PROTEIN CHEMISTRY AND STRUCTURE:

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J. Biol. Chem. 1998, 273:924-931.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 2, Issue of January 9, pp. 924–931, 1998
Printed in U.S.A.

Post-translational Modifications of Endothelin Receptor B from Bovine Lungs Analyzed by Mass Spectrometry*

(Received for publication, August 28, 1997, and in revised form, November 3, 1997)

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A new mild experimental approach for isolation of peptide membrane receptors and subsequent analysis of post-translational modifications is described. Endothelin receptors A and B were isolated on oligo(dT)-cellulose using *N*-(ϵ-maleimidocaproyloxy)succinimide endothelin coupled to a protected (dA)-30-mer. This allowed a one-step isolation of the receptor from oligo(dT)-cellulose via variation solely of salt concentration. The identity of the receptor was confirmed by direct amino acid sequencing of electroblotted samples or by using antibodies against ET_A and ET_B receptors. The method used here is very fast, requires only very mild elution conditions and, for the first time, gave both ET_A and ET_B receptors concurrently in very good yield. Following enzymatic in-gel digestion, MALDI, and electrospray ion trap mass spectrometric analysis of the isolated endothelin B receptor showed phosphorylation at Ser-304, -418, -438, -439, -440, and -441. Further phosphorylation at either Ser-434 or -435 was observed. The endothelin B receptor is also palmitoylated at Cys residues 402 and 404. Phosphorylation of Ser^{304} may play a role in Hirschsprung's disease.

Although it is clear that post-translational modifications of G-protein-coupled receptors are intimately involved in the physiological function of these signal transduction systems, there is as yet relatively little direct evidence for the specific modifications and the relationship of the different modifications to signal transduction processes. These types of processes are not easily amenable to analysis by genomic methods, *i.e.* there is a need for efficient methods to directly analyze these processes at the proteome level. We report here new, efficient methods for rapid isolation of the endothelin B receptor and for highly sensitive analysis of its post-translational modifications via mass spectrometry.

Endothelin, the strongest vasoconstrictor yet known, is a 21-amino acid peptide with physiological effects on cellular development, differentiation, vasoconstriction, and mitogenesis (1, 2). There are 3 different endothelin isoforms, ET-1,[1] ET-2, and ET-3 with different affinity for the two different endothelin receptor subtypes, A and B (3–6). Both receptors are members of the G-protein-coupled receptor superfamily (3–6, 7). The consensus ET receptor topology includes three extracellular domains, three intracellular loops, and a cytoplasmic COOH-terminal tail, separated by seven hydrophobic helical regions thought to span the lipid bilayer. In addition it has been presumed that ET receptors are post-translationally modified by glycosylation of the NH_2 terminus and by phosphorylation and palmitoylation of the cytoplasmic surface. Based on homology with other G-protein-coupled receptors (8–10), there has been speculation regarding possible structures, functional regions, and sites of post-translational modifications for endothelin receptors (11–16). However, as yet there is very little direct evidence for attributes such as the sites and the roles of glycosylation, palmitoylation, and phosphorylation, the location of the endothelin-binding site and the basis for discrimination among the three different endothelin isoforms.

A role of endothelin in disease has recently been demonstrated by the finding that mutated ET_B receptor is associated with Hirschsprung's disease (17–21). In addition, mice lacking the ET-1 gene display severe malformation of large blood vessels, stressing the importance of endothelin during development (22). Endothelin has been shown to be a mitogenic agonist in different cell types (23, 24). The signaling pathway by which ET-1 promotes cell proliferation involves activation of intracellular kinase cascades and transcription factor stimulation. Recently it was suggested that the cytoplasmic tail of ET_B receptor is involved in activation of three distinct mitogen-activated signal transduction pathways requiring extracellular-regulated kinase, c-Jun N-terminal kinase, and p38 kinases (25). Studies conducted by site-directed mutagenesis of ET_A receptor suggested that the third intracellular loop and the COOH-terminal tail are also important for receptor-G-protein coupling (26, 27).

We report here a new method for isolation of endothelin receptor using oligo(dA) covalently linked to endothelin via a specially developed bifunctional cross-linker. Affinity chromatography has been carried out using oligo(dT) columns with mild elution using only changes in salt concentration analogous to methods used for isolation of eukaryotic mRNA. In-gel digestion of electrophoretically purified receptor, subsequent peptide mass fingerprinting by MALDI-TOF or electrospray ion trap mass spectrometry, and fragment analysis by tandem (MS/MS) mass spectrometry have been used to characterize post-translational modifications of this receptor.

EXPERIMENTAL PROCEDURES

Materials

Fresh bovine lungs were obtained at a local slaughterhouse and immediately frozen with liquid N_2. Digitonin, pepstatin, leupeptin, soybean trypsin inhibitor, tosyl-L-phenylalanyl chloromethyl ketone, triethylammonium

* This work was supported by Deutsche Forchungsgemeinschaft Grant Go-639/1-2. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: ET, endothelin; ET_A, endothelin A receptor; ET_B, endothelin B receptor; EMC-ET, *N*-(ϵ-maleimidocaproyloxy)succinimide endothelin; (dA)30–5′-S-EMC-ET, (dA)30–5′-S-*N*-(ϵ-maleimidocaproyloxy)succinimide endothelin; MS/MS, tandem mass spectrometry; TOF, time-of-flight; Bu_4NHSO_3, tetrabutylammonium-hydrogensulfate; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; MES, 4-morpholineethanesulfonic acid; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; MALDI, matrix-assisted laser desorption ionization.

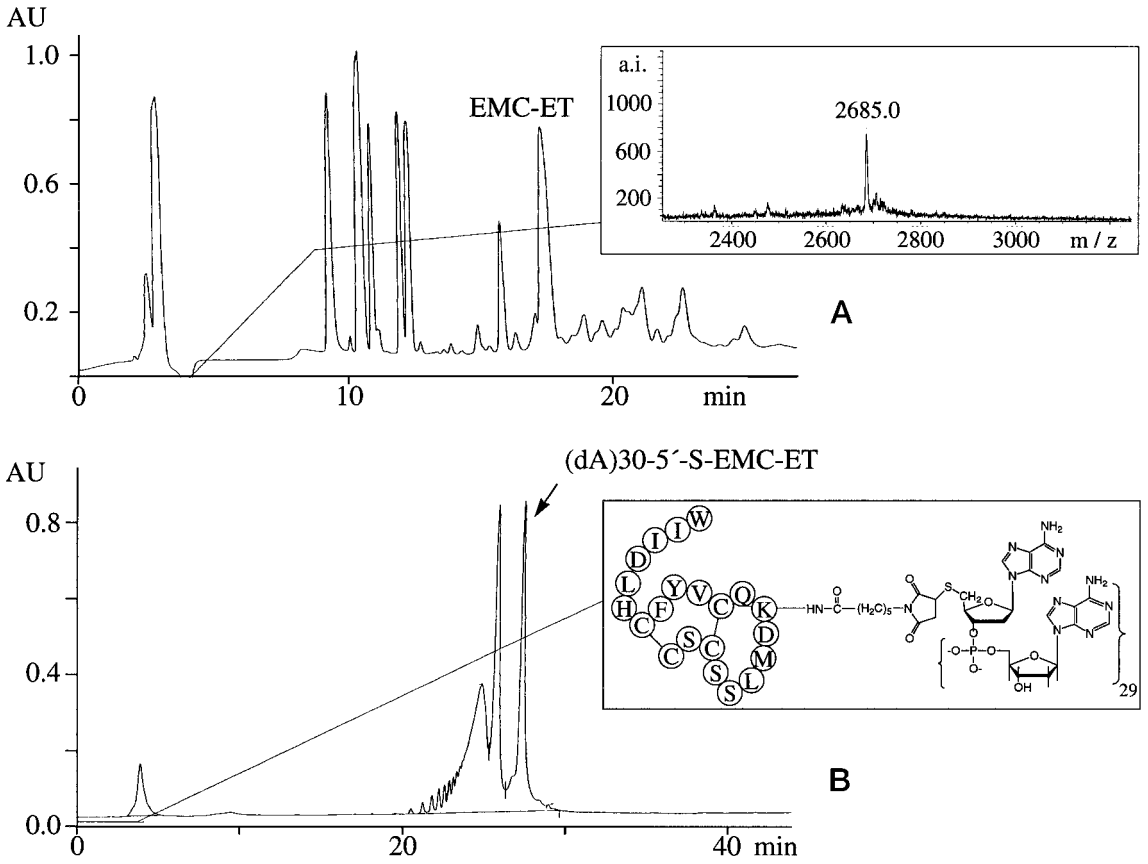

FIG. 1. **Preparation of the (dA)30–5′-S-EMC-ET fishhook.** *A*, purification of EMC-ET. The purification was carried out on a Sephacel C18 column using a gradient of 5–70% acetonitrile in 0.1% trifluoroacetate. *Inset*, MALDI mass spectrometry of EMC-ET; *B*, purification of ((dA)30–5′-S-EMC-ET) on a Sephacel C18 column by applying a linear gradient of 2–70% acetonitrile in 100 mM triethylammonium acetate, 2 mM Bu_4NHSO_3. *Inset*, structure of (dA)30–5′-S-EMC-ET.

acetate, 1,4-dithiothreitol (Microselect for deprotecting the modified oligonucleotide), tetrabutylammoniumhydrogensulfate (Bu_4NHSO_3), and chymostatin were from Fluka Chemie; 1,10-phenanthroline was from Merck; Hepes, *N*-(ϵ-maleimidocaproyloxy)succinimide, phenylmethylsulfonyl fluoride, Chaps, Ellman's reagent, bacitracin, bovine serum albumin, and MES were from Sigma; the Aquapore RP-300A HPLC column and chemicals for sequencing were from Applied Biosystems; human ^{125}I-endothelin-1 (74 TBq/mmol) was from Amersham Corp.; DNase-free RNase A was from Boehringer Mannheim; (dA)30-homomer with and without 5′-modification with 1-*O*-dimethoxytrityl hexyldisulfide was from BioTeZ (Berlin). Other chemicals (Merck and Roth) were of the best grade available. Trypsin was from Promega (Madison, WI). Endothelin-1 was synthesized with Fmoc chemistry as described previously (28–31).

Membrane Preparations

Bovine lung (1.4 kg) was homogenized in a Heavy Duty Blender in 3 volumes of 20 mM Tris-HCl, pH 7.4, which contained 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin, and 10 μg/ml leupeptin (buffer A). The homogenized lungs were centrifuged at 5000 × *g* for 20 min at 4 °C. The supernatants were discarded, the membrane pellets were suspended in 3 volumes of buffer A and washed 3 times by centrifugation. Afterward the membranes were washed twice in 20 mM phosphate buffer, pH 7.4, which contained 500 mM NaCl, 20 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, and the same amount of protease inhibitors as in buffer A (buffer B). The membrane preparations were stored at −70 °C.

Ligand Binding Assays

The binding activity of the solubilized endothelin receptor was tested with ^{125}I-endothelin-1. 10–20 μg of total protein were incubated for 2 h at 20 °C with ^{125}I-endothelin-1 (15,000 cpm) in a total volume of 100 μl of NaCl/P_i, 20 mM sodium phosphate, 150 mM sodium chloride, pH 7.4, containing 1 mM EDTA and 2 mM phenylmethylsulfonyl fluoride. Binding was terminated by gel filtration through Whatman GF/B glass filter sheets precoated with 0.3% (w/v) polyethyleneimine. The filters were washed three times with 4 ml of NaCl/P_i buffer in a filtration device (Hoeffer) and transferred to polystyrene tubes. The filter-bound radioactivity was measured in a γ-counter (Packard).

Synthesis of (dA)30–5′-S-N-(ϵ-Maleimidocaproyloxy) succinimide Endothelin ((dA)30–5′-S-EMC-ET)

Synthesis of N-(ϵ-Maleimidocaproyloxy)succinimide endothelin (EMC-ET)—1 mg (400 nmol) of endothelin-1 was dissolved in 200 μl of 35% acetonitrile in 0.05% trifluoroacetate and further diluted with 50 mM sodium borate, 0.015% Triton X-305, pH 8.2. A 10-fold excess (4 μmol) of the heterobifunctional cross-linker *N*-(ϵ-maleimidocaproyloxy)succinimide dissolved in 200 μl of acetonitrile, was added in 5 portions at 10-min intervals, followed by intensive mixing. After incubation for 2.5 h at room temperature the pH of the reaction mixture was adjusted to 6.0 with 140 mM potassium dihydrogen phosphate to protect the sensitive maleimido group of the cross-linker from hydrolysis (32).

Derivatized endothelin-1 (EMC-ET) was purified on a Sephacel C18 column using a gradient of 5–70% acetonitrile in 0.1% trifluoroacetic acid. The purity of the EMC-ET containing fraction was checked by protein sequencing and mass spectrometry (found MH^+ 2685.0, expected 2685.0). The susceptibility of the maleimido group to Michael addition of thiols was indirectly monitored with cysteine and Ellman's reagent (33). Purified EMC-ET was stored at −20 °C.

Synthesis of (dA)30–5′-S-EMC-ET—To (dA)30–5′-SS-R 1-*O*-dimethoxytrityl hexyldisulfide in 45 μl of 100 mM triethylammonium acetate, 10 μl of freshly prepared 1 M dithiothreitol in 200 mM Tris/HCl, pH 8.0, were added. The solution was mixed briefly then incubated for 3 h at room temperature to achieve quantitative reduction of the disulfide to the free thiol. For complete removal of the dithiothreitol, a gel filtration on PC 3.2/10 column was performed under isocratic conditions (50 mM MES, pH 6.0, 5 mM EDTA). The fraction with the deprotected oligonucleotide (about 200 μl) was immediately mixed with 15 nmol of EMC-ET, which had previously been concentrated in a Speed-vac, and adjusted to pH 6.0 in a volume of 150 μl with 200 mM MES. The reaction mixture was incubated overnight under argon to prevent dimerization of the thiol-containing oligonucleotides. The extent of the reaction was

monitored by gel-retardation on SDS gels of the modified oligonucleotide, compared with the monomer and the dimer of the thiol-modified oligonucleotide.

The (dA)30–5′-S-EMC-ET was purified on a Sephacel C18 column by applying a linear gradient of 2–70% acetonitrile in 100 mM tryethylammoniumacetate, 2 mM Bu_4NHSO_3. Binding of the (dA)30–5′-S-EMC-ET to endothelin receptor was checked by a competitive binding assay against native endothelin-1.

Purification of ET Receptor on Oligo(dT)-cellulose using Oligo(dA)-coupled Endothelin-1

Frozen membranes (100 g) were thawed and suspended in 2 volumes of 20 mM potassium phosphate, pH 7.4, containing 0.40% digitonin, 0.25% Chaps, 500 mM NaCl, 20 mM EDTA, 1 μg/ml RNase, and the same protease inhibitors as buffer A (buffer C). The mixture was gently stirred at 4 °C for 2 h, filtered through 3 layers of miracloth and the filtrate was centrifuged at 100,000 × *g* for 1 h at 4 °C. The supernatants (85 ml) were incubated with 0.5 nmol of (dA)30–5′-S-EMC-ET for 3 h at room temperature, 100 mg of oligo(dT)-cellulose was added and the suspension was gently agitated at 4 °C for 12 h. Oligo(dT)-cellulose was pelleted by centrifugation (4 °C, 1,000 × *g*, 5 min) and packed in a micro column. The column was washed with 10 ml of buffer C at 4 °C and afterward eluted with 10 mM Tris/HCl, pH 7.4, 0.40% digitonin, 0.25% Chaps, 1 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml pepstatin. 200-μl fractions were collected. Fractions containing endothelin receptor were identified by SDS-PAGE and by immunoblot analysis.

SDS-Polyacrylamide Gel Electrophoresis

20 μl of each fraction from the oligo(dT)-cellulose column were mixed with an equal volume of sample buffer containing 6% SDS, 10% (v/v) 2-mercaptoethanol, 20% (v/v) glycerol, 0.01% bromphenol blue, and 250 mM Tris-HCl, pH 6.8, and brought to 95 °C for 2 min. Electrophoresis was performed in 10 or 12.5% polyacrylamide gels in the presence of 0.1% SDS at a constant current of 40 mA for 1.5 h (34). Marker proteins (Bio-Rad) were phosphorylase *b* (95.0 kDa), bovine serum albumin (68.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (30.0 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). The resolved proteins were visualized by silver staining (Sigma Rapid Silver Staining Kit).

Immunoblot Analysis

Immunoblot analysis followed the procedure described by Hagiwara *et al.* (35). 5 μl of the protein sample were loaded per gel lane. Proteins from SDS-PAGE were transferred electrophoretically to a nitrocellulose membrane (Scleicher & Schuell). The blot was incubated with anti-ET_B receptor serum at a 1:5,000 dilution in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20, at room temperature for 1 h. The receptor-antibody complexes were treated with alkaline phosphatase-conjugate goat anti-rabbit immunoglobulin G antiserum (Sigma) at 1:2.000 dilution. The immunoprecipitate was stained with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (ready made solution from Böhringer, Mannheim).

In-gel Tryptic Protein Digestion

After visualization, the gel was destained with a solution of 25 mM ammonium bicarbonate, 50% acetonitrile. The proteins were digested in the gel according to the modified procedure of Hellman *et al.* (36). The resulting peptides were separated using a Hewlett-Packard 1090 HPLC on a Aquapore RP 300 A (2.1 × 250 mm) column in 0.05% trifluoroacetic acid, 3% acetonitrile, 7% 1-propanol, 0.1% (w/v) octyl-β-glucoside using a gradient of 0.05% trifluoroacetic acid, 30% acetonitrile, 70% 1-propanol, 0.1% (w/v) octyl-β-glucoside from 0 to 60% in 50 min and 60–100 in 15 min. The flow rate was 0.4 ml/min and the fractions were monitored at 215 and 280 nm.

Mass Spectrometric Analysis

For MALDI mass spectrometry, samples were dissolved in 5 μl of 50% acetonitrile, 0.1% trifluoroacetic acid and sonicated for few minutes. Aliquots of 0.5 μl were applied onto a target disk and allowed to air dry. Subsequently, 0.3 μl of matrix solution (1% w/v α-cyano-4-hydroxysuccinnamic acid in 50% acetonitrile, 0.1% (v/v) trifluoroacetic acid) was applied to the dried sample and again allowed to dry. Spectra were obtained using a Bruker Biflex MALDI time-of-flight mass spectrometer. MS/MS analyis was carried out using Finnigan Mat (San Jose, CA) LCQ ion trap mass spectrometer. For the interpretation of MS and MS/MS spectra of protein digests we used the Sherpa software (37) and the MS-Fit program available at the www site at the University of California at San Francisco (http://rafael.ucsf.edu/cgi-bin/msfit).

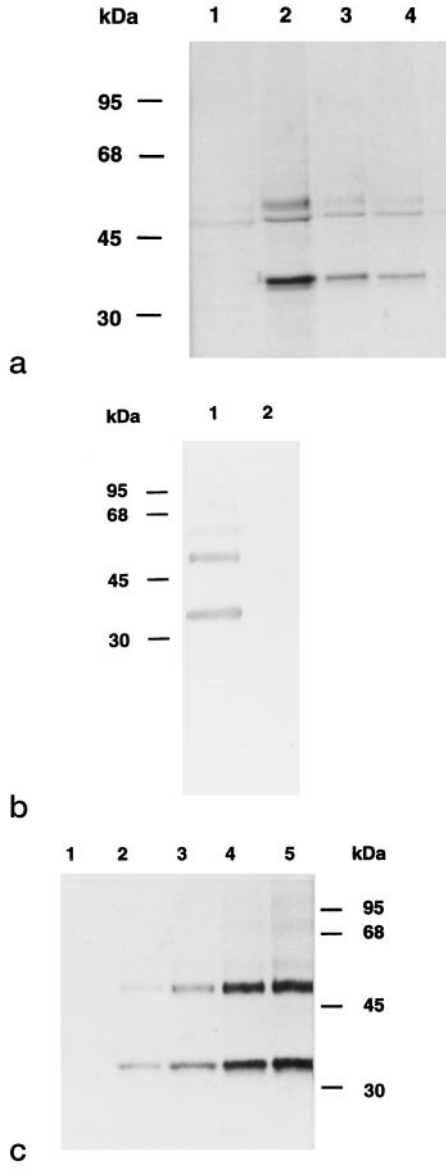

FIG. 2. **Purification of ET_B receptor from bovine lung using the (dA)30–5′-S-EMC-ET/oligo(dT)-cellulose purification system.** *A*, the elution profile of the receptor protein from an oligo(dT)-cellulose column monitored by 10% SDS-PAGE with silver staining. Elution fractions are represented by the *numbered lanes*. Immunoblotting (not shown) revealed that the bands at 34 and 49 kDa correspond to ET_B receptor, while the band at about 54 kDa corresponds to ET_A receptor. *b*, the effect of externally added endothelin-1 on the purification of ET_B receptor. The protein fractions were analyzed on 12.5% SDS-PAGE. *Lane 1*, immunoblot of the purified ET_B receptor contained in fraction 2 from the oligo(dT)-cellulose column. *Lane 2*, immunoblot of material obtained with the identical purification procedures except for the presence 1 mM endothelin-1 as a specific competitor. *C*, the effect of (dA)30–5′-S-EMC-ET concentration on the yield of purified ET_B receptor. Immunoblots of ET_B receptor show the purification of ET_B receptors performed as described under "Experimental Procedures" except that the concentration of (dA)30–5′-S-EMC-ET was variable. Concentration of (dA)30–5′-S-EMC-ET: 0 (*lane 1*), 0.37 nM (*2*), 1.5 nM (*3*), 6.0 nM (*4*), and 24.0 nM (*5*).

Protein Sequencing of the ET_B Receptor

Proteins were electroblotted onto poly(vinylidenedifluoride) membrane, stained with Coomassie Blue, and directly used for protein sequencing. The protein sequence analyses were performed on an Applied Biosystems Procise 494 4-cartridge sequencer (ABI, Foster City). The sequencing results were analyzed using a Macintosh based 610A data system.

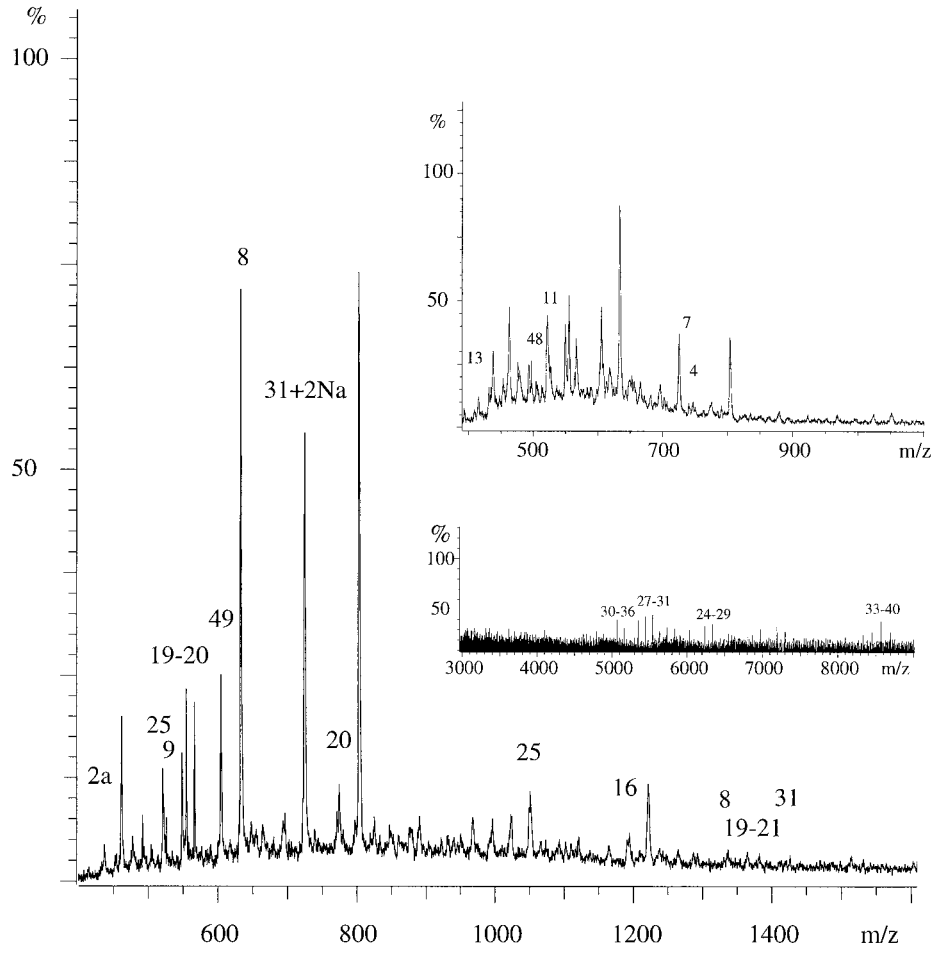

FIG. 3. **MALDI-TOF mass spectrometry of peptides obtained by in-gel trypsin digestion of ET_B receptor.** The peptides correspond to $(M + H)^+$ masses except for peptides 25, 19–20, 8, and 31, which correspond to $(M + 2H)2^+$ masses. Where the same masses are shown, the same peptide is indicated in only one spectra. The *insets* show spectra of partially purified peptides obtained from HPLC.

RESULTS

Isolation of ET_B Receptor Using a New "Fishhook"—We have designed a heterobifunctional cross-linking reagent which in addition to a *N*-hydroxysuccinimide-activated ester also possesses a thiol-selective maleimido group. This linker was first attached to endothelin-1 via the ϵ-amino group of Lys^9. The identity of the *N*-(ϵ-maleimidocaproyloxy)succinimide endothelin (Fig. 1*A*) was checked by MALDI mass spectrometry and protein sequencing. Following HPLC purification, the thiol selective maleimido group was used to attach 30-mer(dA). After purification (Fig. 1*B*), the final product, (dA)30–5′-S-EMC-ET, was subsequently added to a suspension of bovine lung membranes solubilized in digitonin/Chaps. The ET receptors were purified in a manner similar to that used for isolation of eukaryotic mRNA: absorbtion on oligo(dT)-cellulose, washing under conditions which favor formation of a (dA·dT) double helix (high salt concentration) and elution with very low salt concentrations that destabilize a (dA·dT) double helix.

Endothelin A and B receptors were isolated in virtually pure form (Fig. 2*A*) and their identity was confirmed by immunoblotting with antibodies raised against ET_A and ET_B receptors. We estimate that about 30 pmol of ET_B receptor was obtained from 100 g of bovine lung tissue. This is more than twice the yields that we and others have previously reported (38). As expected from our previous experience (39, 40), ET_B receptor was isolated in larger quantities than ET_A (Fig. 2*A*). The specificity of the isolation procedure was further verified by experiments demonstrating that natural endothelin competes with the (dA)30–5′-S-EMC-ET fishhook (Fig. 2*B*) and that the yield of the receptor depended on the concentration of (dA)30–5′-S-EMC-ET (Fig. 2*C*). As previously noted by ourselves and others (38, 39), two ET_B receptor species of 49 and 34 kDa were observed. After electroblotting, sequence analysis gave the NH_2-terminal sequence EEREFP for the ET_B receptor of 49 kDa, which also conforms to previous identification of the NH_2 terminus of the ET_B receptor (38, 39).

MALDI-TOF Mass Spectrometry—From the SDS-PAGE gels, about 4 pmol of Coomassie Blue-stained ET_B receptor were subjected to in-gel tryptic digestion. We took precautions during gel electrophoresis to avoid formation of acrylamide adducts and used only the best and purest chemicals and solvents available throughout the entire purification process. MALDI mass spectrometry was used for initial analysis of the entire mixture of tryptic peptides. This gave predominantly singly charged fragments, which allows easier interpretation of masses observed for peptide mixtures than is the case for spectra generated by electrospray mass spectrometry. From the MALDI mass spectra (Fig. 3), it was possible to identify the entire NH_2-terminal extracellular region and peptides from the second and third extracellular regions. From the cytoplasmic region, peptides from the second, third, and COOH-terminal loops were observed. Peptides containing transmembrane helical regions joined to loop regions could also be observed (Fig. 4, Table I). Half of the total tryptic digest (about 2 pmol) was submitted to HPLC separation using octyl-β-glycoside as a detergent during elution. The separated peptides were collected and subjected to MALDI analysis. As summarized in Fig. 4 and Table I, analysis of the original peptide mixture and the

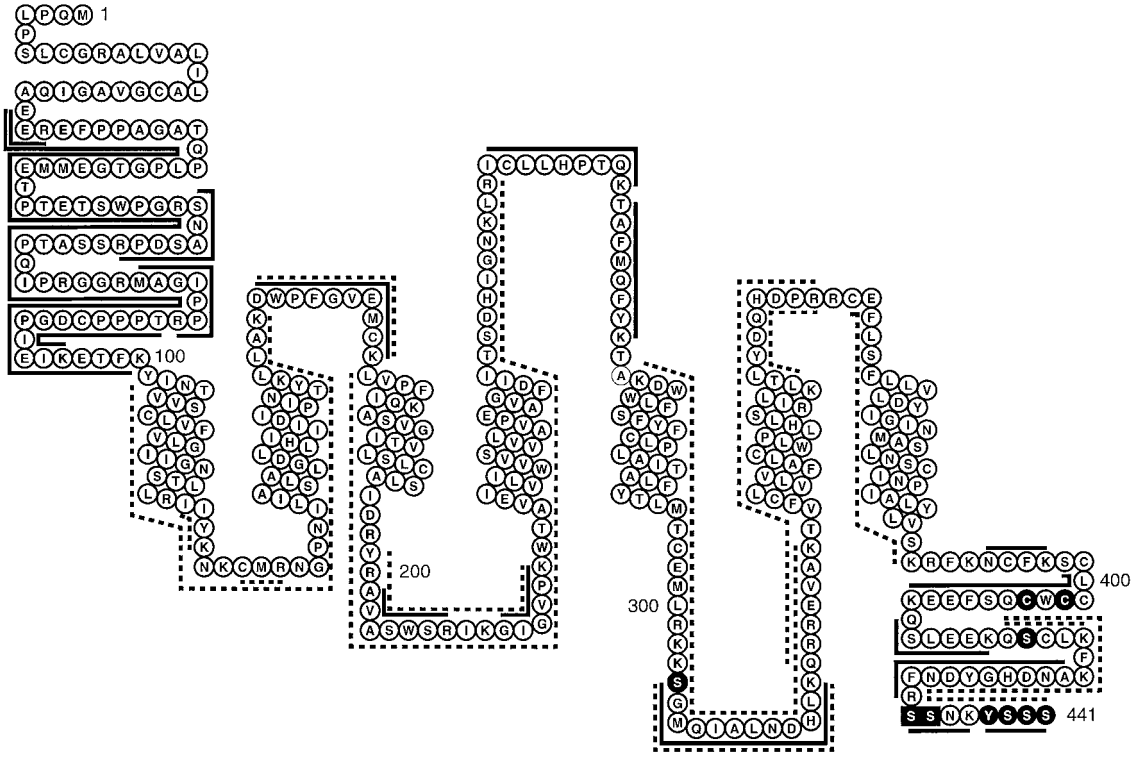

FIG. 4. **Peptides identified by MALDI-TOF mass spectrometry of bovine ET_B receptor.** *Solid lines* denote tryptic peptides identified from peptide mixture, *dotted lines* denote tryptic peptide identified after HPLC separation using octyl-β-glycoside in the elution solvent. *Solid black circles* indicate post-translationally modified amino acid residues. *Squares* indicate phosphorylation at Ser^{434} or Ser^{435}.

HPLC-separated peptides allowed the observation of peptides corresponding to the entire protein sequence including all transmembrane helices. Due to incomplete tryptic cleavage, which is often observed for membrane receptors, two membrane helices with the intervening loop were often recovered in a single peptide, presumably because the attached loops decrease the overall hydrophobicity of the fragment (Fig. 4). The mass measurements were sufficiently accurate (better than 0.1% of the calculated mass) to give clear indications of which peptides were post-translationally modified (Table I). We observed phosphorylation for peptides 31, 45, 48, and 49. Peptides 31, 45, and 48 showed an increased mass of 80 Da, which is characteristic for peptides with single phosphorylation sites. For peptide 49, masses consistent with mono, di-, tri-, and tetra-phosphorylated peptide were observed. For peptide 43, palmitoylation at two sites was suggested by an increase of mass of 476 Da (2 × 238 Da). Although endothelin B receptor contains two consensus glycosylation sites at residues Asn-60 and -118, the masses of peptide 2a-9 (residues 27–100), peptide 4 (50–65), and peptide 10–15 (101–164) were consistent with no glycosylation at these sites.

Electrospray Ion-trap Mass Spectrometry—To confirm the identity of the peptides and to determine the attachment sites of the post-translational modifications, we performed electrospray ion trap mass spectrometry of the unseparated peptide mixture with subsequent MS/MS analysis of selected fragments of interest. We observed that in electrospray-ion trap mass spectrometry the phosphorylated peptides partially lose the H_3PO_4 moiety in the mass spectrometer, thus producing a pair of peaks separated by a mass difference of 98 Da. This has previously been reported for MALDI-ion trap mass spectrometry of phosphorylated peptides (41). In accordance with this, we observed that phosphopeptides could be identified by a pair of masses 80 Da higher (the mass of H_3PO_4 minus H_2O) and 18 Da lower than expected based on the amino acid sequence.

Peptide 31 provides an example of the identification of a phosphorylation site. There were two pairs of peaks separated by 98 Da (m/z 1407.0 and 1307.6 Da). In addition the MS/MS (collision induced dissociation) spectrum of a peptide 31 showed a y ion series, y_{12}, y_{11}, and y_{10}, which was sufficient to identify Ser^{304} in the amino acid sequence $^{304}S(P_i)GMQIALNDHLK^{315}$ (Fig. 5*A*) as the site of phosphorylation. We also confirmed the phosphorylation of peptide 45 which appeared as a double peak of m/z 658.3 and 560.2. MS/MS analysis generated a clear set of y_5, y_4, y_3, y_2 and b_5^*, b_4^*, b_3^* (b_n^* are b_n ions minus H_3PO_4), which confirmed that the only Ser in peptide 45, $^{417}QSCLK^{421}$, namely the Ser^{418}, is phosphorylated. Two peaks of m/z 515.2 Da and 417.8 Da clearly indicated a single phosphorylated residue in peptide 48, $^{434}SSNK^{437}$. However, while the MS/MS spectra of this peptide confirmed the identity of the peptide, the spectra were not sufficient to identify the phosphorylation site as Ser^{434} or Ser^{435}.

The COOH-terminal peptide 49, $^{438}YSSS^{441}$ was observed as mono-, di-, tri-, and tetra-phosphorylated tryptic fragments with m/z 522.2, 602.8, 683.1, and 766.0 Da, respectively, in the MALDI spectra, clearly indicating a very complicated phosphorylation pattern for the last 4 amino acids of the cytoplasmic COOH-terminal tail. The MS/MS results indicated that the monophosphorylated species was phosphorylated only at Ser^{439}.

Palmitoylation of ET_B Receptor—The increase of 476 Da in the mass of peptide 43 indicated that among the four Cys residues clustered in this peptide, two are sites for palmitoylation. The MS/MS analysis generated y_{13}^{2+}, y_{12}^{2+}, and y_{11}^{2+} (m/z 1021.6, 978.5, and 926.0 Da) peaks corresponding to a doubly charged palmitoylated peptide, thereby showing that Cys^{399} in peptide $^{398}SCLCCWCQSFEEK^{410}$ is not palmitoylated (Fig. 5*B*). Fragments y_7^{2+} and y_9^{2+} (m/z 555.6 and 819.1) showed that Cys^{402} and Cys^{404} are palmitoylated, but not Cys^{401}. Confirmation of palmitoylation of Cys^{404} was obtained from the set of

TABLE I
MALDI mass spectrometry analysis of ET_B receptor peptides

Peptide[a]	Residues	Expected mass MH^+	Measured mass	
			Peptide mixture	Separated peptide
2a	27–30	433.2	433.4	432.2
2a–9	27–100	7901.9	7978.4[b]	
4	59–65	746.3	746.7	
7	78–84	741.4	740.3	
8	85–96	1266.6	1265.2	
9	97–100	524.2	525.7	
10–15	101–164	7069.6		7092.6[c]
11	124–127	536.3	537.5	
13	130–132	409.1	409.4	
16	165–174	1211.5	1210.5	1211.0
17–24	175–252	8458.1		8456.2
19–20	199–207	1095.6	1095.2	
19–21	199–209	1336.7	1337.6	1338.4
19–26	199–269	7983.5		7585.9
20	201–207	776.4	774.8	
22	210–215	570.3	571.8	
24–29	251–302	6346.7	6342.1	
25	253–261	1052.5	1052.1	
27–29	270–302	4025.8	4025.6	4023.0
27–31	270–315	5462.6	5542.8[d]	
28–31	273–315	5162.2		5163.2
30–36	303–345	4991.1	5069.9[d]	
31	304–315	1326.6	1406.4[d]	1407.0[d]
32–37	316–355	4793.8		4797.4
33–40	318–391	8582.4	8575.9	
38–39	356–390	3935.7	4006.4[e]	
42	394–397	511.2	511.5	582.5[e]
43	398–410	1566.8	2041.2[f]	
44–45	411–421	1292.6		1294.5
45	417–421	578.3		656.2[d]
47	424–433	1208.5		1206.1
48	434–437	435.2	515.2[d]	
49	438–441	443.1	522.1[d]	522.1[d]
			602.8[d]	
			683.1[d]	
			766.0[d]	

[a] Tryptic hydrolysis at all Lys and Arg residues would yield 49 peptides. Di- and tripeptides with mass < 400 Da are not shown. Longer peptides arising from incomplete tryptic hydrolysis are shown as the component peptides. Peptide 2a starts at the NH_2 terminus observed for ET_B receptor by NH_2 terminal sequencing. Although peptides corresponding to all potential cleavage sites were not observed, peptides corresponding to the complete sequence 27–441 were measured.
[b] 2-Mercaptoethanol adduct.
[c] Na^+ ion.
[d] phosphate group.
[e] Cys acrylamide adduct.
[f] palmitoylation.

doubly charged *b* ions, in particular ions b_6^{2+} of *m*/*z* 467.2 and b_7^{2+}, *m*/*z* 638.3 Da. The difference of 170 Da between these two doubly charged *b* ions (1/2 of 341 Da for palmitoylcysteine) confirms that of the 3 Cys residues in the sequence 398–403, only one is palmitoylated.

DISCUSSION

In the work reported here the special fishhook, (dA)30–5′-S-EMC-ET, allowed for a rapid, very mild single step isolation of ET_B receptor by absorbtion to oligo(dT)-cellulose and elution in a small volume of very low salt buffer. The fishhook has been constructed in such a way that it should be applicable to the isolation of other peptide hormone receptors, which we are currently testing. After this single step procedure, the receptor is identified by SDS-gel electrophoresis and the protein from the gel band is subjected to tryptic fragmentation followed by analysis of the peptide mixture by MALDI and electrospray mass spectrometry.

Mass fingerprinting of tryptic peptides is becoming a standard procedure for rapid identification of proteins in proteome analysis (42, 43). The essence of this method is that treatment with trypsin produces a limited number of peptides and that the identification of a surprisingly small number of such peptides suffices to identify the protein in sequence data banks without the need for detailed analysis of potentially very complex mass spectroscopic fragmentation patterns. In the present work we have found that mass fingerprinting of tryptic fragments of ET_B receptor by MALDI mass spectroscopy also provided an efficient method to screen the entire protein sequence for the presence of post-translational modifications. Peptides covering the entire receptor sequence could be detected (Fig. 4) with adequate mass accuracy (better than 0.1%, Table I) to identify sequential regions containing post-translational modifications. Although it would in principle be possible to use only electrospray ion trap mass spectrometric analysis of the peptide mixture with subsequent MS/MS sequencing of each peptide, the prior use of MALDI mass spectrometry to identify the singly charged peptide masses of interest resulted in a dramatically simplified interpretation of the data. It should be stressed that in the present study, as is the general case with mass spectroscopy of peptides and proteins, a large number of spectra were acquired under different ionization/fragmentation conditions to obtain adequate mass information on all peptides. In this regard, we also found that MALDI analysis of membrane peptides was facilitated by appropriate HPLC of these peptides. Inefficient peptide separation due to aggregation and the loss of very hydrophobic peptides from integral membrane proteins in RP-HPLC is a well known problem. We used octyl-β-glycoside in separation solvents, which definitively helped in the recovery of transmembrane peptides, but most of the peptides were found in several HPLC fractions. This clearly means that noncovalent aggregation of peptides is still a problem during reverse phase-HPLC. However, this was of no major concern in the present work since MALDI showed that such aggregates dissociated into their peptide components. The presence of octyl-β-glycoside to keep the membrane peptides soluble resulted in much better signal intensity for these peptides.

Our analysis of peptides from ET_B receptor showed that we were able to recover peptides from the entire sequence of the receptor. MALDI, electrospray ion trap mass spectrometry, and MS/MS analysis revealed that ET_B receptor is phosphorylated at Ser^{304}, Ser^{418}, Ser^{438}, Ser^{439}, Ser^{440}, and Ser^{441}. Furthermore, another phosphorylation at Ser^{434} or Ser^{435} was observed. The ET_B receptor is also palmitoylated at two sites identified to be at Cys^{402} and Cys^{404}. A few peptides were observed both with and without phosphorylation. Given the distinctive fragmentation patterns of these post-translational modifications during mass spectroscopy, we believe this reflects the existence of species of ET_B receptor with different patterns of post-translational modifications. At the present state of knowledge about G-protein-coupled receptors, the large number of post-translational modifications observed for the ET_B receptor was unanticipated and suggests that the number and diversity of post-translational modifications of such receptors, together with concomitant roles in signal transduction pathways, may be much more complex than presently realized.

Phosphorylation and palmitoylation at carboxyl-terminal sites are known to influence the signal transduction in some G-protein-coupled receptors (8–14). The COOH-terminal tail in all known ET_B receptors is identical and with the exception of a few amino acid exchanges, all known ET_A receptors also have identical COOH-terminal tails. However, there is almost no homology in the COOH-terminal region between ET_A and ET_B receptors (3, 4, 7, 44–46). Among the very few strongly conserved amino acids are Ser^{418} and Ser^{439} as well as Cys^{402} and Cys^{404} which we found to be phosphorylated and palmitoylated,

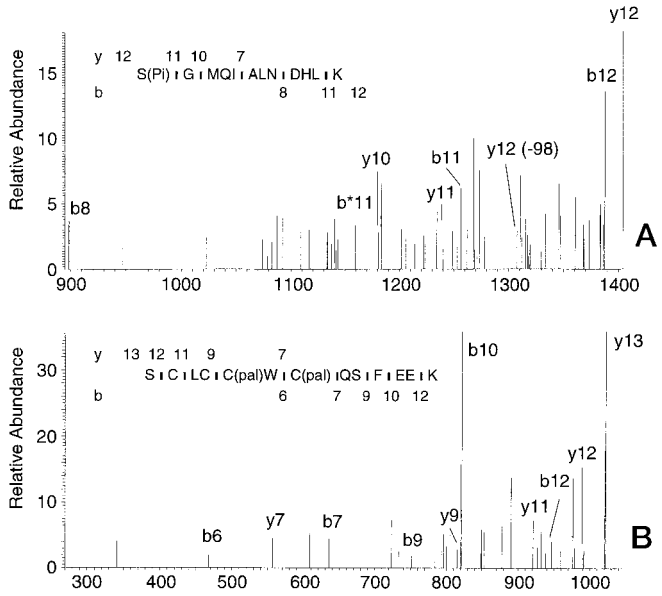

FIG. 5. **Post-translational modifications of tryptic peptides 31 and 43.** *A*, MS/MS spectrum of phosphopeptide 31, S(P_i)GMQIALNDHLK $(M + H)^+$. The notation b^* denotes corresponding b_n ions minus 98 (H_3PO_4), which confirms the phosphopeptide. *B*, MS/MS spectra of palmitoylated peptide 43, SCLCC(Pal)WC(Pal)QSFEEK $(M + 2H)2^+$.

respectively. Those positions may be post-translationally modified in the ET_A receptor as well. However, phosphorylation of Ser^{304} and Tyr^{438} is possible only for the ET_B receptor since these positions are replaced by Gly or Asp, respectively, in the sequence of ET_A receptor. It seems likely that different post-translational modifications of ET_A and ET_B receptors will correlate with different signal transduction pathways and we are therefore currently analyzing post-translational modifications of the ET_A receptor.

We suggest that phosphorylation at Ser^{304} of ET_B receptor may be particularly important in the development of Hirschsprung's disease (17–21). Hirschsprung's disease is characterized by the absence of autonomic ganglion cells in the terminal bowel. It is the most common cause of congenital obstruction with an incidence of 1 in 5000 live births. It has been shown that the endothelin-induced signaling pathway is crucial for the development of enteric ganglia and that this pathway is genetically compromised in Hirschsprung's disease. The analysis of the ET_B receptor gene in patients with Hirschsprung's disease demonstrated two mutations which resulted in stop codons producing truncated and nonfunctional ET_B receptor (20) and a single site mutant that replaced Ser^{304}, which is phosphorylated (this work) and strongly conserved in all known ET_B receptors, with Asn (47). This suggests that phosphorylation of Ser^{304} may play a particularly important role in receptor-mediated signal transduction.

The site-directed mutagenesis of α_2- and β_2-adrenoreceptors and endothelin receptor A as well as mass spectrometric investigations of rhodopsin showed that those receptors are palmitoylated at Cys residues in the COOH-terminal tails (14, 8, 27, 48). It is believed that the covalently bound palmitic acid residue becomes intercalated in the membrane bilayer. Prevention of palmitoylation of the β-adrenergic receptor produced functional uncoupling of the receptor from the adenylyl cyclase pathway, rapid desensitization in response to its ligand, and increased basal phosphorylation (49). For α_2-adrenergic receptor the signal transduction pathways were not affected, but prevention of palmitoylation caused a decrease of ligand-promoted down-regulation of the receptor. In the case of nonpalmitoylated bovine rhodopsin, an increase in signal transduction activity was observed (50). Site-directed mutagenesis of ET_A receptor (27) has shown that non-palmitoylated ET_A receptor shows no change in ligand binding affinity or stimulation of adenylyl cyclase. However, the lack of palmitoylation was reported to affect phosphatidylinositol hydrolysis by phospholipase C activation after stimulation by endothelin-1. Furthermore, it was observed that the mutated ET_A, in contrast to wild type ET_A, failed to show a ligand-induced transient increase in cytoplasmatic calcium concentration. We have obtained direct evidence that ET_B receptor is palmitoylated at Cys^{402} and Cys^{404}. The cluster of Cys residues, $^{399}CLCCWC^{404}$, is highly conserved in endothelin receptors of the B type. Together with the above results on other receptors, this suggests that palmitoylation plays a role in the regulation of the ET_B receptor, although no experimental evidence has so far been reported.

The isolation and direct analysis of the chemical structure of membrane receptors has traditionally been a notoriously difficult task. Known difficulties have included low available amounts, limitations in tryptic or other digestion methods, poor peptide separations, very poor recovery of membrane helical fragments, difficulties in obtaining reliable information on post-translational modifications by protein chemical sequencing, etc. As a consequence, the previously available direct protein analyses of membrane receptors were generally limited to proteins that were readily available in larger quantities. Apart from pioneering work on rhodopsin (11, 12), there are only a very few scattered reports in the literature on direct observation of post-translational modifications of G-protein-coupled membrane receptors (13–15). The present results indicate that with the development of new methods for rapid, mild isolation of as little as 1–2 pmol of membrane receptors on gels and the combination of highly sensitive MALDI and electrospray ion trap mass spectrometry, direct evaluation of the sites and types of post-translational modifications of membrane receptors is poised to become a routine characterization. The next challenge will be to correlate different patterns of post-translational modifications with different functional states.

Acknowledgments—We thank Prof. Hagiwara of the Tokyo Institute of Technology, Japan, for a generous gift of antisera against bovine ET_B receptor. We thank Drs. M. Görlach and S. Fischer-Frühholz for helpful discussions and E. Nyakatura for excellent technical help.

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