Functional Diversity of Endothelin Pathways in Human Lung Fibroblasts May Be Based on Structural Diversity of the Endothelin Receptors

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ABSTRACT: Posttranslational modifications of the endothelin receptors A and B from human lung fibroblasts were investigated before and after stimulation of the cells with $(dA)_{30}$-5′-S-EMC-endothelin-1. The patterns of phosphorylation and palmitoylation of both receptors were much more complicated than expected. In both the stimulated and the unstimulated states, multiple isoforms differing in the number and location of posttranslational modifications were present. MS analyses suggested rapid changes in these isoforms following stimulation. Overall, the ETA receptor was modified at 20 sites (15 phosphorylation, five palmitoylation sites) and ETB at 17 sites (13 phosphorylation, four palmitoylation sites). Part of the structural diversity involved hypermodification of short sequence regions, and it is suggested that this could represent a mechanism for incremental modulation of receptor activity. It is postulated that the observed structural diversity over disparate parts of the receptor sequences forms the basis for parallel stimulation of different signaling pathways at spatially and functionally distinct ET receptors differing in posttranslational modifications.

One of the most challenging endeavors of modern biological science is the current effort to understand the function of living cells at the molecular level (i.e., how do cells achieve finely tuned control of growth and division, metabolism, shape, and movement in response to dynamic internal and external cues). Despite an unprecedented proliferation in the quantity and quality of the available information, large gaps in our knowledge still exist. The introduction of highly parallel technologies embodied in various "omics" approaches has begun to give a more global view of cellular signaling and response systems but at the same time has demonstrated that these cellular systems are much more complicated than initially anticipated.

Many cellular functions depend on various kinds of receptor-mediated signaling. The G-protein-coupled receptors (GPCRs)[1] are one of the largest protein superfamilies in the human genome and constitute a major proportion of cellular receptors, but at present many GPCRs are orphans (i.e., the relevant physiological ligand is unknown). GPCRs link the cell to its environment by receiving stimuli, transmitting this signal to the cell, and initiating and regulating the response. It is no surprise that these receptors are involved in numerous disorders and that, with kinases, they predominate as targets for the development of drugs.

The classical paradigm for GPCR signaling was essentially linear and sequential (*1*). However, more recent evidence shows that the signaling pathways of GPCRs are very complex. Both classical GPCR pathways and G-protein-independent pathways can be activated, and responses can involve the cross-regulation of many specific pathways, including cross-talk between different GPCRs as well as interactions with other signaling pathways (*2*). Interactions with adaptor and scaffolding proteins can enhance the activation of proteins in the signal cascades, ensure specificity by bringing only the necessary proteins into close proximity, and effect a compartmentalization of the signaling cascade (*3*, *4*). GPCRs may undergo dimerization and even higher-order oligomer formation (*5*), which can contribute to the diversity of signaling by altering the specificity of agonist and antagonist interactions (*6*). Although the evidence is inconclusive at present, there are increasing indications that in a single cell different molecules of a given GPCR may contribute in different ways to the cellular response upon stimulation.

Many aspects of cellular life are regulated via protein phosphorylation. Phosphorylation plays a major role in intracellular communication during development, in physiological responses, in homeostasis, and in the functioning of the nervous and immune systems. This very sophisticated cellular regulatory mechanism involves phosphorylation/dephosphorylation of as much as 30% of all cellular proteins, including the GPCR superfamily. Stimulation of GPCRs typically triggers a cascade of phosphorylation events on other cellular proteins, which in turn feed back to modifications of GPCRs that are critical to their functional roles. In the companion to this paper (*7*), we present evidence from studies of rapid changes in the cellular phosphoproteome that

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[1] Abbreviations: MEM, minimum essential media, with Earle's salts, w/o L-Glu; NEAA, nonessential amino acids; FBS, fetal bovine serum; ET-1, endothelin-1; $(dA)_{30}$-5′-S-EMC-ET, $(dA)_{30}$-5′-S-*N*-(ϵ-maleimidocaproyloxy)succinimide-endothelin-1; MALDI-TOF MS, matrix-assisted laser desorption ionization-time-of-flight mass spectrometry; ESI-ion trap MS, electrospray ionization-ion trap mass spectrometry; ETA and ETB receptors, endothelin A and endothelin B receptors; GPCR, G-protein-coupled receptor.

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ETA and ETB receptors engage a wide variety of cellular signaling pathways upon stimulation of human lung fibroblasts (*7*).

Given their low cellular abundance and status as integral membrane proteins, direct characterization of posttranslational modifications of GPCRs has been limited. Apart from pioneering work on rhodopsin (*8*, *9*), there are still only a few scattered reports in the literature involving direct observation by mass spectrometry of posttranslational modifications of GPCRs (*10*−*16*). Much of what is presently known about posttranslational modification of GPCRs is largely based on indirect methods. These include genetic substitution of amino acid residues that are possible sites of modification (e.g., Ser, Thr, Tyr for phosphorylation, or Cys for acylation) and the in vitro phosphorylation/dephosphorylation by kinases or phosphatases of synthetic peptides derived from the receptor amino acid sequences. There are indications that these types of indirect studies may give misleading results (*17*, *18*), and in some cases, it has been observed that genetic substitutions (e.g., Ser to Ala) can lead to the phosphorylation of alternative residues in protein sequences with at most very moderate influences on protein activity (*19*).

We have developed methods for rapid, mild isolation of membrane receptors that, combined with the high sensitivity of MALDI and electrospray ion trap mass spectrometry, greatly facilitate the direct evaluation of the sites and types of posttranslational modifications of GCPRs (*13*, *14*). The present investigations of the posttranslational modifications of the ETA and ETB receptors of human lung fibroblasts both prior and after stimulation reveal an unsuspected number of isoforms involving differently posttranslationally modified receptors. We suggest that the multiple phenotypes observed for individual protein sequences may correspond to another level of diversification in the function of endothelin receptors and that this is coupled to the wide diversity in the types of responses elicited by endothelin stimulation.

MATERIALS AND METHODS

Materials. The cell line CCD19Lu was from the European Collection of Cell Cultures (Salisbury, UK). Trypsin-EDTA, antibiotic/antimycotic, minimal essential medium (MEM), HBSS, nonessential amino acids (NEAA), and L-glutamine were from GibcoBRL (Eggenstain, Germany). FBS was from PerbioScience (Tattenhall, UK). HPLC-grade acetonitrile, methanol, and acetic acid were from BDH (Poole, UK). Duracryl (30% acrylamide, 0.8% BIS) was from Genomic Solutions (Huntingdon, UK). TFA was from Perkin-Elmer (Wellesley, MA). DMF was from Fluka Chemie (Gillingham, UK). The terminal transferase 3′-labeling kit was from New England Biolabs (Hitchin, UK). Modified trypsin was from Promega (Madison, WI). Oligo-dT cellulose and α-^{32}P-ddATP were from Amersham (Amersham, UK). $(dA)_{30}$-5′-SS-R 1-*O*-dimethoxytrityl hexyldisulfide was from BioTeZ (Berlin, Germany). All other chemicals were from Sigma-Aldrich (Gillingham, UK) and of the best grade available.

Cell Culture. The human lung fibroblast cell line CCD19Lu was grown in MEM containing 2 mM L-Glu, 1% NEAA, 0.8% antibiotic/antimycotic solution, and 10% FBS. Cells were grown at 37 °C, 5% CO_2 in 75 cm^2 dishes.

Synthesis of $(dA)_{30}$-5′-S-EMC-ET. Synthesis of $(dA)_{30}$-5′-S-EMC-ET was carried out using a modification of the procedures of Roos et al. (*13*). A total of 0.5 g of oligo-(dT)-cellulose powder was added to 1 mL of 0.1 M NaOH and packed in a column that was rinsed with water until the column effluent had a pH of <8. The column was equilibrated with 5 column volumes of loading buffer (50 mM sodium citrate, pH 7.5, 0.5 M LiCl, 1 mM EDTA, 0.1% (w/v) SDS) and transferred to a 15 mL tube. The 140.6 OD (3.02 mg) of DMT-hexyl-SS-$(dA)_{30}$ (BioTeZ, Berlin-Buch GmbH) was dissolved in 4 mL of loading buffer and mixed with the oligo(dT) cellulose. The mixture was shaken at room temperature for 2 h, then 1600 μL of 1 M DTT and 80 μL of 200 mM tributyl phosphine (TBP) in DMF were added, and shaking continued for 3 h at RT. The suspension was then transferred to a column, repacked, and washed with 10 column volumes of reaction buffer (50 mM sodium citrate, pH 7.0, 0.5 M LiCl, 1 mM EDTA, 0.1% (w/v) CHAPS). The oligo(dT) cellulose was resuspended in 3 mL of reaction buffer and transferred to a 15 mL tube. A solution of *N*-(ε-maleimidocaproyloxy)succinimide (EMC, 10 mg in 1 mL of 90% EtOH containing 0.5 M LiCl) was added, and the tube was flushed with argon and shaken overnight at RT.

The suspension was repacked into a column and washed with 20 column volumes of reaction buffer. The material was then resuspended in 3 mL of loading buffer and transferred to a tube. A total of 1 mg of ET-1 was dissolved in 1 mL of loading buffer and added to the suspension. The suspension was shaken overnight at RT. The slurry was repacked into a column and washed with 20 column volumes of washing buffer (50 mM sodium citrate, pH 7.0, 0.5 M LiCl, 1 mM EDTA, 0.05% (w/v) CHAPS). A total of 3 mL of elution buffer was added (10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.05% (w/v) CHAPS), and elution fractions of 1.5 mL were collected.

Calcium Response of Human Lung Fibroblasts to Stimulation with $(dA)_{30}$-5′-S-EMC-ET. Binding of ET by ET receptors is known to produce a rise in intracellular calcium (*20*). This property was used to test the function of the synthetic ligand. Cells were grown on sterile cover-slips in a 12 well plate with 1 mL of medium each (prepared as described under cell culture), by which time they were 90% confluent. The cells were then incubated for 30 min with serum and antibiotic-free medium. The medium was then aspirated and replaced with calcium buffer (127 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 0.5 mM NaH_2PO_4, 2 mM $CaCl_2$, 5 mM $NaHCO_3$, 10 mM glucose,10 mM HEPES, 0.1% BSA) containing the red fluorescent dye Fluo-3 (4 μL/mL), and the cells were incubated at 37 °C for 30 min.

Three experiments were conducted: (i) stimulation with $(dA)_{30}$-5′-SS-R 1-*O*-dimethoxytrityl hexyl disulfide (the poly dA tail), (ii) stimulation with ET-1, and (iii) stimulation with $(dA)_{30}$-5′-S-EMC-ET. The fluorometer was allowed to take 10 readings to establish a baseline, and then 1.5 mL of 3.3 mM $(dA)_{30}$-5′-SS-R 1-*O*-dimethoxytrityl hexyl disulfide, 50 nM ET-1 (final concentrated), or 50 nM $(dA)_{30}$-5′-S-EMC-ET (final concentrated) was added. Readings were taken from seven, six, and seven cells, respectively.

Affinity Purification of Endothelin Receptors on Oligo-(dT)-Cellulose Using Oligo(dA)-Coupled Endothelin-1. Affinity purification of endothelin receptors was carried out by a modification of the procedures of Roos et al. (*13*). Twelve p100 dishes of CCD19Lu at 90% confluence were washed twice with HBSS and incubated at 37 °C, 5% CO_2

for 30 min in serum-free medium. The cells were then cooled on ice for 15 min. A total of 100 pmol of $(dA)_{30}$-5′-S-EMC-ET was added to each dish. The stimulated group was incubated at 37 °C. The unstimulated group remained on ice. After 2 min, all cells were washed with ice-cold buffer P. A total of 3 mL of buffer S (20 mM potassium phosphate, pH 7.4 containing 0.4% digitonin, 0.25% CHAPS, 500 mM NaCl, 20 mM EDTA, 1 mg/mL RNase, protease inhibitors as stated previously plus 10 mg/mL pepstatin) was added to each dish. The cells were incubated on ice for 15 min and then scraped from the dishes and homogenized on ice in a Dounce homogenizer. The homogenates were shaken at 4 °C for 1 h and then spun down for 45 min at 4 °C, 13 000 rpm (Sorvall, SS34). The supernatants were rotated overnight at 4 °C with 40 mg of oligo-dT cellulose.

The following day, the samples were spun down at 2000 rpm, 4 °C for 10 min. The pellets were resuspended in 1400 μL of buffer S, loaded into empty microcolumns (Amersham), and pelleted for 5 min at 2500 rpm, 4 °C. The columns were washed at 4 °C with 4 mL of buffer S and then eluted three times with 10 mM Tris/HCl, pH 7.4 containing 1 mM EDTA, 0.4% digitonin, 0.25% CHAPS, and protease inhibitors as buffer S.

SDS−Gel Electrophoresis. Eluates were mixed 1:1 with a 2X Laemmli sample buffer (Sigma) and incubated at 95 °C for 2 min. Thirty mL of each sample was run on a 10% polyacrylamide gel and silver stained.

In-Gel Digestion. Major bands were selected from stimulated and unstimulated samples, and a small square of gel from each band was digested as follows. The gel was reswollen in 50 mM NH_4HCO_3 for 3 × 15 min and then destained in 50 mM sodium thiosulfate and 15 mM potassium ferricyanide. After washing in 3 × 100 mL of ddH_2O, the gel was dried for 3 × 10 min in AcN. The liquid was removed, and the gel was dried in a speedvac for 30 min. The dry gel was incubated at 56 °C in 100 mM NH_4HCO_3 containing 10 mM DTT for 30 min, then dehydrated in AcN as stated previously, and dried in the speedvac for 30 min. The gel pieces were incubated overnight at 30 °C in a mixture of 5 mL of modified trypsin (75 ng/mL) and 5 mL of 70% DMF.

MALDI-TOF Mass Spectrometry. A total of 0.5 mL of the tryptic digest was applied to the target plate and allowed to air-dry, then overlaid with 0.5 mL of a saturated solution of α-cyano-4-hydroxysuccinamic acid in 50% AcN, 0.1% TFA v/v. Spectra were taken on a Biflex III MALDI-TOF spectrometer (Bruker, Germany) calibrated with a mixture of five known peptides. The spectrometer was in reflector mode, detector voltage 1.8 kV, laser attenuation 70%. Peaks were identified manually using Bruker DataAnalysis software. The peaks identified were compared with theoretical receptor digests generated by the Protein Prospector MS-Digest tool (http://www.jpsl.ludwig.edu.au).

ESI-Ion Trap Mass Spectrometry. Tryptic digest samples were diluted 1:5 with ddH_2O, then 1:10 in 50% methanol, 0.5% acetic acid. Samples were subjected to MS/MS analysis using a Finnigan-Matt LCQ. Collision energies ranged between 25 and 55%. Spectra were compared to theoretical fragmentations of the peptides identified by MALDI-TOF analysis using MS-Product (http://www.jpsl.ludwig.edu.au), and the sites of phosphorylation or pamlitoylations were determined where possible.

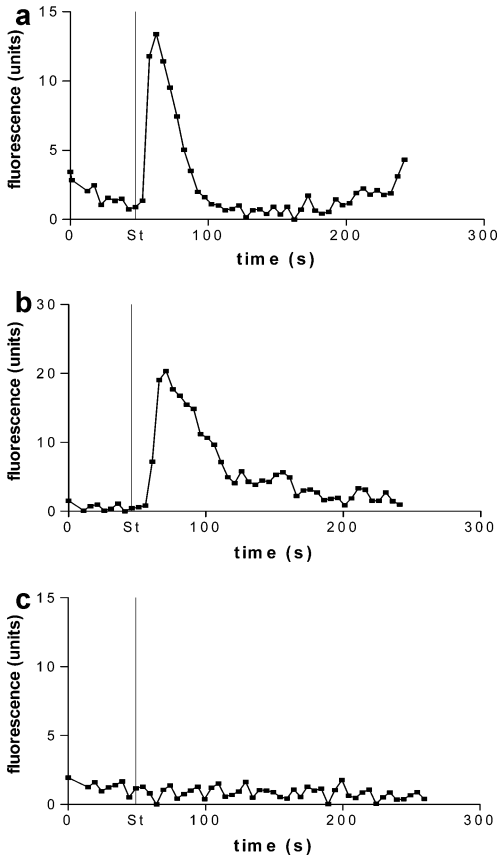

FIGURE 1: Calcium imaging studies for CCD19Lu cells. The cells were stimulated with (a) 50 nM ET-1, (b) 50 nM $(dA)_{30}$-5′-S-EMC-ET-1, or (c) 50 nM $(dA)_{30}$-5′-SS-R 1-*O*-diethoxytrityl hexyldisulfide (unreacted oligonucleotide control), and the fluorescence of the red fluorescent dye Fluo-3 was monitored vs time. The line labeled St shows the point of stimulation.

RESULTS

We have isolated endothelin receptors A and B from the human lung fibroblast cell line CCD19Lu, which expressed both endothelin receptors. The isolations were carried out using a modified synthesis of a fishhook that we described previously (*13*). The fishhook consists of poly-dA (dA_{30}) joined by a linker to the ϵ-amino group of Lys-9 of ET-1. It can be recovered from solutions of cellular lysates by chromatography on immobilized poly-dT under very mild conditions. The fishhook was labeled with α-^{32}P-ddATP at the 3′ end using the terminal transferase, and its specific binding to fibroblast membranes was measured. This served both to confirm the expression of ET receptors in CCD19Lu cells and to verify that binding of the fishhook to these receptors was virtually indistinguishable from binding of ET-1. In addition, measurement of the calcium response of the cells to $(dA)_{30}$-5′-S-EMC-ET-1 has shown that the synthetic ligand was able to produce a response from the cells similar to ET-1 and that this effect is not caused by the oligonucleotide alone (Figure 1). To use this fishhook to obtain samples of ETA and ETB receptors from both stimulated and unstimulated cells, we have taken advantage of the fact that at 4 °C, ET-1 does not activate downstream processes. Stimulated cells were exposed at 37 °C for 2 min, while unstimulated cells were retained at 4 °C. Subsequently, ETA and ETB receptors were recovered as described (Materials and Methods) and then subjected to further

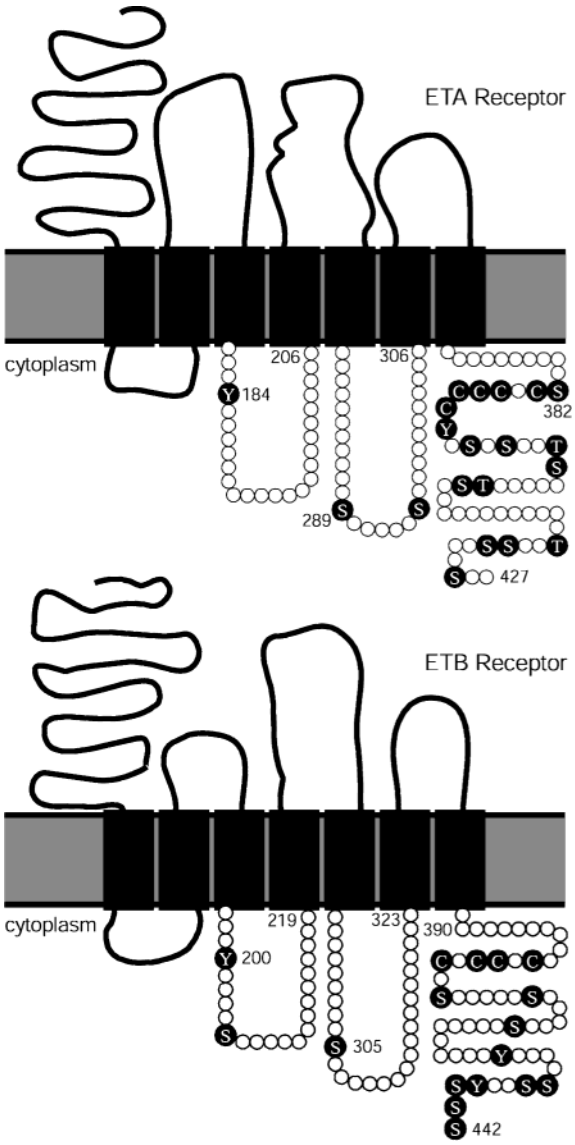

FIGURE 2: Summary of all sites of phosphorylation (S, Y, T) or palmitoylation (C) of ETA and ETB receptors in the stimulated receptor, the unstimulated receptor, or both.

purification and separated via PAGE gel electrophoresis. These gels (not shown) indicated that $(dA)_{30}$-5′-S-EMC-ET-1 has a very high selectivity for the endothelin receptors.

In the following, we describe the experimental MS evidence used to identify the posttranslational modifications for both stimulated and unstimulated forms of the receptors, first for ETA and subsequently for the ETB receptor. Figure 3 shows the orientation of these receptors in the membrane and a summary of all the sites that have been observed to undergo palmitoylation or phosphorylation in either the stimulated or unstimulated states of the receptors. Consideration of the differences between the simulated and the unstimulated receptors and of the functional significance of the observed modifications, as well as a comparison of these modifications to information obtained for other GPCR receptors, is deferred to the Discussion.

MS Analysis of Posttranslational Modifications of Unstimulated and Stimulated ETA Receptors. As summarized in Figure 2, a large number of sites showing either phosphorylation or palmitoylation of the ETA receptor could be identified by MS. There were two complications in the interpretation of the MS data. First, the observed peptides made it clear that for both the unstimulated and the stimulated forms of the ETA receptor, there were multiple forms of the receptor differing in the sites and degrees of the posttranslational modifications. For example, the sequence 378−392 in the C-terminal region of the unstimulated ETA receptor showed a variant with no posttranslational modifications at all, a variant with phosphorylation of all three possible phosphorylation sites, and a variant with no phosphorylation but attachment of five palmitoyl chains (Figure 3). An even more complicated pattern of modifications was observed for the sequence 378−392 in the stimulated receptor, where a total of nine different sequence variants were observed (Figure 3). Peptides from other regions of the sequence also indicated the presence of multiple species for the ETA receptor. The existence of multiple forms of the ETA receptor and of regions with very heavy degrees of modification complicated the determination of the exact residues that were modified, especially in peptides with only partial occupation of the sites.

A second complication in the interpretation of the MS data was that different peptides were recovered from the stimulated and unstimulated forms of the ETA receptor (Figure 3). Since the peptides were produced by tryptic fragmentation of the receptor, this is likely to be a consequence of the different patterns of posttranslational modifications of the receptor following stimulation (e.g., phosphorylation can affect the sites of tryptic hydrolysis (*21*)). Nonetheless, differences could be established between the sites and the degree of modification of the second and third intracellular loops as well as the C-terminus of the ETA receptor in the stimulated and unstimulated states (see Discussion). Furthermore, the volatilization of phosphorylated peptides is often reduced. This may have contributed to differences in the sequence coverage between the stimulated and the unstimulated receptors, and in some cases, this appeared to limit the amount of peptide available for MS/MS sequencing experiments to determine the exact sites of the posttranslational modifications.

Altogether, the MS evidence indicated that there was a total of 20 sites modified in the ETA receptor, five sites with palmitoylation and 15 sites with phosphorylation (Figure 2). All of these sites could be conclusively established from the MS data. Although the sites were clearly defined, it was not always possible to ascertain which of the sites were occupied in peptides with only partial modification of the sites. For example, the sequencing information obtained for the peptide (378−392 + 2P + 1Palm) did not locate the site of palmitoylation, and we could not definitively establish whether the second phosphorylation was at Tyr-389 or Ser-391 (Figure 3).

MS Analysis of Posttranslational Modifications of Unstimulated and Stimulated ETB Receptors. Similar complications were encountered for the ETB receptor (Figure 4). For the C-terminal sequence 435−442, six different peptides differing in the degree or sites of phosphorylation were observed for the unstimulated receptor, and a complex pattern with multiple phosphorylations was also observed for the stimulated receptor (Figure 4).

Evidence for multiple forms of both the stimulated and the unstimulated forms of the ETB receptor was also obtained at several other regions of the sequence (Figure 4). Similar

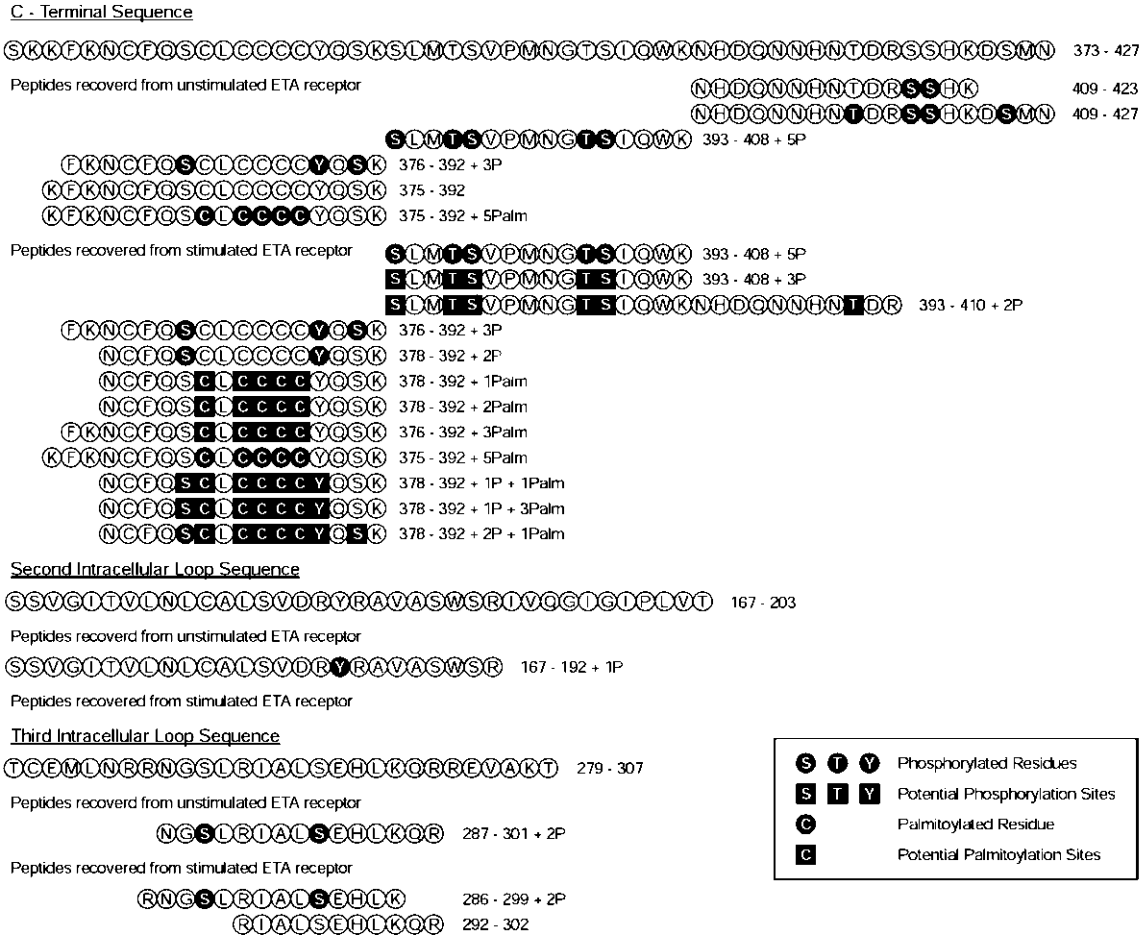

FIGURE 3: Peptides showing the patterns of posttranslational modifications that were observed by MS for the ETA receptor. For some of the peptides, especially those with partial modification of the possible sites, sequencing by ESI-ion trap MS was used to establish the sites of modification.

to the ETA receptor, the ETB receptor showed different tryptic fragmentation patterns for the stimulated and unstimulated receptor (Figure 4).

For the ETB receptor, a total of 17 sites for posttranslational modifications were established, four sites for palmitoylation and 13 sites for phosphorylation (Figure 2). Among these sites, there was some uncertainty about the location of the two sites located in the second intracellular loop. This loop was recovered with phosphorylation at two of the three possible sites (Tyr-200, Ser-205, and Ser-207) for both the stimulated and the unstimulated receptor (Figure 4). We were unable to obtain MS/MS sequencing data for this loop, and the assignment of the two sites is based on the tryptic fragmentation patterns. We cannot conclusively exclude phosphorylation at Ser-207.

DISCUSSION

The present experiments were initiated with the goal of correlating posttranslational modifications of the ET receptors with functional states. Such modifications are a product of the networks and fluxes characteristic of cellular signaling and regulation systems, and conversely, observation of the modifications can provide a means to monitor the state of cellular networks. As a first step in this direction, we have been able to demonstrate the presence of multiple species of the endothelin receptors A and B and to show that the patterns of posttranslational modifications change upon stimulation of the receptors. The large number of diverse species for both the stimulated and the unstimulated receptors was a surprise. In the first part of the following, we discuss how the observed modifications of the two receptors relate to previous information about ET and other GPCR receptors. Subsequently, we consider the general implications of the large number of different ET receptor isoforms that were observed.

Posttranslational Modifications of the C-Terminus. Clustering of cysteine residues in the C-terminal tail is a conserved feature across species and receptor types in many members of the GPCR family, and palmitoylation of these residues is thought to be involved in several important receptor processes. Palmitoylation of cysteines is thought to dock the palmitoylated region to the cell membrane to produce a fourth intracellular loop (*22*). In several cases, evidence has been obtained that palmitoylation of the C-terminus modifies the phosphorylation pattern (*23*, *14*). In addition, there is evidence from site-directed mutagenesis studies that for the ETB receptor and other GPCRs, the degree of C-terminal palmitoylation may act as a selector for coupling with different G-proteins and therefore for different pathways of intracellular signaling (*24*−*26*).

ETB receptors showed the most striking change in receptor palmitoylation. For residues 399−411, unstimulated receptors were highly palmitoylated, and stimulated receptors showed no evidence for palmitoylation (Figure 4). This rapid

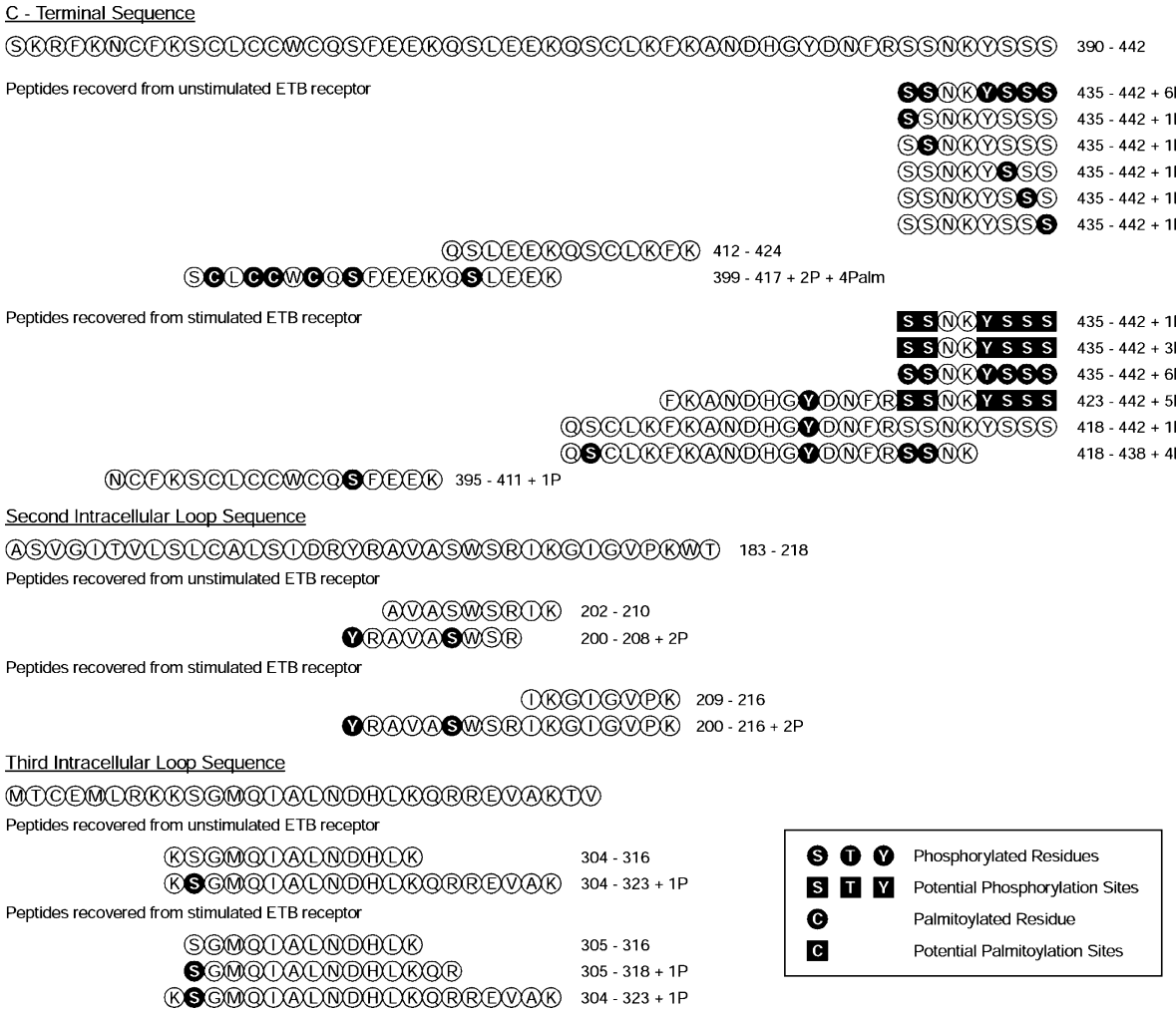

FIGURE 4: Peptides showing the patterns of posttranslational modifications that were observed by MS for the ETB receptor. For some of the peptides, especially those with partial modification of the possible sites, sequencing by ESI-ion trap MS was used to establish the sites of modification.

depalmitoylation may represent an unraveling of a fourth internal loop upon stimulation, exposing a greater region of the tail sequence to interactions with other proteins, including kinases and phosphatases. This fits well with the greater diversity of phosphate modification observed in the C-terminal sequence 435−442 for the stimulated receptor as compared to the unstimulated receptor (Figure 4).

For the ETA receptor, three distinct species were observed for the unstimulated receptor at residues 376−392: (i) a variant with no palmitoylation and full phosphorylation at all three possible phosphorylation sites, (ii) a variant with no phosphorylation and five palmitoyl chains, and (iii) a variant with no posttranslational modifications at all (Figure 3). Given the evidence cited previously that palmitoylation of the C-terminal region can lead to different G-protein interactions for GPCRs, these variants are suggestive of different functional entities for unstimulated ETA receptors. Following stimulation, a variant with full palmitoylation and no phosphorylation as well as a variant with phosphorylation and no palmitoylation continued to be observed (Figure 3). In the stimulated state, a diverse combination of species with intermediate levels of palmitoylation and phosphorylation was also seen. In general, more palmitoylation correlated with less phosphorylation for the stimulated ETA receptor (Figure 3).

In ETB receptors, palmitoylation seemed to be an all or nothing phenomenon, whereas in ETA receptors, numerous intermediate species between the extremes of full palmitoylation or full phsophorylation were observed. Some of these differences between the two types of receptors might reflect a cross-section of a dynamic process that proceeds more rapidly for the ETB receptor than for the ETA receptor. However, the presence of three distinct species for the unstimulated ETA receptor indicates that this cannot be a complete explanation of the observed differences.

Extensive phosphorylation of the C-termini was observed for both ET receptors in both the stimulated and the unstimulated states. Phosphorylation has been shown to be a component of receptor internalization (*27*) and desensitization (*28*−*31*); in particular, it is important in mediating the interaction with arrestins. In addition, there is evidence that the phosphorylation of GPCR intracellular loops is involved in an interaction with other signaling complex proteins (*32*).

In the ETA receptor, changes in the phosphorylation of the C-terminus were seen between the unstimulated and the stimulated receptor (Figure 3). Apart from the region discussed previously that is both palmitoylated and phosphorylated (residues 376−392), there are clearly species that show a decrease in phosphorylation for residues 393−408 in the stimulated receptor as compared to the unstimulated

receptor. In the region 409−427, two distinct species differing in phosphorylation at Thr-417 were detected for the unstimulated receptor. Comparison of the unstimulated and stimulated receptor was not possible for residues 409−427 because too few peptides from this region were recovered for the stimulated receptor.

In the ETB receptor, there is a high concentration of potential phosphorylation sites at the very end of the C-terminus (435−442). It was this region that demonstrated the greatest variability between stimulated and unstimulated receptor (Figure 4). Fully phosphorylated variants and singly phosphorylated variants were present in stimulated and unstimulated receptors, but in the stimulated state, variants with zero, one, two, three, five, and six phosphates were recovered. Phosphorylation of Tyr-431 seems to be a fixture in the stimulated ETB receptor. Unfortunately, the corresponding region of the unstimulated receptor was not recovered. For the unstimulated receptor, Ser-413 was observed in both unmodified and phosphorylated forms, while for the stimulated ETA receptor, a similar phenomenon was observed at Ser-419 (Figure 4).

Posttranslational Modifications of the Second Intracellular Loop. This region was of interest because it contains the conserved DRY motif that has been identified in other GPCRs as being important in the interaction between receptor and G-protein (*33*−*36*). Phosphorylation of this motif has been reported for the bradykinin B2 receptor (*14*). In the unstimulated ETA receptor, Tyr-200 of the corresponding DRY motif was phosphorylated, but no information was obtained for the stimulated ETA receptor. The second intracellular loop of the ETB receptor was recovered in both stimulated and unstimulated states, and in both cases, it was found to be phosphorylated at two of three possible sites. The MS data were not conclusive, but the tryptic fragmentation pattern suggested that Tyr-200 and Ser-205 were the phosphorylation sites. Phosphorylation at Ser-207 could not, however, be definitively excluded. For the unstimulated ETB receptor, a variant lacking phosphorylation at both Ser-205 and Ser-207 was also observed (Figure 4).

Posttranslational Modifications of the Third Intracellular Loop. The third intracellular loops were isolated from both receptors in both states. For ETA, receptor residues 280−307 contain only two possible phosphorylation sites, and peptides with phosphorylation at both Ser-289 and Ser-305 were observed for both the stimulated and the unstimulated states. For the stimulated state, a peptide without phosphorylation at Ser-305 was also observed. For the ETB receptor, residues 298−325 contain only a single possible phosphorylation site. Ser-305 was observed both unmodified and phosphorylated in both the stimulated and the unstimulated states. It has been postulated (*34*) that GPCRs may exist in two unstimulated states, one of which is coupled to the G-protein even though there is no ligand bound. The existence of two forms of the ETB receptor, with Ser-305 of the third intracellular loop both unmodified and phosphorylated, might indicate that one of these states is bound to the G-protein in both stimulated and unstimulated receptors.

The observance of differently modified peptides is good evidence for changes in the posttranslational modifications of the ET receptors upon stimulation. However, it should be kept in mind that the MS methods detect the existence of differently modified peptides but do not give any indication of their relative abundance. For this reason, caution is warranted in interpreting some of the present results in terms of changes between stimulated and unstimulated receptors. For example, the previous results suggest at first sight that the third intracellular loop of the ETB receptor does not change its phosphorylation state during the first 2 min of stimulation. Peptides with and without phosphorylation of Ser-305 are observed both before and after stimulation. This might suggest that phosphorylation/dephosphorylation of the third loop is not important in the receptor/G-protein interaction since data from GPCRs linked to ion channels suggests that coupling occurs in less than a second (*37*). However, in the absence of quantitative data, it is theoretically conceivable that there is an equilibrium between an unstimulated state and a stimulated state (rare in the absence of ligand), which shifts in the presence of a stimulatory ligand. The MS results might then detect trace amounts of the disfavored state both before and after the addition of the stimulatory ligand. It is also conceivable that the receptor/G-protein interaction occurs so quickly that the third loop has returned to its resting state at the 2 min time point.

Diversity of Receptor Forms. It is clear from the present data that for both stimulated and unstimulated states of ETA and ETB receptors, there exist multiple covalent forms differing in the sites of posttranslational modifications. For example, for the unstimulated ETB receptor, six different forms of the C-terminal sequence 435−442 were identified (Figure 5). With the present data it is not possible to specify exactly how many different receptor species exist, but some estimates can be made. For example, for the unstimulated ETB receptor, two different forms of the sequence 412−417 were also observed (Figure 4). This indicates that there are at least six forms of the C-terminal loop of unstimulated ETB receptor (if modifications at residues 395−424 and 435−442 are correlated), but there could be as many as 12 distinct species for the C-terminal loop (no correlation at 395−424 and 435−442) (Figure 5). In the case of the stimulated ETB receptor, the six peptides identified for residues 418−442 (Figure 4) are not sufficient to exactly specify the different C-terminal species. These six peptides could be combined to give a maximum of nine distinct species (Figure 5). On the other hand, a minimum of five C-terminal sequences could give rise to the observed peptides (Figure 5) (i.e., the present evidence indicates five to nine different C-terminal species for the stimulated ETB receptor). Similar estimates of a possible number of different species can also be made for the second and third internal loops of the receptors (Table 1).

Overall, 20 sites of posttranslational modifications have been found for the ETA receptor and 17 sites for the ETB receptor. If modification of these sites was completely uncorrelated, there would be over 1 million (ETA) or over 130 000 (ETB) different covalent species possible (Table 1). The present data are not suggestive of such high numbers of receptor species, indicating that some level of correlation of modifications at distinct sequence locations occurs. The present data clearly indicate that there are a minimum of three to nine distinct species for the different receptors (Table 1). These numbers should be regarded as absolute minima since, in the present experiments, the complexity of the

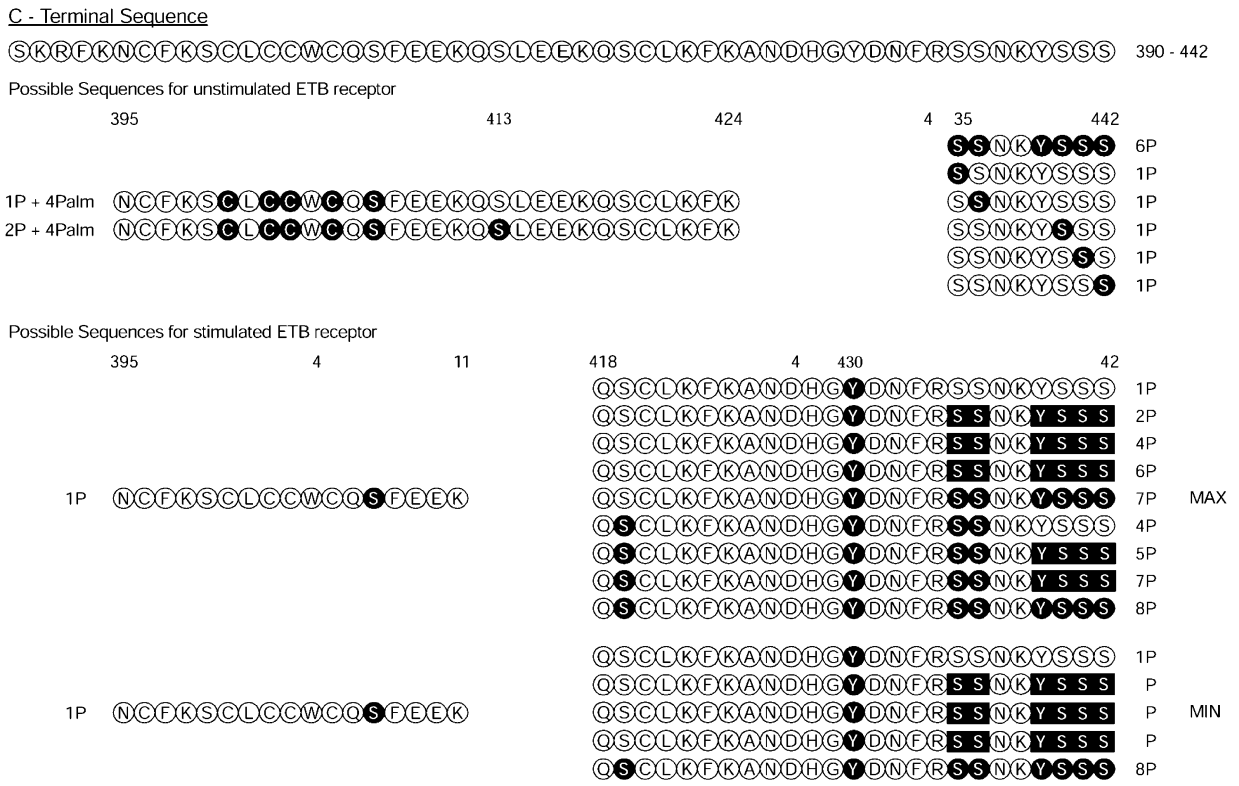

FIGURE 5: Possible sequences for the C-terminal region of ETB receptor based on the observed peptides.

Table 1: Possible Numbers of Distinct Endothelin Receptor Species

	second loop		third loop		C-terminal		overall			
receptor	min[a]	max[b]	min[a]	max[b]	min[a]	max[b]	min[c]	med[d]	max[e]	comb[f]
ETA unstimulated	1	1	1	1	3	12	3	12	12	1.05×10^6
ETA stimulated	1	1	2	4	9	27	9	18	108	1.05×10^6
ETB unstimulated	2	4	2	2	6	12	6	24	96	1.31×10^5
ETB stimulated	1	1	2	2	5	9	5	10	18	1.31×10^5

[a] The minimum number of receptor species that is consistent with the peptides observed in the indicated sequence region. [b] The maximum number of receptor species that is consistent with the peptides observed in the indicated sequence region. Calculated by assuming no coordination (other than that observed in the individual peptides) for posttranslational modifications at different locations within each indicated region of the receptor sequence. [c] The minimum possible number of receptor species assuming total coordination of posttranslational modifications over the entire receptor sequence. [d] A medium number of receptor species calculated by assuming full coordination of posttranslational modifications within the two loops and the C-terminal sequnce but no coordiation between the two loops and the C-terminal sequence. [e] The maximum number of receptor species calculated by assuming that the only coordination between different sites of posttranslational modification is that seen in the observed peptides. [f] The combinatorial number of possible receptor species assuming that each site is modified completely independently.

mixtures of differently modified ETA and ETB receptors has prevented us from obtaining complete sequence coverage of all the different variants. Another reason to regard these numbers as absolute minima is that they would require full correlation in posttranslational modifications over residues 184−425 (ETA) or residues 200−442 (ETB), and the present experiments do not provide any evidence in favor of (or against) this degree of correlation over widely disparate regions of the receptor sequences. If correlation in the modification of different sequence regions is less strict, then there could be substantially larger numbers of different receptor species (Table 1). At present, the estimates in Table 1 are purely illustrative (apart from the minimum numbers), but they serve to show that there could be large numbers of structural isoforms and that determining the exact number and form of the isoforms of the ET receptors will be a very complex task.

What could be the source and significance of such diversity in posttranslational modifications of ET receptors? Clearly, much more work will be necessary to elucidate this, but some possible explanations can be considered already.

First, for the stimulated receptors, it is possible that some of the diversity simply represents a cross-section of a dynamic process and that many intermediate species would disappear at longer times after stimulation. In the present experiments, the widely varying degrees of palmitoylation and phosphorylation observed for residues 378−392 of the stimulated ETA receptor (Figure 3) could be an example of this phenomenon. Further data would be needed to test this. However, given that the unstimulated ETA receptor also shows multiple variants (Figure 3, Table 1), this type of explanation is not sufficient to explain all of the observed diversity.

Second, there is the possibility that some of the different modified forms of the receptors are functionally equivalent. For example, for the unstimulated ETB receptor, the sequence 435−442 was observed in a hyperphosphorylated form with five serines and Tyr-439 phosphorylated. Five

further forms of this region were observed in which Tyr-439 was not phosphorylated and any one of the five seines was phosphorylated (Figure 4). It may be that one functional unit for this region is simply the phosphorylation of any one of the five serines. At first, this appears somewhat counter-intuitive since it is normally assumed that the substrate specificities of kinases and phosphatases lead to the phosphorylation/dephosphorylation of specific residues determined by the protein sequence. However, recent evidence from hyperphosphorylated Net-1, which plays a regulatory role in exit from mitosis in yeast, suggests that mechanisms of this kind may exist. In that case, it was shown that Ser to Ala substitutions of amino acid residues subject to phosphorylation simply led to the phosphorylation of alternative, proximal serine residues in the protein sequence with no major effects on functional activity (*19*). This suggests that in sequence regions that can be heavily modified, it might be the number of modifications rather than the exact residues involved that is the relevant functional signal. This might even allow for incremental modulation of activity/response by the control of the level of modification of such sequence regions.

Third, the present data might be a consequence of the observation of many cells at different stages of the cell cycle. We attempted to minimize this potential problem by using cells that had been simultaneously cultured and by looking at two different time points. While this possibility cannot be completely excluded with the present data, the observation of multiple isoforms in both stimulated and unstimulated ET receptors makes it unlikely that the degree of variety we detected is due simply to the observation of an average over many cell states. At the least, this possibility would suggest that there are strong changes in posttranslational modifications of ET receptors during the cell cycle (which we do not exclude). Since these variants would presumably have functional significance, the putative influence of the cell cycle would then simply provide an explanation for at least some of the diverse forms of the ET receptors that have been observed.

Finally, it is possible that many of the different forms represent functionally different ET receptors. Individual GPCRs are known to stimulate many signaling pathways and to have many actions. In the companion paper (*7*), evidence that ET-1 elicits many diverse functional actions in human lung fibroblasts has been obtained by measurements of rapid changes in the phosphoproteome following stimulation. It is known that endothelin receptors are able to interact with several different G-proteins and that GPCRs in general are able to interact with non-G-proteins as well. It is therefore possible that not every receptor is doing the same thing at the same time. Local conditions in different parts of the cell, for example, local concentrations of scaffolding proteins and components of signaling complexes, could allow the assembly of different complexes that might require different posttranslational modifications for assembly and might produce different changes in posttranslational modifications following stimulation. It is known that the phosphorylation state of the GPCR intracellular loops can affect their interaction with intracellular proteins, and it may be that the different posttranslationally modified forms that have been observed offer specificity for a variety of signaling complexes. This would suggest that the wide variety of actions initiated at the ET receptors (*7*) might be based on the parallel stimulation of different signaling pathways at spatially and functionally distinct ETA and ETB receptor subtypes rather than on a very complex branching of downstream signaling pathways following stimulation of a single functional type of ET receptor.

The wide variety of different posttranslationally modified forms of the ET receptors observed in both stimulated and unstimulated states suggests that this last possibility is worthy of further consideration. The large number of different modified forms of ETA and ETB receptors was a surprise for us, and it will clearly be challenging to unravel the significance of this diversity. Elucidation of the patterns of modifications over the full protein sequence for individual subtypes of posttranslationally modified ETA and ETB receptors would provide one means to further investigate this hypothesis. It seems that the level of complexity involved is probably beyond the analytical capability of indirect methods such as genetic substitutions or in vitro modification experiments. We are therefore currently developing methods for subfractionation of ETA and ETB receptors according to their type/degree of posttranslational modification with a view to performing full sequence analysis by more sophisticated, direct MS analyses involving top-down, in situ fragmentation of whole protein molecules in the mass spectrometer (*38*).

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SUPPORTING INFORMATION AVAILABLE

MS/MS spectra of phosphopeptide 167−192 and tryptic phosphopeptide 409−423. This material is available free of charge via the Internet at http://pubs.acs.org.

NOTE ADDED AFTER ASAP

The Supporting Information paragraph was added to the version published on the Web 11/01/03. The new version was posted 11/13/2003.

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