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Covalent Binding of Arachidonate to G Protein α Subunits of Human Platelets*

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The α subunits of GTP-binding regulatory proteins (G proteins) are subject to lipid modifications required for anchorage to membrane and/or interactions with other proteins. With the knowledge that α subunits are palmitoylated, which we demonstrate here for human platelets, we sought to determine whether these subunits also bind arachidonate and myristate in a covalent, post-translational manner. All α subunits examined were found to incorporate radioactivity upon incubation of human platelets with [^{3}H]palmitate, [^{3}H]arachidonate, and [^{3}H]myristate. The identity of [^{3}H]palmitate and [^{3}H]arachidonate as covalently bound fatty acids was confirmed by high pressure liquid chromatography following alkaline methanolysis. With [^{3}H]myristate, however, the bound fatty acid proved to be [^{3}H]palmitate, presumably generated by a 2-carbon chain elongation. Protein-bound [^{3}H]palmitate and [^{3}H]arachidonate were released by hydroxylamine at neutral pH, implying a thioester linkage between protein and fatty acid. Thus, post-translational modifications of G protein α subunits include palmitoylation and arachidonoylation, but not myristoylation. Given the different physical properties of saturated and unsaturated fatty acids and the large-scale release of arachidonate during platelet activation, changes in arachidonate incorporation may serve as an important regulator of α subunit function.

The α subunits of GTP-binding regulatory proteins (G proteins)[1] are subject to lipid modifications required for anchorage of the subunits to membranes and/or interactions with other proteins. Members of the α_i family (α_i, α_o, α_z, and α_t; Ref. 1), but not those of the α_s and α_q (and probably α_{12}) families, are *N*-myristoylated (2–5). *N*-Myristoylation is the attachment of myristate (C14:0) to an NH_2-terminal glycine through an amide bond, and occurs during or immediately following translation (6, 7). Replacement of the glycine with alanine in α_i, α_o, and α_z abrogates *N*-myristoylation and, in addition, prevents attachment of these subunits to membranes (2–4). *N*-Myristoylation additionally supports a high affinity interaction between α_o and $\beta\gamma$ (8), a role that presumably extends to other members of the α_i family.

Almost all α subunits are palmitoylated upon overexpression in COS, HEK 293, and Sf9 cells (α_t is an exception) (4, 5, 9–11), as are α subunits endogenous to COS, PC12, and NG108 cells (9, 12). Palmitoylation is the attachment of palmitate (C16:0) typically to a cysteine through a thioester bond (6). In contrast to *N*-myristoylation, palmitoylation is distinctly a post-translational modification and is reversible. Palmitoylation of α subunits appears to occur at one or more cysteines near the NH_2 terminus, *i.e.* at Cys^3 for α_o and α_s (5, 9–11), and Cys^9 and/or Cys^{10} for α_q (11). Substitution of serine for Cys^3 in α_s and Cys^9/Cys^{10} in α_q disrupts attachment of the subunits to membrane and coupling of a co-expressed receptor with adenylyl cyclase and phospholipase C, respectively (11). Anchorage of α_o is less dramatically affected by a similar mutation (9), suggesting that the myristate already present provides an alternate means of attachment. Consistent with this inference is the finding that α_t/α_s and α_t/α_q chimeras that are subject to *N*-myristoylation, but not palmitoylation, are able to anchor to membrane (11). The relevance of palmitoylation to interactions with $\beta\gamma$ has not been explored.

Human platelets have been used extensively in studies of signal transduction (13), and their potential to carry out fatty acid acylation of proteins has been clearly established (14–16). A variety of proteins in platelets are subject to both myristoylation and palmitoylation (16). Since protein synthesis in platelets is negligible, the myristoylation differs from *N*-myristoylation, *i.e.* its binding to protein is not closely tied to translation. Indeed, both myristate and palmitate are incorporated into platelet proteins through linkages sensitive to hydroxylamine, implying thioester rather than amide bonds. This finding is consistent with observations elsewhere that myristate, stearate (C18:0), and oleate (C18:1) can substitute for palmitate at a single cysteine (17–20). Myristoylation, however, is clearly subordinate to palmitoylation in platelets. More recently, a similar set of proteins in platelets was found to incorporate the polyunsaturated fatty acids arachidonate (C20:4) and eicosopentaenoate (C20:5), also through thioester bonds (21).

In the present studies, we have used platelets to examine fatty acid acylation of G protein α subunits in a setting unrelated to overexpression or protein synthesis. We find that all α subunits examined covalently bind arachidonate, as they do palmitate, but not myristate. Binding of both arachidonate and palmitate is sensitive to hydroxylamine and possibly occurs at the same site. Thus, G protein α subunits are the first proteins to be specifically identified as substrates for arachidonoylation. The finding is significant in view of the functional roles now accorded palmitoylation (*i.e. S* fatty acid acylation), the physical differences between palmitate and arachidonate, and the large-scale release of arachidonate from phospholipids during platelet activation. The need to evaluate *S* fatty acid acylation

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[1] The abbreviations used are: G protein, GTP-binding regulatory protein; BSA, bovine serum albumin; HPLC, high pressure liquid chromatography; PGE_1, prostaglandin E_1; PAGE, polyacrylamide gel electrophoresis.

of α subunits by measurements extending beyond palmitate is underscored.

EXPERIMENTAL PROCEDURES

Platelets and Radiolabeling—Human platelets were prepared and incubated with ^{3}H-labeled fatty acids essentially as described earlier (16, 21). Platelet-rich plasma was obtained by centrifugation (120 × *g*, 15 min) from blood anticoagulated with acid-citrate-dextrose containing 0.18 μM prostaglandin E_1 (PGE_1). Platelets were pelleted from the platelet-rich plasma (1300 × *g*, 15 min), then washed and resuspended in solution A (140 mM NaCl, 2.5 mM KCl, 0.1 mM $MgCl_2$, 10 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, 1 mg/ml glucose, 10 mM HEPES, pH 7.4) containing 3.6 mg/ml fatty acid-free bovine serum albumin (BSA), 1 unit/ml apyrase, and 0.3 μM PGE_1 at 37 °C. [^{3}H]Myristate (34 Ci/mmol), [^{3}H]palmitate (47 Ci/mmol), and [^{3}H]arachidonate (200 Ci/mmol) (DuPont NEN or American Radiolabel Co.) in ethanol were dried under nitrogen, solubilized by mixing in solution A containing BSA, apyrase, and PGE_1, and added to an equal volume of platelet suspension. The platelet count in the final suspension was approximately 3 × 10^8 cells/ml, and radioactivity was 250 μCi/ml. The concentrations of myristate, palmitate, and arachidonate were 7.5, 5.5, and 1.2 μM, respectively. The platelets were incubated for 2 h, pelleted, and washed twice in solution A as a preliminary to analysis of radiolabel incorporation.

Immunoprecipitation and Blotting—Platelets incubated with radiolabeled fatty acids were solubilized in 0.5% sodium dodecyl sulfate (SDS), 50 mM sodium phosphate, pH 8.0, and 2 mM EDTA at 90 °C for 5 min. Samples were then supplemented at 4 °C to achieve 50 mM sodium phosphate, pH 7.2, 0.5% SDS, 1% sodium deoxycholate, 1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 1% aprotinin, and 200 μg/ml leupeptin. Selected α subunit-directed antisera were added (1:50), followed 18 h later by protein A-Sepharose (22). Precipitates were collected and washed in buffered 0.5% Triton X-100. The antisera used in this study were generated in rabbits using conjugated peptides. The peptides corresponded to: (i) the COOH-terminal 10 residues common to α_{i1} and α_{i2} (antiserum 0116, which recognizes $\alpha_{i1} \sim \alpha_{i2} > \alpha_{i3}$ and is analogous to antisera 8729/30 described previously; Refs. 23 and 24); (ii) the COOH-terminal 10 residues of α_q (0946, which recognizes α_q and probably α_{11}; Ref. 25); (iii) residues 111–125 of α_z (2919; Ref. 23); and (iv) the COOH-terminal 10 residues of α_s and α_{13} (1190 and 120, respectively).

Western blotting was carried out using α subunit-directed antisera (1:100), biotinylated goat anti-rabbit IgG, avidin-conjugated horseradish peroxidase, and hydrogen peroxide/4-chloro-1-naphthol as described previously (22).

Analysis of Protein-bound Fatty Acids—Incorporation of ^{3}H-labeled fatty acids into subunits was analyzed either by fluorography following SDS-polyacrylamide gel electrophoresis (SDS-PAGE) or by high pressure liquid chromatography (HPLC) following release of fatty acids by alkaline methanolysis. For SDS-PAGE, immunoprecipitates were solubilized at room temperature in sample buffer containing 1% SDS, 50 mM Tris·HCl, pH 6.8, 10% glycerol, 0.08% bromphenol blue, and 0.1% β-mercaptoethanol. Gels were stained, destained, and soaked in Amplify® (Amersham Corp.) according to the manufacturer's specifications before drying and fluorography (15). In some experiments, destained gels were treated overnight with 1 M hydroxylamine, pH 7.0, or, as a control, 1 M Tris·HCl, pH 7.0.

For release and analysis of protein-bound fatty acids by HPLC (21), immunoprecipitated subunits were first extracted with vigorous vortexing once with acetone, three times with chloroform/methanol (2:1), and once with methanol. Dried, delipidated subunits were subjected to alkaline methanolysis, and the released fatty acids (>95% protein-bound radioactivity) were extracted with hexane, dried, redissolved in methanol, and analyzed. Separation of fatty acids and fatty acid methyl esters was achieved by reverse-phase HPLC using a Microsorb C18 column (4.6 mm × 30 cm, Rainin). The fatty acids were eluted with 40–90% (v/v) acetonitrile from 0 to 22:45 min, 90–94% from 22:45 to 25 min, 94% from 25 to 40 min, 94–97% from 40 to 41 min, and 97% from 41 to 55 min, in 17 mM phosphoric acid at a flow rate of 1 ml/min. The absorbance profile of eluted compounds was monitored at 205 nm, and retention times of radioactive alkaline methanolysis products were determined by coelution with unlabeled fatty acid and fatty acid methyl ester standards (Sigma).

RESULTS

We first addressed whether G protein α subunits were subject to palmitoylation in human platelets. Platelets were incubated with [^{3}H]palmitate, and α subunits were subsequently immunoprecipitated. As shown in Fig. 1, incorporation of radiolabel was clearly evident by fluorography following SDS-PAGE for the several α subunits examined. Depicted in the figure are the one or more subtypes of α_i ($\alpha_{i2} > \alpha_{i3} > \alpha_{i1}$ in platelets; Ref. 22), α_q and/or α_{11} (referred to as α_q), α_z, and α_{13}. Labeling of α_s was also observed in separate experiments following longer times of exposure (not shown). Differences in fluorographic intensities probably reflect the relative abundance of subunits and/or properties of the antibodies used, as implied by a similar pattern of intensities achieved by Western blotting (not shown). The identity of the incorporated radiolabel as [^{3}H]palmitate was confirmed for several of the subunits (*i.e.* α_q, α_z, and α_{13}) by HPLC analysis following alkaline methanolysis (see below).

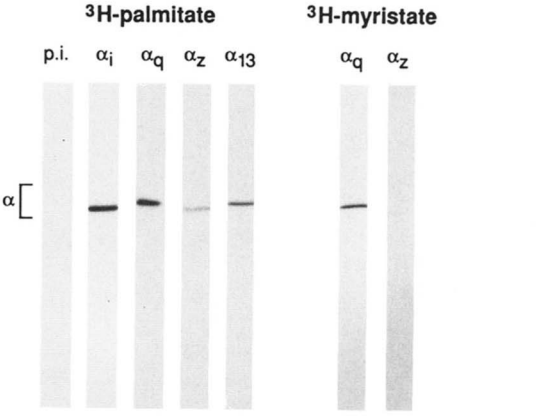

FIG. 1. **Incorporation of [^{3}H]palmitate and [^{3}H]myristate into G protein α subunits.** Washed, PGE_1-treated platelets were incubated with [^{3}H]palmitate or [^{3}H]myristate (250 μCi/ml) in 3.6 mg/ml BSA for 2 h at 37 °C. Platelets were washed and solubilized in 0.5% SDS at 90 °C, and immunoprecipitation was carried out with antisera 0116 (α_i), 0946 (α_q), 2919 (α_z), and 120 (α_{13}); 0116 preimmune serum (*p.i.*) served as a control for specificity. Illustrated are fluorographs following subjection of the immunoprecipitates to SDS-PAGE. Times of exposure were 6 and 28 days for [^{3}H]palmitate and [^{3}H]myristate, respectively.

The possibility that myristate might also be incorporated into α subunits was similarly examined. α_q and α_z (the only subunits tested) incorporated low amounts of radiolabel upon incubation of platelets with [^{3}H]myristate (Fig. 1, *right panel*; note the 28-day exposure time). The bound fatty acid, however, was not [^{3}H]myristate, as anticipated, but rather [^{3}H]palmitate, as determined by HPLC analysis for α_q (not shown). The [^{3}H]palmitate was presumably generated through a 2-carbon chain elongation, which was demonstrated previously to occur, albeit modestly, in human platelets (16, 26). Thus, post-translational myristoylation is not evident for G protein α subunits.

Several, though not all, palmitoylated proteins in human platelets can incorporate polyunsaturated fatty acids such as arachidonate (21). Fig. 2 suggests that G protein α subunits are among these proteins. All α subunits examined: α_i, α_q, and α_z (also α_{13}, not shown), incorporated radiolabel upon incubation of platelets with [^{3}H]arachidonate. Differences in labeling among subunits paralleled those occurring with [^{3}H]palmitate. The identity of the incorporated fatty acid as arachidonate was validated by HPLC by demonstrating the formation of [^{3}H]methyl arachidonate upon alkaline methanolysis (Fig. 3, *upper panel*). Here, immunoprecipitated α_q yielded [^{3}H]methyl arachidonate alone. Similar results were obtained with α_{13} and α_z (not shown). The profile obtained for α_q when platelets were incubated with [^{3}H]palmitate is shown in the *lower panel*. Recovery of radioactivity by HPLC was approximately 70%.

The covalent nature of [^{3}H]palmitate and [^{3}H]arachidonate incorporation was implied by the stability of the radiolabel association with the protein during SDS-PAGE and extraction

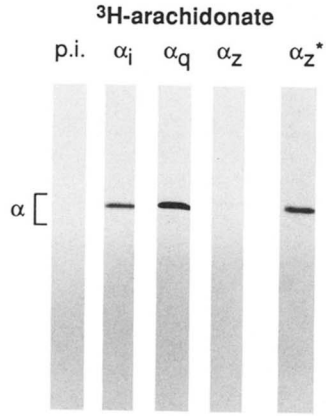

FIG. 2. **Incorporation of [^{3}H]arachidonate into G protein α subunits.** Platelets were incubated with [^{3}H]arachidonate (250 μCi/ml) as described in Fig. 1. Immunoprecipitation was carried out with antisera 0116 (α_i), 0946 (α_q), and 2919 (α_z); 0116 preimmune serum (*p.i.*) served as a control. Illustrated is a fluorograph following SDS-PAGE. Time of exposure was 6 days except for α_z*, which represented a 31-day exposure.

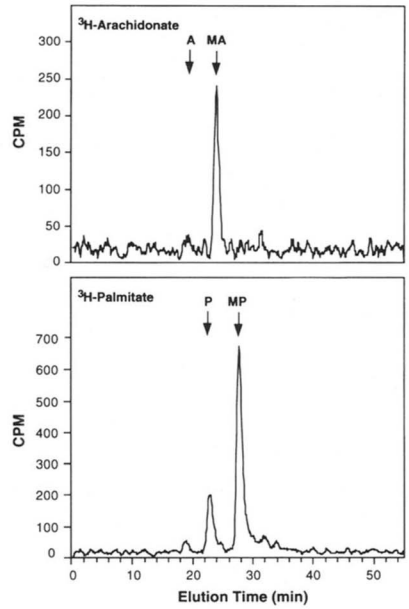

FIG. 3. **Confirmation of fatty acid identity by HPLC.** α_q immunoprecipitates from [^{3}H]arachidonate- or [^{3}H]palmitate-labeled platelets were extracted with acetone, chloroform/methanol, and methanol, then subjected to alkaline methanolysis to release incorporated radiolabel. Analysis of fatty acids and fatty acid methyl esters was achieved by reverse-phase HPLC using acetonitrile gradients. Shown are elution profiles for radioactivity, together with positions at which fatty acids and fatty acid methyl ester standards elute (*A*, arachidonate; *MA*, methyl arachidonate; *P*, palmitate; *MP*, methyl palmitate).

with organic solvents. That the covalent bond was an ester was indicated by sensitivity to alkaline methanolysis. The nature of the linkage was explored further using hydroxylamine, which at neutral pH cleaves thioester bonds (27). As shown in Fig. 4, greater than 80% of the [^{3}H]arachidonate incorporated into α_q was sensitive to hydroxylamine, as was a similar percentage of [^{3}H]palmitate. Similar data were obtained for α_z. The sensitivities of incorporated arachidonate and palmitate to hydroxylamine were comparable to those observed previously for total platelet protein (21). These data are consistent with the linkage of both palmitate and arachidonate primarily to cysteine residues.

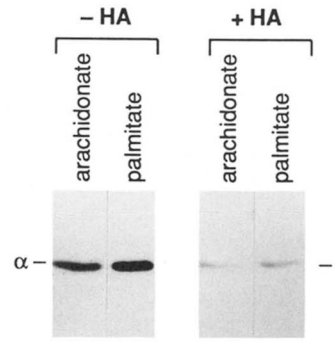

FIG. 4. **Sensitivity of incorporated [^{3}H]arachidonate and [^{3}H]palmitate to neutral hydroxylamine.** α_q immunoprecipitates from [^{3}H]arachidonate- or [^{3}H]palmitate-labeled platelets were subjected to SDS-PAGE, and stained/destained gels were treated with 1 M Tris·HCl, pH 7.0 (*–HA*), or with 1 M hydroxylamine, pH 7.0 (*+HA*), for 12 h. Gels were washed and processed for fluorography.

DISCUSSION

Our results show that G protein α subunits are specific substrates for arachidonoylation and that palmitoylation can occur for α subunits in a context unrelated to overexpression. Arachidonoylation and palmitoylation are necessarily post-translational, since platelets are anucleate cells that synthesize little, if any, protein. Previously, palmitoylation of endogenous subunits had been demonstrated for α_s, α_i, and α_q in PC12 and NG108 cells (9), and for α_s and α_i in COS cells (12). Our observations are in agreement with these, and we now extend the findings to α_z and α_{13}.

Fatty acid specificity in post-translational acylation is evident. Although most proteins that are palmitoylated in platelets can also incorporate myristate, apparently at the same site (16), the α subunits do not, at least within the limits of detection. In our experiments, the low amount of label incorporated into α subunits using [^{3}H]myristate was [^{3}H]palmitate, presumably generated through chain elongation. This observation is not inconsistent with the previous interpretation of myristoylation as a subordinate post-translational modification (16), but rather represents an extreme of the selectivity for palmitate relative to myristate. These findings do not preclude an amide-linked myristate for α_z and subtypes of α_i, since *N*-myristoylation probably occurs upon synthesis of the subunits in megakaryocytes and would not show up as incorporated ^{3}H in these experiments.

Virtually all of the α subunits examined (α_i, α_q, α_z, and α_{13}) incorporated [^{3}H]arachidonate in intact, resting platelets at a concentration of added fatty acid (1.2 μM) approximating that bound normally to albumin (28). Issues of stoichiometry were not approached. Esterification of arachidonate into phospholipids such as phosphatidylcholine and phosphatidylinositol, which is known to be more rapid than esterification of other fatty acids (29), may limit its availability for protein acylation. We have not yet examined regulation of palmitoylation and/or arachidonoylation of α subunits by agonists, which has recently been reported for α_s in the context of palmitoylation (12). However, platelets release large amounts of arachidonate in response to thrombin (30), suggesting that arachidonoylation of proteins may indeed be subject to regulation during platelet activation.

Several, though not all, palmitoylated proteins in human platelets can incorporate polyunsaturated fatty acids (21). In preliminary experiments, [^{3}H]arachidonate- and [^{3}H]palmitate-labeled forms of α_q, when treated with CNBr, gave rise to a labeled 6-kDa peptide anticipated for that encompassing Cys^9 and Cys^{10} (data not shown). It is conceivable, therefore, that arachidonate may be incorporated as an alternative to palmi-

tate at the same site(s). Proteins that incorporate palmitate indeed exhibit a propensity to incorporate other fatty acids. However, the reported alternatives have been limited to myristate (rarely), stearate, and oleate. P-selectin from platelets, for example, has been demonstrated to contain palmitate and even greater amounts of stearate (20). Bovine rhodopsin, in one study (17), was found to contain palmitate, stearate, and oleate consistently, and arachidonate sporadically at very low levels. In another study (19), only palmitate and stearate were detected. Human erythrocyte Band 3 protein contains myristate, palmitate, oleate, and stearate (18). Thus, the observation that G protein α subunits can bind arachidonate is a novel finding. Whether incorporation occurs by virtue of a relaxed specificity of a platelet transferase for fatty acids or represents a property otherwise unique to platelets remains to be determined. Platelet proteins that incorporate arachidonate also incorporate eicosopentaenoate (21), an observation that may prove true for α subunits.

Incorporation of different fatty acids at the same site is reminiscent of the data presented by Neubert *et al.* (31) and Kokame *et al.* (32) for lipid modifications of α_t falling under the rubric of *N*-myristoylation. α_t contains a consensus sequence for *N*-myristoylation, but the lipids incorporated are C12:0, C14:1, and C14:2 predominantly. The presence of C12:0 or C14:0 at the NH_2 terminus makes a severalfold difference in interactions of α_t with $\beta\gamma$ (32) and possibly interactions of α_t with membrane. Palmitoylation is critical to the anchorage of subunits such as α_s and α_q, and consequently (or perhaps independently) to interactions of these subunits with receptors and/or effectors (11). We anticipate that the difference between C16:0 and C20:4 will prove significant in this regard, and possibly in interactions with $\beta\gamma$.

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