The palmitoylation status of the G-protein $G_o1\alpha$ regulates its avidity of interaction with the plasma membrane

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Plasmids containing cDNAs encoding either the wild-type guanine-nucleotide-binding protein $G_o1\alpha$ or the palmitoylation-negative cysteine-3-to-serine (C3S) mutant of $G_o1\alpha$ were transfected into Rat 1 cells, and clones stably expressing immunoreactivity corresponding to these polypeptides were isolated. Clones C5B (expressing wild-type $G_o1\alpha$) and D3 (expressing the mutant form) were selected for detailed study. Immunoprecipitation of whole cell lysates of each clone labelled with either [^{3}H]palmitate or [^{3}H]myristate demonstrated incorporation of [^{3}H]myristate into both wild-type and the C3S mutant of $G_o1\alpha$, but that incorporation of hydroxylamine-sensitive [^{3}H]palmitate was restricted to the wild type. When membrane and cytoplasmic fractions were prepared from cells of either the C5B or D3 clones, although immunodetection of wild-type $G_o1\alpha$ was observed only in the membrane fraction, the C3S mutant was present in both membrane and cytoplasmic fractions. Furthermore, a significant proportion of the C3S $G_o1\alpha$ immunoreactivity was also detected in the cytoplasmic fraction if immunoprecipitation of recently synthesized $G_o1\alpha$ was performed from fractions derived from cells pulse-labelled with [^{35}S]Trans label. Pretreatment of cells of both clones C5B and D3 with pertussis toxin led to complete ADP-ribosylation of the cellular population of $G_o1\alpha$ in both cell types, irrespective of whether the polypeptide was subsequently found in the membrane or cytoplasmic fraction following cellular disruption. By contrast, separation of membrane and cytoplasmic fractions before pertussis-toxin-catalysed [^{32}P]ADP-ribosylation allowed modification only of the membrane-associated $G_o1\alpha$ (whether wild-type or the C3S mutant). This labelling was decreased substantially by incubation of the membranes with guanosine 5′-[$\beta\gamma$-imido]triphosphate. No cytoplasmic G-protein β subunit was detected immunologically, and the non-membrane-associated C3S $G_o1\alpha$ from D3 cells migrated as an apparently monomeric 40 kDa protein on a Superose 12 gel-filtration column. Membrane-associated wild-type and C3S $G_o1\alpha$ appeared to interact with guanine nucleotides with similar affinity, as no alteration in the dose–response curves for guanine-nucleotide-induced maintenance of a stable 37 kDa tryptic fragment was noted for the two forms of $G_o1\alpha$. Chemical depalmitoylation of membranes of clone C5B with neutral 1 M hydroxylamine caused a release of some 25–30 % of each of $G_o1\alpha$, $G_i2\alpha$ and $G_q\alpha/G_{11}\alpha$ from the membranes. Equivalent treatment of D3 cells caused an equivalent release of $G_i2\alpha$ and $G_q\alpha/G_{11}\alpha$, but was unable to cause any appreciable release of the CS3 form of $G_o1\alpha$, which was membrane-bound.

INTRODUCTION

Heterotrimeric guanine-nucleotide-binding proteins (G-proteins) [1,2] play central roles in the transmission of information between plasma-membrane serpentine receptors and effector systems which include enzymes responsible for the rate of production and degradation of intracellular secondary messengers and a variety of classes of ion channels. Although the primary sequences of serpentine receptors [3] and of many effector moieties [4] contain stretches of predominantly hydrophobic residues, which are consistent with these elements representing trans-plasma-membrane-spanning helices, the same is not true of any of the subunits of G-proteins. It is well established that G-protein γ subunits are members of the so-called CAAX-motif-containing family of proteins, which also includes many low-molecular-mass G-proteins and the nuclear lamins [5–7]. In all of these proteins, a complex series of modifications occurs at the C-terminal CAAX motif which causes, sequentially, prenylation of the cysteine residue (with either a farnesyl or a geranylgeranyl group, depending on the identity of the final amino acid of the motif), peptidase cleavage of the three C-terminal amino acids and finally carboxylmethylation of the now C-terminal prenylated cysteine [5–7]. These modifications are essential for the membrane attachment of these proteins [5–7], and, in the case of G-protein γ subunits, which form a physiologically non-dissociating complex with the β subunit, prenylation of the γ subunit is important for maintaining this complex at the membrane.

G-protein α subunits of the G_i-like family are modified by the co-translational addition of myristate [7–9] to their N-terminal glycine, and, although this also plays a role in membrane attachments of these polypeptides, it may be more important in protein–protein interactions and in the regulation of the signalling function of the α subunits [10,11]. Moreover, as α subunits of the G_s, G_q and G_{12} families of G-proteins do not have an N-terminal sequence consistent with them acting as high-affinity substrates for N-myristoyltransferase, but nevertheless these proteins are found at the plasma membrane of cells, myristoylation cannot itself represent a definitive membrane attachment signal, particularly as a number of myristoylated proteins, such as the catalytic subunit of cyclic AMP-dependent protein kinase, have predominantly cytoplasmic locations [12].

We [13] and others [14–17] have recently demonstrated that all of the widely expressed G-protein α subunits can also be modified by the post-translational addition of palmitate. At least in the cases of $G_o1\alpha$ [13] and $G_s\alpha$ [15], mutational analyses have demonstrated this to be likely to occur via a thioester link to

Abbreviations used: p[NH]ppG, guanosine 5′-[$\beta\gamma$-imido]triphosphate; GDP[S], guanosine 5′-[β-thio]diphosphate.

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a cysteine residue at position 3. The role of palmitoylation of G-protein α subunits in their association with the plasma membrane and with $\beta\gamma$ subunits remains controversial [15–17], and to date has been poorly explored. Thus in the present paper we have stably expressed either wild-type $G_o1\alpha$ or a palmitoylation-defective (C3S) mutant of this protein in Rat 1 cells to examine these questions.

We demonstrate that: (1) all of both wild-type and the C3S mutant of $G_o1\alpha$ act as a substrate for pertussis-toxin-catalysed ADP-ribosylation *in vivo*; (2) disruption of the cells results in a substantial fraction of the C3S mutant, but not wild-type $G_o1\alpha$, being recovered in the cytoplasmic fraction; (3) after cellular disruption, the C3S $G_o1\alpha$ in the cytoplasmic fraction is not a substrate for pertussis-toxin-catalysed [^{32}P]ADP-ribosylation, whereas the fraction which remains associated with the membrane is; (4) the C3S $G_o1\alpha$ present in the cytosol after cellular disruption migrates as a 40 kDa α subunit monomer in a sizing column; (5) membrane-associated wild-type and C3S $G_o1\alpha$ bind the guanine nucleotide analogue guanosine 5′-[$\beta\gamma$-imido]triphosphate (p[NH]ppG) equivalently, and both interact with the $\beta\gamma$ complex; (6) treatment of cell membranes containing wild-type $G_o1\alpha$ with hydroxylamine causes release of some 30% of this polypeptide, whereas equivalent treatment of membranes containing C3S $G_o1\alpha$ is unable to cause significant release of this protein.

These data demonstrate that the palmitoylation state of a G-protein α subunit alters the avidity of interaction of the polypeptide with the plasma membrane, but that the palmitoylation status of the protein is not sufficient in isolation to determine fully the membrane association of the polypeptide.

MATERIALS AND METHODS

Materials

[^{32}P]NAD$^+$ (> 800 Ci/mmol) and [9,10-^{3}H]myristate were from New England Nuclear/DuPont. [9,10-^{3}H]Palmitate was from Amersham International. [^{35}S]Trans label was from ICN. Prestained lactate dehydrogenase was from Sigma. All reagents for tissue cultures were from GIBCO. DOTAP reagent was from Boehringer Mannheim. All stock laboratory chemicals were purchased from either Sigma or BDH and were of the highest quality available.

Generation of Rat 1 cell clones expressing wild-type and C3S $G_o1\alpha$

A cDNA encoding $G_o1\alpha$ was subcloned into plasmid pcEXV-3 [18]. Generation of the serine-to-cysteine at codon 3 mutant (C3S) of $G_o1\alpha$ has previously been described [13]. Wild-type and the C3S mutant of $G_o1\alpha$ in plasmid pcEXV-3 were transfected along with pSV2 neo in a 10:1 ratio into Rat 1 fibroblasts by using DOTAP reagent (Boehringer Mannheim), following the manufacturers' instructions. Colonies expressing the neomycin-resistance gene were selected for by incubation with 700 μg/ml geneticin sulphate. Resistant colonies were expanded and assessed for expression of $G_o1\alpha$ immunoreactivity (see the Results section).

Cell growth and maintenance

Rat 1 fibroblasts and colonies generated from these cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) newborn-calf serum, penicillin (100 units/ml) and streptomycin (100 μg/ml) in 5% CO_2 at 37 °C. Transfected colonies expressing $G_o1\alpha$ in addition to the above were grown in geneticin sulphate (700 μg/ml) until expanded, then subsequently grown in the presence of geneticin sulphate for one in every four passages. Cells were grown in 75 cm^2 tissue-culture flasks and were harvested just before confluency.

Membrane preparation

Membranes were prepared from cells by homogenization with a Teflon-on-glass homogenizer and differential centrifugation completed as described previously [19]. After homogenization, P200 and S200 fractions were generated from postnuclear supernatants by centrifugation at 200000 *g* for 30 min at 4 °C.

Incorporation of [^{3}H]palmitate, [^{3}H]myristate and [^{35}S]Trans label into $G_o1\alpha$

For fatty acid labelling, cells were fed with the minimum volume of fatty acid (FA) medium: DMEM supplemented with 5% (v/v) dialysed newborn-calf serum and 5 mM sodium pyruvate, with antibiotics as above. Cultures were labelled with 200 μCi/ml [9,10-^{3}H]palmitate or [9,10-^{3}H]myristate dissolved in ethanol at a stock concentration of 10 μCi/μl. For amino acid labelling DMEM (lacking methionine and cysteine) was used, supplemented with 5% (v/v) dialysed newborn-calf serum and antibiotics. For long labelling periods this was supplemented with 5% (v/v) normal growth medium. [^{35}S]Trans label was added at 10–50 μCi/ml.

Immunodetection of $G_o1\alpha$

(a) Immunoblotting

Cell membranes were resolved by SDS/PAGE on either 10% (w/v) acrylamide/0.8% (w/v) bisacrylamide gels or 12.5% (w/v) acrylamide/0.0625% (w/v) bisacrylamide gels containing a linear 4–8 M urea gradient and transferred to nitrocellulose (Costar). Blots were probed with anti-peptide antisera as previously described [20]. A variety of different $G_o\alpha$-specific antisera were used in this study. The characterization of these primary antisera, OC2 [21], IM1 [21], ON1 [21] and an antiserum (1028) generated against a trpE fusion protein with the 93 C-terminal amino acids of $G_o1\alpha$ [13] has been described previously, as has the generation of the anti-(G-protein β subunit) antiserum BN [22].

(b) Immunoprecipitation

Cells, labelled as above, were washed twice with ice-cold PBS, collected in PBS and spun down. The supernatants were discarded and the pellets resuspended in 0.2 ml of 1% (w/v) SDS containing protease inhibitors (0.067 trypsin-inhibitor unit/ml aprotinin and 0.2 mM phenylmethanesulphonyl fluoride). After breakage of DNA by repeated pipetting and boiling for 4 min, 0.8 ml of Mix 1 [1.25% (w/v) Triton X-100, 190 mM NaCl, 6 mM EDTA, 50 mM Tris/HCl, pH 7.5] plus protease inhibitors as above was added to each sample. The samples were centrifuged in a microfuge for 10 min at 4 °C, and supernatants were transferred to fresh tubes. Then 20 μl of a 1:1 suspension of Protein A–Sepharose (Pharmacia) beads prewashed three times in Mix 2 (4 parts of Mix 1 plus 1 part of 1% SDS) were added to each sample and left for 2 h at 4 °C with continuous rotation. After centrifuging for 10 min in a microfuge, the supernatants were transferred to fresh tubes and a sample was withdrawn to determine the total radioisotope incorporation into cell proteins. To the remaining samples 15 μl of antiserum 1028 or ON1 or preimmune rabbit serum was added, and the samples were incubated overnight at 4 °C with continuous rotation. Immuno-

precipitates were washed three times with Mix 2, once with 50 mM Tris/HCl (pH 6.8) and then dissolved in Laemmli loading buffer containing 20 mM dithiothreitol, followed by SDS/PAGE. Bands were detected by fluorography using preflashed Kodak XAR-5 film. To test for ester-linked fatty acid, gels were treated for 60 min at room temperature with 1 M hydroxylamine/HCl, or 1 M Tris/HCl, as a control, at pH 7.2.

Pertussis-toxin-catalysed ADP-ribosylation

For pertussis-toxin-catalysed ADP-ribosylations *in vivo*, cells at 70% confluency were incubated with 25 ng/ml pertussis toxin (Proton Products, Porton Down, Wiltshire, U.K.) for 16 h. Cells were then harvested and particulate and soluble fractions prepared as described above. ADP-ribosylations *in vitro* were carried out as previously described [23] before samples were immunoprecipitated and resolved as described above.

Gel filtration

A sample of the S200 fraction derived from cells of clone D3 was applied to a Superose 12 gel-filtration column (Pharmacia). The column was then washed with a buffer containing 10 mM Tris/HCl, pH 7.4, 0.1 mM EDTA and 150 mM NaCl; 500 μl fractions were collected and precipitated with a final concentration of 20% (w/v) trichloroacetic acid, and analysed by SDS/PAGE and immunoblotting. Molecular-size standards [β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), BSA (68 kDa), carbonic anhydrase (24 kDa), cytochrome c (13.3 kDa)], were chromatographed under identical conditions. Blue Dextran was used to define the void volume of the column.

Limited trypsin treatment

For limited tryptic proteolysis, membrane samples were preincubated with either 100 μM guanosine 5′-[β-thio]diphosphate (GDP[S]) or 1–100 μM p[NH]ppG (Boehringer) in 0.1 mM EDTA/10 mM Tris/HCl, pH 7.5, at 37 °C for 20 min. Samples were then incubated with tosylphenylalanylchloromethane-treated trypsin (Sigma) at a ratio of 1:60 (w/w) trypsin to membranes, at 30 °C for 15 min, and the digestion was stopped on ice by addition of 25 μg of soybean trypsin inhibitor (Sigma). Samples were then precipitated with trichloroacetic acid, resolved by SDS/PAGE (10% gels) and immunoblotted as described above.

Hydroxylamine treatment of cell membranes

A 100 μg portion of cell membranes was treated with 1 M hydroxylamine, pH 8.0 (or 1 M Tris/HCl, pH 8.0, as control) for 4 h at 25 °C before being fractionated by centrifugation at 100000 *g* for 30 min at 4 °C. Particulate fractions were boiled for 5 min in SDS/PAGE sample buffer, whereas soluble fractions were precipitated with trichloroacetic acid as described above. Samples were then resolved by SDS/PAGE and immunoblotted as described above.

RESULTS

cDNAs encoding rat $G_o1\alpha$ and a mutant in which cysteine-3 was replaced by serine (C3S) in plasmid pcEXV-3 [13] were transfected along with pSV2 neo in a 10:1 ratio into Rat 1 fibroblasts. Colonies expressing the neomycin-resistance gene were identified by incubation in the presence of 700 μg/ml geneticin sulphate. Geneticin-resistant colonies were expanded and assessed for expression of $G_o\alpha$ immunoreactivity by using the $G_o\alpha$-specific antisera IM1 [21] (Figure 1a) or OC2 [21] (results not shown).

Parental Rat 1 cells demonstrated no detectable $G_o\alpha$ immunoreactivity, but varying levels of $G_o\alpha$ immunoreactivity were detected in a number of individual clones transfected with either wild-type or C3S $G_o1\alpha$ (Figure 1a). Levels of immunoreactivity varied considerably between individual clones. Clones C5B (transfected with wild-type $G_o1\alpha$) and D3 (transfected to express the C3S mutant) (Figure 1a) were selected for further study, as both cell lines exhibited good expression of $G_o\alpha$ immunoreactivity. Antiserum IM1 also identified a 66 kDa polypeptide in membranes of clone D3 cells (see Figure 2a), but not other transfectants. The nature of this polypeptide is unknown, but it was not identified by the other anti-G_o antisera used in this study.

Since both of the anti-(peptide $G_o\alpha$) antisera OC2 and IM1 were generated against peptide sequences which are conserved between $G_o1\alpha$ and $G_o2\alpha$ and have been shown to identify both splice variants non-selectively [21], we confirmed that the observed $G_o\alpha$ immunoreactivity in these clones corresponded to $G_o1\alpha$ by separating membranes of these clones under SDS/PAGE conditions which are able to resolve $G_o1\alpha$ and $G_o2\alpha$ (Figure 1b).

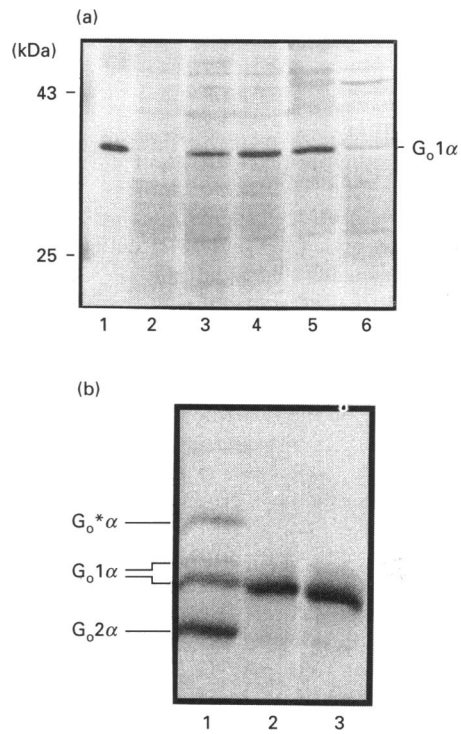

Figure 1(a) Expression of wild-type and C3S $G_o1\alpha$ in Rat 1 cells; (b) the $G_o\alpha$ isoform expressed is $G_o1\alpha$

(**a**) Membranes (50 μg, except in lane 1) from (1) rat frontal cortex (5 μg) positive control for $G_o\alpha$ immunoreactivity, (2) parental Rat 1 fibroblasts and clones of Rat 1 fibroblasts transfected with plasmids incorporating a cDNA for wild-type $G_o1\alpha$ (3, 4) or C3S $G_o1\alpha$ (5, 6) were resolved and immunoblotted for the presence of $G_o\alpha$ by using antiserum IM1 [21] as primary reagent. Clones C5B (wild-type $G_o1\alpha$) (lane 4) and D3 (C3S $G_o1\alpha$) (lane 5) were selected for detailed study. Molecular-mass markers corresponding to 25 and 43 kDa are shown for reference. (**b**) Membranes of neuroblastoma × glioma hybrid NG108-15 cells (100 μg) (1) and of clones C5B (2) and D3 cells (3) (75 μg) were resolved in SDS/PAGE containing a 4–8 M urea gradient and immunoblotted with antiserum IM1 as primary antiserum. Under these gel conditions, three forms of $G_o\alpha$ can be observed in NG108-15 cells, but only a single form, which migrates identically with $G_o1\alpha$, was detected in the clones transfected with either wild-type or C3S $G_o1\alpha$. Only a fraction of this gel is displayed, to allow display of the separation of the $G_o\alpha$ isoforms, and as the urea gradient alters the apparent molecular mass of standard proteins substantially, no estimate of molecular mass is given.

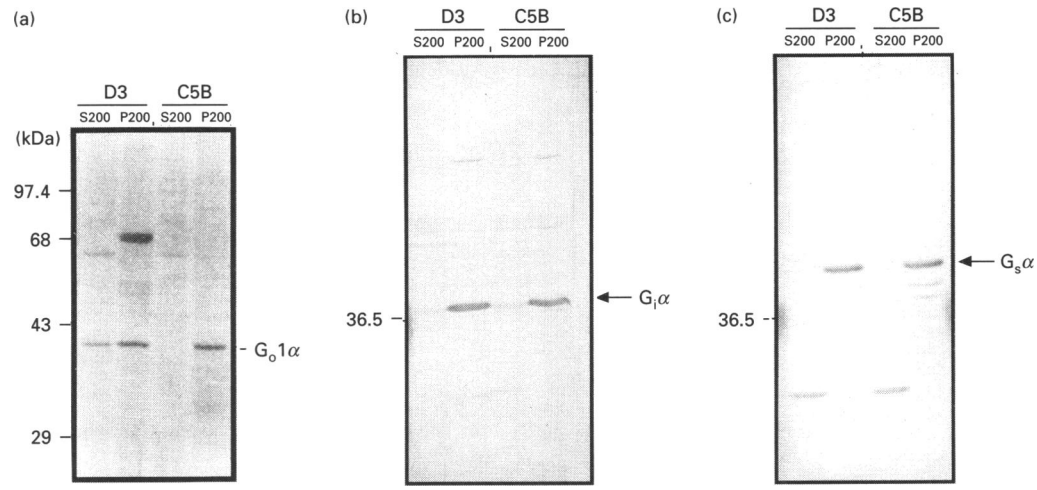

Figure 2 Immunoblot analysis of particulate and cytosolic partitioning of wild-type and C3S mutant forms of $G_o1\alpha$

Cells of clone C5B (wild-type $G_o1\alpha$) or clone D3 (C3S $G_o1\alpha$) were separated into particulate (P200) and cytosolic (S200) fractions, resolved by SDS/PAGE and immunoblotted with antiserum IM1 to detect the presence of $G_o1\alpha$ (**a**), with antiserum SG to detect '$G_i\alpha$' (a combination of $G_i1\alpha$ and $G_i2\alpha$) (**b**) or with antiserum CS to detect $G_s\alpha$ (**c**). Only insignificant fractions of the 'G_i' and G_s immunoreactivity was located in the S200 fractions of either cell line, but, although wild-type $G_o1\alpha$ was virtually entirely associated with the P200 fraction, a significant fraction (25–40% in individual experiments) of the mutant C3S $G_o1\alpha$ immunoreactivity was in the S200 fraction.

As NG108-15 cells express $G_o1\alpha$, $G_o2\alpha$ and $G_o{}^*\alpha$ (a third form of $G_o\alpha$ which has been purified from brain [24]) [21], membranes of these cells were used as positive controls. Immunoblotting NG108-15, C5B and D3 cell membranes with antiserum IM1 demonstrated that both C5B and D3 membranes expressed a form of $G_o\alpha$ which migrated identically with the polypeptide which has previously been shown [21] to correspond to $G_o1\alpha$ in NG108-15 membranes. Although antiserum IM1 also identified the presence of each $G_o2\alpha$ and $G_o{}^*\alpha$ in NG108-15 cells, no immunoreactive polypeptides corresponding to either of these forms were observed in the two Rat 1-fibroblast-derived clones. Equivalent immunoblots of membranes from both C5B and D3 cells and of rat brain (which also expresses each of $G_o1\alpha$, $G_o2\alpha$ and $G_o{}^*\alpha$) with antiserum OC2 produced equivalent conclusions (results not shown).

Pulse-labelling of both C5B and D3 cells with [^{35}S]Trans label resulted in equivalent total incorporation of ^{35}S into newly synthesized protein in both cell types (results not shown). As we have demonstrated previously after transient expression of wild-type and C3S $G_o1\alpha$ in COS cells [13], a ^{35}S-labelled polypeptide of some 38 kDa which was not immunoprecipitated with normal rabbit serum was specifically immunoprecipitated from both cell lines with the $G_o1\alpha$ fusion-protein-directed antiserum 1028 (results not shown). Immunoprecipitation of $G_o1\alpha$ from separated S200 cytosolic and P200 membrane fractions of such ^{35}S-labelled cells showed marked differences between the distribution of wild-type and C3S mutant $G_o1\alpha$ (results not shown). Newly synthesized ^{35}S-labelled wild-type $G_o1\alpha$ was located primarily in the P200 membrane fraction, whereas most of the C3S mutant was located in the cytoplasmic fraction. Differences in membrane-associated and cytoplasmic localization of wild-type and the C3S mutant of $G_o1\alpha$ after cellular disruption were also recorded when steady-state immunologically detectable protein was measured by immunoblotting cytoplasmic and membrane fractions (Figure 2). Essentially all of the wild-type $G_o1\alpha$ was in the membrane fraction (Figure 2a), whereas a substantial proportion of C3S $G_o1\alpha$ was present in the cytoplasmic fraction (Figure 2a). Equivalent immunoblots of membrane and cytoplasmic fractions from C5B and D3 cells showed no differences in distribution between the cell lines for $G_i\alpha$ (Figure 2b) or $G_s\alpha$ (Figure 2c), with the vast majority of each G-protein being membrane associated. These results demonstrated that the altered distribution of $G_o1\alpha$ between the wild-type and C3S-mutant-expressing cells was not a general artefact based on the specific clones examined.

Cells of both clones C5B and D3 were incubated with either [^{3}H]palmitate or [^{3}H]myristate, and subsequently $G_o1\alpha$ was immunoprecipitated from cell lysates with antiserum 1028. SDS/PAGE and fluorography of these samples demonstrated incorporation of [^{3}H]myristate into both the wild-type and C3S mutant of $G_o1\alpha$ in a manner which was resistant to treatment of the gels with hydroxylamine (results not shown). Incorporation of [^{3}H]palmitate into $G_o1\alpha$ was primarily restricted to the wild-type, as previously described after transient transfection of COS cells [13], but weak labelling of the mutant polypeptide was also observed in D3 cells incubated with [^{3}H]palmitate (results not shown). This incorporation into the C3S mutant was not reversed by hydroxylamine treatment, however, whereas virtually all of the incorporation from [^{3}H]palmitate in the wild-type $G_o1\alpha$ was removed (results not shown), suggesting that incorporation from [^{3}H]palmitate into C3S $G_o1\alpha$ protein was due to the metabolic interconversion of the added [^{3}H]palmitate label to [^{3}H]myristate. This again is exactly the pattern of labelling that we have observed previously after expression of these constructs in COS cells [13].

When either clone C5B or clone D3 was incubated with pertussis toxin (25 ng/ml, 16 h), all of the cellular population of $G_o1\alpha$ became ADP-ribosylated (Figure 3). As a measure of this, we took advantage of the observation that addition of ADP-ribose to members of the family of pertussis-toxin-sensitive G-proteins slows appreciably their mobility through SDS/PAGE. In SDS/PAGE gels containing a linear gradient (4–8 M) of urea, this difference in mobility is exaggerated in comparison with standard gels (results not shown). In particulate and supernatant fractions isolated from C5B cells which had been pretreated with pertussis toxin, all of the immunodetectable wild-type $G_o1\alpha$ was

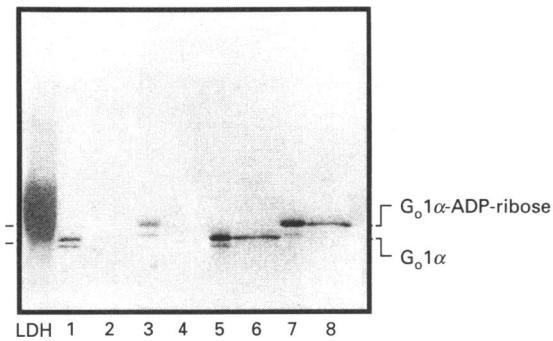

Figure 3 Pertussis-toxin treatment of both clone C5B and D3 cells produces ADP-ribosylation of the full cellular population of $G_o1\alpha$

Cells of clone C5B (wild-type $G_o1\alpha$) (lanes 1–4) and clone D3 (C3S $G_o1\alpha$) (lanes 5–8) were treated with pertussis toxin (25 ng/ml, 16 h) (lanes 3, 4, 7, 8) or vehicle (lanes 1, 2, 5, 6), and particulate (lanes 1, 3, 5, 7) and cytoplasmic (lanes 2, 4, 6, 8) fractions were generated. These were resolved by SDS/PAGE incorporating a 4–8 M gradient of urea and subsequently immunoblotted with the $G_o\alpha$-specific antiserum ON1 [21]. Prestained lactate dehydrogenase (LDH) was used to monitor the migration of the sample. ADP-ribosylated $G_o1\alpha$ migrates more slowly through such gels than non-modified $G_o1\alpha$. The immunologically detected polypeptide which migrated more rapidly than $G_o1\alpha$ may represent a proteolytic fragment. It too was clearly modified by pertussis-toxin treatment of the cells, as measured by its slower mobility after treatment.

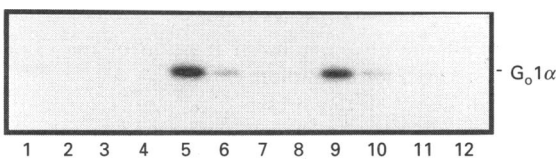

Figure 4 Pertussis toxin catalyses [^{32}P]ADP-ribosylation of membrane-bound forms of both wild-type and C3S $G_o1\alpha$, but not cytoplasmic C3S $G_o1\alpha$

Particulate (1, 2, 5, 6, 9, 10) and cytosolic (3, 4, 7, 8, 11, 12) fractions of cells of parental Rat 1 (1–4) and clones C5B (5–8) and D3 (9–12) were generated and pertussis-toxin-catalysed [^{32}P]ADP-ribosylation was performed with thiol-activated pertussis toxin in the absence (1, 3, 5, 7, 9, 11) or presence (2, 4, 6, 8, 10, 12) of p[NH]ppG (100 μM). Samples were subsequently recovered and immunoprecipitated with antiserum ON1, resolved by SDS/PAGE and autoradiographed.

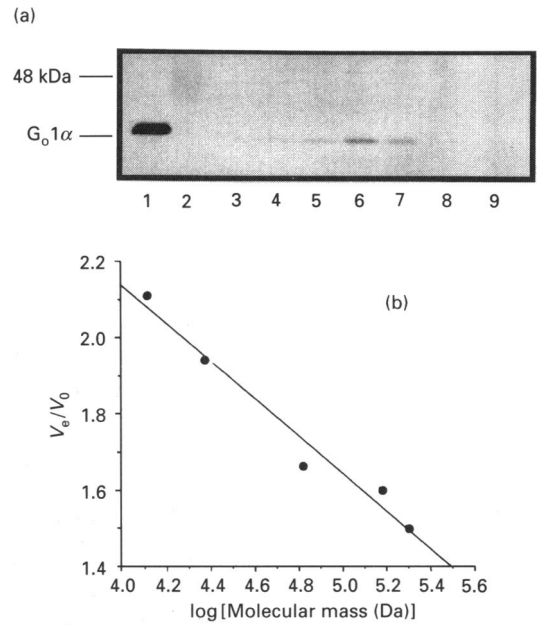

Figure 5 Cytoplasmic C3S G_o1 exists as an isolated α subunit

A sample of the S200 fraction derived from cells of clone D3 was applied to a Superose 12 gel-filtration column (Pharmacia). The column was then eluted with a buffer containing 10 mM Tris/HCl, pH 7.4, 0.1 mM EDTA and 150 mM NaCl; 500 μl fractions were collected and precipitated with a final concentration of 20% (w/v) trichloroacetic acid and analysed by SDS/PAGE, followed by immunoblotting for $G_o\alpha$ (**a**). Lane 1, rat brain (10 μg) as positive control for the immunoblot; lane 2, molecular-mass markers; lanes 3–9 represent individual fractions from this elution ranging from V_e 12.0 ml (lane 3) to 15.0 ml (lane 9). Lane 6 (V_e = 13.5 ml) represented the peak of $G_o1\alpha$ immunoreactivity. Only fractions close to the peak of $G_o1\alpha$ immunoreactivity are shown, but no immunoreactivity corresponding to this polypeptide was detected in any of the fractions which are not displayed. Molecular-size standards [β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), BSA (68 kDa), carbonic anhydrase (24 kDa), cytochrome c (13.3 kDa)] were chromatographed under identical conditions to provide a calibration for the position of elution of C3S G_o1 (**b**). Blue Dextran was used to define the void volume of the column. $G_o1\alpha$ was eluted with a V_e/V_0 ratio of 1.88 (corresponding to 40.7 kDa).

in the membrane fraction and migrated with decreased mobility through a urea-gradient gel in comparison with that derived from untreated cells (Figure 3). In fractions isolated from pertussis-toxin-pretreated D3 cells, both the C3S $G_o1\alpha$ which could be immunodetected from the membrane and that from the supernatant migrated with decreased mobility (Figure 3). These results suggest that the supernatant form either must be cycling between the membrane and cytoplasm or is released from the membrane during homogenization (see below and the Discussion section). A second $G_o\alpha$ immunoreactive band detected in the membrane fractions also migrated more slowly after pertussis-toxin treatment (Figure 3). This polypeptide may represent a proteolytic fragment of $G_o1\alpha$.

By contrast, if membrane and supernatant fractions were prepared from untreated D3 cells, immunoprecipitation with the anti-G_o antiserum ON1 after pertussis-toxin-catalysed [^{32}P]ADP-ribosylation *in vitro* demonstrated that, whereas membrane-associated C3S $G_o1\alpha$ was a target for modification (Figure 4, lane 9), the C3S $G_o1\alpha$ present in the supernatant fraction was not (Figure 4, lane 11). As expected, wild-type $G_o1\alpha$ in the membrane fraction of clone C5B cells was also a substrate for pertussis-toxin-catalysed [^{32}P]ADP-ribosylation (Figure 4, lane 5). Addition of the poorly hydrolysed GTP analogue p[NH]ppG (100 μM) to membranes of both C5B and D3 cells decreased substantially thiol-activated pertussis-toxin-catalysed incorporation of [^{32}P]ADP-ribose into the membrane-associated forms of both wild-type and the C3S mutant $G_o1\alpha$ (Figure 4, lanes 5 versus 6 and 9 versus 10), suggesting that both the wild-type and C3S mutant of $G_o1\alpha$ were able to interact with the $\beta\gamma$-subunit complex.

As it has been demonstrated that $G_o\alpha$ is a very poor substrate for pertussis-toxin-catalysed ADP-ribosylation in the absence of $\beta\gamma$ complex [25], we assessed whether the supernatant form of C3S $G_o1\alpha$ recovered after homogenization of D3 cells existed in isolation or in association with $\beta\gamma$ complex. Gel filtration on a Superose 12 column demonstrated the supernatant C3S $G_o1\alpha$ to migrate as a 40 kDa polypeptide (Figure 5). Furthermore, no G-protein β-subunit immunoreactivity could be detected in the cytoplasmic fraction (results not shown). To assess further the interaction of wild-type and C3S $G_o1\alpha$ with $\beta\gamma$ complex, and to measure the ability of both wild-type and C3S $G_o1\alpha$ to bind guanine nucleotides, we performed a series of limited trypsin-treatment experiments on membranes of both C5B and D3 cells in the presence and absence of guanine nucleotides. In the

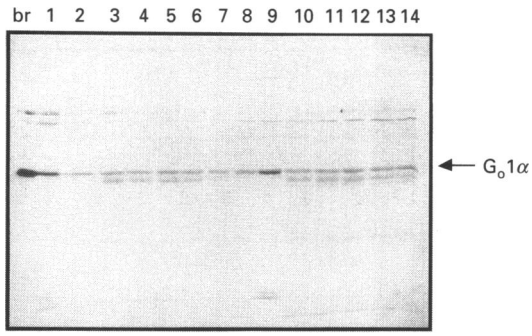

Figure 6 Limited trypsin treatment of wild-type and CS3 $G_o1\alpha$

Membranes (80 μg) from either clone C5B (wild-type $G_o1\alpha$) (lanes 1–7) or clone D3 (CS3 $G_o1\alpha$) (lanes 8–14) were incubated for 20 min at 37 °C in 10 mM Tris/HCl/0.1 mM EDTA, pH 7.4, in the presence of no guanine nucleotide (lanes 1, 9), 100 μM GDP[S] (lanes 2, 8) or p[NH]ppG at 100 μM (lanes 3, 10), 50 μM (lanes 4, 11), 10 μM (lanes 5, 12), 5 μM (lanes 6, 13) or 1 μM (lanes 7, 14). Tosylphenylalanylchloromethane-treated trypsin [1:60 (w/w) ratio trypsin: membrane protein] (samples 2–8 and 10–14) or water (samples 1, 9) was subsequently added and digestion performed for 15 min at 30 °C. At the termination of the incubation, soybean trypsin inhibitor (25 μg) was added, and samples were recovered by trichloroacetic acid precipitation and resolved by SDS/PAGE along with rat brain cortex membranes (10 μg) (br) to act as a positive immunological control. Proteins transferred from the gel to nitrocellulose were probed with the anti-$G_o\alpha$ antiserum OC2.

Table 1 Hydroxylamine treatment releases a substantial fraction of membrane-associated wild-type, but not C3S, $G_o1\alpha$

Membranes (50 μg) from clone C5B (wild-type $G_o1\alpha$) or clone D3 (C3S $G_o1\alpha$) were treated with either 1 M hydroxylamine, pH 8.0, or 1 M Tris/HCl, pH 8.0, for 4 h at 25 °C. Samples were subsequently recovered and separated into particulate and supernatant fractions, resolved by SDS/PAGE and immunoblotted to detect the presence of $G_i1\alpha + G_i2\alpha$, $G_q\alpha + G_{11}\alpha$ or $G_o1\alpha$. The samples were then quantified by densitometry. Release from the particulate fraction into the supernatant fraction produced by 1 M hydroxylamine treatment minus that produced by 1 M Tris/HCl is displayed as a percentage of the total G-protein pool in particulate + supernatant fractions. Data are presented as means ± S.E.M.; $n = 4$ from independent experiments.

	Release produced by 1 M hydroxylamine (% of total)	
G-protein	Clone C5B	Clone D3
$G_o1\alpha$	21.6 ± 4.7	6.0 ± 2.3
$G_i\alpha$	20.3 ± 0.3	15.2 ± 0.2
$G_q\alpha/G_{11}\alpha$	24.1 ± 5.9	20.8 ± 5.2

presence of GDP[S] (100 μM), both forms of $G_o1\alpha$ were proteolysed without the retention of a clipped form in which only the extreme N-terminus is removed (Figure 6). However, in the presence of p[NH]ppG concentrations above 1 μM, a stable 37 kDa form of $G_o1\alpha$ was generated. No obvious differences in the dose-dependence of p[NH]ppG for the detection of the clipped form was observed between wild-type $G_o1\alpha$ and C3S $G_o1\alpha$ (Figure 6).

Hydroxylamine treatment can be used to cause chemical depalmitoylation. When membranes from clone C5B were treated with 1 M hydroxylamine, pH 8.0, for 4 h and the membranes and supernatant fractions were subsequently recovered by centrifugation, it was observed that some 20% of the total levels of wild-type $G_o1\alpha$ and the α subunits of the phosphoinositidase C-linked G-proteins G_q/G_{11} and of the inhibitory G-protein G_i2 were released from the membrane (Table 1). Equivalent treatment of membranes of D3 cells caused an equivalent release of G_q/G_{11} and G_i2, but there was little release of the membrane-associated fraction of C3S $G_o1\alpha$ (Table 1).

DISCUSSION

The demonstration that the purified α subunit of G_o did not reconstitute effectively into phospholipid vesicles unless $\beta\gamma$ subunits were present [26] suggested that a key role in the maintenance of G-protein α subunits at the plasma membrane would be their interaction with the $\beta\gamma$ complex. It is believed that, on agonist activation of a receptor, exchange of GTP for GDP on the G-protein α subunit probably results in a physical dissociation of the α subunit from $\beta\gamma$. If the sole membrane anchorage of the α subunit was to be provided by its interaction with $\beta\gamma$, then it would be expected that receptor activation would cause the α subunit to migrate to the cytoplasm. Some recent studies by Levis and Bourne [27], which extend earlier work by Insel and co-workers [28] and by Rodbell [29], suggest a transitory alteration of the cellular location of at least G_s upon agonist stimulation of a receptor. Furthermore, a number of experiments have demonstrated a guanine nucleotide and/or receptor agonist-dependent release of G-proteins from a membrane fraction (see [30], for example). However, a series of other experiments which have utilized the maintained presence of poorly hydrolysed analogues of GTP have failed to produce a rapid transfer away from the membrane of a significant fraction of the G-protein population (see [31], for example).

The identification of the presence of myristate on the N-terminus of the G_i-like G-proteins offered a potential solution, particularly as mutation of the target glycine to alanine in $G_i1\alpha$ prevents myristoylation and has been reported to result in a diminution of membrane association of the expressed polypeptide [8,9]. However, G-proteins of the G_s family of G-proteins, although having a glycine residue at position 2, do not have serine at position 6, which appears to be essential for high-affinity recognition by N-myristoyltransferase [5]. We [13] and others [14–17] have recently demonstrated that many of the widely expressed G-protein α subunits are modified close to the N-terminus by the addition of thioester-linked palmitate. At least for $G_o1\alpha$ [13] and $G_s\alpha$ [15], mutation of cysteine-3 prevents incorporation of palmitate, and this amino acid is thus likely to be the target for modification, although potential caveats to this interpretation have been noted [15].

In this paper we have addressed a variety of questions relating to the importance of palmitoylation of $G_o1\alpha$ in regulating interactions of this G-protein both with the membrane and with the $\beta\gamma$ subunit, and in pertussis-toxin-catalysed ADP-ribosylation of this protein. To do so we have expressed stably in Rat 1 fibroblasts either wild-type $G_o1\alpha$ or a C3S palmitoylation-negative mutant of this polypeptide (Figure 1). As we have noted previously, after transient expression in COS cells, although this mutant is not palmitoylated, it remains a substrate for N-linked myristoylation [13]. This is the first report on the role of G-protein-attached palmitate which has used cell lines in which stable expression of a non-palmitoylated mutant of a G-protein has been achieved, rather than by simply using transient expression [13–15].

Initial studies involving homogenization of cells expressing either form of $G_o1\alpha$ and separation into particulate and cytosolic fractions, followed by immunoblotting to detect the distribution between these fractions of the steady-state level of this polypeptide, demonstrated an essentially exclusive membrane lo-

cation for wild-type $G_o1\alpha$. However, in such protocols a considerable fraction of the cellular C3S mutant of $G_o1\alpha$ was present in the cytosolic fractions (Figure 2). Such results are consistent with the concept that the palmitoylation status of a G-protein alters the avidity of interaction with the plasma membrane. Related studies in which cells were pulsed with [^{35}S]Trans label, separated into membrane and cytosol fractions and then immunoprecipitated with a $G_o\alpha$ antiserum to detect the presence of recently synthesized $G_o1\alpha$ confirmed the cytoplasmic location of a considerable fraction of C3S $G_o1\alpha$ after cellular disruption. However, it may be that cellular disruption is responsible for the appearance of C3S $G_o1\alpha$ in the cytoplasm. After transient transfection of either wild-type or a cysteine-3–alanine mutation of $G_s\alpha$ in COS cells, Degtyarev et al. [15] have reported both forms of this protein to be targeted to the particulate fraction of the cells. It may thus be that the palmitoylation status of a G-protein, even of one which is not also modified by the addition of myristate, does not define membrane association in an all-or-none manner. The issue of the role of palmitoylation in membrane association of G_s is currently controversial, however, as Wedegaertner et al. [17], by contrast, have recently reported that palmitoylation is required for the membrane attachment of $G_s\alpha$ and $G_q\alpha$, both of which are palmitoylated but not myristoylated.

Evidence that the bulk of the steady-state levels of both wild-type and C3S $G_o1\alpha$ are initially present at the membrane of the transfected Rat 1 cells was provided in a set of experiments designed initially to analyse whether both forms of the protein would act as substrates for the ADP-ribosyltransferase activity of pertussis toxin. Addition of ADP-ribose to the G_i family of G-proteins (of which G_o1 is a member) can result in a substantial decrease in the mobility of the polypeptide in SDS/PAGE. After treatment of the cells expressing wild-type $G_o1\alpha$ with pertussis toxin, immunoblotting of membrane fractions demonstrated that all of the cellular wild-type $G_o1\alpha$ had been modified, as it all migrated more slowly than that in membranes from untreated cells. Similar treatment of Rat 1 cells expressing C3S $G_o1\alpha$ also resulted in decreased mobility of the entire cellular population of C3S $G_o1\alpha$, whether it was present in the particulate or cytosolic fractions after disruption of the cell. As $G_o\alpha$ does not act as a substrate for pertussis-toxin-catalysed ADP-ribosylation in the absence of $\beta\gamma$ complex [25], then such data argue that the $G_o1\alpha$ found in the cytosolic fraction after cell disruption is associated with $\beta\gamma$, or was at the membrane before cell disruption, or was caught in the process of cycling between these compartments. To address these possibilities, we concurrently immunoblotted cytosolic fractions from cells expressing wild-type and C3S $G_o1\alpha$ for $G_o\alpha$ and for G-protein β-subunit, but were unable to detect the presence of β subunit in these fractions. Furthermore, when we chromatographed a cytosolic fraction from disrupted cells expressing C3S $G_o1\alpha$ on a Superose 12 gel-filtration column, this polypeptide migrated as a 40 kDa species (Figure 5), indicating its likely existence as a free monomeric α subunit. To address this point further, we performed pertussis-toxin-catalysed [^{32}P]ADP-ribosylation *in vitro* on membrane and cytosolic fractions prepared from both wild-type and C3S $G_o1\alpha$-expressing cells. Immunoprecipitation of $G_o\alpha$ after such treatments (to prevent interference from G_i1, G_i2 and G_i3, which are endogenously expressed by these cells, are also substrates for pertussis-toxin-catalysed ADP-ribosylation and migrate in similar position in SDS/PAGE [32]) demonstrated that both membrane-associated wild-type and C3S $G_o1\alpha$ were substrates for this reaction, but that the cytosolic C3S $G_o1\alpha$ was not. Furthermore, as pertussis-toxin-catalysed [^{32}P]ADP-ribosylation of the membrane-bound forms of both wild-type and C3S $G_o1\alpha$ was decreased to a similar extent by inclusion of p[NH]ppG in the assay, such experiments also provided suggestive evidence that both forms were able to interact equivalently with $\beta\gamma$ subunits. These data thus eliminate the first possibility, but cannot distinguish between the other two.

To address whether there were substantial differences in the ability of the wild-type and C3S forms of $G_o1\alpha$ to bind guanine nucleotides, we took advantage of a well-studied assay in which the trypsin-treatment pattern of $G_o\alpha$ (and of other G-proteins) is dependent on the nature of the bound guanine nucleotide (see [33], for example). In the presence of a poorly hydrolysed analogue of GTP, it is possible to produce a stabilized fragment of the G-protein which, although N-terminally clipped by some 2 kDa, is relatively resistant to further tryptic cleavage [33]. If the bound nucleotide is an analogue of GDP, the proteolytic process continues and the clipped form does not accumulate. When such experiments were performed with membranes containing either wild-type or C3S $G_o1\alpha$, the dose/effect curves for p[NH]ppG-induced protection of the 37 kDa trypsin-clipped form were very similar, indicating that the two forms have a similar ability to bind guanine nucleotides.

Thioester-linked palmitate can be removed from proteins by treatment with neutral 1 M hydroxylamine [34], and this has been used to define that the incorporation of [^{3}H]palmitate into G-proteins is via such a linkage [13,15,17]. To explore further the role of palmitoylation in interaction of $G_o1\alpha$ with the membrane fraction, membranes of cells transfected to express either wild-type or C3S $G_o1\alpha$ were exposed for 4 h to either 1 M hydroxylamine, pH 8.0, or 1 M Tris/HCl at the same pH as a control. After these treatments, samples were separated into particulate and supernatant fractions, and these were immunoblotted to detect the presence of various G-protein α subunits. Using the release from the particulate fraction produced by hydroxylamine minus any produced by the treatment with Tris/HCl as a measure of release produced by depalmitoylation, we noted that in cells expressing wild-type $G_o1\alpha$ some 25–30% of the total of this polypeptide and of the α subunits of G_q/G_{11} and G_i2 was released. However, although hydroxylamine treatment of membranes expressing the C3S mutant of $G_o1\alpha$ caused release of an equivalent proportion of both G_q/G_{11} and G_i2, little or none of the membrane-associated CS3 $G_o1\alpha$ was released. Obviously, as the C3S mutant is not palmitoylated, it would not be expected to be affected by hydroxylamine treatment of the membranes. It is an intriguing but currently unresolved issue as to why similar proportions of G-proteins which are in addition myristoylated (e.g. G_i2), as well as those which are only palmitoylated (G_q/G_{11}), were released from the particulate fraction by such treatment if myristoylation does indeed play a key role in membrane association of these polypeptides. However, as noted above, a recent report has indicated a key role for palmitoylation in the membrane attachment of both $G_s\alpha$ and $G_q\alpha$ [17], a suggestion supported by the data in the present paper.

An area which has received limited attention to date is the role of the cytoskeletal architecture of the cell in maintaining polypeptides involved in signal transduction cascades in their appropriate locations [35,36]. It is now clear, however, that many such polypeptides are associated with the cytoskeleton, and these interactions may be regulated by cellular activation. It will clearly be of interest to examine the role of lipid acylation in such interactions in future studies.

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