5. In intact cells, the incorporation of label into material comigrating with phosphatidylcholine is often great enough to overwhelm the signal from palmitoyl-CoA. If this is the case, aliquots of lipid extract can be enriched for palmitoyl-CoA by phase partitioning. Redissolve the dried lipid extracts in 1.1 ml chloroform/methanol/10 m*M* Tris, pH 7.4 (5:5:1, v/v/v), then add 0.5 ml chloroform and 0.275 ml water and split the phases as described above for the synthesis of [^{3}H]palmitoyl-CoA. Transfer the phases and dry separately in a Speed-Vac concentrator. The upper aqueous phase will be substantially enriched in palmitoyl-CoA (see Fig. 2).

Measurement of Protein Synthesis and Glycosylation

1. Cells are prepared and incubated under the same conditions as for the relevant protein palmitoylation experiment, but instead of [^{3}H]palmitate, [^{35}S]cysteine (Amersham, Arlington Heights, IL, 50–500 μCi/ml) or [^{3}H]mannose (DuPont NEN, 50 μCi/ml) are used as the radiolabel.
2. After the appropriate labeling period, incubations are quenched by the addition of an equal volume of 20% (w/v) ice-cold trichloroacetic acid (TCA), and the samples are placed on ice for 60 min.
3. Cells grown on monolayers are then scraped and transferred to polypropylene vials.
4. Insoluble material is pelleted (14,000 *g* for 15 min at 4°) and the supernatant discarded.
5. The pellet is washed with the same volume of 1% (w/v) TCA, and then twice with 1/10 volume ether (presaturated with water), with centrifugation as above in between each wash.
6. Redissolve the pellet in a minimum volume of 100 m*M* Tris-buffered 1% SDS (pH 7.4) with heating.
7. Determine incorporation of radioactivity by counting in a scintillation counter after addition of an appropriate water-tolerant scintillant.

[24] Dynamic Palmitoylation of G-Protein-Coupled Receptors in Eukaryotic Cells

By MICHEL BOUVIER, PETER CHIDIAC, TERENCE E. HEBERT, THOMAS P. LOISEL, SERGE MOFFETT, and BERNARD MOUILLAC

Introduction

Fatty acylation of proteins in eukaryotic cells has been found to be a more frequent modification than originally anticipated. Three general

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classes of modification via acylation can be distinguished: N-terminal myristoylation, C-terminal prenylation, and palmitoylation of cysteine residues. It has been proposed that these modifications may contribute to the targeting and anchoring of modified proteins to distinct biological membranes.[1] The observation that integral membrane proteins can also be fatty-acylated, even though they are anchored in the membrane through one or more transmembrane domains, has led to speculation that such acylation may also serve other purposes. Evidence has been found to support the involvement of fatty acylation in protein–protein interaction,[2] protein trafficking,[3,4] and protein phosphorylation.[5]

Observations that many proteins involved in signal transduction are fatty-acylated have raised the intriguing possibility that the modifications may play a regulatory role in the process. In particular, proteins involved in G-protein-mediated signaling appear to be subject to the entire spectrum of the posttranslational modifications. At least six G-protein-coupled receptors have been shown to be palmitoylated. These include the visual pigment rhodopsin,[6] the β_2-adrenergic receptor (β_2-AR),[7] the α_{2A}-adrenergic receptor,[8] the serotonergic 5-HT_{1b} receptor,[9] the luteinizing hormone receptor,[9a] and the dopamine D_1 receptor.[10] In each of the receptors, a cysteine residue located in the carboxyl tail approximately 12 residues from the seventh transmembrane domain has been shown to be thioesterified by a palmitic acid. Such palmitoylation is likely to be a general phenomenon, since cysteine residues located in a similar position are found in the vast majority of the G-protein-coupled receptors sequenced to date (Table I). Although γ subunits of the heterotrimeric G proteins have been found to be isoprenylated by the addition of geranylgeranyl residues,[11]

[1] G. James and E. N. Olson, *Biochemistry* **29,** 2623 (1990).
[2] C. J. Marshall, *Science* **259,** 1865 (1993).
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[4] J. A. Thissen and P. J. Casey, *J. Biol. Chem.* **268,** 13780 (1993).
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[6] Y. A. Ovchinnikov, N. G. Abdulaev, and A. S. Bogachuk, *FEBS Lett.* **230,** 1 (1988).
[7] B. F. O'Dowd, M. Hnatowich, M. G. Caron, R. J. Lefkowitz, and M. Bouvier, *J. Biol. Chem.* **264,** 7564 (1989).
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[9] G. Y. Ng, S. R. George, R. L. Zastawny, M. Caron, M. Bouvier, M. Dennis and B. F. O'Dowd, *Biochemistry* **32,** 11727 (1993).
[9a] N. Kawate and K. M. J. Menon, *J. Biol. Chem.* **269,** 30651 (1994).
[10] G. Y. Ng, B. Mouillac, S. George, M. Caron, M. Dennis, M. Bouvier, and B. O'Dowd, *Eur. J. Pharmacol.* **267,** 7 (1994).
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TABLE I
CLASSIFICATION OF MAMMALIAN G-PROTEIN-COUPLED RECEPTORS[a]

Receptors containing potentially palmitoylated cysteine	Receptors lacking putative palmitoylation site
Adenosine A_1, A_{2a}	Adrenergic α_{2c}
Adrenergic β_1, β_2,[b] β_3, α_{1a}, α_{1b}, α_{1c}, α_{2a},[b] α_{2b}	Parathyroid
Adrenocorticotropic hormone	Prostaglandin EP_1
Angiotensin AT_1, AT_2	Serotonin 5-HT_{5a}, 5-HT_{5b}
Bombesin BB_2	Somatostatin SS_3
Calcitonin gene-related peptide	Thromboxane A_2
Dopamine D_1,[b] D_2, D_3, D_4, D_5	
Endothelin ET_a	
Follicle-stimulating hormone	
Growth hormone-releasing hormone	
Histamine H_2	
Luteinizing hormone[b]	
Melanocortin-stimulating hormone	
Muscarinic acetylcholine M_1, M_2, M_3, M_4, M_5	
Opioid μ, δ, κ	
Oxytocin	
Platelet-activating factor	
Prostaglandin EP_2, EP_{3a}, EP_{3b}, EP_{3c}, EP_{3d}	
Rhodopsin[b]	
Serotonin 5-HT_{1a}, 5-HT_{1b},[b] 5-HT_{1c}, 5-HT_{1d}, 5-HT_{1e}, 5-HT_2, 5-HT_6, 5-HT_7	
Somatostatin SS_1, SS_2, SS_4	
Tachykinin NK_1, NK_2, NK_3	
Thrombin	
Thyroid-stimulating hormone	
Thyrotropin-releasing hormone	
Vasopressin V_{1a}, V_2	
Vasoactive intestinal peptide	

[a] According to the presence or absence of a cysteine in a position similar to the palmitoylated cysteine of the β_2-AR.
[b] Palmitoylation has been experimentally demonstrated.

most α subunits have been found to be both myristoylated[12] and palmitoylated.[13,14] Also, regulatory kinases such as the cAMP-dependent protein kinase (PKA)[15] and a G-protein-coupled receptor kinase (GRK)[16] have been found to be myristoylated and prenylated, respectively.

[12] S. M. Mumby, R. O. Heuckeroth, J. I. Gordon, and A. G. Gilman, *Proc. Natl. Acad. Sci. U.S.A.* **87,** 728 (1990).
[13] M. E. Linder, P. Middleton, J. R. Hepler, R. Taussig, A. G. Gilman, and S. M. Mumby *Proc. Natl. Acad. Sci. U.S.A.* **90,** 3675 (1993).
[14] M. Y. Degtyarev, A. M. Spiegel, and T. L. Z. Jones, *Biochemistry* **32,** 8057 (1993).
[15] C. H. Clegg, W. Ran, M. D. Uhler, and G. S. McKnight, *J. Biol. Chem.* **264,** 20140 (1989).
[16] J. Inglese, W. J. Koch, M. G. Caron, and R. J. Lefkowitz, *Nature (London)* **359,** 147 (1992).

The specific roles played by the modifications in signaling remain the subject of intense investigation, but the following concepts are emerging. Acylation of the G-protein subunits and regulatory kinases is believed to play an important role in the targeting to proper membrane locations[17] and may influence the ability to form functional multimeric complexes.[18] For the receptors, which are integral membrane proteins composed of seven transmembrane domains, palmitoylation has been proposed to promote the formation of an additional cytoplasmic loop.[7] This in turn has been suggested to affect the coupling,[7] phosphorylation,[5] and even internalization[19] of some receptors.

Palmitoylation, unlike other lipid modifications, is truly posttranslational rather than cotranslational,[20] and moreover it has been shown to be reversible.[21] This suggests the possibility of regulatory cycles of palmitoylation–depalmitoylation that may be involved in signal transduction. In this respect, studies have confirmed that palmitoylation of receptors[9,10,22] and of G-protein α subunits[23,24,24a,24b] can be regulated on biological activation. Although the nature of the enzymes which are specifically involved in regulating the palmitoylation sate of the proteins is still unknown, a palmitoyl-protein thioesterase that cleaves palmitate from H-ras has been purified and cloned.[25,25a]

Studying the dynamics of receptor palmitoylation is essential for elucidating the role of the modification in regulating signaling. However, this presents a number of technical problems which are linked to the low abundance of the receptors in native systems, the nature of the modification, and the lack of knowledge of the enzymatic processes involved. We have developed a number of tools and approaches to deal with some of these problems. These are discussed in the following sections of this chapter.

[17] J. A. Pitcher, J. Inglese, J. B. Higgins, J. L. Arriza, P. J. Casey, C. Kim, J. Benovic, M. M. Kwatra, M. Caron, and R. J. Lefkowitz, *Science* **257,** 1264 (1992).

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[24b] P. B. Wedegaertner and H. R. Bourne, *Cell.* **77,** 1063 (1994).

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Palmitoylation of G-Protein-Coupled Receptors in Native and Heterologous Systems

The abundance of rhodopsin in rod outer segments makes it feasible to study the palmitoylation of the G-protein-coupled receptor in cells where it occurs endogenously.[6] However, posttranslational modifications are difficult to observe in tissues expressing other members of the receptor family owing to the relatively low levels of native expression associated with such proteins. This is particularly true for palmitoylation because of the relatively low specific activity of [^{3}H]- or [^{14}C]palmitate in comparison with the [^{32}P]P_i used to detect phosphorylation, for example. One way to circumvent the problem is to study fatty acylation in heterologous systems expressing higher levels of receptor. Palmitoylation of a G-protein-coupled hormone receptor was first demonstrated in Chinese hamster fibroblasts (CHW cells) transfected with the human β_2-AR cDNA and stably expressing 2 pmol of the receptor per milligram of membrane protein.[7] Even at that level of expression, purification of the ^{3}H-palmitoylated receptor from large quantities of cells ($\sim 10^8$), followed by several weeks of exposure, is necessary for the autoradiographic detection of the labeled receptor. Although such a protocol can be used to detect major differences among experimental conditions, quantitation of the relatively subtle dynamic changes associated with palmitate incorporation and turnover requires a more refined system.

Spodoptera frugiperda (Sf9) cells infected with recombinant *Autographica californica* nuclear polyhedrosis viruses (baculoviruses) have been used successfully in many cases to express large quantities of proteins. Proteins expressed using the system (unlike those produced using bacterial expression systems) have largely been found to maintain normal characteristics, including specific posttranslational modifications such as palmitoylation and phosphorylation. Sf9 cells can transiently express up to 50 pmol β_2-AR per milligram of protein after infection with a recombinant baculovirus encoding the receptor.[22,26,27] The detection of relatively small differences in β_2-AR palmitoylation is more feasible with the insect system than with mammalian cells, since more receptors can be purified from a given volume of cells and thus time-dependent changes can be detected on autoradiograms after relatively short durations of exposure.

Construction of Recombinant Baculovirus. To prepare the recombinant baculovirus encoding the human β_2-AR, the plasmid vector pTZ18R (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) containing the entire hu-

[26] E. M. Parker, K. Kameyama, T. Higashijima, and E. M. Ross, *J. Biol. Chem.* **266,** 519 (1991).
[27] S. T. George, M. A. Arbabian, A. E. Ruoho, J. Kiely, and C. C. Malbon, *Biochem. Biophys. Res. Commun.* **163,** 1265 (1989).

man β_2-AR sequence is digested with *Eco*RI–*Hin*dIII[28] and filled in by Klenow DNA polymerase. The β_2-AR cDNA fragment is then placed into the baculovirus recombination vector pJV*ETLZ* at the *Nhe*I site by blunt-end ligation to yield the pJVβ_2-AR construct. Transfer of the β_2-AR and β-galactosidase coding sequences from pJVβ_2-AR to the baculovirus genome is achieved by homologous recombination in Sf9 cells. The recombinant baculovirus is purified by successive plaque assays using β-galactosidase detection.[22]

Culture and Infection of Sf9 Cells. Sf9 cells are maintained at 27°–28° either in serum-supplemented or serum-free media. The experiments described here were carried out on cells grown in Grace's insect medium (GIBCO, Grand Island, NY) supplemented with fetal bovine serum (FBS; 10%, v/v). As outlined below, metabolic labeling with [^{3}H]palmitate should be carried out at minimal levels of serum, and thus the contents of the medium may need to be modified before the experiment is started. Sf9 cells can be grown either as monolayers (in plastic petri dishes or T flasks) or in suspension [in spinner bottles (Bellco, Vineland, NJ) or in Erlenmeyer flasks placed in an orbital shaker] in the presence of an agent which inhibits tearing of cell membranes caused by agitation (e.g., pluronic acid).

Cells should be infected with baculovirus when they are in a logarithmic phase of growth. With cells cultured in serum-supplemented Grace's medium, optimal conditions for infection are found at a density of 1×10^6 to 2×10^6 cells/ml for cells in suspension and near confluence for attached cells (2×10^6 to 4×10^6 cells/ml). At a multiplicity of infection (MOI) of approximately 5 recombinant baculovirus molecules per Sf9 cell, expression of β-adrenergic receptor should be evident 24 hr after exposing the cells to the virus. The presence of the receptor in membranes from infected cells can be detected by the specific binding of a radiolabeled β_2-AR antagonist (e.g., [^{125}I]iodocyanopindolol, [^{125}I]CYP), and receptor function can be assessed with appropriate agonists (e.g., isoproterenol-stimulated adenylate cyclase activity). After 48 hr of infection, the level of receptor expression is found to be maximal or near-maximal, typically around 25 pmol/mg protein for cells in suspension and about half that for attached cells. This difference may be due to the decreased accessibility of the attached cells to the virus. To obtain comparable receptor levels, cells grown as monolayers thus tend to require a higher MOI than do cells in suspension. The proportion of viable cells after 48 hr, as assessed by the ability of cells to exclude trypan blue, is greater than 90%. Further incubation, however, leads to substantial cell morbidity without substantial gain in the level of

[28] B. K. Kobilka, R. A. F. Dixon, T. Frielle, H. G. Dohlman, M. A. Bolanowski, I. S. Sigal, T. Z. Yang-Feng, V. Francke, M. G. Caron, and R. J. Lefkowitz, *Proc. Natl. Acad. Sci. U.S.A.* **84,** 46 (1987).

expression. Therefore, we and others have chosen 48 hr as an optimal time of infection. Additional details about baculovirus and Sf9 cells are found in O'Reilly *et al.*[29]

Charcacterization of Human β_2-Adrenergic Receptor Expressed in Sf9 Cells. When using a heterologous expression system to study a given receptor, an appropriate assessment of the properties of the receptor is required. Although characterization of the β_2-AR in Sf9 cells has been carried out, similar studies would be needed before such a system could be used to study the palmitoylation of other receptors. The binding affinities of β-adrenergic agonists and antagonists determined using the baculovirus expression system are consistent with those reported in mammalian systems.[22,26,30] The agonist isoproterenol stimulates adenylate cyclase activity in a dose-dependent manner in membranes derived from Sf9 cells infected with baculovirus encoding either human β_2-AR[22] (Fig. 1) or avian β-AR.[26] Whereas isoproterenol dose–response curves are similar in appearance in membranes from Sf9 and mammalian cells, maximal stimulation of cAMP production occurs at lower agonist concentrations in Sf9 cells, presumably owing to the relatively large number of β_2-AR typically observed in such preparations.

Similar to observations in mammalian cells, treatment of β_2-AR-expressing Sf9 cells with isoproterenol leads to an increase in the intracellular level of cAMP.[30] Furthermore, short-term (5–30 min) agonist treatment is associated with a time-dependent decrease in maximal agonist-induced stimulation in membranes (Fig. 1A). Similar changes in maximal activity in mammalian systems have been attributed to receptor desensitization.[31] These agonist-induced decreases are accompanied by apparent decreases in agonist potency and by increased receptor phosphorylation (Fig. 1B), supporting the notion that β_2-AR desensitization in Sf9 cells is similar to that in mammalian cells. Moreover, desensitization in Sf9 cells appears to involve phosphorylation of the receptor by at least two protein kinases. As in mammalian cells, partial desensitization and phosphorylation are observed after treatment of β_2-AR-expressing Sf9 cells with the PKA activator dibutyryl-cAMP,[31a] suggesting that a second enzyme, possibly a G-protein-coupled receptor kinase (GRK), is also involved. Thus, both the activation of second messenger systems and the desensitization of G-pro-

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[30] P. Chidiac, T. E. Hebert, M. Valiquette, M. Dennis, and M. Bouvier, *Mol. Pharmacol.* **45,** 490 (1994).

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[31a] S. St. Onge, B. Mouillac, P. Chidiac, and M. Bouvier, manuscript in preparation.

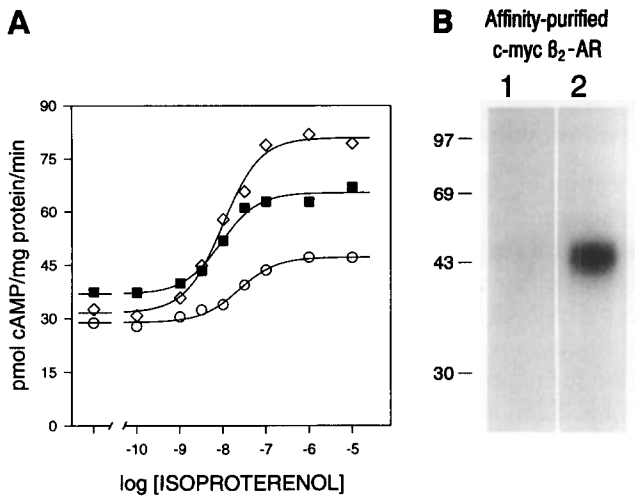

FIG. 1. Effect of isoproterenol treatment of β_2-AR-expressing Sf9 cells on agonist-stimulated adenylate cyclase activity and receptor phosphorylation. (A) Sf9 cells infected for 48 hr with recombinant baculovirus encoding the human β_2-AR were treated with vehicle (0.1 m*M* ascorbate) for 30 min (◇) or with 1 μM isoproterenol for 15 (■) or 30 min (○). Membrane adenylate cyclase activity was assayed and data were analyzed as described.[30] (B) Sf9 cells infected for 48 hr with β_2-AR-encoding baculovirus were labeled for 120 min with [^{32}P]P_i and subsequently exposed to 1 μM isoproterenol for 15 min. β_2-AR were purified by affinity chromatography. Lane 1, Untreated cells; lane 2, isoproterenol-treated cells. Molecular weight markers are indicated to the left of Lane 1.

tein-coupled receptors in β_2-AR-expressing Sf9 cells are comparable to those in mammalian systems.

In addition to the β_2-AR, several other G-protein-coupled receptors have been expressed in the baculovirus/Sf9 system. Pharmacological as well as biochemical properties similar to those found in mammalian systems were reported.[9,10,32] In spite of the functional similarities, however, it should be noted that the glycosylation of integral membrane proteins expressed using the baculovirus/Sf9 system differs from that found in mammalian expression systems owing to the incomplete processing of N-linked oligosaccharides in the insect cell line.[33] Although this leads to decreases in the apparent molecular weights of G-protein-linked receptors in Sf9 cells compared to mammalian cells (see Mouillac *et al.*[22] and references cited therein), β_2-AR function, which is relatively unaffected by deglycosylation, appears to be normal. However, in a receptor where function or ligand binding is affected by changes in the attached sugar residues, some functional differ-

[32] S. K.-F. Wong, E. M. Parker, and E. M. Ross, *J. Biol. Chem.* **265,** 6219 (1990).
[33] V. A. Luckow and M. D. Summers, *Bio/Technology* **6,** 47 (1988).

ences between receptors expressed in mammalian and Sf9 cells might be anticipated.

Dynamic Palmitoylation of β_2-Adrenergic Receptor

Incorporation of [^{3}H]palmitate has been demonstrated for a number of proteins in mammalian cells. These include several proteins involved in signal transduction such as a 64-KDa growth factor-sensitive protein in the BC_3H1 muscle cell line,[34] the transferrin receptor,[21] the insulin receptor,[35] p21N-RAS,[36] GAP-43,[37] and several G-protein-coupled receptors.[6-10] However, as outlined above, the study of the dynamic regulation of palmitate incorporation in mammalian cells has been difficult. Because palmitoylation of proteins has been shown to occur normally in baculovirus-infected Sf9 cells, this system offers an alternative to study the dynamics of protein palmitoylation.

Metabolic Labeling

Optimization of Labeling of β_2-Adrenergic Receptor for Dynamic Studies. Before one can characterize the regulation of receptor acylation, an analysis of the labeling kinetics must be undertaken. This is especially true when labeling cells with a compound such as [^{3}H]palmitate. Indeed, the large cellular pool of palmitate and rapid turnover of the fatty acid prevents isotopic equilibrium of the cellular palmitate and palmitoylated proteins.

To study the kinetics of β_2-AR palmitoylation, 100 ml of Sf9 cells are grown in spinner flasks and infected with the β_2-AR-encoding baculovirus as described above. At 30 hr postinfection, cells are transferred to serum-free Grace's medium. At 47 hr cells are counted and brought up to 1% (v/v) FBS at a density of approximately 50 million cells/20 ml. At 48 hr, [9,10-^{3}H]palmitic acid (60 Ci/mmol) dissolved in 50 μl of dimethyl sulfoxide (DMSO) is added to the culture at a final concentration of 0.2 mCi/ml. After various times, the labeling is terminated by centrifugation at 500 *g* for 5 min at 4° followed by two washes with ice-cold PBS. The cells are then disrupted by sonication in 10 ml of ice-cold buffer containing 5 m*M* Tris-HCl, pH 7.4, 2 m*M* EDTA, plus a protease inhibitor cocktail (5 mg/ml leupeptin, 10 mg/ml benzamidine, and 5 mg/ml soybean trypsin inhibitor). Lysates are centrifuged at 500 *g* for 5 min at 4°, the pellets are sonicated as before and spun again, and the supernatants are pooled. The pooled

[34] G. James and E. N. Olson, *J. Biol. Chem.* **264,** 20998 (1989).
[35] J. Hedo, E. Collier, and A. Watkinson, *J. Biol. Chem.* **262,** 954 (1987).
[36] A. I. Magee, L. Gutierrez, I. A. McKay, C. J. Mafshall, and A. Hall, *EMBO J.* **6,** 3353 (1987).
[37] J. P. H. Skane and I. Viràg, *J. Cell. Biol.* **108,** 613 (1989).

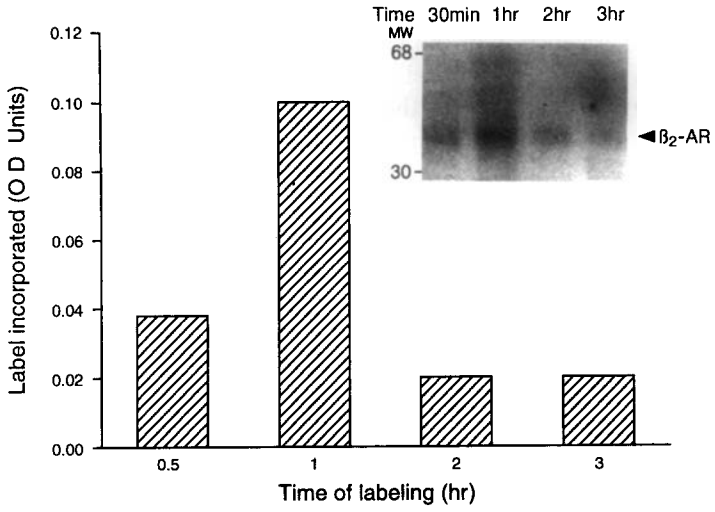

FIG. 2. Tritiated palmitate incorporated into β_2-AR purified by affinity chromatography on alprenolol-Sepharose. Sf9 cells were metabolically labeled for the times indicated with tritiated palmitic acid as described in the text. Bars represent a densitometric scan of the fluorogram shown in the inset. Molecular weight markers are indicated to the left of Lane 1 of the inset.

supernatant is then centrifuged at 45,000 *g* for 20 min, and the pellets are washed twice in the same buffer. The receptor is solubilized in digitonin and purified as described below.

Following purification, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) is carried out under nonreducing conditions (the thioester bond of cysteine to palmitate is somewhat sensitive to reducing agents such as dithiothreitol and 2-mercaptoethanol) on 10% slab gels.[38] Equal amounts of receptor, as determined by a soluble radioligand binding assay using [^{125}I]CYP as the radioligand, are loaded into each lane. To allow fluorographic detection of the incorporated palmitate, fixed gels are incubated in Enlightening (DuPont, Mississauga, Canada) for 30 min, dried, and exposed to Kodak (Rochester, NY) XAR-5 film at −70° for several days. Using this protocol, incorporation of tritiated palmitate can be detected in purified proteins (in the case of the β_2-AR, ~5 pmol of receptor) after 1 to 2 weeks of exposure.

Figure 2 shows the results of a densitometric scan of an autoradiogram representing different times of labeling. As seen in Fig. 2, label incorporation peaks at 1 hr and declines sharply thereafter. This indicates clearly that turnover of the incorporated palmitate is rapid. The observed decrease

[38] U. K. Laemmli, *Nature (London)* **227,** 680 (1970).

in labeling is most likely due to a decrease in specific activity of the palmitate resulting from exhaustion of the labeled palmitate in poorly defined metabolic pathways. Thus, a 1-hr labeling period is selected for optimal labeling. One caveat to be considered is that the specific activity of the donor pool of palmitate is not at equilibrium. This must be taken into account when the dynamics of palmitate incorporation are studied.

Characterization of Incorporated Radiolabel. The most direct way to identify the nature of the incorporated fatty acid would certainly be analysis by mass spectrometry.[39] However, a simpler approach requiring considerably less purified protein can satisfactorily confirm the identity of the [^{3}H]palmitate incorporated. Unlike the N-terminal amide linkage or the C-terminal thioether linkage of myristoylation and prenylation, respectively, thioesterification of cysteine by palmitic acid is sensitive to neutral hydroxylamine. Sensitivity to hydroxylamine can be determined by treating either the soluble purified protein or, alternatively, the polyacrylamide gel following electrophoresis. Solubilized receptors (as described below) can be treated with 1 *M* hydroxylamine, pH 7.0, for 12 hr at 22°. Alternatively, fixed SDS–polyacrylamide gels can be incubated with 1 *M* hydroxylamine, pH 7.0, for 16 hr, washed extensively, dried, and exposed to Kodak XAR film at −70°. This technique also has the advantage of distinguishing between covalently attached [^{3}H]palmitate and nonspecifically adsorbed tritiated lipid.

Covalent attachment of the palmitate can also be confirmed by assessing the resistance of the labeling to organic extraction. Noncovalently bound lipids can be extracted from the purified receptor in a mixture of chloroform/methanol (2:1, v/v). The extract is mixed vigorously, and after a 30-min incubation at room temperature the protein is pelleted by centrifugation at 4500 *g* for 15 min at room temperature. This extraction is repeated twice and then twice with chloroform/methanol/water (1:1:0.3, v/v/v) and finally with methanol alone. After each extraction step, the mixture is incubated for 10 min at room temperature. The pellets can then be solubilized in nonreducing SDS–PAGE sample buffer.

Analysis of the tritiated lipids incorporated into the β_2-AR can also be performed using ascending chromatography. The receptor band is excised from polyacrylamide gels, homogenized in 0.1 *M* $(NH_4)HCO_3$, pH 7.7, and digested with trypsin (0.3 mg/ml) for 15 min at 37°. The digest is acidified and extracted with hexane. The extract is lyophilized and treated with 1 *M* KOH for 12 hr at 37° to cleave the attached lipid. Finally, the extract is dried under nitrogen and applied to a silica gel thin-layer chromatography

[39] D. I. Papac, K. R. Thornburg, E. E. Bullesbach, R. K. Crouch, and D. R. Knapp, *J. Biol. Chem.* **267,** 16889 (1992).

(TLC) plate along with tritiated lipid standards in parallel lanes. The chromatogram is developed with hexane/ethyl acetate/acetic acid (80:20:1, v/v/v).

Receptor Purification

To characterize the incorporation of labeled palmitate into the β_2-AR, it is necessary to purify the receptor. Following metabolic labeling carried out as described above, β_2-AR is solublized from membranes in 12 ml of a buffer containing 100 m*M* NaCl, 10 m*M* Tris-HCl, 5 m*M* EDTA, pH 7.4, 0.3% (w/v) digitonin, and a protease inhibitor cocktail with mild agitation at 4° for 90 min. Nonsolubilized material is removed by centrifugation at 45,000 *g* for 20 min at 4°. At this point, either of two procedures can be used to purify the metabolically labeled β_2-AR.

Affinity Purification of β_2-Adrenergic Receptor. Solubilized β_2-AR can be purified by affinity chromatography using a Sepharose matrix coupled to the β-adrenergic antagonist alprenolol.[40] Alprenolol-Sepharose columns (6 ml of gel per column) are equilibrated with 5 bed volumes of buffer A (100 m*M* NaCl, 10 m*M* Tris-HCl, 2 m*M* EDTA, pH 7.4, 0.05% (w/v) digitonin, and protease inhibitors). Solubilized receptor is loaded on the column and shaken gently for 2 hr at room temperature to allow binding of the receptor to the matrix. The supernatant is then allowed to flow through, and the columns are placed at 4° and washed with 25 ml of a buffer containing 500 m*M* NaCl, 50 m*M* Tris-HCl, 2 m*M* EDTA, pH 7.4, 0.05% (w/v) digitonin, and protease inhibitors. The original ionic strength is restored by washing with 50 ml of buffer A. After the columns are returned to room temperature, receptors are eluted with buffer A containing 60 μM alprenolol. The eluate is concentrated with Centriprep and Centricon cartridges (Amicon, Danvers, MA) down to 50 μl. Recovery of β_2-AR after affinity purification is measured by soluble binding after desalting on a Sephadex G-50 gel filtration column to remove alprenolol. The purified sample can then be prepared for electrophoretic analysis.

Until relatively recently, Sepharose-alprenolol stationary phase affinity chromatography of digitonin-solubilized receptor was the only available method to purify the β_2-AR. Although this method is commonly used, yields rarely exceed 30%.

Immunopurification of c-myc-Tagged β_2-Adrenergic Receptor. Several attempts to raise antibodies against native G-protein-coupled receptors have failed to produce high-affinity antibodies which could be used in immunoaffinity purification procedures. To circumvent this problem, an

[40] J. L. Benovic, R. G. L. Shorr, M. G. Caron, and R. J. Lefkowitz, *Biochemistry* **23,** 4510 (1984).

epitope-tagged receptor is constructed. Eleven amino acids from the c-*myc* protein are inserted immediately before the initial methionine at the NH_2 terminus of the β_2-AR. The site of epitope insertion is chosen because that domain of the β_2-AR does not appear to be involved in ligand recognition or signal transduction. The recombinant baculovirus encoding the epitope-tagged β_2-AR is constructed as described above with the following modifications. Two complementary oligodeoxynucleotides encoding the c-*myc* epitope (5′ AATTC^ATGGAGCAAAAGCTCATTTCTGAAGAG-GACTTGAAT^GC 3′ and 3′ G^TACCTCGTTTTCGAGTAAAGACT-TCTCCTGAACTTAC^GGTAC 5′) are inserted between *Eco*RI and *Nco*I sites in the pTZβ_2-AR. The pharmacological and biochemical properties of the epitope-tagged β_2-AR are found to be indistinguishable from those of the wild-type receptor when expressed in Sf9 cells.[22] The presence of the epitope allows visualization of the receptor by immunoblotting and quantitative immunoprecipitation.

Following metabolic labeling of Sf9 cells expressing the c-*myc* β_2-AR and solubilization of the receptor as described above, the tagged receptor can be immunoprecipitated using the mouse anti-c-*myc* monoclonal antibody (9E10). A crucial factor for optimal immunoprecipitation is to reduce the concentration of the detergent digitonin to the minimum required to maintain the receptor in solution. Removal of digitonin and concentration of the solublized receptor can be accomplished by dialysis using Centriprep cartridges (Amicon). A final volume of 700 μl of an ice-cold solution containing 100 m*M* NaCl, 10 m*M* Tris-HCl, pH 7.4, 2 m*M* EDTA, and a protease inhibitor cocktail (buffer B), which does not exceed 0.05% digitonin, is used to perform the immunoprecipitation. Then, purified anti-c-*myc* 9E10 antibody (at a 7:1 antibody to receptor molar ratio) is added to the concentrate and agitated for 2 hr at 4°. Eighty microliters of buffer B and 250 μl of anti-mouse immunoglobulin G (IgG) agarose (Sigma, St. Louis, MO; at an 11:1 secondary to primary antibody molar ratio) are then added. The reaction proceeds for 10 to 15 hr at 4° under gentle agitation. The immunoprecipitate is centrifuged at 12,000 rpm in a microcentrifuge for 10 min. The pellet is rinsed three times with 1 ml of buffer B. Finally, the pellet is resuspended in 200 μl of nonreducing SDS-PAGE loading buffer and incubated at room temperature for 30 min, after which the receptors are released from the matrix by sonication. Yields can be estimated by comparing the number of receptors determined in soluble binding assays performed before and after immunoprecipitation.

With the immunoaffinity method, yields are dramatically higher than those generally obtained by affinity chromatography, and up to 85% of the solubilized receptor can be recovered. Use of this purification technique considerably shortens the time of exposure required to detect the palmitoyl-

ated receptor. In the best cases, less than 1 week of exposure is sufficient to visualize the labeled receptor. A similar epitope-tagging approach followed by immunoprecipitation was successfully used to study the palmitoylation of the serotonin 5-HT_{1b}[9] and the dopamine D_1[10] receptors. Theoretically, this approach could be used to study the posttranslational modifications of any cloned receptor for which no other purification technique is available.

Dynamic Regulation of Palmitate Incorporation into β_2-Adrenergic Receptor

As discussed above, the lability of the thioester bond suggests that dynamic regulation of palmitate may occur and play a role in receptor function. An intriguing possibility is that biological activation of the receptor could modulate its palmitoylation state. To assess this possibility, the effect of agonists on palmitate incorporation into the receptor can be studied. One approach is to prelabel the cells with [^{3}H]palmitate for a given period (e.g., 1 hr to obtain optimal labeling) and then challenge the cells with an agonist such as isoproterenol for a short period (e.g., 15 min). The receptor can then be purified as described above and the level of palmitoylation assessed by autoradiography. In a previous study we demonstrated that, using such a protocol, isoproterenol (10^{-6} *M*) led to a specific increase in the incorporation of [^{3}H]palmitate in the receptor without changing the palmitoylation level of total membrane protein.[22] This observation can be interpreted in two ways: agonist increases the stoichiometry of

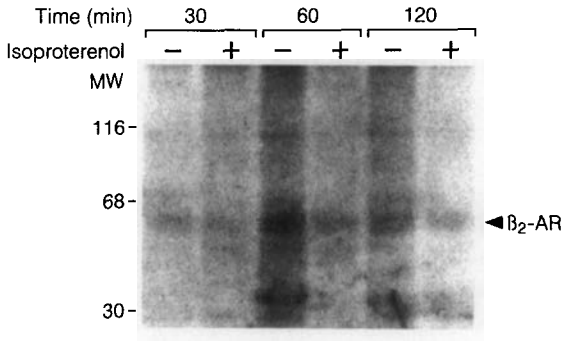

FIG. 3. Effect of isoproterenol on [^{3}H]palmitate incorporation into β_2-AR purified by immunoprecipitation using anti-c-*myc* antibodies. Sf9 cells were metabolically labeled with tritiated palmitate in the presence or absence of the β_2-agonist isoproterenol for the times indicated. Agonist was added 5 min after labeling began and remained for the duration of the label period. Two picomoles of purified receptor was loaded in each lane. Molecular weight markers are shown to the left of the first lane.

palmitoylation or, alternatively, agonist treatment increases the turnover rate of palmitate linked to the receptor. Modulation of receptor palmitoylation by agonist can also be observed in mammalian cells expressing the human β_2-AR,[5] but kinetic studies are considerably more difficult using those cells. In a similar approach, three other groups observed comparable increases in the apparent incorporation of palmitate into the α subunit of G_S following its stimulation.[23,24,24a,24b]

A different approach to study the influence of receptor activation on palmitoylation is to treat the cells with the agonist over the entire course of the labeling period. For these studies, labeling of Sf9 cells expressing the c-*myc* β_2-AR is performed as described above. After 5 min of labeling, agonist (10^{-6} *M*) is added to the cells and incubation continued for various lengths of time. As shown in Fig. 3, a treatment of 30 min with isoproterenol reduces the incorporation of tritiated palmitate into the β_2-AR by approximately 35% relative to the control condition. After 1 hr the reduction reaches above 65%. The results are more consistent with an effect of the agonist on the turnover rate of palmitate linked to receptor than a change in the stoichiometry of receptor palmitoylation. It should be remembered that the experiments cannot be carried out at isotopic equilibrium of the palmitate donor pools. Therefore, although it is clear that activation of the β_2-AR modulates its palmitoylation, caution should be exercised when making kinetic interpretations of changes in tritiated palmitate incorporation.

[25] Palmitoylation of G-Protein α Subunits

By MAURINE E. LINDER, CHRISTIANE KLEUSS, and SUSANNE M. MUMBY

Introduction

A family of guanine nucleotide-binding regulatory proteins (G proteins) serve in signal transduction systems to link receptors exposed at the cell surface to intracellular effectors such as enzymes and ion channels.[1] On activation by receptor, a heterotrimeric G protein dissociates into a GTP-bound α subunit and a $\beta\gamma$ complex which are then able to modulate effector activity. Many α subunits are acylated at (or near) their amino termini by myristate and/or palmitate. G-protein γ polypeptide chains are prenylated

[1] J. R. Hepler and A. G. Gilman, *Trends Biochem. Sci.* **17,** 383 (1992).

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