N-terminal binding domain of Gα subunits: involvement of amino acids 11–14 of $G\alpha_o$ in membrane attachment

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Heterotrimeric guanine nucleotide binding proteins (G-proteins) transmit signals from membrane receptors to a variety of intracellular effectors. G-proteins reversibly associate with components of the signal transduction system, yet remain membrane attached throughout the cycle of activation. The Gα subunits remain attached to the plasma membrane through a combination of factors that are only partially defined. We now demonstrate that amino acids within the N-terminal domain of Gα subunits are involved in membrane binding. We used *in vitro* translation, a technique widely utilized to characterize functional aspects of G-proteins, and interactions with donor–acceptor membranes to demonstrate that amino acids 11–14 of $G\alpha_o$ contribute to membrane binding. The membrane binding of $G\alpha_o$ lacking amino acids 11–14 (D[11–14]) was significantly reduced at all membrane concentrations in comparison with wild-type $G\alpha_o$. Several other N-terminal mutants of $G\alpha_o$ were characterized as controls, and these results indicate that differences in myristoylation, palmitoylation and βγ interactions do not account for the reduced membrane binding of D[11–14]. Furthermore, when membrane attachment of $G\alpha_o$ and mutants was characterized in transiently transfected ^{35}S-labelled and [^{3}H]myristate-labelled COS cells, amino acids 11–14 contributed to membrane binding. These studies reveal that membrane binding of Gα subunits occurs by a combination of factors that include lipids and amino acid sequences. These regions may provide novel sites for interaction with membrane components and allow additional modulation of signal transduction.

INTRODUCTION

Heterotrimeric guanine nucleotide binding proteins (G-proteins), made up of Gα and Gβγ subunits, transmit signals from plasma membrane receptors to a variety of intracellular effector enzymes and ion channels. Agonist-liganded hormone receptors cause Gα subunits to release GDP. The Gα subunits then bind GTP, dissociate from Gβγ, and both subunits remain activated until the intrinsic GTPase activity of Gα hydrolyses GTP to GDP (reviewed in [1–3]). During the cycle of activation, G-protein α and βγ subunits participate in reversible protein–protein interactions while remaining membrane attached. In addition to interactions of G-proteins with other components of the signal transduction system, G-proteins have been described as components in large molecular mass complexes and in association with other proteins [4–11]. Furthermore, proteins that regulate nucleotide hydrolysis (regulators of G-protein signalling) have recently been described [12,13].

The keys to signal transduction through G-proteins are the conformational changes induced in Gα upon GDP/GTP exchange. The regions of Gα that are conformationally sensitive to the bound nucleotide are known from crystal structures of Gα subunits and Gαβγ heterotrimers [14–20]. Regions of Gα that are conformationally sensitive to the state of activation are most likely to be involved in interactions with other components and potentially provide sites for regulation. The N- and C-termini, and switch regions I–IV are the major regions of Gα that undergo conformational changes. The N-terminus of Gα subunits is important to membrane binding for several reasons: (1) it contains the site(s) for lipid modifications (reviewed in [21]), (2) it is predicted to face the plasma membrane [16,20], and (3) it interacts with the more hydrophobic Gβγ subunits [22]. Detail of Gα N-terminal structure is incomplete; in several crystallized Gα subunits the N-terminus is either absent or disordered [14,17,18], but in crystallized heterotrimers [16,20] and in GDP-bound $G\alpha_{i1}$ [17], the N-terminus is α-helical. However, in all crystal structures analysed so far, the N-terminal lipid modifications are missing and the structure of the N-terminus in the activated conformation is unknown.

Some Gα families are myristoylated and palmitoylated near the N-terminus ($G\alpha_o$, $G\alpha_i$, and $G\alpha_z$), while other families are only palmitoylated ($G\alpha_s$, $G\alpha_q$) (reviewed in [21]). Many studies have demonstrated that these modifications affect Gα membrane binding [23–29], but other factors also play a role [22,30]. In several of these studies, mutant Gα subunits that were not lipid modified were still partially detected in the membrane fraction of transfected cells. Furthermore, activation of Gα by agonist-liganded-receptor leads to transient depalmitoylation [25,27,30,31] and dissociation from Gβγ. Taken together, these findings suggest that other features of Gα are likely to contribute to membrane attachment.

We have previously characterized a series of N-terminal mutations in $G\alpha_o$ for interactions with βγ using *in vitro* translation, a technique well established to yield functional G-protein subunits (reviewed in [32]). Gα subunits translated *in vitro* are a uniform population of molecules that exchange guanine nucleo-

Abbreviations used: G-proteins, guanine nucleotide binding proteins; D[11–14], $G\alpha_o$ protein deleted of amino acids 11–14; MDCK, Madin–Darby canine kidney; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; GTP[S], guanosine 5′-[γ-thio]triphosphate; DTT, dithiothreitol.

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tides, reversibly associate with $\beta\gamma$ and are myristoylated [33–35]. Palmitoylation does not occur *in vitro*, probably due to the absence of palmitoyltransferase in the rabbit reticulocyte lysate. This allows membrane binding *in vitro* to be studied in the absence of palmitoylation, a transient, but critical intermediate state of most Gα subunits. Since there are multiple factors that contribute to membrane binding of Gα, important regions may be identified *in vitro* and then analysed in intact cells. We used this combined approach to demonstrate that four amino acids near the N-terminus of Gα_o (amino acids 11–14) are involved in membrane binding.

EXPERIMENTAL

Mutations

Wild-type rat Gα_o cDNA was obtained from Dr. R. R. Reed (Johns Hopkins University) and all mutations were made by PCR and cloned into Bluescript (Stratagene) as previously described [33]. D[11–14]C2A and C2A (see Table 1) utilized an *Xba*I site in the 5′-primer and the PCR product was digested with *Xba*I/*Pst*I. Gα_o in Bluescript was digested with *Xba*I/*Pst*I and the appropriate fragments were ligated and cloned using standard techniques. All cDNA sequences were confirmed by T7 double stranded DNA sequencing (U.S. Biochemical Corp.).

Translation *in vitro*

cDNAs (1 μg) for wild-type or mutant Gα_o in Bluescript were transcribed and translated *in vitro* (final volume 50 μl) using a coupled reticulocyte lysate system (TNT system, Promega, Madison, WI, U.S.A.) and [^{35}S]methionine (Amersham; 20 μCi/reaction) as previously described [36].

Binding to acceptor membranes

Acceptor membranes were prepared from cultured Madin–Darby canine kidney (MDCK) cells grown to confluence in Dulbecco's Modified Eagle's Medium (DMEM) containing 5 % (v/v) fetal bovine serum (FBS). Cells were scraped, sonicated at 4 °C in buffer A [50 mM Tris/HCl (pH 7.6)/6 mM $MgCl_2$/75 mM sucrose/1 mM dithiothreitol (DTT)/1 mM EDTA/soy and lima bean trypsin inhibitors (1 μg/ml)/3 mM benzamidine]. The cell homogenate was ultracentrifuged at 100000 ***g*** for 1 h at 4 °C, and the particulate fraction was washed and resuspended by sonication in NTE buffer [10 mM Tris/HCl (pH 7.4)/100 mM NaCl/1 mM EDTA]. MDCK cell membrane concentration was determined by the method of Bradford according to the manufacturer's directions (Bio-Rad), and aliquots were stored at −70 °C and used as acceptor membranes. To assay for membrane binding, *in vitro* translated proteins (2–10 μl) were incubated with acceptor membranes (0–5 mg/ml) in NTE and 5 μM GDP (final volume 50 μl) at 25 °C for 30 min. NTE (150 μl) was added and the mixture centrifuged at 100000 ***g*** for 1 h at 4 °C to resolve soluble and particulate fractions. The particulate fraction was resuspended in 200 μl of NTE, and equal volumes of soluble and particulate fractions were analysed by SDS/PAGE (11 % w/v gels) [24], stained with Coomassie Blue, soaked in ENHANCE (Du Pont–New England Nuclear), dried and then exposed to Fuji AR film for 1–2 days at −70 °C. The percentage of the total in each fraction was determined by desktop scanning with a Hewlett Packard ScanJet and quantified using NIH Image 1.59/fat (Wayne Rasband, NIH, Bethesda, MD, U.S.A.).

Sucrose density gradient centrifugation

Sucrose density gradients (5–20 %, w/v) were made up in 50 mM Tris/HCl, pH 7.6, 1 mM EDTA, 1 mM DTT, 0.3 % (v/v) Triton X-100, 1 mM $MgCl_2$, and 100 μM GDP or 20 μM guanosine 5′-[γ-thio]triphosphate (GTP[S]). Samples of *in vitro* translated Gα subunits (10–20 μl) were mixed with purified Gα_o (1 μg) and $\beta\gamma$ (3 μg) from bovine brain [18] and incubated with either GDP or GTP[S] for 15 min at 30 °C. Markers were added [100 μg of BSA ($s_{20,w} = 4.3$), ovalbumin ($s_{20,w} = 3.5$) and carbonic anhydrase ($s_{20,w} = 2.9$)] and the mixture centrifuged at 52000 rev./min for 16 h at 4 °C as previously described [37]. Gradient fractions were analysed by SDS/PAGE as described above. Position of the peaks of marker proteins and radiolabelled proteins were determined by laser densitometry of autoradiograms or of the stained gels.

Transient transfections

Each cDNA listed in Table 1 was excised from Bluescript and cloned into eukaryotic expression vector pcDNA 3 (Invitrogen) using *Hin*dIII/*Xba*I or *Xba*I/*Apa*I sites depending upon the orientation in Bluescript. COS cells were maintained in DMEM with 10 % (v/v) fetal calf serum and when 50–80 % confluent were transfected in P-60 dishes with 4 μg of DNA using LipofectAMINE® (GibcoBRL). Cells were incubated with the DNA for 3 h in serum-free Opti-MEM I (GibcoBRL). FBS was then added to a final concentration of 4 % (v/v) and the incubation was continued for a total time of 24 h. The cells were washed with fresh media and kept in DMEM containing 10 % FBS until analysis at 48–72 h.

Metabolic labelling, subcellular fractionation and immunoprecipitation

For ^{35}S-labelling, transfected COS-7 cells were incubated for 30 min in DMEM containing 5 % (v/v) dialysed serum but without methionine and cysteine. The medium was changed and Trans [^{35}S]-Label® (> 1000 Ci/mmol, ICN Radiochemicals) was added to a final concentration of 200 μCi/ml. The incubation was continued for 3 h and the cells processed as described below. For labelling with [^{3}H]myristic acid, cells were incubated with [9,10(n)-^{3}H]myristic acid (53 Ci/mmol; Amersham Life Science) for 16 h at 200 μCi/ml in DMEM/10 % FBS. For [^{3}H]palmitic acid labelling, the cells were prepared by incubation in serum-free DMEM for 2 h followed by 30 min in 50 μg/ml cycloheximide (Calbiochem) in serum-free media. Cycloheximide prevents protein synthesis and co-translational myristoylation. Metabolic labelling was done for 30 min with 500 μCi/ml [^{3}H]palmitic acid ([9,10(n)-^{3}H]palmitate, 52 Ci/mmol, Amersham) and 50 μg/ml cycloheximide. Cells were washed with cold PBS, scraped, and centrifuged at low speed for 5 min. The pellets were resuspended in 500 μl of buffer A, frozen and thawed three times in liquid nitrogen and passed 15 times through a 27 gauge needle. After spinning for 5 min at low speed, the homogenates were ultracentrifuged at 100000 ***g*** for 1 h at 4 °C to prepare soluble and particulate fractions. The particulate fraction was washed with buffer A, then incubated at 4 °C for 75 min with 50 mM Tris/HCl (pH 7.4)/1 % Triton X-100. The samples were again ultracentrifuged for 1 h at 100000 ***g*** to obtain solubilized membranes and Triton X-100-insoluble pellet. Immunoprecipitations were done in buffer B [10 mM Tris/HCl (pH 7.4)/1 % Triton X-100/0.1 % (v/v) SDS/1 % sodium deoxycholate] after preclearing with protein A–Sepharose (Sigma). A

Table 1 N-terminal sequences of Gα subunits

Dashes in sequences indicate deletions; dots at the ends represent continuation of sequence. Point mutations are underlined. The N-terminal glycine is essential for myristoylation; Cys-2 is the site for palmitoylation in $G\alpha_o$ and $G\alpha_{i2}$.

	Sequence																									
Name	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26 ...
Rat $G\alpha_o$	G	C	T	L	S	A	E	E	R	A	A	L	E	R	S	K	A	I	E	K	N	L	K	E	D	G...
D[11–14]α_o	G	C	T	L	S	A	E	E	R	A	–	–	–	–	S	K	A	I	E	K	N	L	K	E	D	G...
D[11–14]C2Aα_o	G	A	T	L	S	A	E	E	R	A	–	–	–	–	S	K	A	I	E	K	N	L	K	E	D	G...
C2Aα_o	G	A	T	L	S	A	E	E	R	A	A	L	E	R	S	K	A	I	E	K	N	L	K	E	D	G...
G1A	A	C	T	L	S	A	E	E	R	A	A	L	E	R	S	K	A	I	E	K	N	L	K	E	D	G...
Bovine $G\alpha_t$	G	A	G	A	S	A	E	E	K	H	–	–	–	–	S	R	E	L	E	K	K	L	K	E	D	A...
Rat $G\alpha_{i2}$	G	C	T	V	S	A	E	D	K	A	A	A	E	R	S	K	M	I	D	K	N	L	R	E	D	G...

1:100 dilution of a polyclonal rabbit antiserum to $G\alpha_o$ (R4; generously given by Dr. E. Neer, Cardiology Division, Brigham and Women's Hospital, Boston, MA, U.S.A.) was added overnight at 4 °C. Protein A–Sepharose was added for 1 h, the samples centrifuged, and the pellets washed extensively with buffer B. The immunoprecipitates were eluted with SDS/PAGE sample buffer containing either 5 mM DTT (for [^{3}H]palmitate-labelled samples) or 50 mM DTT (all others). Immunoprecipitated proteins were analysed by SDS/PAGE and autoradiography as described above.

RESULTS AND DISCUSSION

Gα subunit families differ in the factors that promote membrane attachment, and this heterogeneity may provide unique sites for regulation of signalling. Even within the same Gα family, there may be important differences in the factors that contribute to membrane attachment. For example, the pertussis toxin family includes $G\alpha_o$ and $G\alpha_{i1-3}$, which are myristoylated and palmitoylated. However, $G\alpha_t$, another pertussis toxin family member, is not palmitoylated and is modified by a variety of lipids on the N-terminal glycine [38]. We compared a series of N-terminal mutations in $G\alpha_o$ with wild-type $G\alpha_o$ for the ability to interact with membranes *in vitro* and in transiently transfected COS cells. The association of proteins with cellular membranes *in vitro* has been extensively used as a technique to characterize protein–membrane interactions [36,39–43]. Expression of wild-type and mutant Gα subunits in transfected cells has been the main approach to studying the roles of lipid modifications in membrane binding (reviewed in [21]). Table 1 summarizes the N-terminal sequences of wild-type $G\alpha_o$ and mutants that were characterized by acceptor membrane binding *in vitro* and in transfected cell studies.

Lipid modification of proteins on the N-terminal glycine by myristic acid has been extensively studied (reviewed in [44]). Myristate (C14:0) is not sufficiently hydrophobic to guarantee membrane attachment of a protein, and many myristoylated proteins are found in the soluble fraction of cells [44]. Myristate is added co-translationally to the N-terminal glycine by *N*-myristoyltransferase after synthesis of the initial 7–8 amino acids and is probably irreversible. Mutation of the N-terminal glycine to alanine in $G\alpha_o$ or $G\alpha_i$ blocks myristoylation and results in predominantly cytosolic localization in transfected cells (see Figure 5; [26,27,30,45]). The absence of myristoylation also reduces the affinity of Gα for Gβγ [28,33]. We tested wild-type $G\alpha_o$ and G1A (non-myristoylated $G\alpha_o$) translated *in vitro* for the ability to interact with acceptor membranes. *In vitro* translated

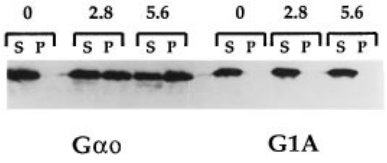

Figure 1 Binding of wild-type $G\alpha_o$ and G1A translated *in vitro* to acceptor membranes

[^{35}S]Methionine labelled, *in vitro* translated $G\alpha_o$ and G1A (5 μl) were incubated without (0) or with two concentrations (2.8 and 5.6 mg/ml) of acceptor membranes prepared from MDCK cells as described in the Experimental section. Samples were incubated at 30 °C for 20 min followed by 100000 ***g*** centrifugation into soluble (S) and particulate (P) fractions and analysed by SDS/PAGE and autoradiography. The gel was exposed for 24 h at −70 °C with two enhancing screens.

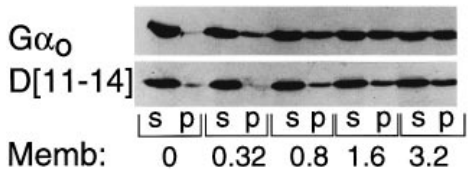

Figure 2 Comparison of $G\alpha_o$ and D[11–14] binding to acceptor membranes

$G\alpha_o$ and D[11–14] were translated *in vitro* and incubated with a range of acceptor membranes (0–3.2 mg/ml). For each concentration of membrane, the samples were separated into soluble (s) and particulate (p) fractions and analysed as described in the Experimental section. Results of five experiments are summarized in Table 2.

$G\alpha_o$ and G1A were incubated at several concentrations of acceptor membranes and separated into soluble and particulate fractions by ultracentrifugation as described in the Experimental section. Figure 1 shows that in the absence of membranes, neither subunit is precipitated at 100000 ***g***. In the presence of two different amounts of acceptor membranes, 50–60% of $G\alpha_o$ associates with membranes and sediments in the particulate fraction. However, G1A (non-myristoylated) remains almost completely soluble which is consistent with results in transfected cells.

N-terminal mutations in $G\alpha_o$ that were previously characterized *in vitro* for βγ binding were studied for interactions with acceptor membranes [33]. Since Gβγ is more hydrophobic than Gα, and may provide some hydrophobic interactions that contribute to anchoring Gα in the membrane [22], we analysed

Table 2 Summary of binding of *in vitro* translated $G\alpha_o$ and D[11–14] to acceptor membranes

Values are the mean ± S.E.M. percentages of subunit associated with the particulate fraction ($n = 5$ for $G\alpha_o$; $n = 4$ for D[11–14]). Binding of *in vitro* translated $G\alpha_o$ and D[11–14] to increasing amounts of acceptor membrane was analysed as described in Figure 2. Autoradiograms were scanned on a Hewlett Packard desktop scanner and the density of each band determined by analysis using NIH Image 1.59/fat (Wayne Rasband, NIH). The percentage bound was calculated using arbitrary units obtained from NIH Image in the following equation: (P units)/(S + P units) × 100, where S and P are soluble and particulate fractions respectively. At each concentration of membrane (except 0), the percentage bound was significantly lower for D[11–14] than for $G\alpha_o$ ($P < 0.005$).

	Binding (%)				
Membrane concentration (mg/ml)	0	0.32	0.8	1.6	3.2
Wild-type $G\alpha_o$	2 ± 2	7 ± 2	27 ± 2	40 ± 1	51 ± 1
D[11–14]	2 ± 2	3 ± 1	8 ± 1	20 ± 1	25 ± 1

Table 3 Relative myristoylation of *in vitro* translated mutant $G\alpha_o$ subunits and sedimentation values

Incorporation of [^{3}H]myristic acid into mutant $G\alpha_o$ subunits translated *in vitro* was determined by parallel *in vitro* translates of each cDNA with [^{3}H]myristic acid and [^{35}S]methionine. The labelled proteins were quantified from autoradiograms (described in Table 2) and results are expressed as the ratio of [^{3}H]myristic acid/[^{35}S]methionine relative to wild-type $G\alpha_o$; $n = 6$ for each protein (mean ± S.E.M.). Wild-type $G\alpha_o$ is defined as 1.0. The $s_{20,w}$ values were determined for C2A and D[11–14]C2A with bovine brain $\beta\gamma$ (3 μg) in GDP or GTP[S] as described in the Experimental section. The peak width for each $G\alpha_o$ subunit was similar to the marker proteins, and the $s_{20,w}$ values for wild-type $G\alpha_o$ and D[11–14] have been previously reported [33]. The values are means ± S.D. or range (when $n = 2$) and the number of experiments is shown in parentheses.

		Sedimentation coefficient $s_{20,w}$ (S)	
Mutation	Relative [^{3}H]myristic acid	GDP	GTP[S]
Wild-type $G\alpha_o$	1.0	–	–
D[11–14]	1.0 ± 0.04	–	–
C2A	0.9 ± 0.14	4.1 ± 0.1 (3)	3.1 ± 0.1 (2)
D[11–14]C2A	1.1 + 0.16	3.9 + 0.1 (2)	3.0 + 0.1 (3)

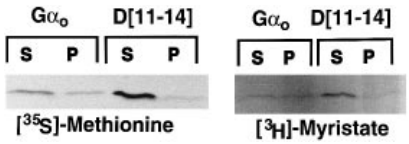

Figure 3 Binding of [^{3}H]myristate- and [^{35}S]methionine-labelled $G\alpha_o$ and D[11–14] *in vitro*

Parallel *in vitro* translates of $G\alpha_o$ and D[11–14] with labelled [^{3}H]myristate or [^{35}S]methionine were incubated with 1.5 mg/ml acceptor membranes under identical conditions as described in the Experimental section. The results of binding with [^{35}S]methionine-labelled proteins are shown on the left and the binding results with [^{3}H]myristate-labelled proteins on the right. The soluble (S) and particulate (P) fractions are shown and the percentage bound was calculated as described in Table 2 legend. The percentage of [^{35}S]methionine- and [^{3}H]myristate-labelled $G\alpha_o$ found in the particulate fraction was similar (30 ± 5%, [^{35}S]; 37 ± 2%, [^{3}H]). The amounts of [^{35}S]methionine- and [^{3}H]myristate-labelled D[11–14] in the particulate fraction were also similar (10 ± 3%, [^{35}S]; 10 ± 5%, [^{3}H]). These values are the mean ± range for two experiments. The [^{35}S]methionine autoradiograms were exposed overnight and the exposure for the [^{3}H]myristate gel was 1.5 months.

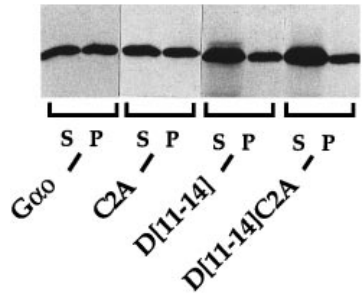

Figure 4 Binding of Cys-2 point mutations *in vitro*

In vitro translated $G\alpha_o$ and D[11–14] were compared with the corresponding Cys-2 point mutations (palmitoylation site). *In vitro* translates (2–5 μl) were incubated with 3 mg/ml acceptor membranes and analysed as described in the Experimental section. The soluble (S) and particulate (P) fractions are shown. In the absence of added membranes, each protein was found only in the soluble fraction (not shown). The percentage bound to the membrane fraction (defined in Table 2) is 58 ± 1 for $G\alpha_o$ ($n = 12$), 50 ± 2 for C2A ($n = 4$), 33 ± 1 for D[11–14] ($n = 12$) and 23 ± 2 for D[11–14]C2A ($n = 5$). Values are means ± S.E.M.

N-terminal mutations that do not affect interactions with $\beta\gamma$. Deletion of amino acids 11–14 of $G\alpha_o$ does not affect $G\beta\gamma$ binding by several techniques [33], and alignment of $G\alpha_o$ with $G\alpha_t$ reveals that amino acids 11–14 of $G\alpha_o$ are not found in $G\alpha_t$ (Table 1). $G\alpha_t$ binds $G\beta\gamma$ without these four amino acids and further supports the notion that they are not required for interactions with $G\beta\gamma$. D[11–14] was compared with wild-type $G\alpha_o$ for binding to a range of acceptor membrane concentrations (Figure 2 and Table 2). At each membrane concentration, there was a significant reduction ($P < 0.005$) in the fraction of D[11–14] that was membrane bound when compared with wild-type $G\alpha_o$ (Figure 2 and Table 2).

The reduced membrane binding of D[11–14] could also occur if there are differences between $G\alpha_o$ and D[11–14] in myristoylation, palmitoylation or $\beta\gamma$ interactions. Since myristoylation is required for membrane binding of $G\alpha_o$ (Figure 1), differences in myristoylation occurring *in vitro* could account for the results in Figure 2. Table 3 compares D[11–14] and wild-type $G\alpha_o$ for the fraction of protein that is myristoylated *in vitro* in comparison to the total amount of translated protein. Parallel *in vitro* translations with [^{35}S]methionine and [^{3}H]myristic acid were done and the autoradiograms quantified for [^{3}H]myristate incorporation as a fraction of [^{35}S]methionine incorporation. The results were normalized to the wild-type control and indicate that D[11–14] is myristoylated *in vitro* like wild-type $G\alpha_o$. Furthermore, we specifically excluded the possibility that the failure of some protein to bind to membranes was due to its lack of myristoylation. Figure 3 compares the binding of *in vitro* translated [^{3}H]myristate-labelled and [^{35}S]methionine-labelled wild-type $G\alpha_o$ and D[11–14]. The percentages of labelled proteins found in particulate and soluble fractions were nearly identical irrespective of whether they were [^{3}H]myristate- or [^{35}S]methionine-labelled. In addition, this was true for both wild-type $G\alpha_o$ and D[11–14] and confirms the finding that there are no significant differences in myristoylation of $G\alpha_o$ and D[11–14] *in vitro*.

To be sure that palmitoylation does not occur during acceptor membrane binding, point mutations in $G\alpha_o$ and D[11–14] were made to block the palmitoylation site at Cys-2 [46] (C2A, D[11–14]C2A; Table 1). C2A and D[11–14]C2A were compared with wild-type $G\alpha_o$ and D[11–14] for binding to acceptor membranes (Figure 4). There was no significant difference in binding of $G\alpha_o$ and C2A to acceptor membranes (Figure 4), nor

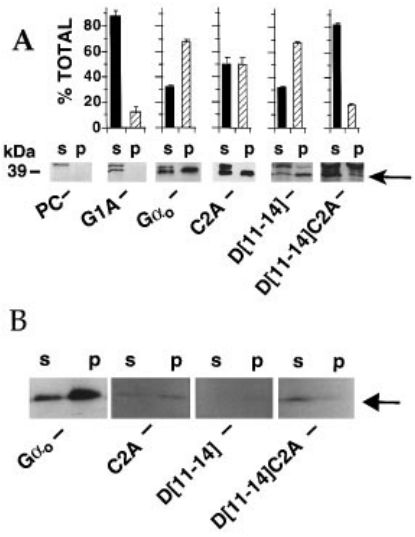

Figure 5 Localization of $G\alpha_o$, D[11–14], C2A and D[11–14]C2A transiently transfected in COS cells

(**A**) $G\alpha_o$ and mutants in pcDNA 3 (4 μg) were transfected and biosynthetically labelled as described in the Experimental section. All experiments included pcDNA 3 without insert and wild-type $G\alpha_o$ as controls. After labelling, cells were scraped, broken, and ultracentrifuged at 100000 ***g*** to yield soluble and particulate fractions. Immunoprecipitations were done using the soluble (s) and Triton X-100-extractable portion of the particulate fraction (p) as described in the Experimental section. A representative immunoprecipitation from ^{35}S-labelled cells is shown for wild-type $G\alpha_o$ and each mutant. The 39 kDa size is noted on the left, and the arrow on the right indicates the position of $G\alpha_o$ subunits. A background band above 39 kDa was seen in most immunoprecipitations, but was not included in the quantification. The results of all experiments are summarized above the autoradiograms. The percentages of total subunit in the soluble and particulate fractions was calculated from arbitrary units obtained after desktop scanning and analysis in NIH Image as described in Table 2 legend. Values are plotted as the mean $\pm$ S.E.M. ($n = 3$ for G1A; 8 for $G\alpha_o$; 3 for C2A; 4 for D[11–14], and 8 for D[11–14]C2A). The difference between C2A and D[11–14]C2A for particulate binding was highly significant ($P = 0.006$). (**B**) Binding of [^{3}H]myristate-labelled $G\alpha_o$ and mutants in transfected cells. The percentage of [^{3}H]myristate-labelled subunits in the particulate fraction was 72% for wild-type $G\alpha_o$; 52% for C2A; 73% for D[11–14] and 20% for D[11–14]C2A. These values are similar to the percentage bound obtained for [^{35}S]methionine-labelled proteins in (**A**). Autoradiograms of [^{35}S]methionine-labelled proteins were exposed for 24–72 h, and [^{3}H]myristate-labelled proteins for 2–6 weeks.

was there a significant difference between D[11–14] and D[11–14]C2A. However, the differences in binding between the full-length proteins ($G\alpha_o$, C2A) and the truncated proteins (D[11–14], D[11–14]C2A) was highly significant ($P < 0.001$). Since *in vitro* translated $G\alpha_o$ has hydrodynamic properties similar to brain-purified $G\alpha_o$, it was expected that C2A and D[11–14]C2A would interact with $\beta\gamma$ in a similar manner to wild-type $G\alpha_o$. This was confirmed by determining the $s_{20,w}$ values for C2A and D[11–14]C2A with $\beta\gamma$ in the presence of GDP or GTP[S] (Table 3). Both *in vitro* translated C2A and D[11–14]C2A reversibly associated with exogenous $\beta\gamma$ and sedimented as a heterotrimer in GDP, but as a monomer in GTP[S]. The $s_{20,w}$ values in Table 3 are similar to expected values obtained previously with *in vitro* translated $G\alpha_o$ and brain-purified proteins [33,47]. Consistent with these results, the fraction of *in vitro* translated C2A and D[11–14]C2A that is myristoylated was similar to wild-type $G\alpha_o$ (Table 3).

To determine the involvement of amino acids 11–14 of $G\alpha_o$ in membrane attachment in transfected cells, G1A, $G\alpha_o$, D[11–14], C2A, and D[11–14]C2A (Table 1) were localized in transiently transfected COS cells (Figure 5). Cells were labelled with [^{35}S]methionine/cysteine, fractionated into soluble and particulate fractions as described in the Experimental section and immunoprecipitated with polyclonal anti-$G\alpha_o$ antibody. The majority of $G\alpha_o$ proteins found in the particulate fraction were Triton X-100-soluble, consistent with the absence of aggregation. For $G\alpha_o$ and each mutant, 10–20% of the total $G\alpha_o$ subunit in COS cells was Triton X-100-insoluble (not shown). The Triton X-100-insoluble fraction of $G\alpha_o$ subunits could result from misfolding, aggregation, or association with specialized membrane domains (such as caveolae). Since the physiological state of $G\alpha_o$ subunits in the detergent-insoluble fraction is unknown, it was not included in calculating the percentage of $G\alpha_o$ that is membrane-attached in COS cells. Figure 5 shows the results of particulate (p; Triton X-100-extractable) versus soluble (s; cytosolic) localization for the $G\alpha_o$ subunits. The top of Figure 5(A) quantifies the percentage of the total accessible $G\alpha_o$ or mutants found in the soluble and particulate fractions for all experiments ($n = 3$–8; see legend to Figure) and the bottom shows a representative autoradiogram of the immunoprecipitated fractions. As reported by others, G1A (non-myristoylated) is predominantly soluble and C2A (non-palmitoylated) is approximately evenly distributed in the soluble and particulate fractions. D[11–14] is found predominantly in the particulate fraction, like wild-type $G\alpha_o$, due to the contribution from palmitoylation that occurs in COS cells at the plasma membrane. [^{3}H]Palmitate labelling was not detected in C2A and D[11–14]C2A, and [^{3}H]palmitate-labelled $G\alpha_o$ and D[11–14] were found exclusively in the particulate fraction (not shown). However, when palmitoylation is blocked, D[11–14]C2A localizes almost completely in the soluble fraction. This is significantly different from the percentage of C2A in the particulate fraction of transfected cells ($P = 0.006$). Table 1 shows that D[11–14]C2A and C2A differ only in amino acids 11–14, and these results reveal that amino acids 11–14 are involved in membrane attachment in transfected cells. The subcellular localization of the myristoylated subunits is shown in Figure 5(B). [^{3}H]Myristate-labelled subunits were localized to the soluble and particulate fractions as described in the Experimental section. Similar to results obtained *in vitro* (Figure 3), the myristate-labelled subunits have a distribution similar to the [^{35}S]methionine/cysteine-labelled proteins (Figure 5A). This finding confirms that differences in myristoylation among the $G\alpha_o$ subunits do not account for the differences in membrane binding in transfected cells.

The involvement of amino acids 11–14 of $G\alpha_o$ in membrane binding is the first demonstration that amino acid structure can influence membrane attachment of $G\alpha$ subunits. This is interesting not only with regard to a role in maintaining membrane attachment during the signalling cycle, but also because it may provide another point for regulating $G\alpha$ function. The techniques used in these studies cannot distinguish the component(s) (protein, lipid, etc.) in the membrane that interact with these amino acids. Other approaches will be needed to quantify binding and determine the affinities of the mutant $G\alpha_o$ subunits for the membrane. Nevertheless, these results clearly demonstrate *in vitro* and in transfected cells that amino acids 11–14 of $G\alpha_o$ are involved in membrane attachment. Since $G\alpha$ subunits are in dynamic equilibrium with many membrane components during the cycle of $G\alpha$ activation, the relative importance of various membrane binding factors is likely to vary throughout the cycle. Amino acids 11–14 of $G\alpha_o$ are highly conserved in the $G\alpha_i$ family (Table 1) and a similar role in membrane attachment is likely for members of the $G\alpha_i$ family. Sequence comparisons of the N-termini of $G\alpha_o$ with $G\alpha_s$, $G\alpha_z$, or $G\alpha_q$ do not identify amino acid stretches that are homologous to 11–14 of $G\alpha_o$. Since $G\alpha_s$ and $G\alpha_q$ families contain a single, reversible lipid modification (palmitate), it is probable that amino acid sequence(s) will be important contributors to membrane attachment. Some studies

have suggested that the C-terminus of $G\alpha_s$ may contain amino acid sequence important for membrane attachment [48,49], but another study failed to find this effect [50]. The N-terminus of $G\alpha_q$ contains a hydrophobic region that is distinct from palmitate [51], but it is not yet known if this hydrophobic region contributes to membrane binding.

Lipid modifications and protein sequence(s) can act in concert to promote membrane binding for other signalling molecules. Some members of the Src-related family of tyrosine kinases utilize myristoylation and palmitoylation or myristoylation and a polybasic region within the N-terminus for membrane binding (reviewed in [52]). Myristoylated alanine-rich protein kinase C substrate utilizes a polybasic domain and a single lipid (myristate) [53]. Our results support a similar, although more complicated, set of factors for the membrane attachment of $G\alpha$ subunits. A combination of one or more lipids, in addition to protein sequence(s), and interactions with $G\beta\gamma$ all contribute to membrane binding of $G\alpha$. For $G\alpha_o$ and $G\alpha_i$ subunits, two lipid modifications (one of which is reversible) in combination with amino acid sequence(s) potentially provide multiple sites for modulation of membrane interactions. The diversity in mechanisms of membrane attachment among $G\alpha$ subunits may be important to the wide range of cellular responses modulated by G-proteins. Future studies will be aimed at identifying regions in other $G\alpha$ subunits important for membrane binding, and will test the hypothesis that unique interactions with other membrane components occur through these sites.

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