0026-895X/95/050818-07$3.00/0

MOLECULAR PHARMACOLOGY, 48:818–824 (1995).

Agonist-Stimulated Phosphorylation of the Carboxyl-Terminal Tail of the Secretin Receptor

FATIH OZCELEBI, MARTIN H. HOLTMANN, RONALD U. RENTSCH, RAMMOHAN RAO, and LAURENCE J. MILLER

From the Center for Basic Research in Digestive Diseases, Mayo Clinic and Foundation, Rochester, Minnesota 55905

Received March 30, 1995; Accepted July 31, 1995

SUMMARY

The secretin receptor belongs to a recently recognized family of G protein-coupled receptors that lack the sequence motifs typical of the β-adrenergic receptor family. Because our understanding of the regulatory mechanisms for these receptors is largely based on the latter group, we have begun to explore these mechanisms in the secretin receptor. In the present study, we focused on receptor phosphorylation, a key mechanism of receptor desensitization. Secretin receptor phosphorylation was demonstrated in intact transiently transfected COS cells and a stable receptor-bearing Chinese hamster ovary cell line in response to stimulation with native agonist. Secretin phosphoreceptor migrated on a sodium dodecyl sulfate-polyacrylamide gel at M_r 57,000–62,000 in its native state and at M_r 42,000 after deglycosylation, similar to the receptor that had been affinity-labeled with ^{125}I-[Tyr^{10},*p*-NO$_2$-Phe^{22}]-secretin-27. Phosphorylation occurred rapidly in a secretagogue concentration-dependent manner, with 0.1 μM secretin eliciting a 7.2-fold increase in phosphorylation after 2 min. One-dimensional phosphopeptide mapping after cyanogen bromide cleavage revealed a single band of M_r 9400, corresponding in size to the carboxyl-terminal tail domain. This identification was confirmed with a truncation mutant in which potential sites of phosphorylation in the tail were eliminated and no agonist-stimulated phosphorylation was observed. Phosphoamino acid analysis of the secretin phosphoreceptor demonstrated predominance of phosphothreonine over phosphoserine (3.2:1), with no phosphotyrosine observed. Three distinct carboxyl-terminal truncation mutants were constructed to each eliminate a subset of potential phosphorylation sites, and differential levels of phosphorylation were observed. Appropriate biosynthetic processing, expression on the cell surface, and signaling for each of these constructs were ensured by demonstration of ligand binding and cAMP responsiveness. Thus, receptors in the recently described secretin receptor family are phosphorylated in response to agonist stimulation in a manner analogous to the β-adrenergic receptor, likely representing an important molecular mechanism for receptor desensitization.

Phosphorylation is a key mechanism for the regulation of G protein-coupled receptors, implicated in uncoupling receptor from G protein, binding arrestin-like proteins, and possibly acting as a signal for the sequestration, internalization, and resensitization of these molecules (1). These themes have been extensively studied and are best developed for receptors in the β-adrenergic receptor family, representing the largest group in this superfamily.

With the cloning in 1991 of cDNAs encoding receptors for calcitonin (2) and parathyroid hormone (3), it became clear that these receptors belong to a distinct family of molecules that have some similarities to the existing G protein-coupled receptors yet many differences. Hydropathic analysis suggests the presence of seven transmembrane helices, and there is clear physiological evidence of signaling through association with G proteins (4). Nevertheless, there is <12% sequence identity between this group and other members of this superfamily, and the highly conserved signature sequences for the β-adrenergic receptor family are not present (4).

In 4 years, this family has grown substantially and now includes receptors for secretin, vasoactive intestinal polypeptide, glucagon, glucagon-like peptide-I, and growth hormone-releasing hormone (4). Within this group are clear new sequence motifs, including a large amino-terminal predicted ectodomain incorporating six conserved cysteine residues (4). Although there is physiological evidence for desensitization of members of this receptor family (5–7), it is not clear how the molecular and cellular mechanisms for this will relate to those for the β-adrenergic receptor family. There have been no previous reports of direct demonstration of the phosphorylation of a receptor in this family.

In the present study, we focused on a typical member of the

This work was supported by grants from the National Institutes of Health (DK46577 to L.J.M.) and from Studienstiftung Des Deutschen Volkes (to M.H.H).

ABBREVIATIONS: CHO, Chinese hamster ovary; CHO-SecR, secretin receptor-bearing Chinese hamster ovary cell line; SDS, sodium dodecyl sulfate; endoglycosidase F, endo-β-*N*-acetylglucosaminidase F; KRH, Krebs-Ringers-HEPES; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

secretin hormone receptor family, the receptor for secretin (8). This cDNA was originally cloned from a neural cell line (8), and we subsequently also cloned an identical receptor cDNA from rat pancreas (9). Secretin is a gastrointestinal and neural peptide hormone known to have physiological actions to stimulate bicarbonate secretion from pancreatic and biliary ducts and regulate pancreatic exocrine secretion (10). This receptor, too, is known to undergo physiological desensitization (5).

We established a CHO cell line that stably expresses the rat secretin receptor, and we developed methodology to allow the rapid partial purification of the phosphorylated receptor so that all radioactivity that migrates on an SDS-polyacrylamide gel in the M_r 57,000–62,000 region represents phosphoreceptor. This provided an appropriate system with which to study secretin-stimulated phosphorylation of its receptor in an intact cell. The domain of phosphorylation was defined as the carboxyl-terminal tail by one-dimensional phosphopeptide mapping and receptor mutagenesis. Truncation mutants transiently expressed in COS cells allowed further localization of the sites of phosphorylation. The shortest such truncation mutant was not phosphorylated in response to secretin, despite the maintenance of normal agonist binding and cAMP responses, thus providing an ideal tool with which to dissociate receptor phosphorylation from other regulatory processes.

Materials and Methods

Reagents. Synthetic rat secretin-27 was purchased from Peninsula Laboratories (Belmont, CA). The analogue of this peptide, [Tyr^{10}-*p*-NO_2-Phe^{22}]-secretin-27, was synthesized as we previously reported (9). All other reagents were analytical grade.

Cell lines. CHO cells and COS-7 cells were acquired from American Type Culture Collection. CHO-SecR was established as described previously (9). CHO cells were cultured in Hams F12 medium with 5% Fetal Clone 2 (HyClone Laboratories, Logan, UT) on Falcon plasticware, whereas COS cells were cultured in Dulbecco's modified Eagle's medium with 5% Fetal Clone 2 on the same plasticware. COS cells were transfected using a modified DEAE-dextran protocol (11), with the DNA constructs prepared as will be described. Recombinant receptor-bearing COS cells were harvested 48–72 hr after transfection through washing with phosphate-buffered saline, scraping into a conical tube, and pelleting at 1000 rpm for 2 min. Receptor-bearing CHO-SecR cells were harvested similarly in preparation for the experiments.

Secretin receptor phosphorylation. Control and receptor-bearing COS and CHO-SecR cells were prepared in suspension in phosphate-free KRH medium containing 25 mM HEPES, pH 7.4, 104 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 2.5 mM D-glucose, 0.2% bovine serum albumin, 0.01% soybean trypsin inhibitor, essential amino acids, nonessential amino acids, and glutamine.

The cellular ATP pool was radiolabeled by incubating cells from each 100-cm^2 dish with 2 mCi $H_3{}^{32}PO_4$ in 0.4 mL phosphate-free KRH in 100% oxygen atmosphere at 37° for 60 min. Cells were then stimulated with different agonists as noted. Incubations were terminated by the addition of 5 mL iced inhibitor buffer containing 25 mM HEPES, pH 7.4, 104 mM NaCl, 10 mM NaF, 2 mM EDTA, 2 mM EGTA, 20 mM sodium pyrophosphate, 0.1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/mL leupeptin.

Secretin phosphoreceptor purification. Secretin receptor was purified after allowing it to be phosphorylated in response to agonist stimulation of the intact cell. After stopping phosphorylation with inhibitor buffer, cells were pelleted by centrifugation for 5 min at 2000 × *g*, and the supernatant was discarded. Then, 4-mL inhibitor buffer containing 40% sucrose was added to each tube, and cells were disrupted by sonication for 11 sec at setting 7 with a Sonifier Cell Disrupter (Heat Systems Ultrasonics, Plainview, NY). The homogenates were transferred into 10-mL polycarbonate tubes and overlayered with inhibitor buffer containing 20% sucrose, followed by centrifugation at 225,000 × *g* for 1 hr. The membrane fraction forming at the interface between the 20% and 40% sucrose layers was aspirated, diluted with inhibitor buffer, and pelleted at 225,000 × *g* for 25 min.

Membranes from each tube were solubilized in 1.5 mL inhibitor buffer containing 1% Triton X-100 for 45 min at 4°. The insoluble material was removed by centrifugation at 100,000 × *g* for 25 min, and the supernatant was used as source of receptor for further purification.

The soluble receptor-bearing supernatant was mixed with wheat germ agglutinin-agarose and agitated for 1 hr. The proteins that did not bind to the lectin were washed three times with inhibitor buffer containing 0.1% digitonin. The wheat germ agglutinin-bound glycoproteins were separated by 10% SDS-polyacrylamide gel electrophoresis according to the method of Laemmli (12). Gels were subjected to autoradiography with Kodak XAR-5 film at room temperature for 1–2 hr. This clearly demonstrated the receptor band, which was excised, homogenized with a glass Dounce homogenizer in 1 mL distilled water, and agitated for 45 min. Receptor was eluted from the gel pieces into distilled water, and this was dried under vacuum. Volume was brought to 100 μL with distilled water, and the sample was reduced with 5 mM dithiothreitol for 1 hr at 37° under nitrogen. Receptor was then alkylated with 20 mM iodoacetamide for 2 hr at room temperature in the dark. The sample was subsequently desalted by chromatography on a 0.5 × 8 cm Sephadex G-50 column that was equilibrated with 50 mM ammonium bicarbonate and 0.01% digitonin. The receptor-bearing fraction was collected and dried under vacuum.

Receptor was then separated as an intact glycoprotein on an SDS-polyacrylamide gel, or it was deglycosylated with endoglycosidase F before electrophoresis. This was accomplished by treating with enzyme at 37° overnight. The core protein was also detected by autoradiography and eluted from the gel and desalted as described.

One-dimensional phosphopeptide mapping of the secretin receptor. The core protein of the secretin receptor was eluted from the SDS-polyacrylamide gel, desalted by chromatography, and equilibrated with 50 mM ammonium bicarbonate and 0.01% digitonin. The desalted receptor was reduced with 5 mM dithiothreitol for 1 hr at 37° under nitrogen in 600 μL volume. The sample was then cleaved with cyanogen bromide overnight at room temperature under nitrogen and dried under vacuum.

Products of cyanogen bromide cleavage were separated on a urea SDS-polyacrylamide gel system adapted from the method of Swank and Munkres (13). In this nondiscontinuous gel system, a Laemmli-like stacking gel was added, and the separating gel incorporated 8 M urea, 0.1% SDS, and 12.5% acrylamide (incorporating 10% bisacrylamide).

Phosphoamino acid analysis. Phosphoamino acid analysis of the core protein of the secretin receptor was performed using thin layer chromatography according to the method of Nairn and Greengard (14). Quantification was performed by densitometric analysis of autoradiographs using National Institutes of Health image software.

Secretin receptor mutagenesis. The wild-type secretin receptor cDNA that we previously cloned from a rat pancreatic library (9) was also used for transient expression studies and as a template for mutagenesis. Truncation mutagenesis was performed using polymerase chain reaction, introducing novel restriction sites for ease of subcloning (15). The final products were ligated into the *Bam*H1 and *Hind*III sites of pBK-CMV (Stratagene, La Jolla, CA). These truncation constructs corresponded to secretin receptor residues 1–398, 1–413, and 1–417. The correct sequence of these constructs was confirmed by DNA sequencing according to the dideoxynucleotide chain termination method of Sanger *et al.* (16).

Biological activity studies. Intracellular cAMP levels were assayed with an [^{3}H]cAMP assay kit provided by Diagnostic Products Corporation (Los Angeles, CA). This was performed with cells in suspension that were lifted as described. For the assay, cells were suspended in KRH buffer with 0.2% bovine serum albumin, 0.01% soybean trypsin inhibitor, and 1 mM 3-isobutyl-1-methyl-xanthine. The assay was performed as recommended by the manufacturer. For this, cells were stimulated at 37° for 10 min. This reaction was stopped by adding perchloric acid and putting the cells on ice. After adjusting the pH to 6 with $KHCO_3$, cell lysates were centrifuged at 4° for 10 min at 2000 rpm, and the supernatants were used for the cAMP radioimmunoassay. All assays were performed in duplicate and repeated at least three times in independent experiments.

Radioligand binding and affinity labeling studies. The secretin analogue that we previously established and validated (9), [Tyr^{10},*p*-NO_2-Phe^{22}]-secretin-27, was radioiodinated and purified to 2000 Ci/mmol as we previously described (9). In standard receptor binding assays, 1–10 μg of receptor-bearing cell membranes was incubated with 3–5 pM radioligand in KRH medium. Steady state binding was achieved after 60 min at room temperature. At that point, bound and free radioligands were separated by centrifugation and washing. Nonspecific binding was determined in the presence of 1 μM secretin and represented <22% of total binding.

For affinity labeling, similar methodology was used, except for greater amounts of radioligand (75–100 pM) and membrane (50–100 μg). Washed membranes were photolyzed in 12 × 75-mm borosilicate tubes 5.7 cm from the 300-nm lamps of a Rayonet model RP-100 apparatus (Southern New England Ultraviolet, Hamden, CT) for 30 min at 4°. These conditions were fully validated in this laboratory (9). The affinity labeled receptor was then solubilized with SDS sample buffer, separated by electrophoresis on a Laemmli SDS-polyacrylamide gel, and visualized by autoradiography.

Statistical analysis. Values are given as mean ± standard error. When appropriate, differences were assessed using the Mann-Whitney nonparametric test of unpaired values, with $p < 0.05$ considered to be statistically significant.

Results

Stimulation of secretin receptor on receptor-bearing CHO-SecR cells or transiently transfected COS cells with native hormone resulted in a clear increase in the phosphorylation of this receptor. This can be seen in Fig. 1 in a representative autoradiograph of an SDS-polyacrylamide gel used to separate the partially purified products of phosphorylation experiments. Shown are lanes representing products of purification of the receptor-bearing cells that were not exposed to hormone (basal state) and those that were exposed to 1 μM secretin. Phosphosecretin receptor migrating at the M_r 57,000–62,000 range can be seen in the lanes representing the receptor-bearing COS and CHO-SecR cells that were stimulated with hormone. Secretin stimulated a 7.2 ± 1.4-fold increase in the phosphorylation of this protein compared with its basal state of phosphorylation. Neither cell line, when untransfected, had any saturable binding of secretin radioligand or expressed any receptor phosphorylation response to secretin when stimulated under identical conditions.

To be certain that the M_r 57,000–62,000 band represented the secretin receptor, this phosphoprotein was deglycosylated with endoglycosidase F to yield a band migrating at M_r 42,000, as expected from our previous work (9) (Fig. 1). The migration of intact and deglycosylated phosphoreceptors were the same as that we reported for the specific affinity labeled receptor (9).

The time course and concentration dependency of agonist-stimulated receptor phosphorylation can be seen in Fig. 2. Phosphorylation in response to secretin occurred rapidly, reaching a peak by 2 min. This was followed by a plateau existing for at least 30 min. Phosphorylation of the secretin receptor above its basal state could be observed with secretin concentrations as low as 10 pM peptide. The intensity of the phosphorylation of this protein was increased in a concentration-dependent manner up to 0.1 μM secretin.

We used one-dimensional phosphopeptide mapping after cyanogen bromide cleavage to gain insight into the domain of the secretin receptor that was phosphorylated in response to agonist stimulation. The theoretical sites of such cleavage are shown in Fig. 3. Fragments with phosphorylated residues exposed to the cytosolic milieu range M_r 2800 to M_r 10,900. When the secretin phosphoreceptor was cleaved with cyanogen bromide and separated on a urea SDS-polyacrylamide gel, only a single band was observed, migrating in the range of M_r 9400 (Fig. 3). This corresponds in size to the carboxyl-terminal tail of the receptor. That domain contains eight serine residues, four threonine residues, and one tyrosine residue, as potential targets, although one of these serine

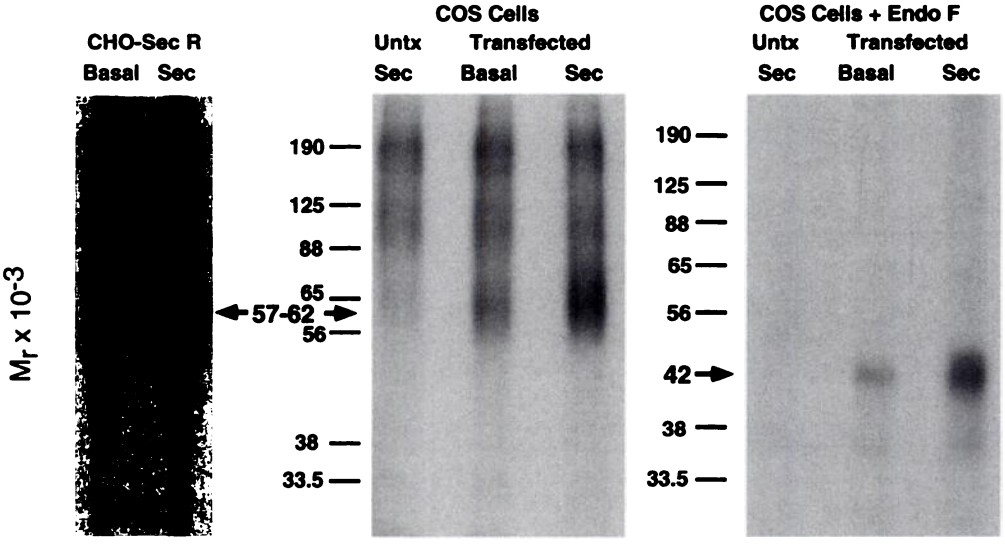

Fig. 1. Secretin receptor phosphorylation. Shown are representative autoradiographs of SDS-polyacrylamide gels used to separate partially purified products of phosphorylation experiments performed in COS cells and CHO-SecR cells. Shown are lanes representing untransfected (*Untx*) and transfected cells in the absence (*Basal*) or presence of stimulation with 1 μM secretin (*Sec*). Left and middle, the M_r 57,000–62,000 band that migrates at the position of the affinity labeled receptor is phosphorylated in an agonist-dependent manner. Right, representative of four independent deglycosylation experiments, demonstrating the expected migration of the core receptor protein at M_r 42,000.

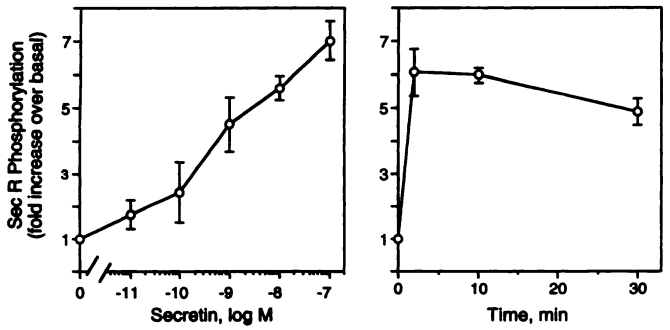

Fig. 2. Concentration dependency and time course of secretin receptor phosphorylation. Receptor phosphorylation occurred rapidly and in a concentration-dependent manner after agonist stimulation of intact cells. Values are given as mean ± standard error from three independent experiments for each.

residues and the tyrosine residue are predicted to reside in the transmembrane domain.

Phosphoamino acid analysis of the secretin phosphoreceptor is also illustrated in Fig. 3, demonstrating that both phosphothreonine and phosphoserine were present, with an average ratio of 3.2:1, respectively. No phosphotyrosine was ever observed.

To test this tentative localization of sites of secretin receptor phosphorylation, mutant receptors with their carboxyl-terminal tail truncated were prepared. These included truncation of the receptor at residue 398, which was able to fully eliminate all cytosolic serines and threonines from the tail of the receptor (which would have been included in the cyanogen bromide fragment), as well as two shorter truncations that partially eliminated potential sites of phosphorylation.

Interpretation of data with these constructs requires a knowledge that they were synthesized appropriately and transported to the cell surface and that they were capable of binding secretin and transmitting appropriate intracellular signals. We were able to be certain of the processing, transport, and signaling of all truncated receptor constructs, based on radioligand binding and cAMP responses (Fig. 4 and Table 1). Appropriate high affinity binding and signaling were intact in all of the truncation mutants. There were no significant differences in any of the binding parameters of COS cells transfected with wild-type or truncated receptor constructs. All demonstrated fully efficacious and concentration-dependent cAMP responses to secretin that were similar to those of wild-type receptor. With such signaling intact, we can assume that appropriate kinases were stimulated in the cells under these conditions as well. Furthermore, shown in Fig. 5 is an affinity labeling experiment with the construct containing the longest truncation deletion in which migration on an SDS-polyacrylamide gel demonstrated the appropriate shift in apparent size of the processed receptor.

Fig. 6 illustrates the secretin-stimulated phosphorylation of the truncation mutants and wild-type secretin receptor expressed in COS cells. Each lane was loaded to contain an equal amount of receptor based on direct binding analysis. Although wild-type receptor was phosphorylated in the expected manner, the mutant with the longest truncation (1–398) was not significantly phosphorylated above its basal level (2 ± 2% of wild-type receptor phosphorylation) (p = 0.4). The two constructs with shorter truncations were phosphorylated substantially less than the wild-type receptor (p = 0.01 for 1–413 and 0.003 for 1–417), suggesting that at least one site of phosphorylation was Ser^{418}, Ser^{420}, or Ser^{425}, distal to these truncations. There was no quantitative difference in the phosphorylation of 1–413 (57 ± 12% of wild-type receptor phosphorylation) and 1–417 (50 ± 10% of wild-type receptor phosphorylation) (p = 0.7), suggesting that residues Ser^{414} and Thr^{415} were not major sites of phosphorylation, whereas Ser^{399}, Ser^{401}, and Ser^{410} and Thr^{404}, Thr^{408}, and Thr^{411} were also candidates for phosphorylation. Phosphoamino acid analysis supports the phosphorylation of at least one of these threonine residues as well (Fig. 3).

Fig. 7 provides further confirmation of the phosphorylation data. The constructs were cleaved with cyanogen bromide before separation on the SDS-polyacrylamide gel. The truncations are clearly apparent as shifts in apparent size.

Discussion

The present study demonstrates the phosphorylation of the secretin receptor that occurs in an intact receptor-bearing cell in response to agonist occupation. This represents the first direct demonstration of phosphorylation of a receptor in the recently recognized secretin receptor family (4). Although functional desensitization has been described for the secretin receptor (5) and for other members of this family (6, 7), it was unclear whether this group of G protein-coupled receptors would use the same molecular mechanisms for desensitization as the extensively studied β-adrenergic receptor family (1).

The agonist-stimulated phosphorylation of the secretin receptor demonstrated in the present study suggests that at least some regulatory themes will be shared with the β-adrenergic receptor family (1). The localization of the sites of phosphorylation of this receptor to the carboxyl-terminal tail will provide important information to facilitate the examination of the possible roles of this domain in signaling, in binding to arrestin-like proteins, and in directing receptor sequestration, internalization, and resensitization.

For the present study, we followed the same strategy we previously reported for the cholecystokinin receptor (17). We chose to work with an intact, receptor-bearing cell. This ensures appropriate conformation and coupling of the receptor, such that only certain domains will be accessible to kinases. Primary sequence analysis can identify consensus sites for common signaling kinases, but the accessibility of those sites needs to be confirmed experimentally. Furthermore, a number of sites that are substrates of these kinases do not follow the most common consensus sequences (18). Direct analysis has the potential to identify all of the sites actually used by the cell for receptor regulation. In contrast, *in vitro* phosphorylation strategies have the potential of phosphorylating more sites than are used in the cell.

This approach is very powerful if an adequate purification scheme can be established. This should ideally be rapid and attempt to minimize artifacts introduced by phosphatase action and proteolysis; this was accomplished for the secretin receptor in the present study.

The use of this scheme for partial purification of the receptor yielded a strong phosphorylation signal within the M_r 57,000–62,000 region of the SDS-polyacrylamide gel when receptor-bearing CHO-SecR cells or transiently transfected receptor-bearing COS cells were stimulated with secretin. A

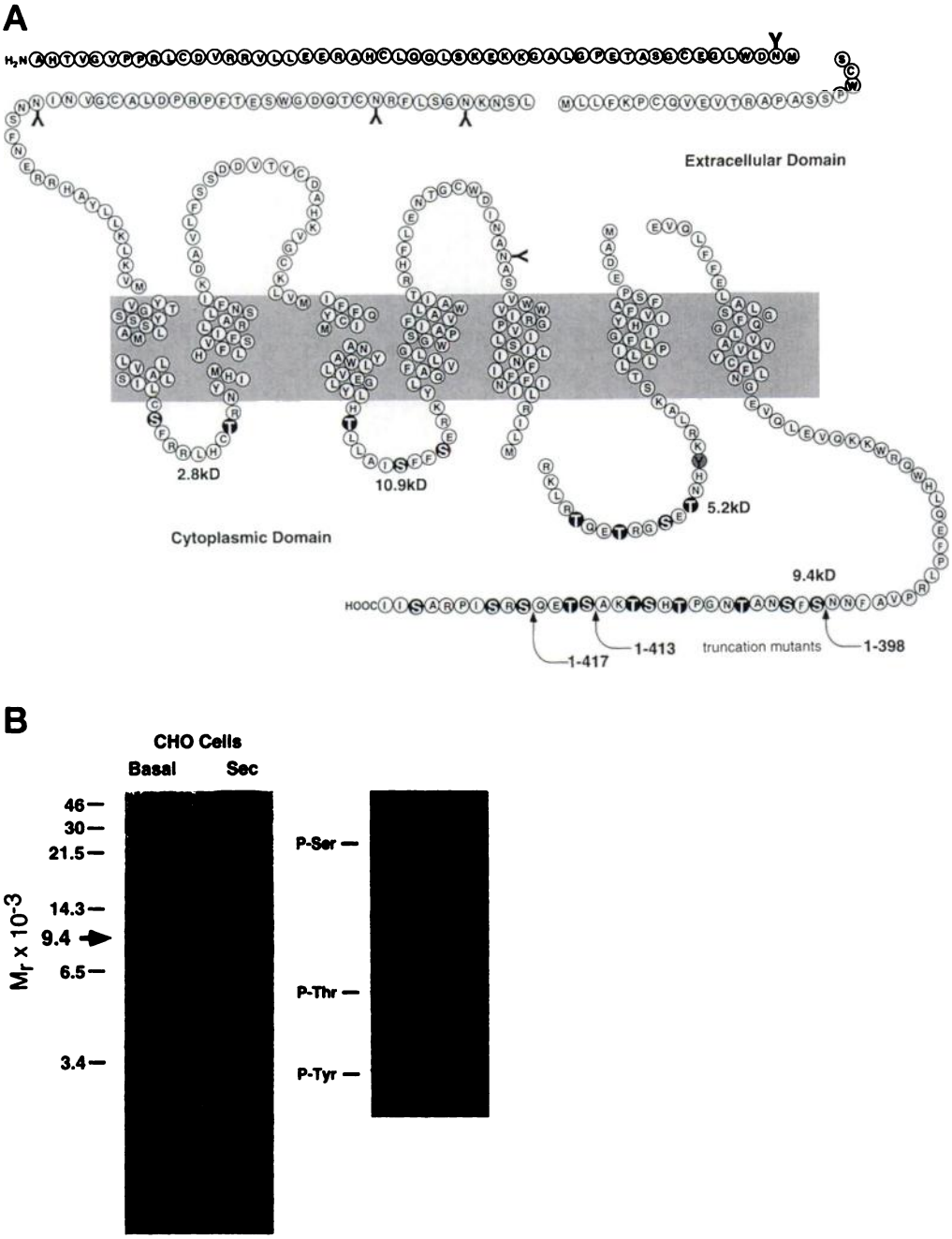

Fig. 3. A, Amino acid sequence predicted for the rat secretin receptor, with theoretical sites of cyanogen bromide cleavage illustrated, and one-dimensional phosphopeptide map and phosphoamino acid analysis of secretin phosphoreceptor. *Gray bar*, predicted transmembrane segments. *Below bar*, cytoplasmic components. *Black*, serine and threonine residues within this domain. *Dark gray*, tyrosine residues in the domain. B, Autoradiograph of an SDS-polyacrylamide gel used to separate cyanogen bromide fragments of the secretin phosphoreceptor, which is typical of four independent experiments. A single phosphopeptide band that was stimulated by secretin (*Sec*) was observed to migrate at M_r 9400. Phosphoamino acid analysis of this revealed that phosphothreonine (*P-Thr*) was most prominent, with approximately one third as much phosphoserine (*P-Ser*) present and no phosphotyrosine (*P-Tyr*) observed. Also indicated are the positions of receptor truncations that were constructed.

much smaller signal was apparent in this region of the gel when unstimulated receptor-bearing cells were analyzed, and no phosphorylation signal was apparent when non-receptor-bearing native CHO cells or COS cells were stimulated with secretin. The radioactivity in the M_r 57,000–62,000 region moved to the M_r 42,000 region after deglycosylation with endoglycosidase F, as expected from our earlier affinity labeling studies with a specific photolabile probe (9).

The apparent size of the single phosphorylated cyanogen bromide fragment of the receptor was strongly suggestive of its representing the carboxyl terminus. Other potential sites of phosphorylation that have access to cytosolic kinases were of substantially different sizes. The direct demonstration that only phosphoserine and phosphothreonine were present focused interest on the region of the tail between residues 398 and 427. This insight also helps focus future studies on specific kinases and groups of kinases. No tyrosine phosphorylation was observed.

Truncation mutagenesis confirmed and further defined the receptor domain that was phosphorylated. The receptor construct with the longest truncation representing residues 399–427, to eliminate all candidate sites of phosphorylation, was not phosphorylated in response to secretin under conditions in which wild-type receptor was clearly phosphorylated. Two constructs with successively shorter truncations that were each designed to eliminate one third of the candidate sites were phosphorylated but yielded signals less intense than that of the wild-type receptor. Comparison of the intensity of these signals suggests that Ser^{399}, Ser^{401}, Ser^{410},

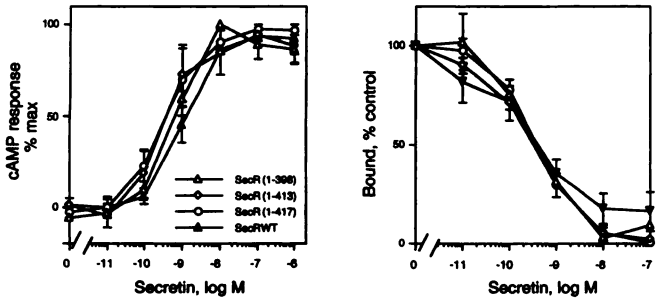

Fig. 4. Signaling and binding characteristics of truncated receptor mutants. Secretin stimulated a concentration-dependent increase in cAMP in COS cells transfected with wild-type (*SecRWT*) and truncated secretin receptor (*SecR*) constructs. These receptor constructs bound secretin with similar characteristics. Values are given as mean ± standard error of three independent experiments performed in duplicate.

TABLE 1
Characterization of secretin receptor constructs

	Binding		Biological activity (cAMP)	
	K_i	B_{max}	EC_{50}	Increase
	nM	*pmol/mg*	*nM*	*multiple of basal*
Wild-type	3.3 ± 0.4	3.1 ± 1.4	1.0 ± 0.4	2.4 ± 0.2
1–417	3.7 ± 0.9	1.9 ± 0.7	0.8 ± 0.6	2.5 ± 0.2
1–413	3.0 ± 1.1	1.6 ± 0.5	0.6 ± 0.3	1.7 ± 0.1
1–398	2.5 ± 0.2	1.5 ± 0.3	0.7 ± 0.1	3.0 ± 0.3

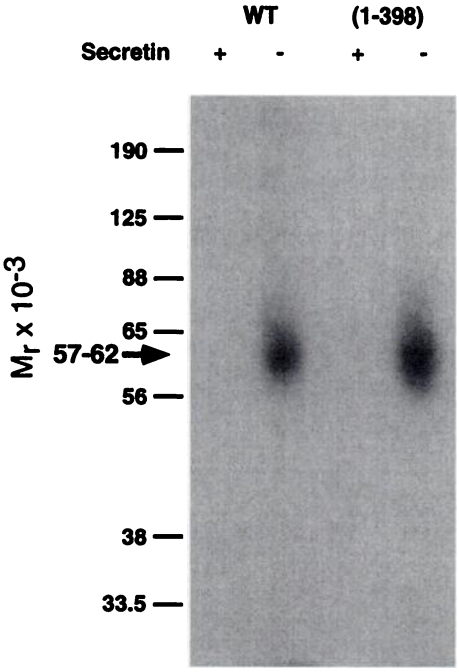

Fig. 5. Expression of truncated receptor construct on cell surface. Affinity labeling of wild-type (*WT*) secretin receptor and truncated secretin receptor (*1–398*) in the absence or presence of competing cold secretin, with products separated on SDS-polyacrylamide gel. Shown is an autoradiogram of such a gel, demonstrating the specific labeling of these receptors and the expected shift in the apparent size of the truncated construct.

Ser^{418}, Ser^{420}, and Ser^{425} and Thr^{404}, Thr^{408}, and Thr^{411} are strong candidates. Determination of the specific sites of phosphorylation must wait for site-directed mutagenesis of these candidate residues.

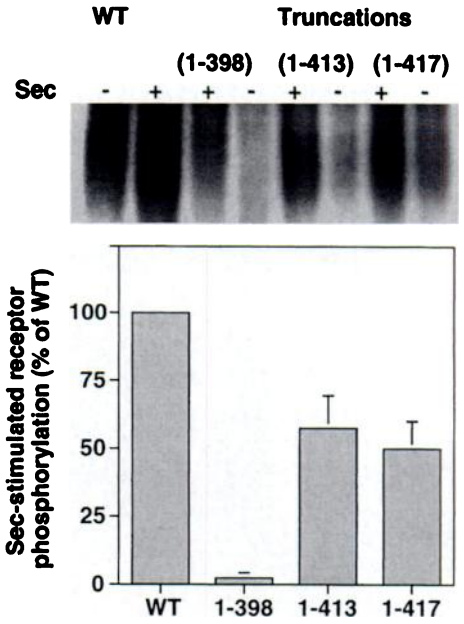

Fig. 6. Secretin (*Sec*)-stimulated phosphorylation of truncated receptor mutants. Shown is an autoradiograph of an SDS-polyacrylamide gel used to separate secretin phosphoreceptor constructs, which is typical of five similar experiments. For each construct, basal and 1 μM secretin-stimulated patterns of phosphorylation are shown. Each lane represents an equal receptor number, based on competition binding on an aliquot of transfected cells. Significant agonist-stimulated phosphorylation was observed with the 1–413 and 1–417 constructs, whereas no consistent phosphorylation above basal was observed with the 1–398 construct. Data are quantified in a bar graph (mean ± standard error). *WT*, wild-type.

Of interest, sequence analysis points to three consensus sites for phosphorylation by protein kinase C, with one each in the first intracellular loop, second intracellular loop, and carboxyl-terminal tail. There is a tyrosine kinase phosphorylation site in the third intracellular loop. All other clear consensus sites are predicted to be located extracellularly. Thus, only one of these strong consensus sites is likely used by the cell, and most sites phosphorylated are not identified by sequence analysis.

Critical controls for the present study included the demonstration that the truncated constructs were synthesized, processed, and delivered to the plasma membrane, as well as functioning there to bind and transmit an appropriate intracellular signaling cascade. This was accomplished by direct affinity labeling of receptor on the plasmalemmal surface of the cells and by performing competition binding and assaying cAMP responses to secretin. These results supported the maintenance of biosynthesis, transport to the cell surface, ligand binding, and signaling by the mutagenized receptors.

This provides evidence that signaling kinases were likely activated in the cells. Furthermore, it provides the interesting insights that G protein coupling (likely G_s) critical for the cAMP response was not disturbed by truncation of the receptor tail to eliminate the sites of phosphorylation. It will be interesting to determine if more severe truncation of the carboxyl-terminal tail will affect G protein association, as this domain is important in several other G protein-coupled receptors (19).

The present study provides the first direct evidence for phosphorylation of a receptor in the secretin receptor family

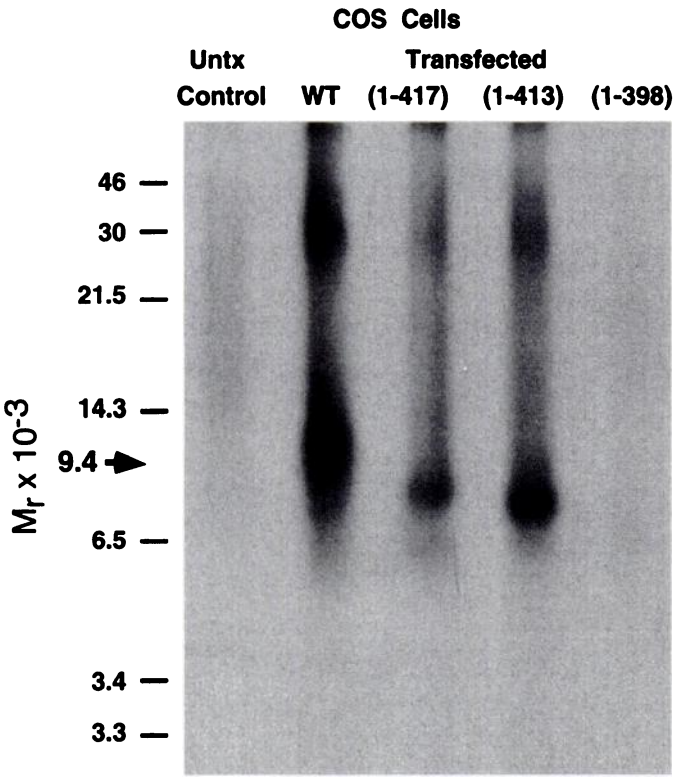

Fig. 7. Cyanogen bromide fragments of secretin phosphoreceptor constructs. Truncations are clearly evident in altered migration of the 1–417 and 1–413 constructs relative to the wild-type (*WT*) receptor. No phosphorylation was observed with the 1–398 construct. *Untx*, untransfected.

and identifies the domain of agonist-stimulated phosphorylation of a serine- and threonine-rich area in the carboxyl-terminal tail. Demonstration that the elimination of this area by truncation does not interfere with cAMP signaling provides useful initial insights into the structural organization involved in signaling and regulation of this receptor.

Acknowledgments

We would like to acknowledge the excellent technical assistance of Eileen Holicky, Elizabeth Hadac, and Delia Pinon and the help of Sara Erickson in preparation of the manuscript.

References

1. Hausdorff, W. P., M. G. Caron, and R. J. Lefkowitz. Turning off the signal: desensitization of beta-adrenergic receptor function. *FASEB J.* **4**:2881–2889 (1990).
2. Lin, H. Y., T. L. Harris, M. S. Flannery, A. Aruffo, E. H. Kaji, A. Gorn, L. F. Kolakowski, H. F. Lodish, and S. R. Goldring. Expression cloning of an adenylate cyclase-coupled calcitonin receptor. *Science (Washington D. C.)* **254**:1022–1026 (1991).
3. Juppner, H., A. Abou-Samra, M. Freeman, X. F. Kong, E. Schipani, J. Richards, L. F. Kolakowski, J. Hock, J. T. Potts, H. M. Kronenberg, and G. V. Segre. A G protein-linked receptor for parathyroid hormone and parathyroid hormone-related peptide. *Science (Washington D. C.)* **254**:1024–1026 (1991).
4. Segre, G. V., and S. R. Goldring. Receptors for secretin, calcitonin, parathyroid hormone (PTH)/PTH-related peptide, vasoactive intestinal peptide, glucagonlike peptide 1, growth hormone-releasing hormone, and glucagon belong to a newly discovered G-protein-linked receptor family. *Trends Endocrinol. Metab.* **4**:309–314 (1993).
5. Katsushima, S., H. Adachi, T. Honda, S. Sato, T. Kusui, S. Onishi, E. Aoki, M. Noguchi, and J. Konishi. Cholecystokinin downregulates receptors for vasoactive intestinal peptide and secretin in rat pancreatic acini. *Am. J. Physiol.* **258**:G395-G403 (1990).
6. Schneider, H.-G., F. Raue, A. Koppold, W. Ruf, and R. Ziegler. Homologous desensitization of calcitonin receptors and calcitonin-dependent adenylate cyclase in T47D cells. *Acta Endocrinol.* **128**:373–378 (1993).
7. Fukayama, S., E. Schipani, H. Jüppner, B. Lanske, H. M. Kronenberg, A.-B. Abou-Samra, and F. R. Bringhurst. Role of protein kinase-A in homologous down-regulation of parathyroid hormone (PTH)/PTH-related peptide receptor messenger ribonucleic acid in human osteoblast-like SaOS-2 cells. *Endocrinology* **134**:1851–1858 (1994).
8. Ishihara, T., S. Nakamura, Y. Kaziro, T. Takahashi, K. Takahashi, and S. Nagata. Molecular cloning and expression of a cDNA encoding the secretin receptor. *EMBO J.* **10**:1635–1641 (1991).
9. Ulrich, C. D., II, D. I. Pinon, E. M. Hadac, E. L. Holicky, A. Chang-Miller, L. K. Gates, and L. J. Miller. Intrinsic photoaffinity labeling of native and recombinant rat pancreatic secretin receptors. *Gastroenterology* **105**:1534–1543 (1993).
10. Mutt, V. Secretin and cholecystokinin. *Adv. Metab. Disord.* **11**:251–320 (1988).
11. Lopata, M. A., D. W. Cleveland, and B. Sollner-Webb. High level transient expression of a chloramphenicol acetyl transferase gene by DEAE-dextran mediated DNA transfection coupled with a dimethyl sulfoxide or glycerol shock treatment. *Nucleic Acids Res.* **12**:5707–5717 (1984).
12. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* **227**:680–685 (1970).
13. Swank, R. T., and K. D. Munkres. Molecular weight analysis of oligopeptides by electrophoresis in polyacrylamide gel with sodium dodecyl sulfate. *Anal. Biochem.* **39**:462–477 (1971).
14. Nairn, A. C., and P. Greengard. Purification and characterization of calcium/calmodulin-dependent protein kinase I from bovine brain. *J. Biol. Chem.* **262**:7273–7281 (1987).
15. Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease. Site-directed mutagenesis by overlap extension using polymerase chain reaction. *Gene* **77**:51–59 (1989).
16. Sanger, F., S. Nicklen, and A. R. Coulson. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467 (1977).
17. Klueppelberg, U. G., L. K. Gates, F. S. Gorelick, and L. J. Miller. Agonist-regulated phosphorylation of the pancreatic cholecystokinin receptor. *J. Biol. Chem.* **266**:2403–2408 (1991).
18. Rich, D. P., H. A. Berger, S. H. Cheng, S. M. Travis, M. Saxena, A. E. Smith, and M. J. Welsh. Regulation of the cystic fibrosis transmembrane conductance regulator Cl^- channel by negative charge in the R domain. *J. Biol. Chem.* **268**:20259–20267 (1993).
19. Inglese, J., N. J. Freedman, W. J. Koch, and R. J. Lefkowitz. Structure and mechanism of the G protein-coupled receptor kinases. *J. Biol. Chem.* **268**:23735–23738 (1993).

Send reprint requests to: Laurence J. Miller, M.D., Center for Basic Research in Digestive Diseases, Guggenheim 17, Mayo Clinic, Rochester, MN 55905.