Palmitoylation in G-protein signaling pathways

The reversible palmitoylation of G proteins and their receptors is involved both in receptor desensitization and in association and disassociation of Gα subunits with the plasma membrane.

Some say that geography is destiny. Cell biologists feel this way, and biochemists are sometimes forced to agree. Exactly where a signaling protein resides in a cell can markedly influence what it does, how well it does it and to whom it does it. In signal transduction pathways, controlled subcellular localization of receptors, G proteins and effectors influences both the fidelity of signaling — who talks to whom — and the long-term control of signaling through receptor endocytosis and down-regulation. Over the past few years, the reversible palmitoylation of both G proteins and G-protein-coupled receptors has been recognized as one way of regulating their localization and activity.

Both G protein α subunits and G-protein-coupled receptors are palmitoylated through thioester linkages to cysteine residues. The receptors, which are intrinsic membrane proteins based on a bundle of seven hydrophobic, membrane-spanning helices, are palmitoylated in their carboxy-terminal cytoplasmic domains, about 12 residues past the end of the seventh helix. Some receptors terminate just after this residue, but others contain extensive carboxy-terminal domains of 150 amino-acid residues or more.

G protein α subunits, which bind GTP and which interact directly with both receptors and effectors, are palmitoylated near their amino termini, usually at residue 3. The most typical mature amino-terminal structure is an *N*-myristoylated glycine followed by an *S*-palmitoylated cysteine. These hydrophobic modifications of Gα subunits are in addition to the prenylation, usually by a geranylgeranyl thioether, of Gγ subunits on the carboxy-terminal cysteine methyl ester.

In the case of the G proteins, which do not have notably hydrophobic amino-acid sequences and which bind at the inner surface of the plasma membrane, palmitoylation and other alkylations increase their overall hydrophobicity and promote their attachment to the membrane. For the already hydrophobic receptors, the differential hydrophobicity contributed by a palmitoyl group is trivial. In either case, why has biology chosen alkylation as a way of increasing hydrophobicity rather than the permanent addition of a few more leucine or valine residues? There are at least two answers.

First, palmitoylation may enhance binding to particular proteins rather than just enhancing membrane attachment. Such recognition of specific cellular structures can change the location of receptors or G proteins in cells. The reversibility of the palmitoylation, triggered directly or indirectly by an agonist, raises the even more enticing prospect that the cell can regulate both the relocalization of signaling proteins and their associations with other proteins.

Regulated turnover of receptor palmitoylation

Ever since the palmitoylation of rhodopsin was first observed in 1988 [1], a small but steady series of papers has described palmitoylation of other G-protein-coupled receptors; nearly all the receptors contain the palmitoylatable cysteine residue after the seventh span. But mutation of this cysteine generally has subtle, if any, effects. An engineered rhodopsin mutant with no cytoplasmic cysteine residues is functionally wild type when measured in an *in vitro* reconstituted assay [2]. Similarly, cysteine missense mutants of β_2-adrenergic [3], D_1 dopaminergic [4] and α_{2A}-adrenergic [5] receptors, which do not become palmitoylated, have all been reported to display unaltered signaling phenotypes when expressed (at fairly high levels) in cultured cells. In another study, a similar cysteine mutation in the β_2-adrenergic receptor caused only subtle changes in G-protein coupling [6,7].

Two observations by Bouvier and co-workers [3,4] made receptor palmitoylation more intriguing by linking it to receptor desensitization, which occurs upon the continued exposure of cells to agonists. Desensitization and subsequent resensitization of G-protein-coupled receptors are the result of a complex set of processes. They include receptor phosphorylation by at least two protein kinases, decreased coupling to G protein (probably caused both by phosphorylation and by the binding of inhibitory proteins known as arrestins), receptor endocytosis and degradation, and (frequently) a decrease in new receptor synthesis.

How does palmitoylation fit in with these processes? Mouillac *et al.* [3] reported that continued exposure of cells to agonist increases the slow, *in vivo* labeling of β_2-adrenergic receptors with [^{3}H]palmitic acid, an observation later confirmed for D_1 receptors [4]. Of even greater interest, a non-palmitoylated mutant receptor (Cys341Gly) displayed several features of desensitization without exposure to agonist. The mutant receptor was active in signaling, but a little less so than the wild type. Unlike the wild-type receptor, however, the mutant was not much uncoupled by continued agonist exposure. The mutant receptor was also more highly phosphorylated

than the wild type in the absence of agonist, but was not phosphorylated much further when agonist was added. Similar observations were made for an analogous mutant D_1 receptor [4].

When these phenomena are put together and some technical inadequacies overlooked, they make a story that links turnover of palmitoyl groups to receptor desensitization. The simplest version of the story is that unstimulated receptor in the plasma membrane is largely palmitoylated. When stimulated by agonist, the receptor not only activates G protein, but also becomes a better substrate for the enzyme palmitoyl-protein thioesterase, which removes palmitoyl groups [8]. Depalmitoylation triggers receptor phosphorylation, consequent uncoupling and (perhaps) later endocytosis. In this scheme, the observed stimulation of labeling with [^{3}H]palmitate in response to continued exposure to agonist reflects some part of the resensitization pathway, perhaps that involved in movement of receptors into or out of endosomes [9]. More detailed speculations can be left to the imagination of the reader.

Now for the hard part. So far, it has not been possible to measure receptor palmitoylation. Labeling with [^{3}H]palmitate is expensive, inefficient and hard to detect — two-week exposure of fluorographs of gels of immunoprecipitates or affinity-purified receptor is the norm. Further, labeling with [^{3}H]palmitate may reflect increased steady-state palmitoylation, unchanged net palmitoylation combined with rapid turnover, or even a net decrease in palmitoylation if thioesterase activity is high. So far, there are no data that speak (even qualitatively) to the loss of palmitate from prelabeled receptor. Second, it is unclear which population of receptor — palmitoylated or unpalmitoylated — is under observation in assays of signaling, phosphorylation or endocytosis. Last, some receptors lack the palmitoylated cysteine yet undergo the cycle of uncoupling, endocytosis and recovery. Thus, although receptor palmitoylation certainly seems to be involved in modulating sensitivity to agonists, and in very interesting ways, its control and mechanism of action remain open questions for the G-protein signaling community.

Gα subunit palmitoylation and depalmitoylation

Like the receptors that regulate them, G protein α subunits also undergo a cycle of depalmitoylation and palmitoylation. Gα subunits are palmitoylated on a conserved cysteine residue, usually found at position 3 [10,11], and palmitoylation enhances Gα binding to membranes. A non-palmitoylated mutant of $G_o\alpha$ is found primarily in the cytosol, and the analogous Cys3 mutants of $G_s\alpha$ and $G_q\alpha$, the wild-type forms of which bind more tightly to membranes than does $G_o\alpha$, are found in cytosol fractions at least to some extent [12,13]. This diminished membrane binding may in fact completely account for the diminished signaling activities noted in some of these mutants [13], because non-palmitoylated Gα subunits are active *in vitro*. $G_i\alpha$, $G_o\alpha$ and $G_q\alpha$ are all largely depalmitoylated during purification, as assessed by their abilities to form disulfides either with mastoparans (short receptor-mimetic peptides [14]) or with another cysteine residue near the carboxyl terminus (T. Higashijima and myself, unpublished data). All, however, remain responsive to receptors and purified $G_s\alpha$ and $G_q\alpha$ efficiently activate their respective effector proteins, adenylyl cyclase and phospholipase C-β.

Like receptors, Gα subunits undergo accelerated depalmitoylation and repalmitoylation when they are activated within the cell during signal transduction. The effects of activation on palmitate turnover on Gα are similar whether activation is by receptors, or is the result of covalent modification by cholera toxin (for G_s) or of a mutation that increases the rate of activation by cellular GTP [12,15,16]. In the most thorough study to date, Wedegaertner and Bourne [16] have clarified the linkage between the activation and palmitoylation cycles, and have indirectly estimated the stoichiometry of palmitate turnover. First, they found that activation increased the rate of depalmitoylation of $G_s\alpha$ that had been prelabeled with [^{3}H]palmitate. Activation promoted complete removal of [^{3}H]palmitoyl groups. They argue convincingly that the kinetics of labeling and the subcellular localization of $G_s\alpha$ are consistent with activation-induced depalmitoylation of membrane-bound $G_s\alpha$. Depalmitoylation allowed the Gα to leave the plasma membrane, and repalmitoylation occurred in the cytoplasm. Because both they and Degtyarev *et al.* [15] had found that a mutation that inhibits dissociation of $G_s\alpha$ from Gβγ also inhibits the palmitate cycle, Wedegaertner and Bourne inferred that dissociation of activated Gα from Gβγ precedes hydrolysis of the palmitoyl group and that depalmitoylation is the decisive event in releasing Gα from the membrane. This idea is supported by the observation that depalmitoylation of detergent-solubilized $G_s\alpha$ was inhibited by Gβγ.

The conclusion of this work, assuming that it is confirmed, is that activation-promoted depalmitoylation of Gα subunits causes their slow transfer to the cytosol. Does this mean that the Gα subunits activate cytosolic targets while there? Probably not. Although G proteins deactivate slowly, all but the slowest hydrolyze their bound GTP in less than 3 minutes at 37 °C; G_s and G_i are much faster than this. Thus, by the time much depalmitoylated Gα gets to the cytosol, it is in the inactive, GDP-bound state. The predominant cytosolic species would thus be inactive. Depalmitoylation is more probably involved in long-term regulation of the signaling activity of Gα subunits, perhaps by altering their associations with other important proteins. Results with the Cys3 mutants are consistent with this idea.

True solubilization of Gα subunits by removal of a palmitoyl anchor may also be an oversimplification. As argued by Resh [17], the hydrophobicity of a palmitoyl group is insufficient to anchor a protein to a bilayer. The observed release might indicate that palmitoylation is

important for binding to another membrane protein, Gβγ perhaps. Alternatively, the observed release may only reflect the massive dilution of cytosol that occurs when cells are homogenized. In cells, depalmitoylated Gα may never leave the membrane but only redistribute laterally. The questions that remain are where the depalmitoylated Gα goes, what it does when it gets there and how it gets palmitoylated again and returned to the signaling switchboard.

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