyarev et al. [33] have shown that binding to the $\beta\gamma$ -subunits was sufficient to localize at the membrane a mutated α_{11} -subunit lacking the palmitoylation site, suggesting that once myristoylated α_{11} had reached the lipid bilayer, the association with membrane-bound proteins could substitute for the addition of palmitate to stabilize the protein-lipid interaction. To determine if a trimeric complex of α_o with $\beta\gamma$ -subunits might by-pass the need of palmitoylation for membrane partitioning, we transfected COS-7 cells with an expression plasmid encoding for α_oC3S , alone or in combination with plasmids coding for β_1 - and γ_2 -subunits. As shown for α_{11} [33], co-expression of $\beta\gamma$ -subunits enabled α_oC3S to gain a membrane avidity comparable with α_o wt (Figure 10A). However, α_oC3S , neither expressed alone nor co-expressed with $\beta\gamma$, showed a substantial co-localization with caveolin-1 in low-density sucrose fractions separated by ultracentrifugation from transfected COS-7 cells (Figure 10B).

Figure 10 Co-expression with $\beta\gamma$ -subunits enhances membrane binding of palmitoylation-defective α_o , but not recovery in TIFF

COS-7 cells, transfected with α_o wt or non-palmitoylated mutant of α_oC3S , alone or in combination with β_1 and γ_2 subunits, were subjected to ultracentrifugation at 200 000 g to obtain soluble (S) and particulate (P) fractions (A) or lysed into 1% cold Triton X-100 and fractionated on flotation sucrose density gradients as described in the text. Partitioning of α_o wt and α_oC3S proteins between subcellular fractions was determined by SDS/PAGE and immunoblotting.

This observation indicates that complex formation with $\beta\gamma$ -dimer can fully substitute for palmitoylation to deliver α_o -subunit to membranes, as previously shown for α_{11} [33], but is unable to contribute to partitioning in TIFF membranes.

DISCUSSION

Recent studies have indicated that point mutations that abolish myristoylation and/or palmitoylation prevent the partitioning of α_{11} -subunits, and other dually acylated proteins (such as Src-like non-receptor tyrosine kinases and endothelial nitric oxide synthase), into low-buoyancy sucrose fractions separated by ultracentrifugation from Triton [15–19] or detergent-free, cell lysates [34]. This has been taken as a suggestion that these polypeptides may be compartmentalized in flattened (rafts) or invaginated (caveolae) specializations of the plasma membrane, rich in cholesterol and glycosphingolipids (here collectively defined as TIFF; [13,14]). Moreover, α -subunits, Src-like non-receptor tyrosine kinases and endothelial nitric oxide synthase, preferably in their inactive forms, interact directly with high molecular mass caveolin oligomers, that may represent the protein skeleton of caveolae membranes [12,35–37].

In agreement with these observations, we demonstrate in the present study that only when palmitate is post-translationally added to myristoylated α_o is the polypeptide co-localized with caveolin-1-containing TIFF of transfected COS-7 cells.

To confirm this, we investigated whether non-palmitoylated α_o delivered to membranes by alternative mechanisms, could also be compartmentalized within caveolin-containing TIFF. Two experimental strategies were employed. The first consisted in the fusion of α_o to a fragment of V_2R corresponding to a truncated receptor mutant originally described in a patient carrying congenital nephrogenic diabetes insipidus [23]. This receptor frag-

ment, containing sufficient information for its membrane insertion and orientation [30], when fused to α_o , is able to serve as a plasma-membrane targeting signal. Moreover, the presence of Endo H-resistant sugar moieties indicated that the chimaeric $V_3TR-\alpha_o$ protein was sorted to the cell surface via the Golgi apparatus. Secondly, co-expression with $\beta\gamma$ -subunits was used to promote the association of non-palmitoylated α_o with membranes. Our results clearly show that, in both instances, the membrane-anchoring functions of lipid modifications could be efficiently substituted, but membrane association was not sufficient for the partitioning of α_o within TIFF, in the absence of lipid modifications. This indicates that the two events, i.e. membrane interaction and partitioning in TIFF, can be dissociated, the latter being a function of the presence of a palmitoyl moiety bound to α_o .

The unique role of palmitoylation in mediating the association of α_o with TIFF membranes is important, since, unlike myristoylation, palmitoylation is a reversible, and hence potentially regulatable, modification. Indeed, experimental proof for the direct or G-protein-coupled-receptor-mediated acceleration of palmitate removal has been obtained for α_s [38–40] and $\alpha_{q/11}$ [41], which contain palmitate as their unique fatty acyl moieties and, more recently, for dually acylated α_i [42]. In addition, mutationally activated α_o , showing a constitutively accelerated rate of depalmitoylation [39], fails to partition within low-buoyancy, caveolin-containing subcellular fractions and is unable to interact with recombinant caveolin-1, as compared with α_{wt} . These findings raise the possibility that, upon regulation of palmitoylation, it may dynamically vary the association of α -subunits with TIFF, and hence the degree of their assembly with other resident signalling partner proteins. However, the *in vivo* relevance of α -subunit association with TIFF still remains to be clarified.

Palmitoylation can specify membrane compartmentalization of dually acylated α -subunits within TIFF, either by acting as a targeting signal and/or through the direct interactions with the lipid or protein components of caveolae. The observation that the attachment of palmitate is an absolute prerequisite for the binding of α_{i1} to caveolin-1 [19] highlights the importance of protein–protein interactions. However, the interactions with lipid components cannot be ruled out.

In conclusion, our results show that both membrane anchoring and partitioning functions are encoded by the lipid modified N-terminal domain of α_o , and that thioacylation of Cys³ is required for association with TIFF. Thus regulated palmitoylation of α -subunits provides a further potential means of fine tuning G-protein-mediated signal transduction.

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