Palmitoylation of Human EndothelinB
ITS CRITICAL ROLE IN G PROTEIN COUPLING AND A DIFFERENTIAL REQUIREMENT FOR THE CYTOPLASMIC TAIL BY G PROTEIN SUBTYPES*

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By site-directed mutagenesis, three cysteine residues (amino acids 402, 403, and 405) in the carboxyl terminus of human endothelinB (ETB) were identified as potential palmitoylation sites. Substitutions of all of the three cysteine residues with serine gave an unpalmitoylated mutant, C2S/C3S/C5S. When expressed in Chinese hamster ovary cells, C2S/C3S/C5S was localized on the cell surface, retained high affinities to ET-1 and ET-3, and was rapidly internalized when bound to the ligand. However, unlike the wild-type ETB, C2S/C3S/C5S transmitted neither an inhibitory effect on adenylyl cyclase nor a stimulatory effect on phospholipase C, indicating a critical role of palmitoylation in the coupling with G proteins, regardless of the G protein subtypes. Truncation of the carboxyl terminus including Cys403/Cys405 gave a deletion mutant A403 that was palmitoylated on Cys405 and lacked the carboxyl terminus downstream to the palmitoylation site. A403 did transmit a stimulatory effect on phospholipase C via a pertussis toxin-insensitive G protein but it failed to transmit an inhibitory effect on adenylyl cyclase. These results indicated a differential requirement for the carboxyl terminus downstream to the palmitoylation site in the coupling with G protein subtypes, i.e. it is required for the coupling with Gi, but not for that with Gq.

One of the post-translational modifications of G protein-coupled receptors (GPCRs) is a covalent attachment of palmitic acid to one or more cysteine residues via a hydroxylamine-thiol ester bond. At least 10 GPCRs, including human endothelinA (ETA), have been experimentally shown to be palmitoylated (1–12). In every case in which the palmitoylation sites were determined, they were located in the carboxy-terminal cytoplasmic tail (2–4, 9, 12).

Substitutions of the cysteine residues gave unpalmitoylated mutants of each GPCR and the role of the modification has been described, to a varying extent, on three aspects of the receptor functions; 1) ligand binding, 2) G protein activation, and 3) intracellular trafficking of the receptor molecule. To date, however, there appears to be no common rule applicable to all GPCRs on any of the three aspects. On ligand binding, the elimination of palmitoylation caused no changes in the binding characteristics of all the GPCRs examined (4, 9, 12–15) except for β2-adrenergic receptor (β2AR). The unpalmitoylated β2AR lacked the GTP-sensitive high affinity state for agonists and this lack was ascribed to its uncoupling from Gq (3, 16). On G protein coupling, no effects of the elimination have been found on the capacities of m2 cholinergic (13), thyrotropin-releasing hormone (14), and luteinizing hormone/human chorionadotropin (9) receptors to activate G proteins whereas opposite effects of it, both enhancement and inhibition, were described for rhodopsin to activate Gs (17) and for β2AR to activate Gi (18), respectively. A differential requirement for the modification between G protein subtypes coupled to the same receptor has been highlighted in a study on human ETA (12). On intracellular trafficking of the receptor molecule, reduced cell surface expression was reported for unpalmitoylated mutants of thyrotropin-releasing hormone, luteinizing hormone/human chorionadotropin, and vasopressin V2 receptors (9, 14, 15). Internationalization of β2AR (18) or αvAR (19) was not affected while that of luteinizing hormone/human chorionadotropin receptors was enhanced by the elimination of palmitoylation (9). Because of these pleiotropic effects described, the functional role of palmitoylation in each GPCR is an open question.

The endothelins (ETs) are a family of potent vasoactive peptides that includes ET-1, -2, and -3 (20, 21). They have a wide variety of biological effects in various tissues and cell types (22) that are mediated by specific GPCR subtypes, ETA and ETB (23, 24). The two subtypes can be pharmacologically distinguished by different rank orders of affinity toward the three ET isopeptides; ETA is ET-1-selective, showing an affinity rank order of ET-1 ≧ ET-2 ≫ ET-3, whereas ETB exhibits similar affinities to all of the three isopeptides (23, 24). Both of them belong to a subfamily of GPCRs with a promiscuous nature that can activate multiple subtypes of G proteins and they can also be distinguished by selective coupling with G protein subtypes; when expressed in CHO cells, ETA couples with members of Gq and Gi families while ETB couples with those of Gq and Go families (25, 26).

The purposes of this current study were to identify potential palmitoylation sites of ETB and to reveal a role of the modification in ETB functions including ligand binding, cell surface expression, internalization, and G protein activation. An additional objective was to reveal a functional role of the carboxy-terminal tail downstream to the palmitoylation site in the receptor functions.

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§The abbreviations used are: GPCR, guanyl nucleotide-binding regulatory protein-coupled receptor; β2AR, β2-adrenergic receptor; CAMP, cyclic adenosine monophosphate; CHO, Chinese hamster ovary; ET, endothelin; FCS, fetal calf serum; G protein, guanyl nucleotide-binding regulatory protein; ICL, intracellular loop; PBS, phosphate-buffered saline; PLC, phospholipase C; PTX, pertussis toxin; DMEM, Dulbecco’s modified Eagle’s medium; IP, inositol phosphates; CHAPS, 3-[3-cholamidopropyl](dimethylamino)-1-propanesulfonic acid; GTPγS, guanosine 5′-3-O-(thio)triphosphate.
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**EXPERIMENTAL PROCEDURES**

**Materials**—Transformer™ site-directed mutagenesis kit from CLONTECH Laboratories, Inc. (San Francisco, CA); Dulbecco’s modified Eagle’s medium (DMEM), Ham’s F-12 medium, fetal calf serum (FCS), lipofectamine, and blasticidin from Life Technologies, Inc. (Tokyo, Japan); pertussis toxin (PTX) from Funakoshi Co. (Tokyo, Japan); [125I]ET-1 (74 TBq/mmol), myo-[3H]inositol (370 GBq/mmol), [35S]Cys/Met or [3H]palmitic acid, COS cells were harvested by incubation in PBS, 1 mM EDTA. The cells from each well were centrifuged and resuspended in 0.5 ml of PBS containing biotinylated ET-1 (100 nM) and incubated at 37 °C for 25 min. The cells were centrifuged and then lysed by incubation for 2 h at 4 °C in 0.5 ml of the lysis buffer (20 mM sodium phosphate buffer, pH 7.4, 130 mM NaCl, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 0.2% CHAPS, and 0.4% digitonin). After removing insoluble materials by centrifugation at 100,000 g for 1 h at 4 °C, 30 µl of avidin-agarose (50% (v/v) slurry in the lysis buffer) was added to the supernatant and the reaction was let go at 4 °C for 16 h. The agarose-avidin-biotin-ET-1 receptor complex was recovered by centrifugation and then extensively washed with ice-cold lysis buffer containing high (0.5M) or low (0.05M) NaCl concentrations. The recovered proteins were subjected to the imaging plates for 2 days for [35S]Cys/Met-labeled proteins or for 14 days for [3H]palmitic acid-labeled proteins and the autoradiographs were developed with a BAS2000 image analyzer (FujiFilm, Tokyo, Japan).

**Cyclic AMP Formation**—Cells at ~50% confluence in 48-well plates were incubated for 16 h with or without PTX (50 ng/ml). The cells were washed with PBS and then incubated at 37 °C for 10 min with 0.3 ml of PBS containing 3-isobutyl-1-methylxanthine (1 mM). They were then stimulated for 10 min with forskolin (100 µM) alone or simultaneously with forskolin and ET-1. The reaction was halted by addition of 10% (v/v) trichloroacetic acid, and the cAMP content in the trichloroacetic acid was measured using a radioimmunoassay kit (Amersham).

**Phosphoinositide Breakdown**—CHO cells in 24-well plates were incubated for 24 h in Ham’s F-12, 10% FCS containing myo-[3H]inositol (5 µCi/ml). Where indicated, PTX (50 ng/ml) was added to the labeling medium for the last 16 h. The cells were washed with Krebs-Henseleit buffer with LiCl (110 mM NaCl, 4.5 mM KCl, 1.3 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 25 mM NaHCO₃, 11.7 mM glucose, 5 mM HEPES, pH 7.4, and 10 mM LiCl) equilibrated with 5% CO₂ and then incubated in 250 µl of the same buffer. ET-1 (50-µl solutions in Krebs-Henseleit) was added at various concentrations and the plates were kept in a CO₂ incubator for 30 min. The reactions were terminated by adding ice-cold 10% perchloric acid (100 µl/well). The following procedures including neutralization of the extracts and separation of [3H]inositol phosphates ([3H]IPPs) by anion-exchange chromatography were done exactly as described (30).

**Measurement of [Ca²⁺]**—CHO cells in 100-mm dishes were incubated for 16 h with or without PTX (50 ng/ml) and dispersed by incubation in PBS, 1 mM EGTA. The following procedures including fura-2-loading and measurement of [Ca²⁺], with a CAF-110 spectrophotometer (Japan Spectroscopy Inc., Tokyo, Japan) were exactly as described (30).

**Cy5 Labeling of ET-1**—ET-1 was labeled with a fluorescent dye Cy5 using a Fluorolink™ Cy5 reactive dye pack (Amersham) according to the manufacturer’s instructions. In brief, 48 nmol of ET-1 in 1 ml of 0.1 mM sodium carbonate buffer, pH 9.3, was applied to a vial containing the dye. The reaction was let go for 3 h at room temperature and the Cy5-labeled ET-1 was separated from naive ET-1 by high performance liquid chromatography using a C18 column as described above for the purification of biotinylated ET-1. The biological activity of Cy5-labeled...
that the cells were metabolically labeled with [3H]palmitic acid. Molecular sizes are indicated on the left (kDa).

ET-1 was verified by its ability to induce a transient increase of [Ca2+]i in CHO cells expressing wtETB (not shown).

In Situ Binding and Internalization of Cy5-labeled ET-1—CHO cells grown on poly-l-lysine-coated glass coverslips were washed with ice-cold binding buffer (140 mM NaCl, 4 mM KCl, 1 mM Na2HPO4, 1 mM MgCl2, 25 mM HEPES, pH 7.4, 11.7 mM glucose, 0.1% bovine serum albumin) and then incubated at 4 °C for 2 h in the same buffer containing 10 nM Cy5-labeled ET-1. After washing with ice-cold binding buffer, the fluorescent images of the cells were obtained with a MRC1024 laser-scanning confocal microscope (Bio-Rad, Osaka, Japan). To facilitate internalization of the bound ligand, the cells were then incubated in the binding buffer at 37 °C and the images were obtained at the time indicated.

Statistical Analysis—Student’s t test was used for the statistical analysis of the results. p values of <0.05 were considered to be significant.

RESULTS

Identification of the Potential Palmitoylation Sites of ETB—When COS cells were transfected with pME/wtETB and then metabolically labeled with [35S]Cys/Met, two radioactive proteins with the average molecular sizes of 52 and 34 kDa were affinity-purified from the cell lysate with biotinylated ET-1 (Fig. 2a). The specificity of either band was verified by their absence in the lysate from vector-transfected cells and by their disappearance in the presence of excess unlabeled ET-1 in the binding step. Kozuka et al. (29) purified endogenous ETB from bovine lung membrane preparations in essentially the same way and proved that the 52- and 34-kDa species correspond to a full-length intact receptor and a proteolytic derivative with amino-terminal truncation, respectively. When the cells were labeled with [3H]palmitic acid, the radioactivity was incorporated into both bands (Fig. 2b), indicating the palmitoylation of ETB.

Potential palmitoylation sites of ETB were then determined by expression and affinity purification of a series of mutant receptors with substitutions of cysteine residues. All the substitution mutant receptors were successfully expressed in and purified from the transfected cells as judged by the recovery of [35S]Cys/Met-labeled proteins (Fig. 2a). Of the four cysteine residues at the carboxyl-terminal juxtamembrane portion of ETB Cys402 is highly conserved among GPCRs and corresponds to the site that was proven to be palmitoylated in some of them (1). However, a single substitution of Cys402 with serine did not inhibit the [3H]palmitic acid incorporation. Neither of Cys403 nor Cys405 affected the incorporation. In contrast, simultaneous substitutions of Cys402 and Cys403 resulted in an apparent decrease and further substitution of Cys405 resulted in a complete disappearance. Thus, we concluded that Cys402, Cys403, and Cys405, but not Cys404 are the potential palmitoylation sites of ETB.

Ligand-binding Properties of the Wild-type and Mutant Receptors Expressed in CHO Cells—That all the mutant receptors with cysteine substitutions were successfully affinity-purified by binding of biotinylated ET-1 to intact cells suggested that palmitoylation is required neither for the ligand binding nor the cell surface expression of ETB. [125I]ET-1 binding assays on intact COS cells expressing the wild-type or mutant receptors failed to reveal any differences in the binding characteristics (data not shown), giving a supportive evidence to the notions. To further confirm these and to explore a functional significance of palmitoylation in the receptor trafficking and signal transduction, the mutant receptors were stably expressed in CHO cells. This cell line was adopted because we had already shown that wtETB, when stably expressed in CHO cells, directly couples with members of both G0 and G1 families to inhibit adenylate cyclase and activate phospholipase C (PLC), respectively (26).

By co-transfecting CHO cells with each expression plasmid and pSVbsr′ and then selecting for resistance against blasticidin, we obtained more than three individual clonal cell lines that stably expressed each receptor construct. [125I]ET-1 binding assays on membrane preparations from various clones gave KD values of 30–150 pM and Bmax values of 0.5–1.7 pmol/mg protein. Representative clones were used for the subsequent study because of the similar receptor densities as listed in Table I. On the selected clones, we also performed competition binding experiments of [125I]ET-1 with ET-1 or ET-3. The IC50 values for ET-1 and ET-3 to inhibit the binding of [125I]ET-1 (25 pm) were similar in the wild-type and all mutated ETB, being in the range from 63 to 110 pm for ET-1 and from 99 to 141 pm for ET-3.

Cell Surface Expression and Internalization of the Wild-type and Mutant Receptors Expressed in CHO Cells—To examine the cell surface expression and internalization, the localization of Cy5-labeled ET-1 in CHO cells expressing wtETB or C2S/C3S/C5S was visualized by confocal microscopy. When CHO/wtETB cells were incubated with the ligand at 4 °C, Cy5-ET-1 showed a homogenous distribution on the plasma membrane. The specific activity of the binding was verified by its disappearance in the presence of excess unlabeled ET-1 and also by its absence in native CHO cells (Fig. 3). Subsequent incubation at 37 °C
elicited internalization of the surface-bound ligand within minutes and, after 30 min, the ligand showed a patchy distribution both below the plasma membrane and around the nucleus, presumably being localized in lysosomes. As shown in Fig. 4, there were no apparent differences between wtETB and C2S/C3S/C5S in the localization of Cy5-ET-1.

**Failure of an Unpalmitoylated Mutant to Activate Gi**—To reveal a functional significance of palmitoylation in the coupling with Gi, we tested the abilities of the mutant receptors to transmit an inhibitory effect on adenylate cyclase (Fig. 5). In CHO/wtETB cells, ET-1 caused a dose-dependent inhibition of forskolin-stimulated cAMP formation with EC50 values of 58 ± 1 μM (mean ± S.E., n = 3) and the maximum inhibition to −40% of control. This effect was abolished by pretreatment of the cells with PTX (50 ng/ml for 16 h) as reported previously (26). An unpalmitoylated mutant C2S/C3S/C5S totally failed to transmit this effect while C2S/C3S did transmit the effect with EC50 values of 63 ± 6 μM (n = 3) and the maximum inhibition to −35%, both of which were comparable with those obtained for wtETB. Also comparable with the effect transmitted by wtETB were those transmitted by C2S, C3S, or C5S (data not shown). These results suggested a critical role of palmitoylation of ETB in the coupling with Gi.

**Failure of an Unpalmitoylated Mutant to Activate Gq**—To reveal a functional significance of palmitoylation in the coupling with Gq, we tested the abilities of the mutant receptors to transmit a stimulatory effect on PLC (Fig. 6). In CHO/wtETB cells, ET-1 caused a dose-dependent stimulation of [3H]IPs accumulation with EC50 values of 2.5 ± 1 nM (n = 3) and the maximum stimulation of 2.5-fold increase. PTX treatment of the cells with PTX (50 ng/ml for 16 h) as reported previously (26). An unpalmitoylated mutant C2S/C3S/C5S failed to transmit this effect while C2S/C3S showed a maximum stimulation of 40% (27). It was, however, left unknown whether the lack of response was due to a lack of palmitoylation or due to that of the carboxyl terminus per se. To resolve the issue and further explore a functional role of the carboxyl terminus, the deletion mutants were expressed and subjected to the same assays as described.

All the three deletion mutants were successfully expressed in and purified from the transfected COS cells as judged by
the recovery of [35S]Cys/Met-labeled proteins (Fig. 8a). [3H]Palmitic acid was metabolically incorporated into Δ403 but not into Δ402 or Δ400 (Fig. 8b), as expected from the notion that Cys402, Cys403, and Cys405, but not Cys400 are the potential palmitoylation sites of ET B.

Ligand Binding and Intracellular Trafficking of the Carboxyl-terminal Deletion Mutants Expressed in CHO Cells—Binding parameters obtained from saturation isotherms with [125I]ET-1 on representative CHO cell clones stably expressing the deletion mutants are listed in Table I. Consistent with our previous results on Ltk<sup>2</sup> cells expressing these mutant receptors (27), all of them retained a high affinity to ET-1 and also that to ET-3 as judged from the competition binding experiments of [125I]ET-1 with ET-3 (data not shown). In situ binding and internalization assays with Cy5-ET-1 on these mutant receptors also failed to reveal any apparent differences from the behavior of wtET<sub>B</sub> (data not shown).

A Palmitoylated Deletion Mutant Δ403 Coupled with G<sub>i</sub> but

Not with G<sub>i</sub>—ET-1-induced signaling was examined in cells expressing an unpalmitoylated mutant Δ402 or a palmitoylated mutant Δ403. ET-1 failed to inhibit forskolin-induced cAMP formation in CHO cells expressing either receptor (Fig. 9), suggesting a lack of coupling with G<sub>i</sub>. ET-1 also failed to stimulate [3H]IP<sub>3</sub> accumulation and to induce a [Ca<sup>2+</sup>]<sub>i</sub> increase in cells expressing Δ402 while it did elicit both responses in cells expressing Δ403 (Figs. 10 and 11). The EC<sub>50</sub> values and the maximum effect for ET-1 to stimulate [3H]IP<sub>3</sub> accumulation in cells expressing Δ403 were 8.7 ± 1 nM (n = 3) and
were stimulated either with forskolin (100 μM) alone or with forskolin and increasing concentrations of ET-1 for 10 min. The contents of cAMP in the trichloroacetic acid-soluble cell extracts were determined by radioimmunoassay. The values were expressed as relative to the forskolin-stimulated formation (100%). Shown are the means ± S.E. of three determinations each done in duplicate. *, p < 0.01; significantly different from the values of cAMP formation stimulated with forskolin alone.

DISCUSSION

We have demonstrated that human ET\textsubscript{B} is covalently modified by thioetherification of palmitic acid and that the potential palmitoylation sites are the cysteine residues at amino acids 402, 403, and 405. The results obtained in the present study, however, did not indicate which of the three potential sites were actually palmitoylated in wtET\textsubscript{B} but suggested that palmitoylation of the individual cysteines was not an independent event but both alternative and hierarchical modifications of the three residues were taking place. Mutation of individual cysteines did not affect the level of \textsuperscript{3}H\textsuperscript{2}H palmitic acid incorporation but that of two caused a significant reduction (Fig. 2b), suggesting that, in the wild-type receptor, not all of the three but two of them are palmitoylated and that, in the single-substitution mutants (C2S, C3S, and C5S), the remaining two cysteines were alternatively palmitoylated. The reduction of \textsuperscript{3}H\textsuperscript{2}H palmitic acid incorporation in the double mutant C2S/C3S was more than 80% on densitometry suggesting the presence of a hierarchical order between Cys\textsuperscript{402}/Cys\textsuperscript{403} and Cys\textsuperscript{405}, i.e. palmitoylation of either Cys\textsuperscript{402} or Cys\textsuperscript{403} may be a prerequisite for the efficient palmitoylation of Cys\textsuperscript{405}. Hierarchical modification of two potential palmitoylation sites has been demonstrated so far only for rhodopsin (5). Identification of the actual palmitoylation sites in wtET\textsubscript{B} as well as verification of the alternative/hierarchical palmitoylation must await further studies that employ chemical or enzymatic methods to detect the modification of individual cysteines.

Also left unaddressed in the present study was the possible regulation of the palmitoylation level by receptor activation as has been described for β\textsubscript{2}AR (31) or D\textsubscript{1} dopaminergic receptor (7). Because the expressed receptors were recovered after \textsuperscript{3}H\textsuperscript{2}H palmitic acid labeling and washing the cells, we could at least conclude that wtET\textsubscript{B} as well as the various mutants were constitutively palmitoylated, without agonist-stimulation. However, because of the use of biotin-labeled agonist in the purification step, we did not assess the effect of receptor activation on the palmitoylation level. Alternative purification procedures including epitope-tagged receptors or specific antibodies are required to pursue the issue.

[\textsuperscript{125}I]ET-1 binding assays on the wild-type and various mutant receptors gave \(K\textsubscript{d}\) and \(B\textsubscript{max}\) values within similar ranges regardless of the presence or absence of palmitoylation, suggesting that the overall integrity of the ligand-binding surface did not depend on the modification. These results are in line with the data obtained in many GPCRs (4, 9, 12–15) except for β\textsubscript{2}AR. In the case of β\textsubscript{2}AR, the lack of palmitoylation eliminated the GTP-sensitive high affinity state of the receptor, secondary to its uncoupling from G\textsubscript{s} (3, 16). There was indeed a 4-fold difference in the \(K\textsubscript{d}\) values of [\textsuperscript{125}I]ET-1 binding to...
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various mutant receptors used in the present study (Table I). It is, however, unlikely that the apparent difference in the affinities was due to the presence or absence of a GTP-sensitive high affinity state of the receptor, because GTP$\gamma$S failed to affect the $[^{125}]$ET-1 binding to wtET$_B$ as well as the various mutant receptors (data not shown). The absence of a GTP-sensitive high affinity state of wtET$_B$ may imply that the receptor does not couple to G proteins prior to agonist binding as has been suggested for ET$_A$ (32). In the present study, a precise reason for the apparent difference in the binding affinities between the various mutants was left unknown. It is, however, at least clear that the differences in the G protein coupling capacities of the various mutants cannot be ascribed to the differences in the ligand binding affinities, because of the all or none feature of the coupling (discussed below).

In situ binding assays with Cy5-ET-1 (Fig. 4) indicated that palmitoylation was not required for the overall sequestration (cell surface expression and internalization) of wtET$_B$. The binding assay used, however, is not quantitative and the intracellular traffic of the receptor molecule from the site of synthesis (endoplasmic reticulum) to the plasma membrane could not be assessed with this assay. Therefore, it is still an open question whether the intracellular traffic of wtET$_B$ is actively regulated by palmitoylation of the receptor molecule.

The most distinct finding of the present study was the failure of unpalmitoylated mutant receptors to activate the G protein-dependent signaling pathways. The unpalmitoylated mutant C2S/C3S/C5S totally failed to transmit an inhibitory effect on adenylate cyclase (Fig. 5) and a stimulatory effect on PLC (Figs. 6 and 7) while C2S/C3S retained the signaling activities comparable with those of wtET$_B$. These results indicated that the presence of either Cys$^{405}$ in the carboxyl terminus of the C5S or the palmitoylation of the carboxyl terminus of C2S/C3S was required for the G protein coupling. Because the specific requirement for the presence of Cys$^{405}$ can be excluded by the unaltered signaling activities of the mutant C5S, the signaling activities of C2S/C3S must be ascribed to the palmitoylation of Cys$^{405}$. The unaltered signaling activities of C5S can in turn be ascribed to the palmitoylation of Cys$^{402}$ and Cys$^{403}$. Therefore, we conclude that palmitoylation of at least one of the three potential sites is required for the G protein coupling, regardless of the G protein subtypes.

The activation of signaling pathways to adenylate cyclase and PLC by various receptors was an all or none phenomenon and the quantitative relationship between the palmitoylation level and the signaling effects was not detected in the present study. Both the inhibition of CAMP formation and the stimulation of $[^{3}H]IPs$ accumulations caused by C2S/C3S were comparable with those by wtET$_B$ (and other single-substitution mutants) (Figs. 5 and 6) despite the apparently reduced palmitoylation level of C2S/C3S (Fig. 2b). A precise mechanism for C2S/C3S to cause the maximum effects is left unknown, however, possible explanations include the intracellular amplification of the signal by sequential interactions of receptor-G protein-effector molecules.

In addition to the critical role of palmitoylation in the G protein coupling, the present study also revealed a differential requirement for the carboxyl-terminal tail downstream to the palmitoylation site by G protein subtypes. The palmitoylated deletion mutant $\Delta 403$ failed to transmit an inhibitory effect on adenylate cyclase (Fig. 9) but did transmit a stimulatory effect on PLC (Figs. 10 and 11) indicating that the carboxyl-terminal tail downstream to the palmitoylation site was required for the coupling with G$_s$ but not for that with G$_i$. To reveal a role of the carboxyl terminus in ET$_B$ signaling, Aquilla et al. (33) constructed a deletion mutant which terminates within the seventh transmembrane domain and showed a lack of a capacity of this mutant to activate various cellular kinases. The critical role of palmitoylation and that of the carboxyl-terminal tail in the G protein coupling described here are consistent with their findings.

The requirement for palmitoylation in the G protein coupling has so far been documented for $\beta_2$AR (16) and ETA (12). In the case of $\beta_2$AR, the decreased coupling of unpalmitoylated mutant receptors was linked to an increased phosphorylation of the carboxyl tail of the receptor and not to the formation of a fourth intracellular loop (ICLIV) (16). Indeed there are as many as 10 putative phosphorylation sites in the carboxyl-terminal tail of ET$_B$. However, both deletion mutants $\Delta 402$ and $\Delta 403$ lacked the potential phosphorylation sites in the carboxyl terminus and the difference of their abilities to activate G$_q$ depended on the presence or absence of the potential palmitoylation site Cys$^{402}$. Therefore, it is unlikely that the lack of a capacity of unpalmitoylated mutants was secondary to an altered phosphorylation state of the carboxyl terminus. Although the data presented does not exclude the possible regulation of G protein coupling by phosphorylation, it favors a structural requirement for the formation of ICLIV in the G protein coupling of ET$_B$.

Comparison of the data obtained here on ET$_B$ and that reported on ET$_A$ (12) revealed some features shared by these receptor subtypes. In both cases, the receptors appeared to be constitutively palmitoylated and palmitoylation was not essential for the ligand binding capacities. Another feature shared by ETA and ET$_B$ is the absolute requirement for palmitoylation in the coupling with G proteins of the G$_q$ family. This is, at present, a feature unique to these receptor subtypes; whether it is shared by any other GPCRs coupled with G$_q$ is a subject for the future study. A distinct difference between ET$_A$ and ET$_B$ lies in the structural basis for the coupling of ET$_A$ with G$_q$ and for the coupling of ET$_B$ with G$_q$. Palmitoylation was required for ET$_B$-G$_q$ interaction but not for ET$_A$-G$_q$ interaction. Using chimeric receptors between ET$_A$ and ET$_B$, we have shown that ICLII of ET$_A$ and ICLIII of ET$_B$ are the major determinants for the selective coupling of each receptor subtype with G$_q$ and G$_i$, respectively (26). The requirement for palmitoylation in ET$_B$-G$_i$ interaction suggested an involvement of ICLVI either in selection or activation of G$_i$. Also suggested from the data obtained from deletion mutants was an involvement of the cytoplasmic free tail. Thus, in the case of ET$_B$-G$_i$ interaction, all of the three intracellular domains of the receptor, ICLIII, VI, and the cytoplasmic free tail appear to be involved.

Recently, a splice variant of human ET$_B$ was identified by molecular cloning (35). It is formed by a substitution of a large part of the carboxyl-terminal tail and the newly identified carboxyl-terminal sequence lacks any potential palmitoylation sites. When expressed in cultured cells, the splice variant retained ligand binding capacities but apparently lacked a capacity to activate G$_q$ proteins, giving rise to a hypothesis that it may represent the “spare” ET$_B$, the presence of which has been predicted by some functional studies (36, 37). An obvious explanation for the failure of this splice variant to activate G$_q$ proteins is a lack of palmitoylation. If this is the case, it raises a possibility of a novel mechanism to adjust cells’ responses by alternative expression of palmitoylation-positive and -negative GPCR variants.

In conclusion, we have identified the potential palmitoylation sites of human ET$_B$ and revealed a critical role of the modification in the coupling with G$_q$ proteins. The relevance of these findings to the functional defects of ET$_B$ variant will be clarified in the future study.
REFERENCES

1. Morello, J. P., and Bouvier, M. (1996) Biochem. Cell Biol. 74, 449–457
2. Orchinnikov, Y. A., Abdulaev, N. G., and Bogachuk, A. S. (1988) FEBS Lett. 230, 1–5
3. O'Dowd, B. F., Hnatowich, M., Caron, M. G., Lefkowitz, R. J., and Bouvier, M. (1989) J. Biol. Chem. 264, 7564–7569
4. Kennedy, M. E., and Limbird, L. (1993) J. Biol. Chem. 268, 8003–8011
5. O'Dowd, B. F., Hnatowich, M., Caron, M. G., Lefkowitz, R. J., and Bouvier, M. (1989) Biochemistry 32, 11727–11733
6. Ng, G. Y. K., Mouillac, B., Caron, M., Dennis, M., and O'Dowd, B. F. (1993) J. Biol. Chem. 268, 1589–1595
7. van Koppen, C. J., and Nathanson, N. M. (1991) J. Biol. Chem. 266, 20118–20123
8. Campbell, P. T., Hnatowich, M., O'Dowd, B. F., Caron, M. G., Lefkowitz, R. J., and Hausdorff, W. P. (1991) Mol. Pharmacol. 39, 192–198
9. Eason, M. G., Racinto, M. T., Theiss, C. T., and Liggett, S. B. (1994) Proc. Natl. Acad. Sci. USA 91, 11178–11182
10. Inoue, A., Yanagisawa, M., Kimura, S., Kasuya, Y., Miyauchi, T., Goto, K., and Masaki, T. (1989) Proc. Natl. Acad. Sci. USA 86, 2863–2867
11. O'Dowd, B. F., Hnatowich, M., Caron, M. G., Lefkowitz, R. J., and Bouvier, M. (1989) Biochemistry 32, 11727–11733
12. O'Dowd, B. F., Caron, M. G., Lefkowitz, R. J., and Hausdorff, W. P. (1991) Mol. Pharmacol. 39, 192–198
13. Morello, J. P., and Bouvier, M. (1996) Biochem. Cell Biol. 74, 449–457
14. O'Dowd, B. F., Caron, M. G., Lefkowitz, R. J., and Hausdorff, W. P. (1991) Mol. Pharmacol. 39, 192–198
15. Morello, J. P., and Bouvier, M. (1996) Biochem. Cell Biol. 74, 449–457
16. O'Dowd, B. F., Caron, M. G., Lefkowitz, R. J., and Hausdorff, W. P. (1991) Mol. Pharmacol. 39, 192–198
17. Morello, J. P., and Bouvier, M. (1996) Biochem. Cell Biol. 74, 449–457
18. O'Dowd, B. F., Caron, M. G., Lefkowitz, R. J., and Hausdorff, W. P. (1991) Mol. Pharmacol. 39, 192–198
19. Eason, M. G., Racinto, M. T., Theiss, C. T., and Liggett, S. B. (1994) Proc. Natl. Acad. Sci. USA 91, 11178–11182
20. Inoue, A., Yanagisawa, M., Kimura, S., Kasuya, Y., Miyauchi, T., Goto, K., and Masaki, T. (1989) Proc. Natl. Acad. Sci. USA 86, 2863–2867
21. Yanagisawa, M., Kurthara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K., and Masaki, T. (1988) Nature 332, 411–415
22. Masaki, T. (1993) Endocr. Rev. 14, 256–268
23. Sano, K., Nakao, K., Otani, T., Itoh, T., Hirose, S., Lodi, K. M., and Hagiwara, H. (1991) J. Biol. Chem. 266, 16892–16896
24. Sugawara, F., Ninomiya, H., Okamoto, Y., Miwa, S., Akiyama, O., Katsury, S., and Masaki, T. (1996) Mol. Pharmacol. 49, 447–457
25. Mouillac, B., Caron, M., Bonin, H., and Bouvier, M. (1992) J. Biol. Chem. 267, 21733–21737
26. Rose, P. M., Krystek, S. H., Jr., Patel, P. S., Liu, E. C., Lynch, J. S., Lach, D. A., Fisher, S. M., and Webb, M. L. (1995) FEBS Lett. 361, 243–249
27. Elshourbagy, N. A., Adamou, J. E., Gagnon, A. W., Wu, H.-L., Pullen, M., and Nambi, P. (1996) J. Biol. Chem. 271, 25300–25307
28. Sano, K., Nakao, K., Otani, T., Itoh, T., Hirose, S., Lodi, K. M., and Hagiwara, H. (1991) J. Biol. Chem. 266, 16892–16896
29. Sugawara, F., Ninomiya, H., Okamoto, Y., Miwa, S., Akiyama, O., Katsury, S., and Masaki, T. (1996) Mol. Pharmacol. 49, 447–457
30. Mouillac, B., Caron, M., Bonin, H., and Bouvier, M. (1992) J. Biol. Chem. 267, 21733–21737
31. Rose, P. M., Krystek, S. H., Jr., Patel, P. S., Liu, E. C., Lynch, J. S., Lach, D. A., Fisher, S. M., and Webb, M. L. (1995) FEBS Lett. 361, 243–249
32. Elshourbagy, N. A., Adamou, J. E., Gagnon, A. W., Wu, H.-L., Pullen, M., and Nambi, P. (1996) J. Biol. Chem. 271, 25300–25307
33. Sano, K., Nakao, K., Otani, T., Itoh, T., Hirose, S., Lodi, K. M., and Hagiwara, H. (1991) J. Biol. Chem. 266, 16892–16896
34. Sugawara, F., Ninomiya, H., Okamoto, Y., Miwa, S., Akiyama, O., Katsury, S., and Masaki, T. (1996) Mol. Pharmacol. 49, 447–457
35. Mouillac, B., Caron, M., Bonin, H., and Bouvier, M. (1992) J. Biol. Chem. 267, 21733–21737
36. Rose, P. M., Krystek, S. H., Jr., Patel, P. S., Liu, E. C., Lynch, J. S., Lach, D. A., Fisher, S. M., and Webb, M. L. (1995) FEBS Lett. 361, 243–249
37. Elshourbagy, N. A., Adamou, J. E., Gagnon, A. W., Wu, H.-L., Pullen, M., and Nambi, P. (1996) J. Biol. Chem. 271, 25300–25307
38. Sano, K., Nakao, K., Otani, T., Itoh, T., Hirose, S., Lodi, K. M., and Hagiwara, H. (1991) J. Biol. Chem. 266, 16892–16896
39. Sugawara, F., Ninomiya, H., Okamoto, Y., Miwa, S., Akiyama, O., Katsury, S., and Masaki, T. (1996) Mol. Pharmacol. 49, 447–457
40. Sano, K., Nakao, K., Otani, T., Itoh, T., Hirose, S., Lodi, K. M., and Hagiwara, H. (1991) J. Biol. Chem. 266, 16892–16896
41. Sugawara, F., Ninomiya, H., Okamoto, Y., Miwa, S., Akiyama, O., Katsury, S., and Masaki, T. (1996) Mol. Pharmacol. 49, 447–457
42. Sano, K., Nakao, K., Otani, T., Itoh, T., Hirose, S., Lodi, K. M., and Hagiwara, H. (1991) J. Biol. Chem. 266, 16892–16896
43. Sugawara, F., Ninomiya, H., Okamoto, Y., Miwa, S., Akiyama, O., Katsury, S., and Masaki, T. (1996) Mol. Pharmacol. 49, 447–457