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Palmitoylation alters protein activity: blockade of G_o stimulation by GAP-43

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The addition of palmitate to cysteine residues enhances the hydrophobicity of proteins, and consequently their membrane association. Here we have investigated whether this type of fatty acylation also regulates protein–protein interactions. GAP-43 is a neuronal protein that increases guanine nucleotide exchange by heterotrimeric G proteins. Two cysteine residues near the N-terminus of GAP-43 are subject to palmitoylation, and are necessary for membrane binding as well as for G_o activation. N-terminal peptides, which include these cysteines, stimulate G_o. Monopalmitoylation reduces, and dipalmitoylation abolishes the activity of the peptides. The activity of GAP-43 protein purified from brain also is reversibly blocked by palmitoylation. This suggests that palmitoylation controls a cycle of GAP-43 between an acylated, membrane-bound reservoir of inactive GAP-43, and a depalmitoylated, active pool of protein.

Key words: GAP-43/growth cone/GTP binding protein/palmitoylation

Introduction

Most types of post-translational protein modification, such as glycosylation, proline hydroxylation, proteolysis and C-terminal amidation, are permanent, or at least long-lived relative to the protein's half-life. Although critical to protein function, their persistence does not allow for dynamic signaling opportunities as does, for example, addition and removal of phosphate. Such a dynamic regulatory cycle may exist for the addition of palmitate. This 16 carbon saturated fatty acid is linked to cysteine residues via a thioester in a number of proteins, including ankyrin, major histocompatibility antigens, transferrin receptor, *ras*-like proteins, proteolipid protein of myelin, viral coat proteins, rhodopsin and the β_2-adrenergic receptor (for review, Sefton and Buss, 1987; Schultz *et al.*, 1988). Unlike other lipid modifications of proteins, such as myristoylation, isoprenylation and addition of glycosyl-phosphatidylinositol, palmitic acid turns over with a much shorter half-life than that of the protein (Staufenbiel and Lazarides, 1986; Magee *et al.*, 1987; Sefton and Buss, 1987; Schultz *et al.*, 1988; Skene and Virag, 1989). This rapid turnover and the variation of palmitoylation levels with extracellular stimuli (Huang, 1989; James and Olson, 1989; Jochen *et al.*, 1991) strongly suggest that addition or removal of palmitate regulates the activity of proteins.

One known function of palmitoylation is the enhancement of membrane binding (Hancock *et al.*, 1989; Pearce *et al.*, 1991). In addition, it is conceivable that palmitoylation might change protein activity by specifically altering an active site or by inducing a more general conformational change. Mutations in the palmitoylated cysteines in the β_2-adrenergic receptor, transferrin receptor and *ras* proteins do alter protein activity (Hancock *et al.*, 1989; O'Dowd *et al.*, 1989; Alvarez *et al.*, 1990; Jing and Trowbridge, 1990). This certainly demonstrates the importance of the palmitoylated domains to protein function, but the interpretation of the results is complicated since the effects could be attributable to the absence of palmitate, to the substituted amino acid residue or to altered subcellular localization. Here, we sought direct evidence of a specific protein activity regulated by palmitoylation.

GAP-43 is a neuronal protein which is highly enriched in the tips of extending processes, a region termed the growth cone (Meiri *et al.*, 1986; Skene *et al.*, 1986; reviewed in Skene, 1989). This localization, the protein's massive induction during nerve regeneration (Skene and Willard, 1981; Benowitz and Lewis, 1983) and its increased gene expression during development (Karns *et al.*, 1987), among other data, have suggested that GAP-43 may control growth cone motility (Skene, 1989; Zuber *et al.*, 1989a; Shea *et al.*, 1991; Strittmatter and Fishman, 1991).

At a molecular level, GAP-43 may function by altering the activity of G_o (Strittmatter *et al.*, 1990). This heterotrimeric GTP binding protein is also enriched in the growth cone membrane, where it is the major non-cytoskeletal protein (Strittmatter *et al.*, 1990). G_o may transduce many of the extracellular signals which determine the extent and direction of axonal growth, some of which are known to bind to G protein-linked receptors (Kater and Mills, 1991; Strittmatter and Fishman, 1991). Agents which alter G protein activity have dramatic effects on neurite outgrowth in tissue culture (Doherty *et al.*, 1991; Strittmatter *et al.*, 1992). The ability of GAP-43 to stimulate GTPγS binding to G_o may alter the response of the growth cone G protein-based signal transduction system to extracellular signals. Whether some of the other properties of GAP-43, such as calmodulin binding (Chapman *et al.*, 1991), phosphorylation by protein kinase C (Lovinger *et al.*, 1985) or its ability to regulate neurotransmitter release (Dekker *et al.*, 1989), are separate functions or are related to G protein coupling is not known. The G protein interacting domain of GAP-43 has been localized to the N-terminal 25 amino acids (Strittmatter *et al.*, 1990).

Palmitoylation of GAP-43 occurs on the only two cysteine residues in the molecule, at positions 3 and 4 (Skene and Virag, 1989), within the region necessary for G protein

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stimulation. This modification probably contributes to the membrane binding of the otherwise hydrophilic GAP-43 protein (Skene and Virag, 1989; Zuber *et al.*, 1989b; Liu *et al.*, 1991). Since palmitoylation affects a domain also important for interaction with G_o, it seemed reasonable that palmitoylation would provide a means to regulate this interaction as well. Our observations here show that GAP-43 palmitoylation blocks its activation of G_o. Thus, we suggest that palmitic acid not only serves to help localize the protein to the plasma membrane, but also to conceal sulfhydryl groups, or otherwise modify GAP-43 conformation, so as to regulate its interaction with G_o.

Results

GAP-43 N-terminal peptides stimulate GTPγS binding to G_o

The activity of G_o can be assessed by quantitating its guanine nucleotide binding characteristics. Previously, we showed that GAP-43 and a peptide composed of the N-terminal 25 residues (1–25) are equally effective in stimulating GTPγS binding to G_o (Strittmatter *et al.*, 1990). To localize this stimulatory domain better within GAP-43, we examined a series of N-terminal peptides of increasing length, from 1–6 through 1–25, for their effect on GTPγS binding to G_o. The 1–20 and 1–25 peptides stimulate GTPγS binding as effectively as does the GAP-43 protein itself (Figure 1). The 1–10 and 1–15 peptides are only slightly less effective stimulators of G_o. The 1–6 peptide stimulates GTPγS binding to G_o much less effectively than intact GAP-43. This localizes the stimulatory region of GAP-43 to its N-terminus and particularly to its first 10 amino acid residues. A peptide of residues 11–25 does not alter GTPγS binding to G_o. Within the N-terminus, the cysteine residues at positions 3 and 4 are required for activity, since a 1–10 peptide with threonines in place of the cysteines is inactive (Strittmatter *et al.*, 1990, and Figure 1).

The importance of free thiol groups in the N-terminus is also emphasized by the instability of these peptides in the absence of dithiothreitol (DTT). With DTT present, the peptides are chemically stable and maintain their ability to stimulate G_o. When the peptides are exposed to conditions without DTT, as was the 1–10 peptide in our previous study (Strittmatter *et al.*, 1990), their elution from reverse phase HPLC is altered, reflecting oxidation of thiol groups, and they no longer stimulate G_o (data not shown).

Palmitoylated peptides do not stimulate GTPγS binding to G_o

To test whether palmitoylation of the N-terminal cysteines of GAP-43 alters interaction with G_o, we synthesized peptides with two palmitate residues stably attached to residue 3, residue 4, or residues 3 and 4 through a propane linkage to the cysteine sulfhydryl. The thioester linkage present in palmitoylated protein was not employed since it would probably be too labile to permit synthesis and isolation of the modified peptide. The lipopeptides with one modified cysteine have a small stimulatory effect on GTPγS binding to G_o at saturating concentrations, ~40% as much as non-palmitoylated 1–25 peptide or GAP-43 protein (Figure 2A). There is no statistically significant difference in the EC_{50} for the monopalmitoylated and non-palmitoylated peptides. The dipalmitoylated peptide has no detectable stimulatory activity, even at concentrations well above those which saturate the non-palmitoylated 1–25 peptide's effect (Figure 2A). The equilibrium GTPγS binding assay in Figure 2A is dependent upon the kinetics of guanine nucleotide binding as well as the thermal inactivation of G_o (Ferguson *et al.*, 1986). To ensure that the two peptides have different effects on the GTPγS binding characteristics of G_o, rather than upon its stability, we determined the initial association rate for GTPγS at higher concentrations of G_o and GTPγS, conditions in which thermal inactivation is insignificant (Strittmatter *et al.*, 1991). GAP-43 and the non-palmitoylated peptide stimulate GTPγS binding to G_o under these conditions, while the dipalmitoylated peptide remains inactive (Figure 2B).

Since the palmitoylated peptides would be predicted to be more hydrophobic than those without the fatty acids, it was conceivable that these peptides might function more effectively in a lipid bilayer than in the detergent solution of the standard assay. The phosphatidylcholine vesicle-incorporated G_o is stimulated by the non-palmitoylated 1–25 peptide, but not by the dipalmitoylated peptide

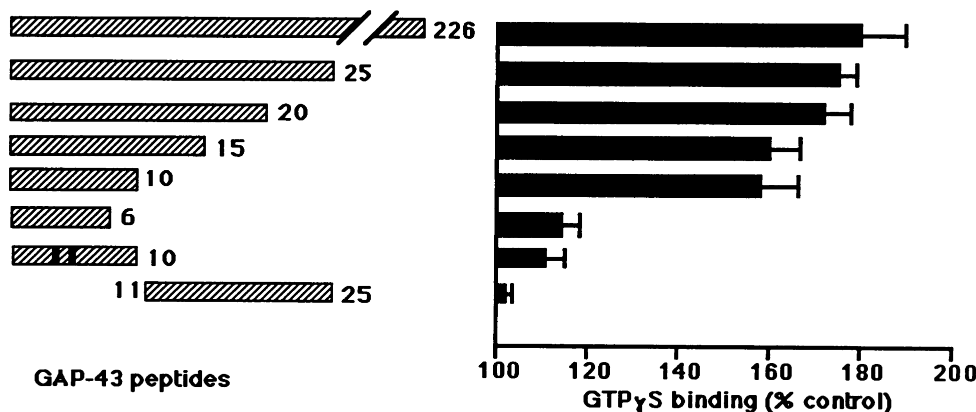

Fig. 1. GAP-43 N-terminal peptides stimulate G_o. GAP-43 N-terminal peptides of the indicated length (left) were added at 100 μM to G_o, and equilibrium GTPγS binding was measured. The level of increase in GTPγS binding, as compared with no addition, is indicated on the right. Native GAP-43 protein (1–226) at 3 μM stimulated binding by 80%. Note that the 1–6 through 1–25 peptides stimulate binding of GTPγS to G_o to varying degrees, but that the 1–10 peptide with threonines in place of cysteines and the 11–25 peptide do not affect G_o. The results are the average of three to six separate determinations, and the standard errors are shown.

(Figure 2C). Thus, palmitoylation blocks the action of the N-terminal peptides on G_o in both detergent solution and lipid vesicles.

Even though the dipalmitoylated peptide does not affect GTPγS binding to G_o, it might still bind to G_o and competitively block GAP-43 action. However, since GAP-43 stimulates GTPγS binding to G_o with exactly the same potency in the presence and absence of peptide (Figure 2D), it is clear that the lipopeptide is not a competitive antagonist for GAP-43. Since the dipalmitoylated peptide does not alter basal or GAP-43-stimulated G_o activity it is unlikely that the dipalmitoyl-propyl modification has a non-specifc inhibitory detergent effect on G_o. It is probable, therefore, that dipalmitoylation prevents binding of the peptide to G_o.

Palmitoylation of GAP-43 protein

The N-terminal peptides and lipopeptides define a GAP-43 domain which activates G_o, and, at least for short peptides, is blocked by the addition of fatty acid residues. Determination of whether this regulation applies to the native protein required a system to reversibly palmitoylate native GAP-43. By obtaining palmitoylated GAP-43 in a thioester linkage we could also test whether the naturally occurring modification, as opposed to the synthetic dipalmitoyl-propyl group, blocks GAP-43 action on G_o. Although it appears that a majority of GAP-43 is palmitoylated *in vivo* (Skene and Virag, 1989), purification removes the fatty acid, due to hydrolysis of thioester bonds during alkali extraction of GAP-43 from membrane fractions (Strittmatter *et al.*, 1991). We tested a variety of different extraction techniques, but none provided palmitoylated GAP-43 in a reproducible manner. However, as shown for rhodopsin (O'Brien *et al.*, 1987), viral glycoproteins (Schmidt and Burns, 1989) and the proteolipid protein of myelin (Bizzozero *et al.*, 1987), it is possible to incorporate palmitate non-enzymatically onto free thiol groups using CoA-palmitate *in vitro*. For GAP-43, we found conditions under which 1 mole of palmitate is incorporated per 1.2 mole of GAP-43 (Figure 3A). Neutral hydroxylamine treatment can release the palmitate, confirming that the addition is via a thioester bond (Figure 3A). The palmitoylated and non-palmitoylated forms can be resolved by hydrophobic chromatography

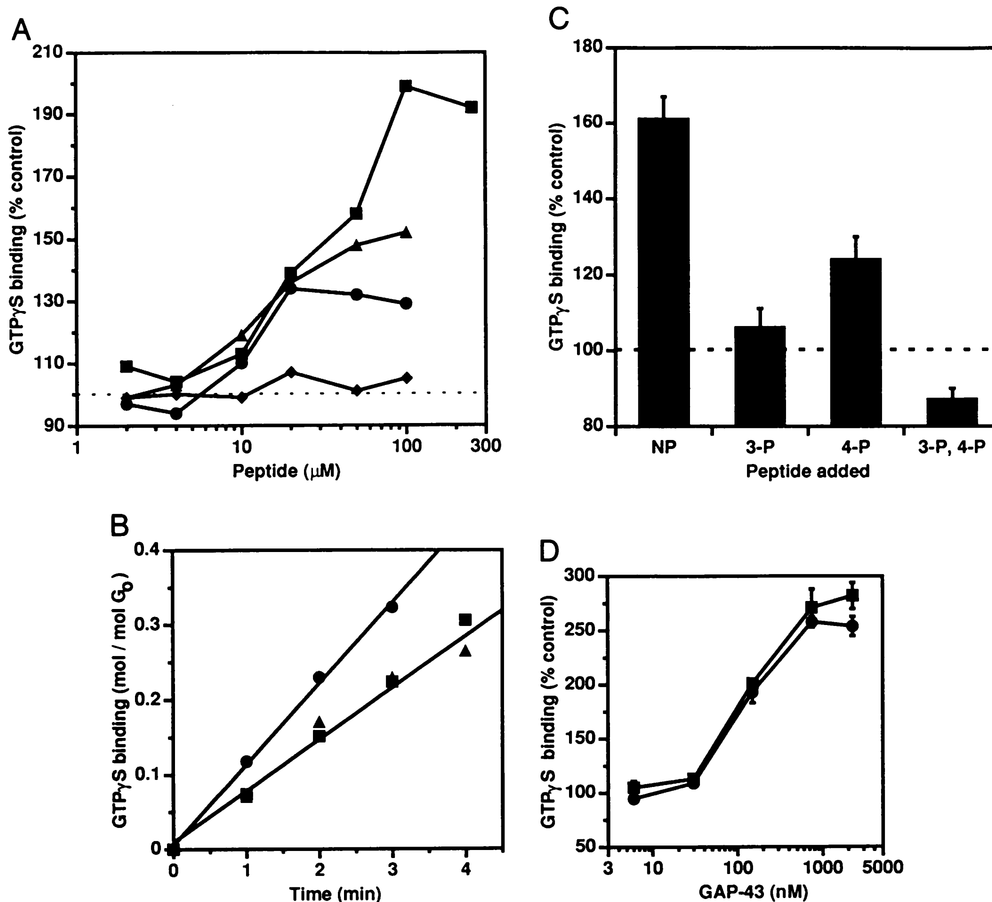

Fig. 2. Palmitoylated peptides are less effective in stimulating G_o. (**A**) An equilibrium G_o–GTPγS binding assay was conducted in the presence of GAP-43 N-terminal peptides of 25 amino acid residues with palmitates linked to cysteines at position 3 (●), position 4 (▲) or positions 3 and 4 (◆) at the indicated concentrations. Note that palmitoylation at a single position diminishes, and at both positions abolishes the stimulation caused by the non-palmitoylated peptide (■). A representative example from three experiments with similar results is shown. (**B**) The initial rate of GTPγS binding to G_o is shown with no addition (■), 100 μM non-palmitoylated 1–25 peptide (●) or 100 μM dipalmitoylated 1–25 peptide (▲). Note that the non-palmitoylated peptide increases the initial rate of binding to G_o, but that the dipalmitoylated form has no effect. (**C**) Equilibrium GTPγS binding to vesicle-incorporated G_o was assayed with the indicated peptides at 100 μM. Note that the non-palmitoylated peptide stimulates GTPγS binding to vesicle-incorporated G_o, but the dipalmitoylated peptide does not increase binding. The singly palmitoylated peptides are intermediate in activity. The standard errors from separate experiments are shown. (**D**) The dipalmitoylated GAP-43 N-terminal peptide (50 μM) was added to G_o–GTPγS equilibrium binding assays with various concentrations of non-palmitoylated GAP-43. The saturation curve for GAP-43 is identical with (●) or without (■) the peptide, showing that the dipalmitoylated peptide does not compete with non-palmitoylated GAP-43 protein. The standard error of the mean for each concentration from separate experiments is shown.

(Figure 3B). After removing non-palmitoylated GAP-43, the palmitoylated preparation has a molar stoichiometry of palmitate to protein of 1.5 to 1, suggesting that this fraction contains an equal mixture of monopalmitoylated and dipalmitoylated GAP-43. That the majority of GAP-43 cysteines are palmitoylated after this purification step can be shown by quantitative reduction in [^{14}C]iodoacetamide labeling of free thiol groups (Figure 3C).

Palmitoylated GAP-43 does not stimulate G_o

The palmitoylated form of GAP-43 has little stimulatory effect upon equilibrium GTPγS binding to G_0, even when present at concentrations above those which saturate the GAP-43 effect (Figure 4A). Similarly, it only minimally affects the initial rate of GTPγS binding to G_0 (Figure 4B). Since the stoichiometry of palmitate incorporation suggests that both monopalmitoylated and dipalmitoylated GAP-43

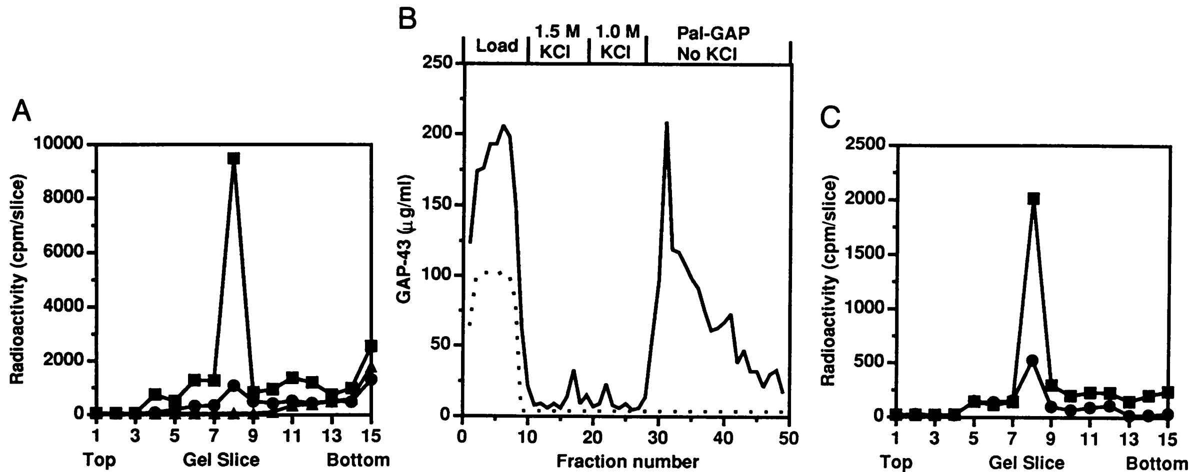

Fig. 3. Palmitoylation of GAP-43 non-enzymatically. (**A**) Polyacrylamide gel electrophoresis demonstrates co-migration of GAP-43 and [^{14}C]palmitate after an *in vitro* palmitoylation reaction. The presence of GAP-43 was detected by Coomassie Brilliant Blue staining in slice 8, and the same fraction contains a peak of radioactivity (■). When GAP-43 was omitted from the sample all radioactivity was at the dye front (▲). If the sample was incubated with 1 M neutral hydroxylamine prior to electrophoresis, then no peak of radioactivity comigrating with GAP-43 was detected (●). (**B**) Palmitoylated GAP-43 was separated from non-palmitoylated GAP-43 on a phenyl-sepharose column. After incubation with [^{14}C]CoA-palmitate, a purified GAP-43 preparation was applied to a phenyl-sepharose resin and eluted with decreasing KCl concentrations. Non-palmitoylated GAP-43 (····) does not bind to this column. The palmitate-modified GAP-43 (———) is separated into two fractions by this procedure. The protein which elutes in the void volume contains no [^{14}C]palmitate, and co-migrates with GAP-43 on SDS–PAGE, and is the non-palmitoylated fraction. The second peak has a molar ratio of protein to palmitate of 1:1.5, when analyzed as in (A). The region indicated (Pal-GAP) was used in further experiments as 'palmitoylated GAP-43'. (**C**) To quantitate free thiol groups in palmitoylated and non-palmitoylated GAP-43, the proteins were incubated with [^{14}C]iodoacetamide, and then analyzed for radioactivity incorporated into protein by SDS–PAGE. Non-palmitoylated GAP-43 (■) incorporates at least four times more radioactivity than does palmitoylated GAP-43 (●), demonstrating that free thiol groups are blocked in the latter preparation. The palmitoylated GAP-43 in this experiment was prepared with unlabeled CoA-palmitate.

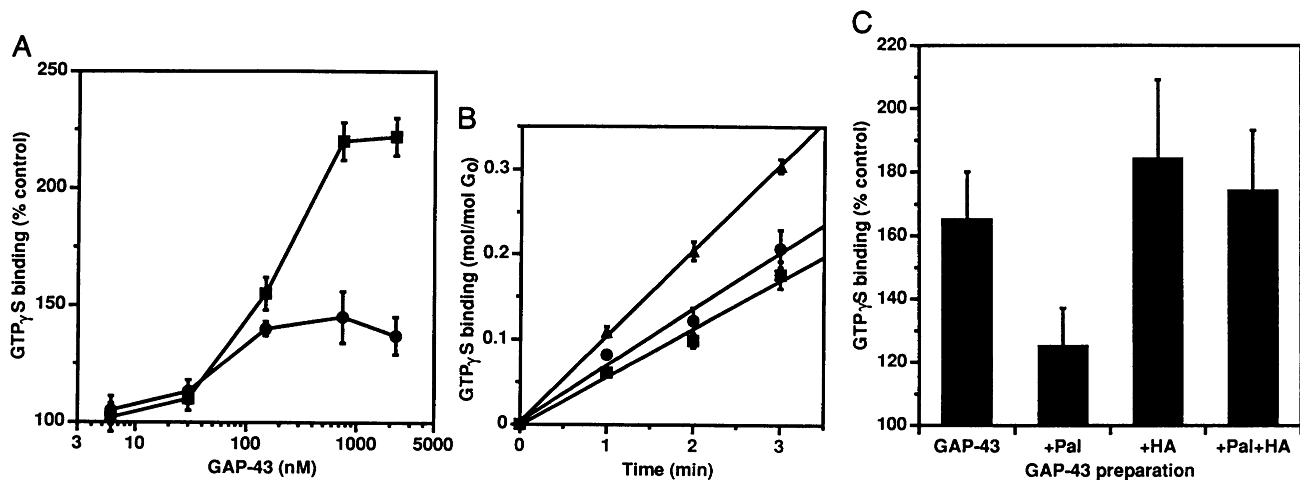

Fig. 4. Palmitoylation blocks GAP-43 stimulation of G_0. (**A**) Equilibrium GTPγS binding to G_0 is shown as a function of GAP-43 (■) or palmitoylated GAP-43 (●) protein concentration. Note that GAP-43 stimulates binding to 250% of control levels, but that the palmitoylated protein produces binding levels which are only 140% of control values. The standard errors for each concentration from separate experiments are shown. (**B**) The initial rate of GTPγS binding to G_0 is shown as a function of time in the presence of no additions (■), 0.75 μM GAP-43 (▲) or 0.75 μM palmitoylated GAP-43 (●). The rate of binding is increased 80% by GAP-43, but only 23% by palmitoylated GAP-43. The standard errors for each time point from separate experiments are shown. (**C**) To examine the reversibility of GAP-43 inactivation by palmitate, the protein was treated with hydroxylamine. Equilibrium GTPγS binding to G_0 in the presence of 0.75 μM GAP-43 protein is shown. The palmitoylated protein is only 30% as effective in stimulating binding as non-palmitoylated GAP-43. When both preparations are treated with 1 M hydroxylamine to cleave thioester bonds, the previously palmitoylated protein is nearly as active as the non-palmitoylated sample. The standard errors are shown.

are present, and since the monopalmitoylated peptides retain some activity, this residual activity in palmitoylated GAP-43 is likely due to monopalmitoylated GAP-43, and dipalmitoylated protein may be completely inactive.

We were concerned that the purification and/or palmitoylation procedure might irreversibly denature GAP-43. Therefore, we sought to demonstrate that palmitoylated GAP-43 can be reactivated by removal of the palmitate. As shown above (Figure 3A), treatment with neutral hydroxylamine cleaves palmitate from GAP-43. Hydroxylamine treatment of the inactive palmitoylated GAP-43 protein also restores its ability to stimulate GTPγS binding to G_o (Figure 4C).

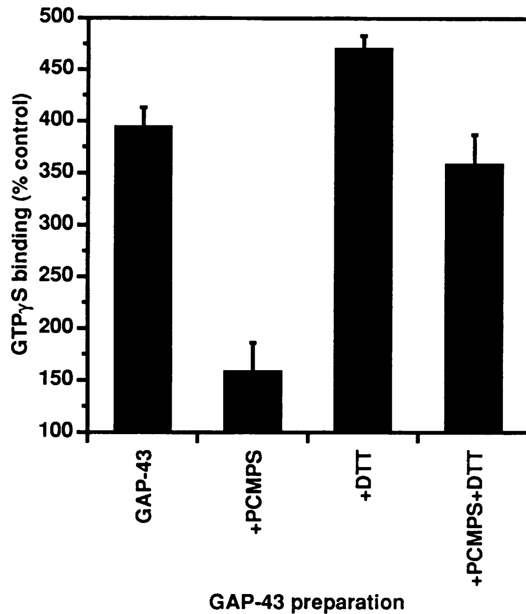

Fig. 5. PCMPS treatment blocks GAP-43 stimulation of G_o in a reversible manner. The level of GTPγS binding to G_o in the presence of 0.75 μM GAP-43 protein is shown. The PCMPS modified protein is only 20% as effective in stimulating binding as non-palmitoylated GAP-43. After treatment with 1 mM DTT to cleave thioester bonds, stimulation of GTPγS binding to G_o is restored. The standard error of the mean from separate experiments is shown.

Other modifications of GAP-43 cysteines block stimulation of G_o

We were curious whether the effect of palmitic acid was specific to this moiety. Therefore, we modified the two GAP-43 cysteine residues with another reagent, *p*-chloromercuriphenylsulfonate (PCMPS), as a selective and reversible modifier of free thiol groups. Treatment of GAP-43 with PCMPS blocks 80% of its stimulatory effect on G_o (Figure 5). Regeneration of the cysteine residues with DTT restores GAP-43 activity.

Discussion

We have investigated whether addition of palmitate to GAP-43 alters its activation of G_o. Synthetic peptides define the GAP-43 region which interacts with G_o to be the N-terminal domain, where palmitoylation occurs. Lipopeptides from this region provide uniform and stable reagents which suggest that fatty acylation of this active site blocks its ability to bind and activate G_o. We have also shown that *in vitro* palmitoylation of the native GAP-43 protein prevents its activation of G_o. Since significant amounts of both non-palmitolyated and palmitoylated GAP-43 appear to exist *in vivo*, and since GAP-43 palmitate turns over rapidly within growth cone preparations (Skene and Virag, 1989), it is plausible that the level of palmitoylation varies within a range that would alter the level of G protein activation.

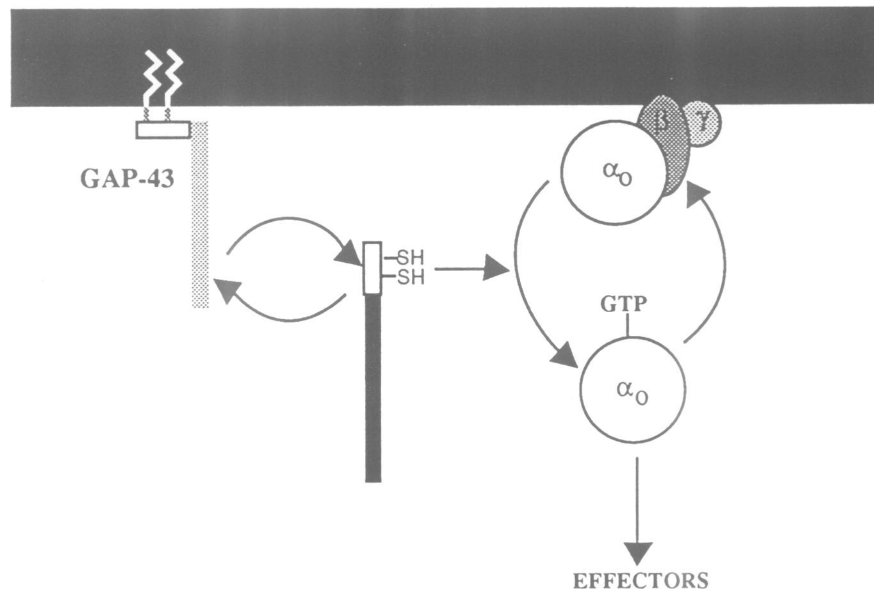

Fig. 6. Palmitoylation as a dynamic regulator of the GAP-43–G_o interaction. A schematic model illustrating the two roles for GAP-43 palmitoylation: enhancement of membrane binding and regulation of interaction with G_o. The palmitoylated form of GAP-43 is more tightly adherent to the membrane. After cleavage of palmitate, non-palmitoylated GAP-43 is free to interact with the α_o subunit and regulate guanine nucleotide exchange, with consequent effects on second messenger systems. The site of interaction of GAP-43 with α_o is likely to overlap with that of the family of receptor proteins with seven transmembrane domains (Strittmatter *et al.*, 1990,1991).

As for other lipid modifications of proteins (Sefton and Buss, 1987; Schultz *et al.*, 1988), the level of GAP-43 palmitoylation is correlated with the membrane association of GAP-43 (Skene and Virag, 1989). The N-terminal domain which contains the two palmitoylation sites is necessary and sufficient for membrane binding (Skene and Virag, 1989; Zuber *et al.*, 1989b; Liu *et al.*, 1991). Thus, palmitoylation may serve two purposes: to concentrate protein at the membrane, and to modify the conformation of the N-terminal, G protein interacting domain. Non-palmitoylated GAP-43 may be the form that interacts with and alters guanine nucleotide binding to G_o, whereas the palmitoylated species may be more important in maintaining a reservoir of inactive protein at appropriate locations (Figure 6). It is unclear whether deacylation results in GAP-43 being freely soluble in the cytosol, or allows local interaction with membrane proteins followed by reacylation before diffusion from the plasma membrane can occur.

The action of GAP-43 in enhancing guanine nucleotide exchange by G proteins resembles that of the G protein-coupled receptors, the family of proteins with seven transmembrane domains which activate G proteins after binding extracellular ligands. Two proteins of this family are known to be palmitoylated: rhodopsin (O'Brien *et al.*, 1987) and the β_2-adrenergic receptor (O'Dowd *et al.*, 1989). The site of palmitoylation is one or two cysteine residues within the cytoplasmic C-terminal tail. These palmitoylated cysteine residues and a few surrounding residues are conserved between many of the sequences in this gene family (O'Dowd *et al.*, 1989) and show a short stretch of sequence similarity with GAP-43 (Strittmatter *et al.*, 1990). Furthermore, this cysteine residue is required for efficient coupling of the β_2-adrenergic receptor with G_s. The observation that reconstitution of purified receptor with G protein is enhanced by incubation with DTT (Florio and Sternweiss, 1989) could conceivably be due to the cleavage of palmitate thioesters.

The small G proteins, which are related to the *ras* gene product, undergo farnesylation of a cysteine residue close to the C-terminus (Hancock *et al.*, 1989). This post-translational modification persists for the life of the protein and is necessary for normal function of *ras* (Glomset *et al.*, 1990; Maltese, 1990). In addition, several members of the *ras* family undergo palmitoylation on nearby cysteine residues (Magee *et al.*, 1987; Hancock *et al.*, 1989). The significance of the later modification is not yet clear, although a dynamic regulatory role has been suggested (Magee *et al.*, 1987; Hancock *et al.*, 1989). Mutations of oncogenic *ras* genes by serine substitution at the palmitoylated cysteines produce products with less transforming activity and lower avidity for the plasma membrane (Hancock *et al.*, 1989). However, whether this is due to replacement of the cysteines *per se*, to a change in palmitoylation status, or to a change in subcellular localization remains to be determined.

If palmitoylation provides a regulatory modification, a highly specific machinery might be predicted to control the level of palmitoylation, as exists for phosphorylation. Cellular extracts from several sources can palmitoylate proteins, but the enzymes responsible are not identified (Berger and Schmidt, 1984, 1985; Slomiany *et al.*, 1984; Mack *et al.*, 1987; Gutierrez and Magee, 1991). It has been suggested that transfer of palmitate from CoA-palmitate to rhodopsin may be non-enzymatic (O'Brien *et al.*, 1987). The inactivation of GAP-43 by palmitoylation places particular emphasis on the depalmitoylation process. Variations in depalmitoylation rates between different proteins and in the presence of agonists have been noted, suggesting the existence of a regulated depalmitoylation mechanism (Staufenbiel, 1988; Huang, 1989). There is no obvious amino acid similarity in the region surrounding palmitoylated cysteines in general (Sefton and Buss, 1987). However, the palmitoylation site of GAP-43, MLCCMRR, shares sequence similarity with that of G protein-linked receptors (Strittmatter *et al.*, 1990) and could conceivably reflect the recognition site of a specific protein palmitoyl transferase.

Materials and methods

[^{35}S]GTPγS (1000 Ci/mmol), CoA-[^{14}C]palmitate (60 mCi/mmol) and [^{14}C]iodoacetamide (21.1 mCi/mmol) were obtained from New England Nuclear. Hydroxylamine, DTT and *p*-chloromercuriphenylsulfonic acid were purchased from Sigma. All unmodified peptides except the 1–6 peptide were prepared as described previously at the Howard Hughes Medical Institute laboratory (Strittmatter *et al.*, 1990). The 1–6 peptide and a second synthesis of the 1–10 peptide were a generous gift of Shigeo Ogino of Sumitomo Chemical Co. Sequences were verified by amino acid sequencing. After synthesis, all peptide solutions contained 1–10 mM DTT. The stability of peptides was monitored by reverse phase HPLC on a C_{18} resin in 0.1% TFA with a 10–60% acetonitrile gradient.

Purification of GAP-43 and G_o

Bovine brain G_o was purified as described previously (Neer *et al.*, 1984). GAP-43 was purified from the brains of 10 day old rats by a modified method (Zwiers *et al.*, 1985; Strittmatter *et al.*, 1991). This purification extracts GAP-43 from membranes with 0.1 N NaOH, so that any endogenous palmitate linked by thioesters is cleaved from GAP-43. We have previously shown that this preparation is not palmitoylated (Strittmatter *et al.*, 1991). The purity of both the G_o and GAP-43 preparations has been demonstrated previously (Strittmatter *et al.*, 1991).

GTPγS binding to G_o

The binding of [^{35}S]GTPγS to G_o was determined as described previously (Strittmatter *et al.*, 1991). For kinetic studies, assays included 20 nM G_o, 50 nM [^{35}S]GTPγS, 0.1% Lubrol PX, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 50 mM Na HEPES, pH 7.5 and any GAP-43 additions. After incubation for 1–10 min at 20°C, the sample was filtered over nitrocellulose, and bound radioactivity was measured. For standard assays, lower concentrations of the reagents were used, 1 nM G_o and 2 nM GTPγS, and 100 μg/ml bovine serum albumin was included in a 30 min incubation at 30°C. In assays of vesicle-incorporated G_o, Lubrol PX was omitted from the assay buffer. In assays of palmitoylated GAP-43 protein, DTT was omitted from the control and experimental tubes to promote stability of the palmitate thioester. Vesicle incorporation of G_o was by the method of Cerione *et al.* (1984) and was confirmed as described previously (Strittmatter *et al.*, 1991).

Synthesis of palmitoylated peptides

GAP-43 lipopeptides were obtained by a method developed for the synthesis of the N-terminal region of a photosynthetic cytochrome from bacteria (Beck-Sickinger and Metzger, 1991; Metzger *et al.*, 1991). Unmodified peptides were produced on a solid phase automated synthesizer using Fmoc strategy and 1-benzotriazole-1,1,3,3-tetramethyluroniumtetrafluoroborate (TBTU)/1-hydroxybenzotriazole activation. To obtain the lipopeptides, the fully protected and resin-bound segments of GAP-43 (5–25 or 4–25) were coupled to Nα-fluorenylmethoxycarbonyl-*S*-[2,3-bis-(palmitoyloxy)-(R)-propyl]-cysteine using TBTU/1-hydroxybenzotriazole chemistry. Deprotection of the Nα-Fmoc group was achieved with morpholin in dimethylformamide (1:1) at 4°C within 60 min. For the dipalmitoylated peptide this cycle was repeated once before the unmodified N-terminal residues were added by the standard method. The identities of the final palmitoylated products were confirmed by amino acid analysis and mass spectrometry. Stock solutions of 1 mM were prepared in PBS, 1 mM DTT by sonication. The 1–25 monopalmitoylated peptides have one unmodified cysteine and one cysteine coupled to two palmitates, while the dipalmitoylated peptide has two palmitates linked to each of two cysteines.

Palmitoylation of GAP-43

Purified GAP-43 protein (3 mg, 20 μM) was incubated with CoA-[^{14}C]palmitate (1.6 mM, 5 mCi/mmol) for 14 h at 37°C in 50 mM Tris–HCl, pH 7.5. In some cases, non-radioactive palmitoylated GAP-43 was produced by substituting unlabeled CoA-palmitate in the same concentration. Inclusion of Lubrol PX (1%) or DTT (1 mM) decreased the incorporation into GAP-43. To assess the degree of palmitoylation, the preparation was boiled in 2% SDS, without β-mercaptoethanol, and electrophoresed through a polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue to detect GAP-43 protein, and then slices of the gel were incubated with 30% H_2O_2 for 16 h at 65°C. Radioactivity was determined by liquid scintillation spectrophotometry, and protein concentration for GAP-43 by Bradford protein assay. A fraction of the radioactivity comigrates with GAP-43 protein during polyacrylamide gel electrophoresis (Figure 3A). Molar stoichiometries were calculated on the basis of a M_r of 24 000 for GAP-43, and the specific activity of the CoA-[^{14}C]palmitate. The molar stoichiometry of palmitate incorporation to GAP-43 protein is 1:1.2, indicating that some, but not all of the GAP-43 is palmitoylated in this reaction. The highest levels of palmitoylation were achieved in the absence of detergent and DTT.

To confirm that [^{14}C]palmitate is attached to GAP-43 by a thioester bond, the preparation was diluted into an equal volume of 2 M hydroxylamine, 2 mM DTT, and then incubated at 37°C for 14 h. This treatment removes essentially all of the radioactivity from GAP-43, as expected for hydrolysis of a thioester bond with this mild treatment (Figure 3A). The depalmitoylated sample was dialyzed against 20 mM Tris–HCl, 1 mM DTT, pH 7.5 to remove the hydroxylamine prior to addition to GTPγS binding assays or before column chromatography.

Separation of palmitoylated from non-palmitoylated GAP-43

In order to test the palmitoylated GAP-43 for G_o stimulation, it was neccessary to separate the different forms of GAP-43. After palmitoylation of GAP-43, the sample (10–200 mg of protein) was mixed with an equal volume of 3 M KCl, and then applied to a 0.4 ml column of phenyl-sepharose (Pharmacia) equilibrated with 2 M KCl, 50 mM Tris–HCl, pH 7.5. In the presence of 1.5 M KCl, untreated GAP-43 does not bind to this resin, but about half of the modified GAP-43 preparation does so. After washing with 4 ml of 50 mM Tris–HCl, 1.5 M KCl, pH 7.5, and 4 ml of 50 mM Tris–HCl, 1.0 M KCl, pH 7.5, palmitoylated GAP-43 was eluted with 20 mM Tris–HCl, pH 7.5, without KCl. The total recovery of protein was 40–75% of that applied to the column. Removal of the non-palmitoylated protein increases the molar ratio of palmitate to GAP-43 protein to 1.5:1, suggesting that this method produces GAP-43 that is both monopalmitoylated and dipalmitoylated. After hydroxylamine exposure no GAP-43 is retained on the resin, as expected for hydroxylamine cleavage of thioester-linked palmitoyl groups.

Labeling of free thiol groups in GAP-43 with iodoacetamide

If the palmitoylation reaction is essentially complete, then the modified GAP-43 should be devoid of free thiol groups. As a measure of free thiol groups in GAP-43, we incubated purified GAP-43 protein (6 μg, 7 μM) or non-radioactive palmitoylated GAP-43 (6 μg, 7 μM) with [^{14}C]iodoacetamide (550 μM, 21 Ci/mmol) for 14 h at 30°C in 50 mM Tris–HCl, pH 8.0. The covalent modification of GAP-43 with [^{14}C]iodoacetamide was quantitated in slices of polyacrylamide gels as described above for the incorporation of [^{14}C]palmitate into GAP-43. Whereas ^{14}C incorporation into non-palmitoylated GAP-43 is easily detected after SDS–PAGE, the radioactivity covalently bound to palmitoylated GAP-43 under the same conditions is $<25\%$ as great (Figure 3C).

p-Chloromercuriphenylsulfonic acid (PCMPS) modification of GAP-43

To modify the free sulfhydryl groups in GAP-43, non-palmitoylated GAP-43 (4 μM) was incubated with PCMPS at a molar ratio of 1:4000 in 50 mM Tris–HCl (pH 8.0) for 14 h at 30°C. Preparations were dialyzed against 20 mM Tris–HCl (pH 7.5) to remove PCMPS prior to addition to GTPγS binding assays. For DTT treatment after PCMPS modification, preparations were dialyzed against 20 mM Tris–HCl, 1 mM DTT, pH 7.5 for 36 h at 4°C.

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