

# Phosphorylation of Cholecystokinin Receptors Expressed on Chinese Hamster Ovary Cells

SIMILARITIES AND DIFFERENCES RELATIVE TO NATIVE PANCREATIC ACINAR CELL RECEPTORS\*

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Fatih Ozcelebi, Rammohan V. Rao, Eileen Holicky, Benjamin J. Madden,  
Daniel J. McCormick, and Laurence J. Miller‡

From the Center for Basic Research in Digestive Diseases and the Department of Biochemistry and Molecular Biology,  
Mayo Clinic and Foundation, Rochester, Minnesota 55905

**Phosphorylation of G protein-coupled receptors is an established mechanism for desensitization in response to agonist stimulation. We previously reported phosphorylation of the pancreatic acinar cell cholecystokinin (CCK) receptor and the establishment of two-dimensional phosphopeptide mapping of its sites of phosphorylation (Ozcelebi, F., and Miller, L. J. (1995) *J. Biol. Chem.* 270, 3435–3441). Here, we use similar techniques to map sites of phosphorylation of the same receptor expressed on a stable receptor-bearing Chinese hamster ovary (CHO)-CCKR cell line. Like the native cell, the CHO-CCKR cell receptor was phosphorylated in response to agonist stimulation in a concentration-dependent manner; however, the time course was quite different. CHO-CCKR cell receptor phosphorylation increased progressively to a plateau after 15 min, while in the acinar cell it peaks within 2 min and returns to baseline over this interval. There were distinct qualitative and quantitative differences in the sites of phosphorylation of the two receptor systems. One site previously attributed to action of a staurosporine-insensitive kinase in the acinar cell was absent in the CHO-CCKR cell. Site-directed mutagenesis was utilized to eliminate predicted sites of protein kinase C action, but only two of four such sites affected the phosphopeptide map of this receptor. Chemical and radiochemical sequencing were performed on these and other phosphopeptides which were present in both the CHO-CCKR cells and agonist-stimulated pancreatic acinar cells to provide direct evidence for the phosphorylation sites actually utilized. Thus, these data support the usefulness and limitations of a model cell system in studying receptor phosphorylation and desensitization.**

Receptor phosphorylation in response to agonist stimulation is a well established mechanism for desensitization, an important and ubiquitous process to protect the cell from overstimulation. This covalent modification of the receptor has been implicated in uncoupling the receptor-G protein interaction, mediating binding of arrestin-like proteins, and even signaling receptor sequestration, internalization, and resensitization (2, 3).

The molecular details of these events, however, are often implied based on indirect data, due to difficulties in the direct identification of phosphorylation sites in sparse membrane proteins. This is often based on the presence of consensus sites in primary sequence analysis, and on the change in receptor behavior observed after truncations, deletions, or substitutions, often of multiple residues. Nonspecific and indirect effects can clearly result from such approaches.

We recently reported the ability to generate a detailed two-dimensional phosphopeptide map for radiochemically pure cholecystokinin (CCK)<sup>1</sup> receptor present as an extremely sparse plasmalemmal protein in the pancreatic acinar cell (1). Because of its sparsity and physicochemical properties, this receptor has been extremely difficult to purify to scale suitable for direct sequencing. The only successful report of such an effort utilized pancreata from 250 rats as source of this receptor (4).

With the cloning of the cDNA encoding this receptor (4), it has been possible to establish tissue culture cell lines expressing large numbers of receptors. We have established a CHO cell line which expresses approximately 25 times the normal receptor density (CHO-CCKR line expressing 125,000 receptors per cell) (5). This type of cell line has been extensively utilized to determine the impact of mutagenesis on the function and regulation of other receptors. It is unclear, however, how closely such a cell line parallels the native cell in these activities.

In this work, we have demonstrated that the CCK receptor expressed on the CHO-CCKR cell line is phosphorylated in response to CCK stimulation. Like the native cell receptor, the predominant domain for phosphorylation was the third intracellular loop; however, the time course of phosphorylation and dephosphorylation of the recombinant receptor was quite distinct, suggesting differences in the equilibrium between relevant kinases and protein phosphatases. Application of two-dimensional phosphopeptide mapping demonstrated both qualitative and quantitative differences in sites of phosphorylation in these two receptor-bearing cells. After mutagenesis of consensus sites was insufficient to fully explain the identity of the sites of phosphorylation, we increased the scale of the phosphopeptide mapping and obtained direct sequence evidence for several sites of receptor phosphorylation utilized by both of these cells.

## MATERIALS AND METHODS

**Reagents**—Synthetic CCK-8 was purchased from Peninsula Laboratories (Belmont, CA). The CCK analogue-agarose affinity resin was synthesized as described (6). Subtilisin was purchased from Boehringer Mannheim (Indianapolis, IN). Other reagents were analytical grade.

<sup>1</sup> The abbreviations used are: CCK, cholecystokinin; CHO, Chinese hamster ovary; HPLC, high performance liquid chromatography; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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‡ To whom all correspondence and reprint requests should be addressed: Center for Basic Research in Digestive Diseases, Guggenheim 17, Mayo Clinic, Rochester, MN 55905. Tel.: 507-284-0680; Fax: 507-284-0762.

**CCK Receptor Constructs and Expression Systems**—The rat CCK-A receptor-bearing CHO-CCKR cell line was established as we described (5). This has been previously characterized to demonstrate that it expresses approximately 125,000 receptors per cell and that agonist occupation results in a full biological response (5). This line was maintained in suspension culture in spinner flasks in Ham's F-12 medium containing 5% Fetal Clone 2 supplement (Hyclone Laboratories, Logan, UT) in a 37 °C humidified incubator containing 5% CO<sub>2</sub>.

The rat type A CCK receptor cDNA we previously cloned (5) was subcloned into the pBK-CMV expression vector (Stratagene, La Jolla, CA), and was mutagenized by the method of Sayers *et al.* (7). Correct sequences of all constructs were confirmed by DNA sequencing using the dideoxynucleotide chain termination method (8). COS-7 and CHO-K1 cells were acquired from ATCC (Rockville, MD), and were cultured in Dulbecco's modified Eagle's medium with 5% Fetal Clone 2 supplement (Hyclone Laboratories). They were transfected with 2–4 µg of DNA using DEAE-dextran or Lipofectin (9). Transient transfectants were harvested for study 48 to 72 h after transfection. Stable receptor-bearing cell lines were established as we described (5).

**CCK Receptor Phosphorylation**—CHO-CCKR cells were harvested from spinner flasks and washed with phosphate-free Krebs-Ringer-Hepes medium containing 25 mM Hepes, pH 7.4, 104 mM sodium chloride, 5 mM potassium chloride, 1.2 mM magnesium sulfate, 2 mM calcium chloride, 2.5 mM D-glucose, essential amino acids, non-essential amino acids, and glutamine. The cellular ATP pool was radiolabeled by adding 10 mCi of H<sub>3</sub><sup>32</sup>PO<sub>4</sub> to cells suspended in 20 ml of phosphate-free Krebs-Ringer-Hepes medium, and incubating them for 30 min at 37 °C in a well oxygenated atmosphere. Aliquots of cells were then incubated with agonists using conditions previously established to provide optimal receptor phosphorylation. As we have described previously (10), incubations were terminated by the addition of inhibitor buffer containing 25 mM Hepes, pH 7.4, 104 mM sodium chloride, 10 mM sodium fluoride, 2 mM EDTA, 2 mM EGTA, 20 mM sodium pyrophosphate, 0.1 µM sodium orthovanadate, 0.1 µM phenylmethylsulfonyl fluoride, and 1 µg/ml leupeptin. The CCK phosphoreceptor was then purified by a modification of the procedure previously described (1). Cells were disrupted by sonication, the plasma membrane-enriched fraction was prepared (5), and receptor was solubilized using 1% digitonin. The supernatant from this procedure was then applied to a CCK analogue (CCK-OPE) affinity column (6), and the eluate was separated on a 10% SDS-polyacrylamide slab gel by electrophoresis using the conditions described by Laemmli (11). The protein of interest was identified by autoradiography of the polyacrylamide gel, and it was eluted by homogenization of the relevant slice with a Dounce homogenizer as we have described (1). We have previously demonstrated the radiochemical purity of the phosphoreceptor at this stage of purification (10).

In experiments requiring chemical rather than radiochemical purity of the CCK phosphoreceptor, the eluted samples were then desalted and buffer was exchanged over a 0.6 × 8-cm size exclusion column (Sephadex G-50) run with buffer containing 50 mM ammonium bicarbonate and 0.01% digitonin. The relevant fractions were then loaded onto a wheat germ agglutinin-agarose affinity column, washed with 0.5 M sodium chloride, and eluted by electrophoresis in SDS-containing buffer. This procedure was then followed by another desalting step and the relevant fractions were dried under vacuum. They were then ready for further purification by HPLC or mapping procedures described below.

**One-dimensional Phosphopeptide Mapping**—Radiochemically pure CCK phosphoreceptor was cleaved by treatment with cyanogen bromide as described (1). Products were then separated on an 8 M urea-SDS gel by electrophoresis (12) to provide information regarding the general domain(s) of receptor phosphorylation.

**Two-dimensional Phosphopeptide Mapping**—For fine mapping of receptor phosphorylation sites, we used subtilisin cleavage and the two-dimensional phosphopeptide mapping system previously described (1). In these experiments, the purified receptor was treated with 20 µl of 1% subtilisin in 50 mM ammonium bicarbonate and 0.5 mM dithiothreitol for 18 h at 37 °C, followed by another addition of 10 µl of subtilisin after 2 h of incubation. The two-dimensional maps were prepared on microcrystalline cellulose thin layer plates 20 × 20 cm and 200 µm thick. Plates were dried and applied to x-ray film or a PhosphorImager plate to generate the map profiles. Each construct was studied a minimum of four times in independent experiments. Only those changes which were consistent in all of these replicates were noted.

**Phosphoamino Acid Analysis**—Individual phosphopeptides on the two-dimensional map which were identified by autoradiography were isolated by scraping and eluted by sonication in 0.1% trifluoroacetic acid followed by centrifugation. Phosphoamino acid analyses were per-

formed by thin layer chromatography by the method of Nairn and Greengard (13).

**Phosphopeptide Purification and Sequencing**—Individual phosphopeptides on the two-dimensional map were also further purified by reversed phase HPLC in preparation for sequencing. This involved pooling material from several maps, drying it under vacuum, and injecting it onto an octadecylsilane column 4.6 × 250 mm, 5 µm, 300 Å (Vydac 218TP54) with the solvent system consisting of 0.1% trifluoroacetic acid as buffer A and 100% acetonitrile in 0.1% trifluoroacetic acid as buffer B. A gradient was utilized which increased the composition of buffer B at 1% per min, from 0 to 40%. The radioactive peaks were identified by γ counting, and were then applied to Sequelon-AA discs (Millipore, Bedford, MA) in preparation for Edman degradation sequencing in an Applied Biosystems System 476 Protein Sequencer (Foster City, CA).

When chemical purity had not been attained, the eluates from the thin layer plates were further purified on a 40% alkaline polyacrylamide gel using the technique of West *et al.* (14). Bands identified by autoradiography were then eluted and again separated by reversed phase HPLC prior to Edman degradation sequencing.

Two types of Edman degradation sequencing were utilized. These included automated pulsed liquid sequencing in the Applied Biosystems Instrument and manual Edman degradation radiochemical sequencing. In peptides having more than one potential site of phosphorylation, the phosphoserines were identified indirectly by modification with an alkanethiol prior to automated Edman sequencing using a method based on the procedure of Meyer *et al.* (15, 16). The manual sequencing was performed after binding the purified peptides to 20 mg of *N*-(2-aminoethyl)-3-aminopropyl glass beads in buffer containing 10 mg/ml 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide for 2 h at room temperature. This was alkalized with triethylamine and dried under vacuum, with the whole cycle repeated three times. Thirty µl of triethylamine:methanol:phenylisothiocyanate (1:7:1) was added and incubated at 50 °C for 5 min. The sample was then dried under vacuum and washed three times with methanol. Twenty µl of trifluoroacetic acid was then added for 5 min at room temperature, before the next cycle was begun. In each cycle, the methanol extract was counted in a γ spectrometer.

## RESULTS

**Phosphorylation of the CCK Receptor in CHO-CCKR Cells**—Demonstration of phosphorylation of a receptor in an intact cell requires methodology to rapidly and efficiently purify the phosphoreceptor from the vast majority of phosphoproteins present in the cell. The methodology we previously established and validated for the pancreatic acinar cell (10) was directly applicable to the CCK receptor-bearing CHO-CCKR cell as well. The key step for this was the specific adsorption of the receptor to an affinity resin incorporating a CCK analogue which efficiently binds to both high and low affinity states of the CCK receptor (CCK-OPE) (6, 17).

Like our previous observations in pancreatic acinar cells (10), agonist stimulation of the CHO-CCKR cells resulted in increased phosphorylation of the CCK receptor (Fig. 1). This occurred in a concentration-dependent manner for both CCK and the phorbol ester, TPA. When stimulated similarly and treated in the same way, no phosphorylation was observed in this region of an SDS-polyacrylamide gel used to separate products of phosphorylation of the parent cell line, CHO-K1 cells (data not shown).

However, unlike the acinar cell experience in which treatment with the protein kinase C inhibitor, staurosporine, inhibited only approximately 50% of receptor phosphorylation stimulated by CCK (18), in the CHO-CCKR cells this treatment reduced receptor phosphorylation by 75 ± 7% (Fig. 2). Of further interest, the time course of CCK receptor phosphorylation in response to CCK stimulation was quite different in the CHO-CCKR cell than in the acinar cell. The phosphorylation of the recombinant receptor occurred rapidly upon agonist stimulation, reaching its maximal level in 15 min and maintaining that level through the 30-min time point, while in the acinar cell receptor phosphorylation peaked within 2 min and returned to its basal state over the same interval (18) (Fig. 3).

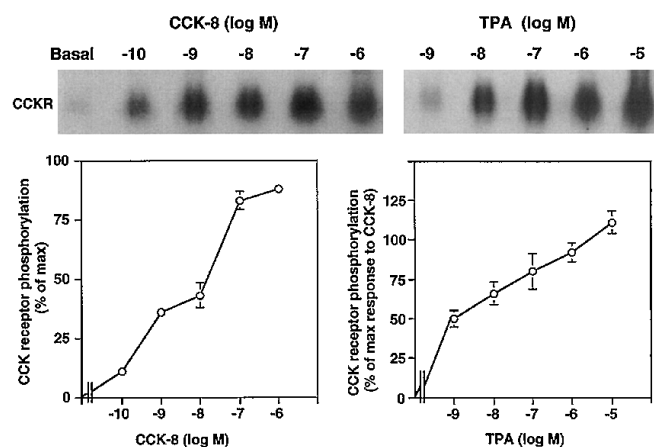


FIG. 1. CCK and TPA stimulated phosphorylation of the CHO-CCKR cell CCK receptor in a concentration-dependent manner. Shown are typical autoradiographs of the  $M_r = 85,000$ – $95,000$  region of a SDS-polyacrylamide gel used to purify the CCK receptor from cells stimulated with the noted secretagogues, as well as means  $\pm$  S.E. for three independent experiments. Basal receptor phosphorylation is considered to represent 0% and the maximal response to CCK is considered to represent 100%.

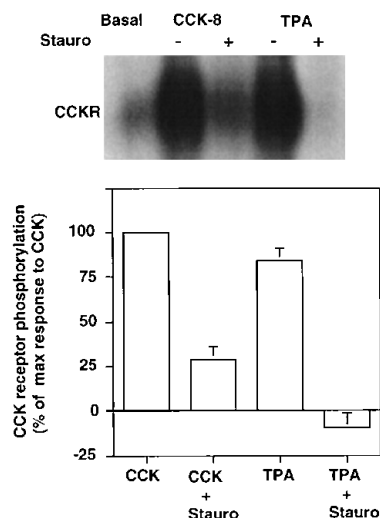


FIG. 2. Staurosporine ( $10 \mu\text{M}$ ) partially inhibited CCK-stimulated CCK receptor phosphorylation, and completely inhibited that stimulated by TPA. Shown is a representative autoradiograph of the  $M_r = 85,000$ – $95,000$  region of a SDS-polyacrylamide gel used to purify the CCK receptor after stimulating the CHO-CCKR cells under the conditions described, as well as means  $\pm$  S.E. for three independent experiments.

**Phosphopeptide Mapping**—To determine the domain of the CCK receptor which was phosphorylated in the CHO-CCKR cells, the purified phosphoprotein was cleaved with cyanogen bromide as we have previously reported (1). Like the acinar cell receptor, the CHO-CCKR cell receptor was phosphorylated predominantly on a  $M_r = 9,900$  band corresponding to the size of the third intracellular loop (Fig. 4) (1). Overexposure of the autoradiograph revealed less than 2% of phosphorylation to be in the range of  $M_r = 4,200$ , corresponding to a serine and threonine-rich domain in the carboxyl-terminal tail of the receptor. The amino acid sequence of these two domains of the CCK receptor are shown in Fig. 4, with the consensus sites for phosphorylation by protein kinase C [(R/K) $_{1-3}$ (X) $_{2-0}$ ]-S\*/T\*(X) $_{2-0}$ (R/K) $_{1-3}$ ] > S\*/T\*(X) $_{2-0}$ (R/K) $_{1-3}$ ] > (R/K) $_{1-3}$ (X) $_{2-0}$ ]-S\*/T\*] noted (19). This very broad definition suggests the presence of 9 potential sites of action of protein kinase C within the third intracellular loop (serines 260, 264, 271, 274, 275, 289, 307, and 314, and threonine 276), and 3 sites within the carboxyl-termi-

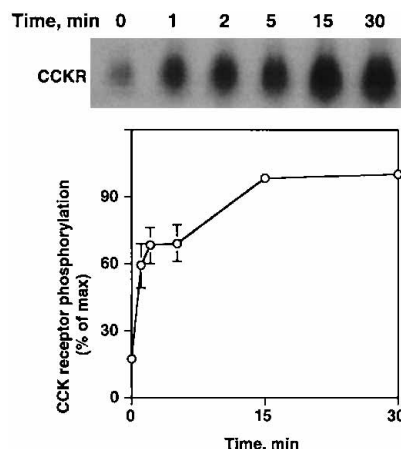


FIG. 3. CCK ( $1 \mu\text{M}$ ) stimulated the rapid and stable phosphorylation of the CCK receptor in the CHO-CCKR cells. Shown is a typical autoradiograph of the  $M_r = 85,000$ – $95,000$  region of a SDS-polyacrylamide gel used to purify the CCK receptor after stimulating the CHO-CCKR cells for the time noted, as well as means  $\pm$  S.E. for four independent experiments.

nal tail (serines 430 and 433, and threonine 424) of this receptor. It should be noted that this is a much broader definition than is used by the most common program to identify such sites, PROSITE (20). That program predicts the phosphorylation of only four residues, representing serines 260, 264, and 275 within the third intracellular loop and threonine 424 within the carboxyl-terminal tail. Site mutants in which each of these residues was changed to an alanine residue were constructed and analyzed (Fig. 5b, below).

We have previously established methodology to separate sites of phosphorylation of the acinar cell CCK receptor by generating a two-dimensional phosphopeptide map after subtilisin cleavage of the radiochemically pure receptor phosphoprotein (1). Fourteen distinct phosphopeptides were reproducibly observed on such a map of the acinar cell CCK phosphoreceptor stimulated by CCK treatment (1). Similar treatment of the CHO-CCKR cells resulted in the phosphorylation of all but one of these phosphopeptides, identified as phosphopeptide 12 (Fig. 5a). Consistent with the staurosporine sensitivity of the receptor phosphorylation observed in Fig. 1, the site missing from the CHO-CCKR maps (phosphopeptide 12) represented one of the sites previously attributed to a staurosporine-insensitive kinase, thought to likely represent the sites of action of a member of the G protein-coupled receptor kinase family (1, 18).

TPA stimulation of the CHO-CCKR cells resulted in a two-dimensional phosphopeptide map which was qualitatively similar to that observed for the CCK receptor on the pancreatic acinar cell after similar treatment (Fig. 5a). Like the experience in the acinar cell, phosphopeptides 4 and 12 were not observed after TPA stimulation, and phosphopeptide 2 was observed only after CCK stimulation, and not in response to TPA stimulation. Of note, phosphopeptide 6, which in the acinar cell was phosphorylated much more heavily in response to TPA than CCK (1), was not observed to be differentially phosphorylated in response to these agonists in the CHO-CCKR cell.

**Characterization of Specific Sites of Phosphorylation**—Phosphoamino acid analysis of each of the CHO-CCKR cell CCK receptor phosphopeptides observed in the two-dimensional phosphopeptide map were determined (data not shown). All of the phosphopeptides incorporated phosphoserine, with two of the peptides also incorporating stoichiometrically significant amounts of phosphothreonine (phosphopeptides 7 and 8). For both of these, quantitation suggested a 1:1 ratio of the two

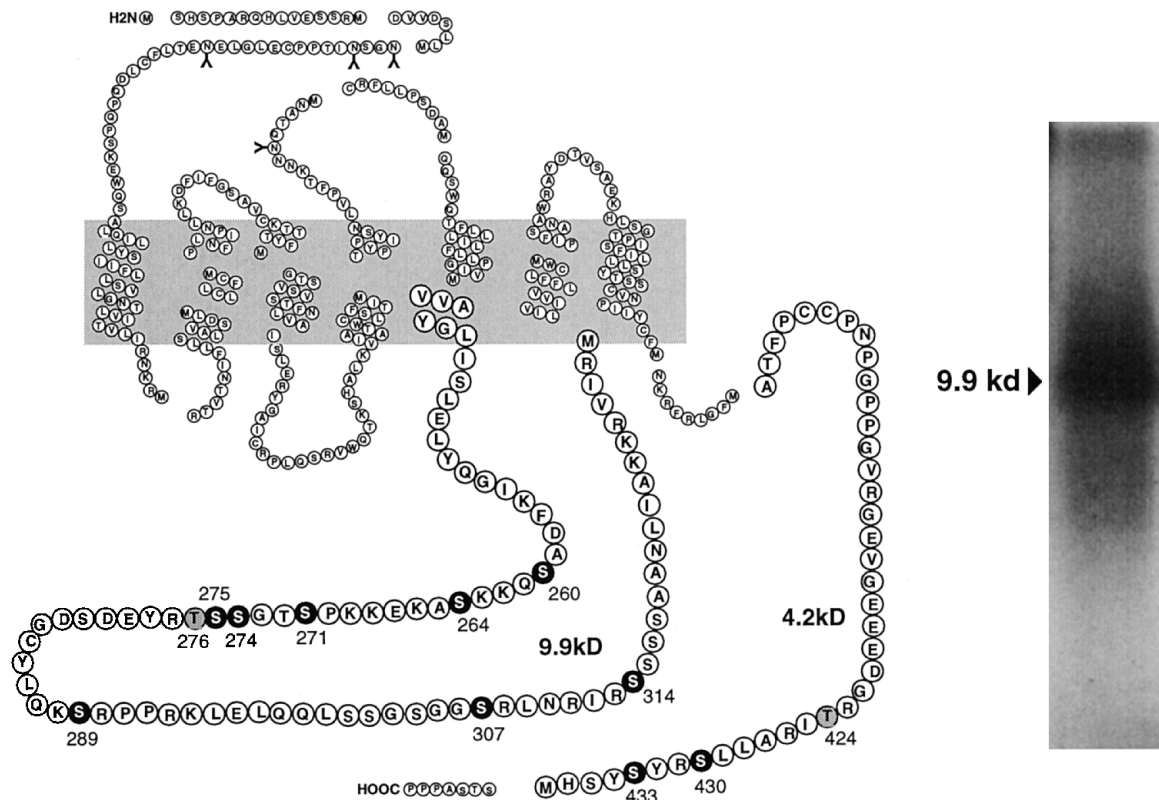


FIG. 4. Shown is a diagram of the predicted amino acid sequence and membrane topography of the rat CCK-A receptor, with predicted sites of cyanogen bromide cleavage noted. The two domains in which data support the possible phosphorylation are accentuated, with consensus sites for protein kinase C action noted. Shown is a representative autoradiograph of a one-dimensional phosphopeptide map of the CCK receptor after cyanogen bromide cleavage which has been separated on a SDS-urea acrylamide gel.

phosphoamino acids.

Since one of the strong consensus sites for protein kinase C action was Thr<sup>424</sup> (T<sup>424</sup>-I-R), sited within the minor phosphopeptide fragment of  $M_r = 4,200$  which was observed after cyanogen bromide cleavage, the possibility that this represented phosphopeptide 7 or 8 was explored by site-directed mutagenesis. Fig. 5b illustrates representative two-dimensional phosphopeptide maps of CCK-stimulated CHO cells expressing a CCK receptor construct in which Ala replaced Thr<sup>424</sup>. In comparing this with the wild type receptor pattern seen in Fig. 5a, there were no consistent differences between the two maps, suggesting that this was not the phosphothreonine observed in phosphopeptides 7 or 8.

The identity of phosphopeptides can also be inferred from analysis of the receptor sequence, since the two-dimensional phosphopeptide map provides information regarding the expected charge of any given spot. Since both phosphopeptides 7 and 8 are on the cathodic side of the site of application, they are expected to be positively charged at the pH of the buffer (3.5) used for thin layer electrophoresis. There are only two additional threonine residues (Thr<sup>272</sup> and Thr<sup>276</sup>) which are present in candidate domains of the CCK receptor and are near serine residues (both phosphoserine and phosphothreonine are present in phosphopeptides 7 and 8). Both of these residues could theoretically be present within basic peptides, although no cleavage sites can be engineered for Thr<sup>276</sup> to theoretically give it the expected charge. The expected charge of K-K-P-S<sup>271</sup>(P)-T<sup>272</sup>(P) would be consistent with the position of these phosphopeptides on the map. Of interest, Thr<sup>272</sup> does not fit any of the established consensus motifs for the action of protein kinase C. Due to the minor nature of these phosphopeptides on the map, this has not yet been directly demonstrated. Ser<sup>275</sup>, which fits the strong consensus motif for protein kinase C

action, was also mutagenized to an Ala residue, but failed to have any consistent effect on the phosphopeptide map. This suggests that this residue is not utilized as a site for phosphorylation. Another predicted site for protein kinase C action was, however, utilized by the cell. Phosphopeptide 6 was postulated to represent K-K-S<sup>264</sup>(P)-A-K, based on similar calculation rationale. Indeed, this was directly confirmed by mutagenesis of Ser<sup>264</sup> to Ala, with elimination of this phosphopeptide after CCK stimulation (Fig. 5b). Also, stimulating this construct with the phorbol ester, TPA, failed to demonstrate phosphopeptide 6 (data not shown).

Several of the major phosphopeptides present on the two-dimensional map were purified to chemical homogeneity and directly sequenced. These are shown in Table I, along with their calculated charges at pH 3.5 and the charge predicted by the map position. A representative example of this process is represented by phosphopeptide 5. This spot was recovered from 7 thin layer plates by scraping, and eluted into 0.1% trifluoroacetic acid upon sonication and centrifugation. The supernatant was then diluted with aqueous buffer A of the reversed phase HPLC system, and injected onto the C-18 column. The elution profile is shown in Fig. 6. The identity of this peak was confirmed by re-running it on a two-dimensional phosphopeptide map to demonstrate its migration at the position of phosphopeptide 5. The peptide in the major radioactive peak was then applied to a Polybrene-coated glass fiber filter which was exposed to automated Edman degradation sequencing, as well as manual cycles of Edman degradation with quantitation of the radioactivity eluted in each cycle (Fig. 6). This confirmed its sequence as D-A-S<sup>260</sup>(P)-Q-K-K-S<sup>264</sup>(P).

For phosphopeptides which were not adequately purified by a single HPLC step, an intermediate step of alkaline polyacrylamide gel electrophoresis was introduced and they were rerun

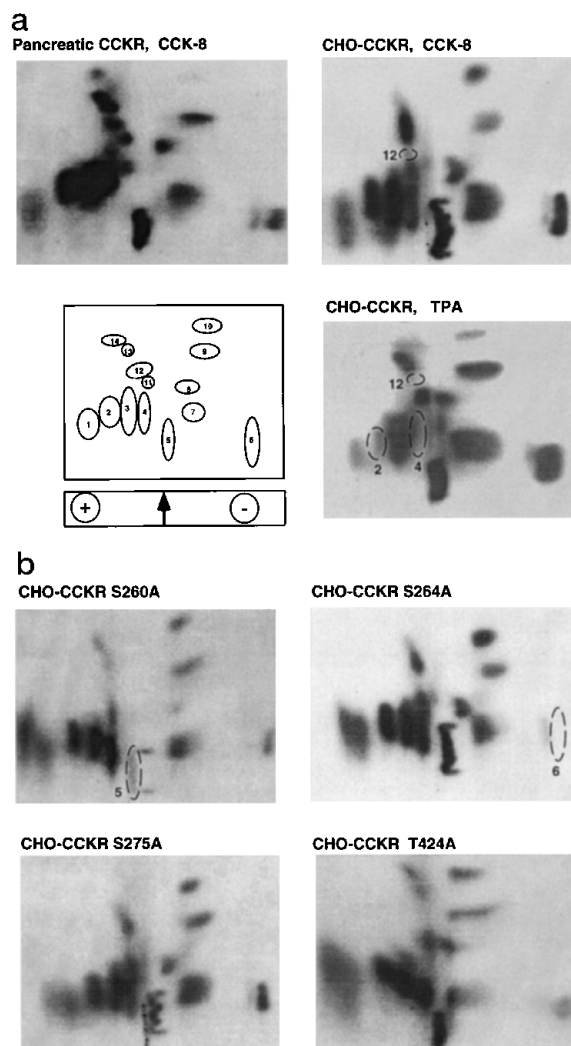


FIG. 5 *a*, shown are typical autoradiographs of two-dimensional phosphopeptide maps of the CCK receptor after subtilisin cleavage, and a numbered key for these maps. These represent the CCK receptor expressed on the pancreatic acinar cell (*a*) and CHO-CCKR cell (*b*) stimulated by CCK, and the CHO-CCKR cell stimulated by TPA (*c*). All of the previously described phosphopeptides are seen on the CCK-stimulated acinar cell map, while only phosphopeptide 12 is missing from the analogous CHO-CCKR cell map, and phosphopeptides 4 and 12 are not seen after stimulating the CHO-CCKR cell with TPA. *b*, shown are typical autoradiographs of two-dimensional phosphopeptide maps (after subtilisin cleavage) of CCK receptor site mutants which were stimulated by CCK. S275A and T424A mutants were not consistently different from control maps (seen in *a*). The S260A mutant consistently reduced phosphopeptide 5 by half of the expected intensity (since this phosphopeptide has two sites of phosphorylation), while the S264A mutant consistently eliminated phosphopeptide 6.

on HPLC (Fig. 7 shows representative data from phosphopeptides 9 and 10).

In addition to radiochemical sequencing, the phosphoserines in phosphopeptides 9 and 10 were specifically identified by their conversion to S-propylcysteine with 1-propanethiol using the method of Madden *et al.* (16) prior to sequencing. The S-propylcysteine residue is compatible with the Edman degradation chemistry and gives an easily identifiable phenylthiohydantoin peak on the ABI 476 protein sequencer. Phosphopeptide 9 was identified as G-G-S<sup>307</sup>(P)-R-L and phosphopeptide 10 was identified as L-S<sup>430</sup>(P)-R-Y using this approach.

#### DISCUSSION

Despite the demonstrated importance of phosphorylation of G protein-coupled receptors as a molecular mechanism for re-

ceptor regulation, there are few examples of the direct demonstration of specific receptor residues which are phosphorylated in the intact cell (21). This relates in large part to the sparsity and extreme hydrophobicity of receptor molecules which make purification difficult. While this problem can be overcome with cell lines which express larger numbers of receptors than native receptor-bearing cells, it is critical to understand that a given site of phosphorylation present in such a cell is also utilized in the native environment. Potential differences exist in the cellular complement of protein kinases and phosphatases, as well as in the microenvironment in which the recombinant receptor might reside. The present approach to this problem was addressed by comparing the two-dimensional phosphopeptide maps of the same receptor in both its native cellular environment and in a receptor-expressing cell line.

Indeed, the CCK receptor expressed on the CHO-CCKR cell line was phosphorylated in response to CCK stimulation in a concentration-dependent manner, much like the native receptor on the acinar cell. The time course of that phosphorylation was different, however. Whereas the native receptor was phosphorylated rapidly and reversibly, peaking within 2 min and rapidly returning to its basal state, the recombinant receptor established and maintained its level of phosphorylation throughout this time interval. Perhaps the receptor phosphatase activity we recently described (22) is not present in the CHO-CCKR cell line. There could also be differences in the cellular complement of kinases which act on the receptor.

Consistent with these possibilities, there were both qualitative and quantitative differences in the phosphopeptide maps of the CCK receptor phosphorylated in the pancreatic acinar cell and in the CHO-CCKR cell. There are important insights to be derived both from these differences and from the extensive similarities proven by the maps. As we have demonstrated, a cell line expressing large numbers of receptor molecules provides an ideal substrate to directly sequence the prominent sites of receptor phosphorylation. When these are sites observed in the native environment as well, we can be certain of their relevance.

The absence of a site of phosphorylation within the same receptor molecule expressed on two different cells could be explained several ways. The intracellular signaling events previously observed suggests that the conformation of the receptor in the plasma membrane was appropriate and that at least some coupling and signaling events were intact. Given the complexity of intracellular signaling events and cross-talk, it is possible that a subset of signaling events was not stimulated in the model cell line. It is also possible that a relevant kinase might have been absent in the cell line. We believe that this is the most likely explanation for the absence of phosphorylation of peptide 12 in the CHO-CCKR cells. This should be an excellent cellular system to introduce candidate kinases in an attempt to phosphorylate the CCK receptor on the appropriate residue.

The sites of CCK receptor phosphorylation by protein kinase C provide important insights. Clearly the enzyme was translocated and activated by CCK and TPA, as previously observed (23, 24). The predicted topology of the receptor based on hydrophobicity and on analogy with other "heptahelical receptors" suggests that there are 12 consensus sites for action by protein kinase C (4 of these were identified by the (S\*/T\*,X, R/K) motif recognized by the PROSITE data base (20)) which are in sites within the third intracellular loop and the carboxyl-terminal tail of the receptor which would be predicted to be accessible. Despite which definition of protein kinase C consensus is chosen, there are several of these sites which are not utilized by these cells, likely reflecting inaccessibility to the activated ki-

TABLE I  
Title

Number	Phosphopeptide Sequence	P-Ser	NH <sub>2</sub> -terminal	COOH-terminal	K	R	D	Calculated charge	Predicted charge
5	DAS <sup>260</sup> (P)QKKS <sup>264</sup> (P)	-2	+1	-0.47	+2	-	-0.27	+0.26	~+0.25
9	GGs <sup>307</sup> (P)RL	-1	+1	-0.47	-	+1	-	+0.53	~+0.5
10	LS <sup>430</sup> (P)RY	-1	+1	-0.47	-	+1	-	+0.53	~+0.5

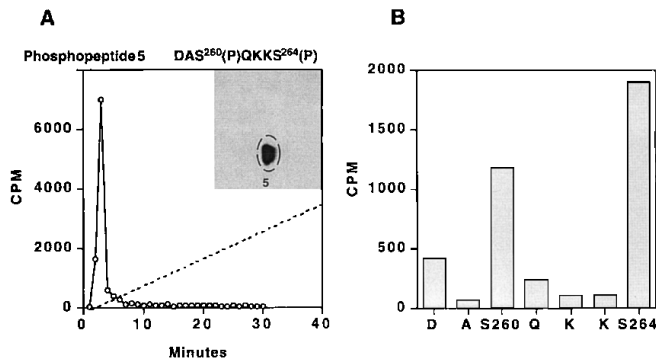


FIG. 6. Purification and radiochemical sequencing of phosphopeptide 5. Shown is the HPLC profile of the final step in purification, with a repeat two-dimensional phosphopeptide map of the product, and the radioactive elution profile of the Edman degradation cycles.

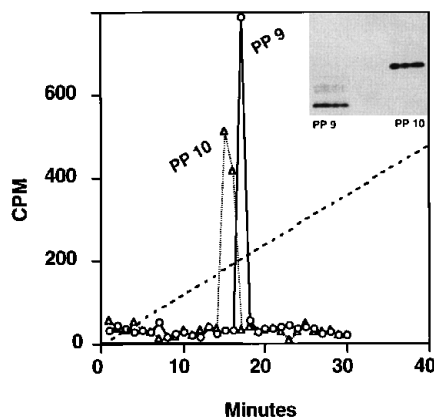


FIG. 7. Purification of phosphopeptides 9 and 10. Shown are HPLC profiles and an autoradiograph of the alkaline polyacrylamide gel used to separate the products.

nase. Currently, there are no meaningful conformational models for the loop regions of receptors in this family. Perhaps such data can be built into a model of these regions in the near future.

The functional impact of phosphorylation of this receptor, and the significance of each site of phosphorylation is clearly of interest. In analogous G protein-coupled receptors, phosphorylation mediates binding of arrestin-like proteins which interfere with G protein-coupling and thereby block initiation of signaling cascades (25). Indeed, in work in preparation,<sup>2</sup> we have demonstrated that desensitization of the stimulated inositol

trisphosphate response occurs rapidly in both pancreatic acinar cells and receptor-bearing CHO-CCKR cells, at the time of initiation of CCK receptor phosphorylation in these cells. Of note, that report demonstrates that this desensitization persists in both types of cells, even after the acinar cell receptor becomes dephosphorylated. This likely reflects the migration of this receptor into the "insulation compartment" we recently described in the acinar cell (26). This represents a postulated mechanism for desensitization in which G protein uncoupling occurs as a result of receptor immobilization in a plasmalemmal compartment depleted in G proteins, rather than requiring receptor phosphorylation to interfere with this step in signaling. It will be quite interesting to determine whether receptor phosphorylation plays any role in directing the receptor into this or other cellular compartments of desensitization.

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<sup>2</sup> R. V. Rao, B. F. Roettger, and L. J. Miller, manuscript in preparation.