
Stable Isotope Applications in Biochemistry* 1132

ibid. 8. Warshel has argued that LBHBs would be anticata-

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Uniformly 15N-labeled (≥95%) ω-lytic protease (*E. c. 3.4.21.12) was expressed by fermentation of wild-type *Lysobacter* enzymes in Cloete-N (Martek Biosciences), a medium containing 15N-enriched peptides and amino acids, which was supplemented with glucose and several vitamins. Purification of ω-lytic protease and assay of enzymatic activity was accomplished as described (18).

Expression of 15N histidine-labeled subtilisin BPN’97 was accomplished by fermentation of a histidine auxotrophic strain of *Bacillus subtilis* DB104 in a minimal medium containing histidine labeled with 15N at both ring positions (18). Purification of labeled subtilin subtilisin BPN’97 was accomplished by dialysis into a 10 mM tris solution (pH 6.0) and subsequent passage over a CM52 cellulose ion-exchange column, with elution of the protein in 0.01 M MES and 0.1 M KCI. Enzyme activity was measured by a colorimetric assay with the substrate succinyl-L-Ala-L-Ala-L-Pro-L-Phe-paraanilinothiolane (18).


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Inhibition of Brain Gz GAP and Other RGS Proteins by Palmitoylation of G Protein α Subunits

Yaping Tu, Jun Wang, Elliott M. Ross*

Palmitoylation of the α subunit of the guanine nucleotide-binding protein Gα inhibited by more than 90 percent its response to the guanosine triphosphatase (GTPase)-accelerating activity of Gz GAP, a Gz-selective member of the regulators of G-protein signaling (RGS) protein family of GTPase-activating proteins (GAPs). Palmitoylation both decreased the affinity of Gz GAP for the GTP-bound form of Gzα by at least 90 percent and decreased the maximum rate of GTP hydrolysis. Inhibition was reversed by removal of the palmitoyl group by dithiothreitol. Palmitoylation of Gzα also inhibited its response to the GAP activity of Gα-interacting protein (GAP), another RGS protein, and palmitoylation of Gzα inhibited its response to RGS4. The extent of inhibition of Gz GAP, GαIP, RGS4, and RGS10 correlated roughly with their intrinsic GAP activities for the Gzα target used in the assay. Reversible palmitoylation is thus a major determinant of Gzα deacti-

vation after its stimulation by receptors, and may be a general mechanism for prolonging or potentiating G-protein signaling.

The α subunits of most heterotrimeric G proteins are modified by irreversible lipid amidation of the NH2-terminus and by addition of a palmitoyl thioester at a nearby, conserved cysteine residue (I, 2). Unlike myristoylation, palmitoylation of Gα subunits is reversible, and bound palmitate turns over rapidly in cells. Although virtually nothing is known of the enzymes that catalyze addition and removal of palmitate, palmitate turnover on G-protein α subunits appears to be regulated coordinately with their activation and deactivation. In the case of Gzα (3, 4) and Gαi (5), substantial depalmitoylation occurs upon receptor-activated activation and repalmitoylation of Gzα coincides at least roughly with deacti-

vation (3). Treatment with cholera toxin, which prolongs activation of Gz by blocking hydrolysis of bound GTP, also promotes turnover of bound palmitate (6). Conversely, palmitate turnover on Gαi and Gzα is decreased by coexpression of excess Gβγ, which inhibits activation (6, 7).

Palmitoylation is involved in anchoring Gα subunits to the membrane or specifying their membrane localization, or both (1–4, 7–9), by increasing their intrinsic hydrophobicity and, at least for Gzα, by increasing affinity for Gβγ (7). Mutation of the palmi-

toylated cysteine of Gαi to alanine also potenti-

ated inhibition of adenyl cyclase in transfected cells (9). Palmitoylation has not yet been linked to alteration of a specific G-protein signaling function, however. It is not required for interaction of Gαi subunits with receptors or effectors in vitro (10), and no effect of palmitoylation on the binding or hydrolysis of guanine nucleotides has been reported. Mutation of the palmitoylatable cysteine residue in Gz or Gαi inhibited signaling (11), but signaling was potentiated by the same mutation in Gα, or GαIP1, the major Gαα subunit in Saccharomyces cerevisiae (9, 12). Although palmitoylation may be responsible for such variable effects on different Gα subunits, these results may also arise from effects of mutating the cysteine residue that are unrelated to palmitoylation (10).

We describe the inhibition of the effects of the major Gz GTPase activating protein, Gz GAP, by palmitoylation of Gzα. Gzα, a relatively rare member of the Gα family that is found in brain, platelets and adrenal medulla, and is therefore suspected to be in-

volved in regulation of secretion (13). Iso-

lated Gzα hydrolyses bound GTP slowly,
such that the half-life of the active, GTP-bound species is about 7 min at 30°C (14, 15). Gα, GAP, which we recently purified from bovine brain, accelerates the hydrolysis of Gα-bound GTP over 200-fold (15). Gα, GAP is a novel member of the RGS family (16), whose members attenuate G-protein signaling at least in part through their GAP activity (17). Gα, GAP is most abundant in tissues that also express Gz. It thus appears to be the major determinant of Gz deactivation and, therefore, of the amplitude and duration of Gz-mediated signals.

To examine the extent of palmitoylation of Gα, on its deactivation, we palmitoylated purified Gα, in vitro (18, 19) and measured the rate of hydrolysis of bound [γ-32P]GTP in the presence and absence of Gα, GAP (15, 20). Fractional autopalmitoylation of purified Gα, in vitro was nearly complete, 80 ± 10% according to total protein or 120 ± 15% according to the number of GTP-γ-S binding sites (n = 6) (Fig. 1) (19). Treatment with either neutral hydroxylamine or dithiothreitol (DTT) removed the palmitate (Fig. 1), consistent with its addition through a thioester bond. [3H]Palmitate could also be completely removed from Gα, by tryptic proteolysis after protection with GTP-γ-S or Al3+/F− (Fig. 1). Because Cys5 is the only cysteine residue before the Arg15→ tryptic cleavage site (21), palmitoylation of Cys5 is unique and nearly quantitative under the conditions used here. There was no difference in the rate of autopalmitoylation of Gα, when it was bound to either GDP or GTP-γ-S (22).

Palmitoylation of Gα, blocked the action of bovine brain Gα, GAP by nearly 90% (87 ± 3.5%, n = 6, in matched experiments) (Fig. 2 and Table 1) (20). Because palmitoylation of Gα, may be incomplete and because GTP bound to residual nonpalmitoylated Gα, will be disproportionately hydrolyzed during a brief GAP assay (Fig. 2A), inhibition of the GAP by palmitoylation of Gα, is underestimated and exceeds 90%. Palmitoylation of Gα, had no effect on the rate at which it hydrolyzed bound GTP in the absence of GAP (Table 1 and multiple control experiments) or on the rate of binding of GTP-γ-S (22).

Palmitoylation of Gα, inhibited its response to Gα, GAP by decreasing both its affinity for the GAP and the maximal rate of hydrolysis of the GAP-Gα, GTP complex. The dependence of GAP activity on the concentration of palmitoylated Gα, was biphasic (Fig. 2A). The first phase indicates the presence of about 10% residual non-palmitoylated Gα, GTP, which displays unaltered substrate kinetics. The second phase reveals that palmitoylated Gα, GTP has both a 7- to 15-fold increase in Michaelis constant (Km) combined with a 50 to 80% decrease in maximum V (Vmax). Essentially identical results were obtained in two other experiments with separately palmitoylated batches of Gα, . The precision of the fit for the palmitoylated component is limited because its concentration could not be increased above the high apparent Km. To confirm the effect of palmitoylation on the affinity of Gα, for the GAP, we measured the ability of palmitoyl-Gα, GTP-γ-S to compete with nonpalmitoylated Gα, GTP in a standard GAP assay (Fig. 2B). The data were again biphasic. They indicate that palmitoylation of Gα, was 92% complete and that palmitoyl-Gα, GTP-γ-S bound to the GAP with an inhibition constant (Ki) of about 75 nM, about 30-fold greater than that of the nonpalmitoylated protein.

Treatment of palmitoylated Gα, with 15 mM DTT restored Gα, GAP activity to more
than 85% of that displayed with Gz, that had not been palmitoylated (Table 1). Restoration of activity is consistent with the removal of more than 90% of the palmitate from Gz by the identical treatment (Fig. 1). Treatment with DTT also restored the affinity of Gz for the GAP (22). Both these effects of DTT on palmitoylated Gz required prolonged incubation at 30°C and were not observed if DTT was simply added to the GAP assay. DTT treatment had no effect on the basal rate of hydrolysis of Gz-bound GTP with or without palmitate, but usually slightly enhanced GAP-stimulated hydrolysis by nonpalmitoylated Gz, (<10%; Table 1) (23).

Gz GAP is a member of the RGS protein family (16), many of which have GAP activity toward members of the Gz and Gz families (17, 24, 25). Palmitoylation of other Gz subunits also inhibited their responses to the GAP activities of RGS proteins (Table 2). The extent of inhibition depended on which Gz was used in the assay. RGS proteins are selective among individual members of the Gz and Gz families, including Gz (24, 25), and fractional blockade of GAP activity by Gz palmitoylation was generally greatest when a GAP was assayed with a good Gz-GTP substrate. For example, GAIP (26) displays somewhat lower GAP activity toward Gz than does brain Gz GAP, and palmitoylation of Gz inhibited the GAP activity of GAIP by about 45% (corrected for substoichiometric palmitoylation). RGS4 (27) and RGS10 (28) are much less active on Gz, and their GAP activities were inhibited by only about 20%. When RGS4 was assayed with Gz substrate, however, activity was inhibited by 65 to 70%. For each RGS protein and Gz substrate, inhibition was reversed by removal of palmitate by DTT.

Table 1. Inhibition of Gz GAP activity after palmitoylation of Gz. Hydrolysis of [γ-32P]GTP bound to Gz (from ST9 cells, 1.8 mCi) was measured over 2 min in the presence or absence of 400 pM Gz GAP (100% is 246 mU) (15, 20). Before binding to [γ-32P]GTP, the Gz was treated in the order shown with Pal-CoA (19), with 15 mM DTT at 30°C for 40 min, or with trypsin after A\textsuperscript{−1} F\textsuperscript{−} protection (21). A\textsuperscript{+1} and F\textsuperscript{−} were removed by gel filtration after binding of [γ-32P]GTP (15).

<table>
<thead>
<tr>
<th>Gz</th>
<th>k_{w0} (min⁻¹)</th>
<th>GAP activity (%)</th>
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<tr>
<td></td>
<td>Control (+GAP)</td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>0.0134</td>
<td>0.259</td>
</tr>
<tr>
<td>Pal-CoA</td>
<td>0.0135</td>
<td>0.059</td>
</tr>
<tr>
<td>DTT</td>
<td>0.0134</td>
<td>0.270</td>
</tr>
<tr>
<td>Pal-CoA + DTT</td>
<td>0.0137</td>
<td>0.235</td>
</tr>
<tr>
<td>Trypsin + DTT</td>
<td>0.0310</td>
<td>0.040</td>
</tr>
<tr>
<td>Pal-CoA + Trypsin + DTT</td>
<td>0.0270</td>
<td>0.033</td>
</tr>
</tbody>
</table>

These data suggest that palmitoylation is a general mechanism for protecting GTP-activated G-proteins against GAP-accelerated deactivation.

Inhibition of the response to GAPs by palmitoylation is apparently highly specific, not simply the result of increased NH₂-terminal hydrophobicity of the Gz substrate (29). In contrast to palmitoylation, myristoylation of the NH₂-terminal amine of Gz enhanced Gz GAP activity. Although myristoylation had no effect on the basal rate of hydrolysis of Gz-bound GTP (22), the response to Gz GAP of 2.2 nM nonmyristoylated Gz-GTP, purified from Escherichia coli, was only 31% that of myristoylated Gz. This difference primarily reflects the lower affinity of nonmyristoylated Gz for Gz GAP, reflected in an increase in K\textsubscript{d} of three- to fivefold relative to myristoylated Gz (Fig. 2B). A similar but smaller difference was observed with RGS4; the GAP accelerated rate for unmodified Gz was 76% that of myristoyl-Gz. Myristoylation of Gz and Gz, also increased their affinities for Gz GAP (15). Because auto-palmitoylation of α subunits requires prior NH₂-terminal myristoylation (18), we were unable to determine the effect of palmitoylation of nonmyristoylated Gz, on its activity as a GAP substrate. Regardless, although palmitoylation of Cys\textsuperscript{1} markedly inhibits the binding of Gz to Gz GAP, myristoylation enhances affinity for the GAP.

Because both palmitoylation and myristoylation occur near the NH₂-terminus, we examined the interaction of Gz GAP with Gz, from which the NH₂-terminal α helix was removed by tryptic cleavage at Arg\textsuperscript{29} (Fig. 1). NH₂-terminal truncation of Gz, essentially abolished GAP activity (Table 1 and Fig. 2). Remaining activity was largely accounted for by incomplete proteolysis, because incubation of trypsin-treated Gz with palmitoyl–coenzyme A (Pal-CoA) further inhibited the low residual GAP activity by about 50% (Table 1). NH₂-terminal proteolysis of Gz\textsuperscript{1} also inhibited its response to the GAP activity of RGS4 (22). As was the case for palmitoyl-Gz, the insensitivity of proteolyzed Gz, to Gz GAP reflected a grossly diminished affinity (K\textsubscript{d} >100 nM; Fig. 2A). Proteolysis reproducibly increased the intrinsic rate at which Gz hydrolyzed bound GTP by two- to threefold (Table 1).

Because three different NH₂-terminal modifications of Gz, α-Gz, and α-Gz—palmitoylation, myristoylation, and proteolysis—all modulate their responses to the GAP activities of several RGS proteins, this region of Gz subunits is apparently crucial for RGS protein recognition. However, no contact between RGS4 and the NH₂-terminus of its Gz substrate was observed in a crystal structure of the RGS4-Gz complex (30). The NH₂-terminus did contact an adjacent RGS4 molecule, but this was judged to be an artifact of crystal packing and it is unlikely that relevant contact could take place even if the NH₂-terminus were freed of packing constraints when in solution. The Gz NH₂-terminus may regulate sensitivity to GAPs but not lie at the protein interface. Alternatively, because only the central portion of RGS4 was defined in the crystallographic structure of the complex (30), it is also possible that the Gz NH₂-terminus binds to the RGS protein outside of its central, conserved domain (17). Such an interaction is consistent with the idea that unconserved regions of RGS proteins are important for the specificity of their interactions with G proteins and is also consistent with our finding that palmitoylation inhibits most strongly when a GAP is assayed with a preferred Gz target.

Because inhibition of the action of RGS proteins by palmitoylation of their Gz substrates correlates roughly with GAP activity and can be virtually complete for a specific RGS-Gz pair, palmitoylation has the capac-

Table 2. Inhibited responses to GAIP and RGS4 after palmitoylation of Gz subunits. GAP activity was assayed as described (15, 20) with either 2.2 nM Gz-[γ-32P]GTP or 5 nM Gz-[γ-32P]GTP as substrate, either palmitoylated or not. The data show percent inhibition by palmitoylation relative to parallel assays with a nonpalmitoylated Gz control at the same concentration. For assays using Gz substrate, the specific activity of each RGS protein is given in standard units (15), with molar amounts of each GAP calculated according to total protein. The maximum specific activity of purified RGS10 was somewhat higher than that of the preparation used for these experiments. For RGS4 and Gz, the assay underestimates GAP activity for kinetic reasons (15, 25), and inhibition of RGS4 by palmitoylation of the Gz-GTP substrate is therefore also underestimated. Assays contained 0.15 mM Gz GAP or GAIP, 6 mM RGS10 or either 1.5 mM or 1.0 mM RGS4 (for Gz or Gz, respectively). GAIP (26), RGS4 (27), and RGS10 (28), all His\textsubscript{6}-tagged, were purified from E. coli (25). Preliminary data indicate that GAIP expressed in E. coli, ST9 cells, or HEK 293 cells displays similar enzymatic properties. Data for Gz were corrected for substoichiometric palmitoylation: 70 and 75% in the preparations from which data were averaged. Data for Gz were not corrected, but palmitoylation of Gz is usually ≥90%. Data are averages of at least two experiments, with duplicate determinations.

<table>
<thead>
<tr>
<th>GAP</th>
<th>Gz-GTP Inhibition (%)</th>
<th>Specific activity (U/pmol)</th>
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<tbody>
<tr>
<td>Gz GAP</td>
<td>87</td>
<td>7.8</td>
</tr>
<tr>
<td>GAIP</td>
<td>37</td>
<td>5.4</td>
</tr>
<tr>
<td>RGS4</td>
<td>22</td>
<td>0.75</td>
</tr>
<tr>
<td>RGS4</td>
<td>69</td>
<td>0.14</td>
</tr>
<tr>
<td>RGS10</td>
<td>20</td>
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</tbody>
</table>
ity to totally inhibit the GAP activity of RGS proteins for their correct cellular targets. Thus, the palmitoylation-depalmitoylation cycle may control both the signal amplitude and the temporal response in G-protein pathways. Palmitoylation can amplify G protein-mediated signals or, alternatively, regulated depalmitoylation could serve as either an off-switch or signal attenuator. Such controls may be G protein-specific, and their complete elucidation awaits better understanding of the control of palmitate addition and removal. Regardless, any of these mechanisms would be compatible with the enhanced binding of palmitoylated Gα to GBγ (7), which would serve to lower background signaling in the absence of stimulation. Regulation of GAP activity may be a major function of Gα palmitoylation.

REFERENCES AND NOTES


15. M. A. Grassie et al., Biochem. J. 302, 913 (1994). A central issue in cognitive neuroscience concerns the functional architecture of the prefrontal cortex and the degree to which it is organized by sensory domain. To examine this issue, multiple areas of the macaque monkey prefrontal cortex were mapped for selective responses to visual stimuli that are prototypical of the brain’s object vision pathway—pictorial representations of faces. Prefrontal neurons not only selectively process information related to the identity of faces but, importantly, such neurons are localized to a remarkably restricted area. These findings suggest that the prefrontal cortex is functionally compartmentalized with respect to the nature of its inputs.