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Role of *N*-Linked Glycosylation on the Function and Expression of the Human Secretin Receptor

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ABSTRACT

Secretin is a 27-amino acid long peptide hormone that regulates pancreatic water, bicarbonate, enzymes, and potassium ion secretion. The human secretin receptor (hSR) is a glycoprotein consisting of 440 amino acids, of which there are 5 putative *N*-linked glycosylation sites at positions Asn^{72}, Asn^{100}, Asn^{106}, Asn^{128} (N-terminal ectodomain), and Asn^{291} (second exoloop). Through functional analysis of the hSR-transfected cells cultured in the presence of various glycosylation inhibitors, it was found that tunicamycin and castanospermine were able to significantly reduce the secretin-stimulated cAMP response. On the other hand, the effects of other inhibitors, swainsonine and deoxymannojirimycin, were much lower, suggesting that the high mannose-type carbohydrate side-chain is essential to the expression of a fully functional hSR. The role of individual *N*-linked glycosylation sites was studied by mutation analysis (Asn to Leu or Ser to Ala) coupled to measurements of cAMP accumulation and extracellular acidification rate. The ED_{50} values of the wild-type receptor in these two assay systems were 0.25 and 0.11 nM, respectively, and mutation at position 100, 106, or 291 did not affect either the ED_{50} values or the maximal responses in the two assays. However, the Asn^{72}Leu and Ser^{74}Ala mutations reduced the maximal responses and increased the ED_{50} values in both assays, suggesting that this site is a true glycosylation signal. This hypothesis was further supported by competitive binding studies, the same mutants were found to be defective in binding with [^{125}I]secretin. To evaluate whether the change in receptor function of the mutants is caused by the change in the process of presenting the receptor to the cell surface, the mutants and the wild-type receptor were tagged with a c-Myc epitope at the C-termini. Using an anti-c-Myc monoclonal antibody and confocal microscopy, all of the mutant receptors were found to be expressed and delivered to the plasma membrane. (*Endocrinology* **140:** 5102–5111, 1999)

SECRETIN IS A hormone produced and secreted from the endocrine S cells in the upper intestine in response to the gastric acid and lipid components of a meal (1–3). It is the most potent physiological stimulant of bicarbonate, water and electrolyte release from pancreatic ductular epithelial cells, and it potentiates enzyme secretion from the acinar cell (4). Secretin is also a putative mitogen to promote pancreatic growth (5–7). In the stomach and gall bladder, secretin inhibits gastric emptying and acid release (8) and stimulates biliary output of water and bicarbonate (5, 9, 10). In addition to its roles in the gastrointestinal tract, secretin increases tyrosine hydroxylase activity and cAMP levels in the rat superior cervical ganglion (11) and binds specifically to rat brain membrane (12), suggesting its role as a neuromodulator. The peptide has also been reported to have cardiac (13, 14) and renal effects (15, 16).

Secretin elicits its biological effects by interacting with specific cell surface receptors. Recently, rat (17) and human (18–20) secretin receptors have been characterized. These receptors belong to a distinct family of guanine nucleotide-binding protein (G protein-coupled)-coupled receptors, including pituitary adenylate cyclase-activating polypeptide (PACAP type 1) vasoactive intestinal peptide-1 (VIP1), VIP2, GH-releasing hormone (GHRH), glucagon, glucagon like peptide-1, and gastric inhibitory polypeptide receptors. The human secretin receptor (hSR) is 440 amino acids in size and contains a putative hydrophobic leader peptide (22 amino acids), a large hydrophilic ectodomain (122 amino acids), 7 transmembrane regions with 3 exoloops and 3 cytoplasmic loops (254 amino acids), and a hydrophilic cytoplasmic tail (42 amino acids). There are also 5 putative *N*-linked glycosylation consensus sequences at positions 72, 100, 106, 128, and 291 (Fig. 1). The glycoprotein nature of rat secretin receptor has been demonstrated using wheat-germ agglutinin affinity chromatography (21) and cross-linking studies in rat gastric gland (22) and pancreatic acini (23).

It is well recognized that glycosylation plays an important role in cell surface receptor functions such as intracellular trafficking (24–26), ligand binding, and signal transduction (27, 28). In the superfamily of G protein-coupled receptors, one of the most well studied examples is the β-adrenergic receptor. It has been shown that glycosylation is important for proper membrane trafficking for this receptor protein (24). However, the role of carbohydrate seems to be different in the secretin receptor family. A mutation study of the PTH receptor showed that glycosylation is not essential to maintaining a normal receptor function (29). As many G protein-coupled receptors are glycoproteins, it is difficult to predict the role of glycosylation in receptor function (25–29). In ad-

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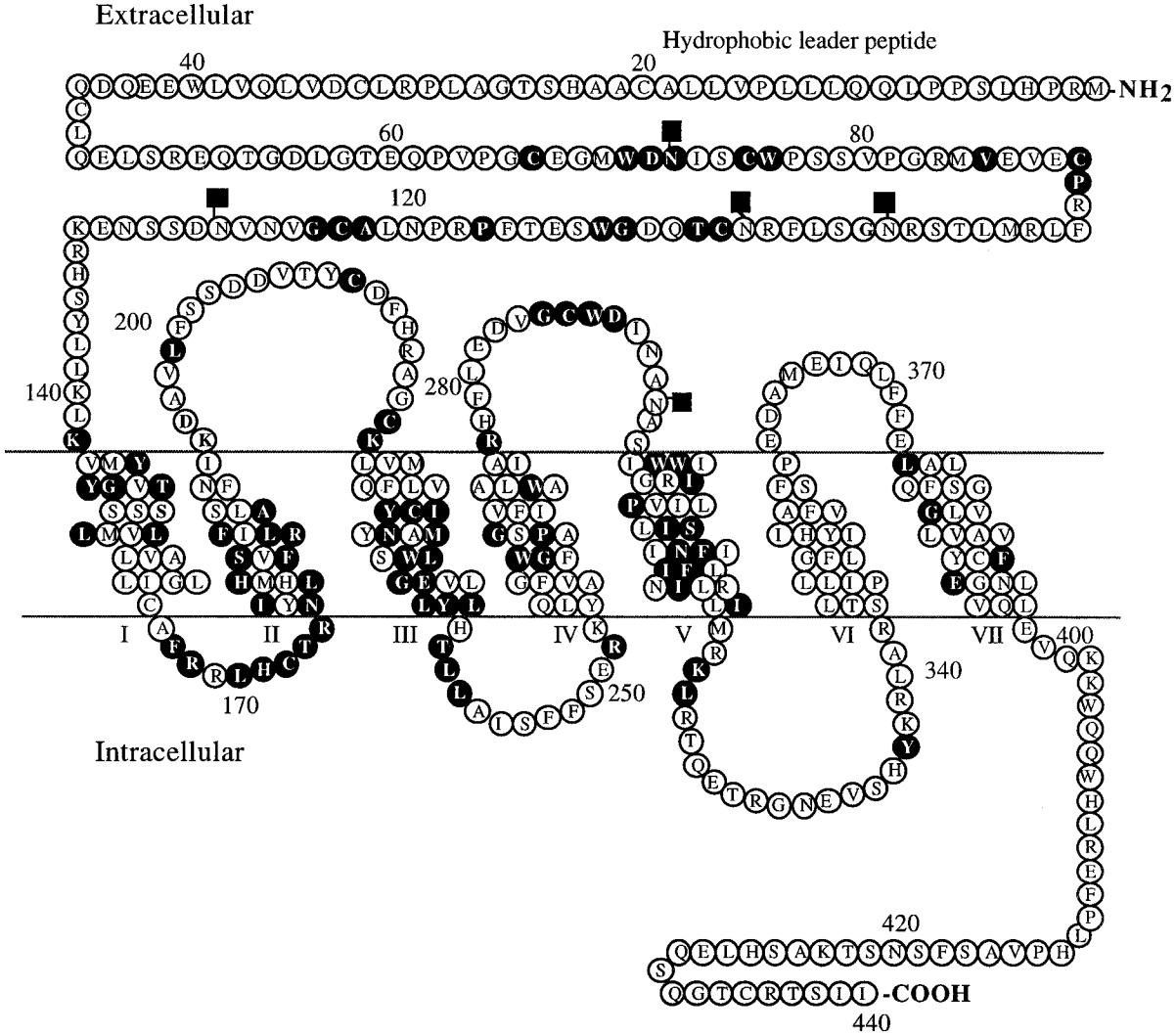

FIG. 1. A diagram of the human secretin receptor. The putative *N*-linked glycosylation sites at residues 72, 100, 106, 128, and 291 are labeled with *boxes*. The transmembrane domains of the receptor are labeled I–VII. Within the receptor, there is a putative hydrophobic leader peptide (22 amino acids), an N-terminal extracellular hydrophilic domain (122 amino acids), and a C-terminal hydrophilic cytoplasmic domain (42 amino acids). *Filled circles* represent amino acids that are conserved with rat secretin receptor, human, rat, mouse, and bovine PACAP type 1 receptor; the human and rat VIP1 receptor; and the human VIP2 receptor.

dition, there is very little information with respect to the function of glycosylation in the secretin receptor family. In this study, we sought to investigate the nature and role of glycosylation in hSR cell surface expression by using various glycosylation inhibitors. Then, five Asn to Leu and two Ser to Ala mutants corresponding to the putative *N*-linked glycosylation sites were constructed. The biological activities of the mutant receptors were investigated. Using the c-Myc epitope tagging technique, we have also localized the wild-type and the mutant receptors to the plasma membrane. Our findings showed that mutation of individual *N*-glycosylation sites seems to have little effect on receptor presentation to the plasma membrane.

Materials and Methods

Reagents

Synthetic human secretin was purchased from Neosystem (Strasbourg, France). Restriction enzymes, enzyme reaction buffers, deoxyribonuclease I, *Taq* polymerase, deoxy (d)-NTPs, MEM, FBS, G418, Lipofectamine, penicillin, streptomycin, and goat antimouse IgG fluorescein isothiocyanate-conjugated antibody were obtained from Life Technologies, Inc. (Grand Island, NY). Anti-c-Myc mouse monoclonal antibody (clone 9E10), swainsonine, deoxymannojirimycin, castanospermine, and tunicamycin were obtained from Roche Molecular Biochemicals (Indianapolis, IN). Iodine-125 was purchased from Amersham Pharmacia Biotech (Arlington Heights, IL). Saponin and IBMX were purchased from U.S. Biochemical Corp. (Cleveland, OH) and RBI (Natick, MA), respectively.

Inhibitor treatment of stably transfected cells

The hSR complementary DNA (cDNA) was previously cloned and functionally characterized in our laboratory (18). The full-length cDNA (1.7 kb) encoding the human secretin receptor was subcloned into an expression vector pRc-CMV (Stratagene, La Jolla, CA). Chinese hamster ovary (CHO) cells and COS-7 cells were kept in MEM supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). A stable cell line was generated by transfecting CHO cells using Lipofectamine (Life Technologies, Inc.). Two days after transfection, the cells were subjected to G418 selection at 500 μg/ml for 2 weeks. For inhibitor treatment, 0.2 million cells were seeded onto 6-well plates

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(Costar, Cambridge, MA) supplemented with 1.7 μg/ml swainsonine, 200 μg/ml deoxymannojirimycin, 200 μg/ml castanospermine, or 1 μg/ml tunicamycin. The cells were incubated with the inhibitors for 30 or 60 h before being stimulated with 0.5 nM secretin. In this experiment, the culture medium was changed every 20 h. In control experiments, the cells incubated with the inhibitors were stimulated with 10 μM forskolin. Conditions for peptide stimulation and cAMP extraction were described previously (18). For RT-PCR, RNA was extracted from the cells incubated with or without glycosylation inhibitors for 60 h. Total RNA was isolated by the guanidium thiocyanate/phenol extraction method (30). Residue genomic DNA was digested with 1 U deoxyribonuclease I in the buffer provided by the manufacturer (Pharmacia Biotech, Piscataway, NJ) for 15 min at 37 C. The messenger RNA (mRNA) was extracted using the PolyATtract mRNA isolation system (Promega Corp., Madison, WI) and was used as the template for first strand cDNA. The RT reaction contained 10 mM dithiothreitol, 0.33 mM dNTPs, 0.2 μg random hexamer, 20 U RNasin (Promega Corp.), and 200 U Moloney murine leukemia virus reverse transcriptase in buffer provided by the manufacturer (Life Technologies, Inc.). The reaction was carried out at 37 C for 2 h. A standard PCR was performed using a pair of hSR-specific primers: hSRF, 5′-ACACAGAGGG CACGGGCAGG CGGACGTCGG; and hSRR, 5′-TGCAGGACCA GCATCATCTG AGAGGGAATT CGC. The PCR contained 50 pmol of each primer, 200 μM dNTPs, and 2.5 U *Taq* DNA polymerase (Life Technologies, Inc.) in the buffer provided by the manufacturer. The reaction conditions were 1 min each at 94, 55, and 72 C for 30 cycles, and the PCR products were analyzed by a 1% ethidium bromide-stained agarose gel.

Construction of hSR mutants by site-directed mutagenesis

The 1.7-kb full-length hSR cDNA was subcloned into the plasmid vector pAlter-1 (Promega Corp.). Single stranded template for site-directed mutagenesis was produced using the R408 helper phage. Mutants were generated using an oligonucleotide-directed method according to the manufacturer's procedure (Promega Corp.). Mutations were designed at positions according to the *N*-linked glycosylation consensus sequences (Asn-X-Ser/Thr) by replacing Asn with Leu. The mismatch oligonucleotides used for Asn mutants were: Asn^{72}Leu, 5′-GGGATGTGGG ACCTCATAAG TGCTGG; Asn^{100}Leu, 5′-CTCACCAGCA GACTTGGTTC CTTGTTC; Asn^{106}Leu, 5′-TCCTTGTTCC GACTCTGCAC ACAGGAT; Asn^{128}Leu, 5′-GGCGTTAATG CGCTCGACTC TTCCAAC; and Asn^{291}Leu, 5′-GACATCAATG CCCTCGCATC CATCTGG. The Ser mutants were constructed by replacing Ser with Ala. The mismatch oligonucleotides used were: Ser^{74}Ala, 5′-TGGGACAACA TAGCATGCTG GCCCTCT; and Ser^{130}Ala, 5′-AATGTGAACG ACGCTTCCAA CGAGAAG. The mutants were confirmed by DNA sequence analysis using a T7 DNA sequencing kit (Pharmacia Biotech). The mutant receptor cDNAs were subcloned into the vector pRc-CMV (Stratagene) for expression studies.

Functional analysis of the glycosylation mutants by cAMP assay

cAMP assay was performed as described previously (18). Briefly, 1 million cells seeded overnight were transfected with the wild-type and mutant hSR cDNA constructs in 6-well plates (Costar) using Lipofectamine according to the manufacturer's protocol. Peptide stimulation and cAMP assays were performed 3 days after transfection as described above. The basal and maximally stimulated cAMP levels were typically, on the average, 4.4 and 23.1 pmol/well, respectively. To control for the transfection efficiency in different transfection experiments, the cells were cotransfected with 1 μg pGL2-Control luciferase reporter plasmid (Promega Corp.). Luciferase assays were performed according to the manufacturer's protocol (Promega Corp.), and luciferase reporter activities were quantified using a luminometer (LUMAT LB 9507, EG&G Berthold, Bad Wildbad, Germany).

Measurement of the extracellular acidification rate

Extracellular acidification rates were measured using the Cytosensor microphysiometer (Molecular Devices, Menlo Park, CA). Transfected CHO cells were seeded into sterile cell capsule cups (Molecular Devices) at a density of 0.6 million cells/capsule in growth medium for 24 h. The internal size of the capsule was 50 μm high and 6 mm in diameter as defined by a spacer and a capsule insert (Molecular Devices). The cells were trapped between two microporus polycarbonate membranes. The assembled capsule cups were loaded into the Cytosensor chambers. The chambers were perfused with the running medium (bicarbonate-free MEM supplemented with 1 mg/ml BSA). The extracellular acidification rates were monitored at 90-sec intervals. A stable basal acidification rate was established before peptide stimulation. The acidification rate response upon peptide stimulation was expressed as a percentage of the basal rate.

Competitive binding assays

The K_d and binding capacity values of the wild-type and mutant receptors (Table 2) were determined by the homologous competitive binding approach using PRISM version 2.0 computer software (GraphPad Software, Inc., San Diego, CA). For the competitive binding experiments, the wild-type and mutant human secretin receptors were expressed transiently in CHO cells using a protocol described previously (18). In brief, CHO cells (0.5 million) were seeded into 6-well plates (Costar) and transfected with 1.5 μg expression plasmid using the Lipofectamine reagent (Life Technologies, Inc.). After 48 h, [^{125}I]human secretin (100,000 cpm) was incubated with the transfected CHO cells expressing the wild-type or mutant secretin receptor in the presence of increasing concentrations (picomolar to micromolar concentrations) of cold peptide for 1 h at 22 C. Human secretin was labeled using ^{125}I according to the method described by Chang and Chey (31). The specific activity of the labeled peptide was 463 cpm/fmol. Peptide was diluted from a stock solution (100 μM) in the binding buffer [50 mM Tris-HCl (pH 7.4), 200 mM sucrose, 5 mM $MgCl_2$, 10 mg/ml BSA, 0.1 mM phenylmethylsulfonylfluoride, and 1 mg/ml bacitracin]. After the incubation, cells were washed twice with ice-cold binding buffer and lysed with 1 M NaOH, and radioactivity was measured in a γ-counter. Nonspecific binding was determined in the presence of 1 μM human secretin. Total specific binding was, on the average, 25–30% (25,000–30,000 cpm) of the total radioactivity added.

Immunofluorescence studies of the c-Myc-tagged receptors

A c-Myc 9E10 epitope EQKLISEEDL (32) was added to the C-termini of the wild-type and mutant hSR cDNAs by PCR using the following primers: TAG-F, 5′-TAGACAAAGC TTCAGAGGGC ACGGGCAGGC GGACGTCGG; and TAG-R, 5′-TAGTGTGGGC CCTTAATTAA GATCCTCTTC GGAGATGAGC TTTTGTTCCA TGATGATGCT GGTCCTGCAG GTGCCCTG. The PCR fragments were purified and subcloned into pBS-SK^+ (Stratagene), and the sequence was confirmed by DNA sequencing. The 3′-extended cDNAs were subcloned in the expression vector pRc-CMV (Stratagene) and were used for transfection. Transfected cells were fixed in PBS containing 0.5% paraformaldehyde for 10 min at 4 C and permeabilized with 0.5% BSA in PBS-saponin [0.1% saponin (wt/vol) in PBS]. Afterward, the cells were incubated with mouse monoclonal anti-c-Myc tag antibody (1:250 dilution) for 20 h at 4 C, washed twice with PBS-saponin, and incubated with the fluorescein isothiocyanate-labeled goat antimouse antibody (1:1000 dilution) for 1 h at room temperature. After washing with PBS-saponin, cells were excited with a laser at 488 nm, and images were obtained using a confocal laser microscope (MRC 600, Bio-Rad Laboratories, Inc., Richmond, CA).

Statistical analysis

All observations were repeated six times, and values are expressed as the mean ± SE. Statistical analyses were carried out using Student's *t* test, and differences were considered significant at $P < 0.01$.

Results

Effects of glycosylation inhibitors on cAMP production in hSR stably transfected CHO cells

The importance and the nature of carbohydrate moieties for the hSR were studied using four different glycosylation inhibitors, swainsonine, castanospermine, deoxymannojirimycin, and tunicamycin, which affect different stages of the

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glycosylation process. Swainsonine is an indolizidine alkaloid, and it is a potent competitor of α-mannosidase. Castanospermine is a plant alkaloid that inhibits α-glucosidases. Deoxymannojirimycin is a mannose analog that specifically inhibits Golgi α-mannosidases IA/IB. Tunicamycin is an antibiotic that blocks *N*-linked glycosylation by preventing the transfer of *N*-acetyl-glucosamine residue to dolichol phosphate, thus inhibiting the synthesis of all *N*-linked glycoproteins (Fig. 2). The cells expressing the hSR, as indicated by RT-PCR (Fig. 3C, positive control lane), cAMP, and acidification rate response (Fig. 4), were cultured for 30 or 60 h in the presence of various inhibitors. The cells were then stimulated with secretin (0.5 nM), and the accumulation of in-

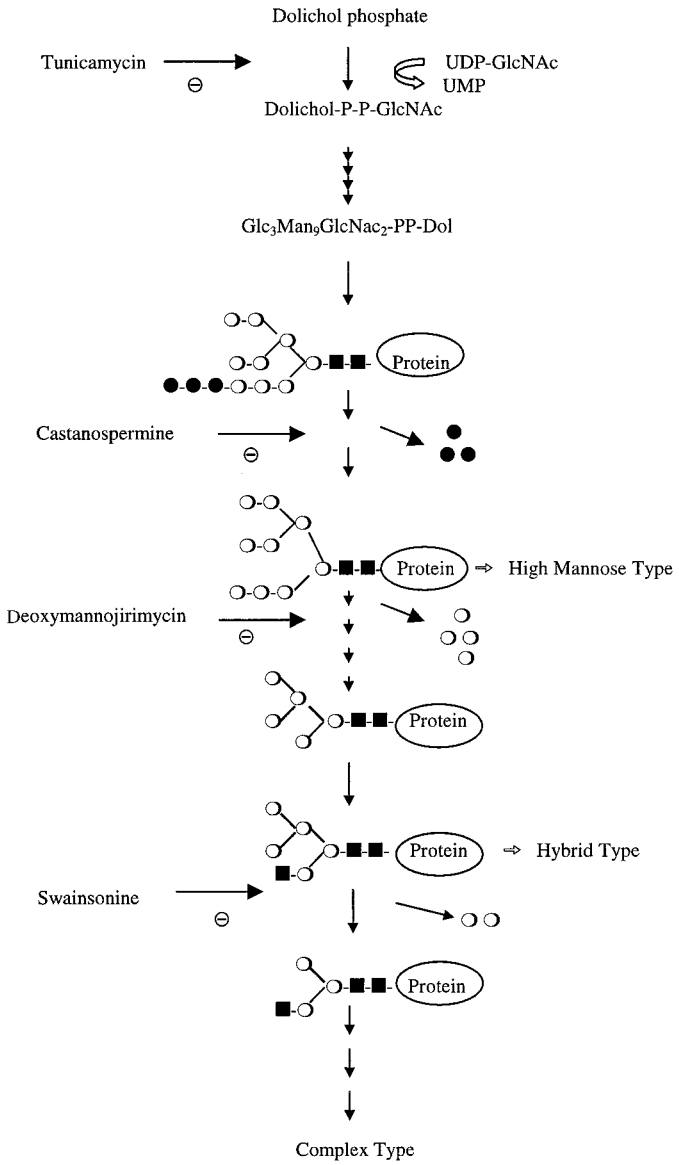

FIG. 2. The *N*-linked glycosylation pathway and the sites of action of glycosylation inhibitors. During glycosylation, sugars are removed and added to the chain to form the complex, hybrid, or high mannose type of oligosaccharide chain. By including these inhibitors in the culture medium, the pattern of glycosylation can be studied. ●, Glucose; ○, mannose; ■, *N*-acetylglucosamine; PP, pyrophosphoryl bridge; Dol, dolichol.

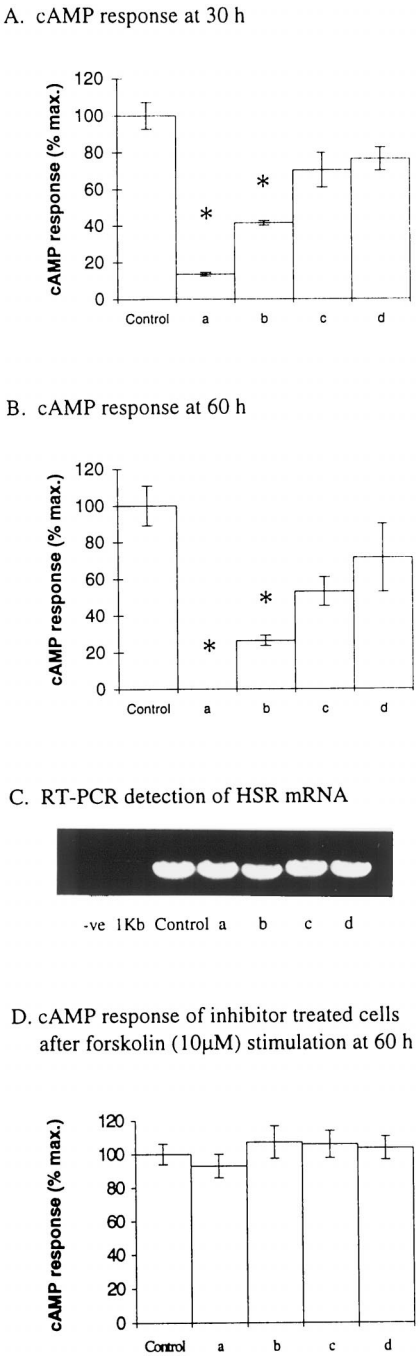

FIG. 3. Effects of glycosylation inhibitors on cAMP accumulation on secretin activation. A and B, cAMP response of the permanently transfected cells after 30 h (A) and 60 h (B) of inhibitor treatment. C, Semiquantitative RT-PCR detection of the hSR mRNA levels in the cells after 60 h of inhibitor treatment. D, Forskolin stimulation of inhibitor treated cells to indicate that the cells can produce cAMP after inhibitor treatment. Cells were cultured with tunicamycin (a; 1 μg/ml), castanospermine (b; 200 μg/ml), deoxymannojirimycin (c; 200 μg/ml), and swainsonine (d; 1.7 μg/ml), and control cells with no inhibitor were added to the culture medium. The basal (0%) and maximal (100%) cAMP responses of the control cells were typically 4.4 and 23.1 pmol/well, respectively. Values are given as the mean ± SE of six independent measurements. Methods for peptide stimulation, cAMP assay, total RNA isolation, and RT-PCR are described in *Materials and Methods*. Statistically significant changes in cAMP response are indicated with an *asterisk*.

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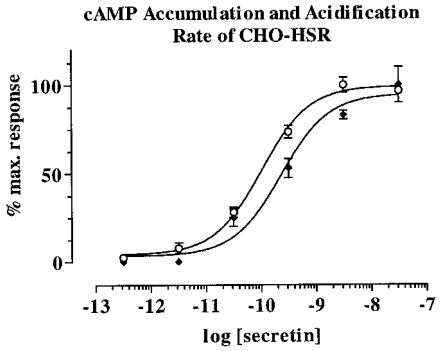

FIG. 4. A comparison of the cAMP accumulation (◆) and acidification rate (○) of hSR in the transfected cells. The response is expressed as a percentage of the maximal cAMP and acidification rate levels. The basal (0%) and maximal (100%) cAMP levels were 4.4 and 23.1 pmol/well, respectively. The peak acidification rate responses were typically 35% above the basal levels immediately before peptide stimulation. Values are given as the mean ± SE of six independent measurements.

tracellular cAMP was measured. Tunicamycin led to a 87% reduction or a total loss of cAMP response after 30 or 60 h of inhibitor treatment, respectively (Fig. 3, A and B, lane a). Besides tunicamycin, castanospermine (Fig. 3, A and B, lane b) also had a significant inhibitory effect on cAMP production in the cells; the responses were reduced by 58% and 78% after 30 and 60 h of treatment, respectively. In contrast, the effect of deoxymannojirimycin or swainsonine was less pronounced and statistically insignificant (Fig. 3, lane c and d). Functional analysis of inhibitor-treated cells using forskolin indicated that these inhibitors do not affect the cAMP signal transduction system (Fig. 3D). Hence, the effects of tunicamycin and castanospermine are probably at the receptor level. In addition, a semiquantitative RT-PCR was performed using mRNA extracted from an equal number of cells (1 million) treated with various inhibitors for 60 h. Our results indicated that inhibitor treatment does not have an adverse effect on the transcription of hSR in the transfected cells. However, we cannot exclude the possibility that these inhibitors may have altered the translation and/or presentation of the receptor to the cell surface, although the morphologies of the cells were unchanged during the study. Taken together, our data suggested that *N*-linked glycosylation is essential to the functional expression of hSR, and glycosylation of the receptor is probably of the high mannose type.

Effect of mutation of the putative glycosylation site on the biological activity of the receptor

We demonstrated the importance of *N*-linked glycosylation to hSR functions. We next sought to investigate the function of individual glycosylation sites by scanning mutation analysis. Five Asn to Leu mutants corresponding to the putative *N*-linked glycosylation consensus sequences of hSR were constructed. The functional role of individual *N*-linked glycosylation sites was studied by measuring intracellular cAMP accumulation and the extracellular acidification rate using a Cytosensor microphysiometer. cAMP generation is the direct measurement of the functional coupling of the receptor to adenylate cyclase. On the other hand, the extracellular acidification rate is an indirect measurement of the metabolic rate, and hence is an indicator of the total downstream effects after ligand-receptor interactions (33, 34). Cytosensor microphysiometry is a recently developed technique that has been used for characterization of a number of G protein-coupled receptors expressed in CHO cells (35, 36). We have also used this technique to monitor the real-time responses and secondary messenger pathways of the hSR (37). A comparison of the cAMP accumulation and acidification rate dose-response curves (Fig. 4) indicated that the Cytosensor bioassay is a more sensitive assay; the ED_{50} values of the wild-type receptor in these two assay systems are 0.25 and 0.11 nM, respectively. In both experiments, the transfection efficiencies were monitored by cotransfection with the luciferase pGL-2 control vector (data not shown). In the negative control experiments, the vector alone-transfected cells showed neither secretin-dependent cAMP nor acidification responses (data not shown).

Mutation at position 100, 106, or 291 did not alter ED_{50} values or maximal responses in the two assays (Fig. 5), indicating either that glycosylation at these sites does not affect receptor function or these sites are not glycosylated. However, the Asn^{72}Leu mutation significantly increased the ED_{50} values (3.1 and 0.51 nM for the cAMP and Cytosensor measurements, respectively; see Table 1) and reduced the maximal responses (50.1% and 35.2%, respectively; Table 1). To show that these effects are related to the changes in receptor glycosylation, a second mutant (Ser^{74}Ala) corresponding to the same glycosylation consensus was constructed and analyzed. By cAMP assays, this mutant was also found to be defective, there was a 6-fold increase in the ED_{50} value (1.5 nM) and a 50% reduction in the maximal response (Fig. 6 and Table 2). In fact, there was no significant difference in the cAMP responses between these two mutants, further suggesting that they have the same defect. The diminished activities of the mutants could be due to a reduction in receptor presentation to the cell surface, binding affinity, or G protein coupling. We next investigate the binding affinities of the wild-type and mutant receptors using competitive binding assays (Fig. 6 and Table 2). Consistent with the cAMP and Cytosensor studies, the Asn^{72}Leu and Ser^{74}Ala mutants were also defective in binding to the radiolabeled human secretin. The K_d values were increased (from 0.24 to 2.2 and 1.0 nM, respectively), whereas the binding capacities were reduced (from 606 to 104 and 107 fmol/10^6 cells, respectively). Taken together, all of these results strongly indicate that this site (position 72–74) is a true glycosylation signal, and glycosylation at this site is required for the interaction of the peptide with the receptor.

Interestingly, the Asn^{128}Leu mutation led to enhanced maximal responses (177.0% and 172.6%, respectively; Table 1), but no significant change in ED_{50} values (Table 1). When the mutant was subjected to competitive binding study, there were no significant differences between the mutant and the wild-type receptors in the K_d and binding capacity values. The reason for the discrepancy between our functional and binding data is unclear at the moment. It is possible that the augmented cAMP and Cytosensor responses reflect differences in the levels of receptor expression. Future experimentation is needed to further investigate this observation. In

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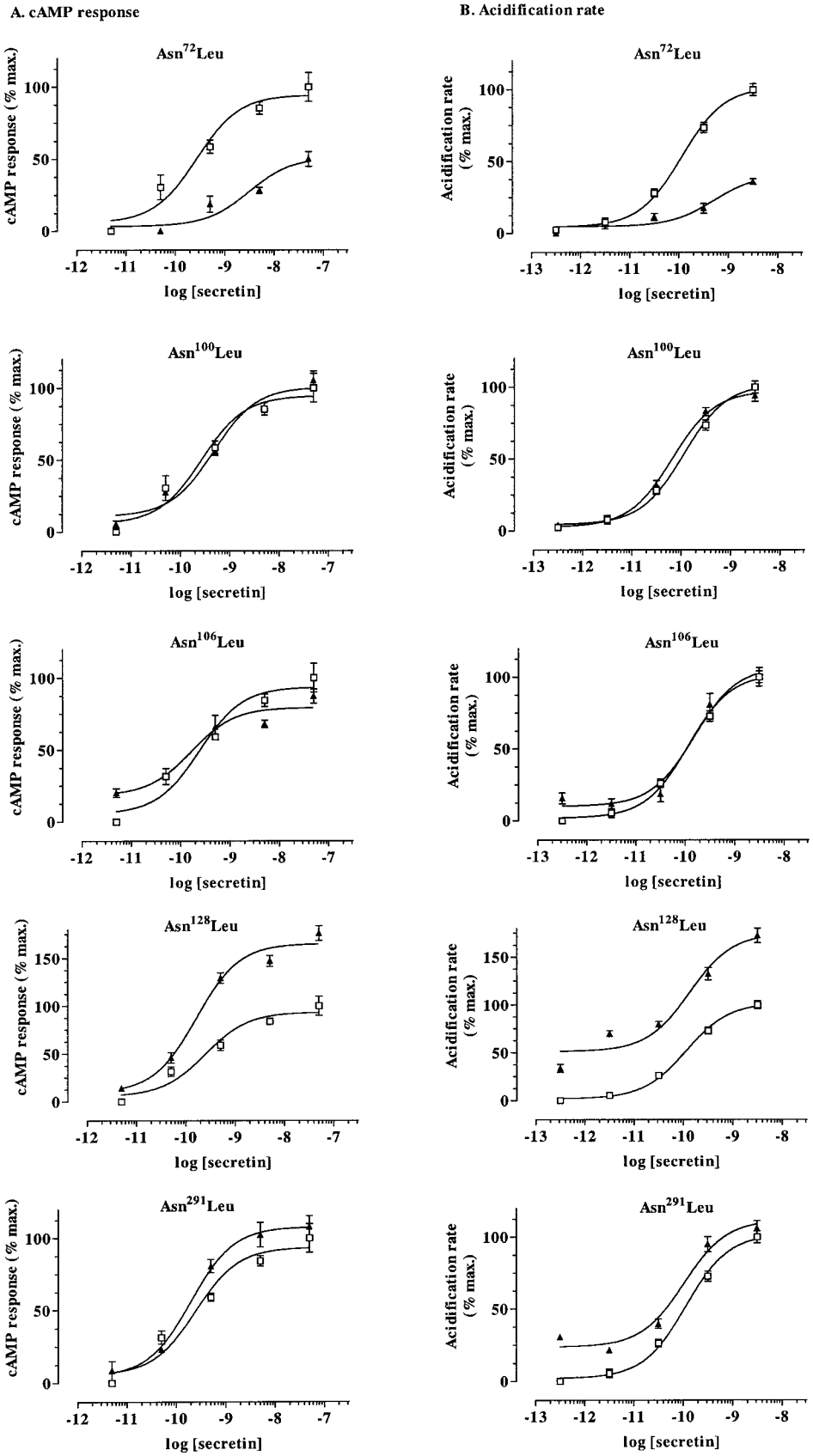

FIG. 5. Effect of mutation on the dose-dependent increase in cAMP accumulation (A) and the extracellular acidification rate (B) of the transfected cells. The basal and maximal cAMP levels of the wild-type receptor were typically 4.4 and 23.1 pmol/well, respectively. The peak acidification rate responses of the wild-type receptor were 35% above the basal levels before peptide stimulation. The scale of the *y*-axis is different for the Asn^{128}Leu mutant due to the fact that this mutant had elevated cAMP and Cytosensor responses. Each *data point* was expressed as a percentage of the maximal response of the wild-type receptor and presented as the mean ± SE of at least six independent measurements. □, Wild-type receptor; ▲, mutant receptors.

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TABLE 1. Biological activities of the wild-type (WT) and mutant hSRs in the transfected cells

	WT	$Asn^{72}Leu$	$Asn^{100}Leu$	$Asn^{106}Leu$	$Asn^{128}Leu$	$Asn^{291}Leu$
ED_{50} of cAMP response (nM) ± SE	0.25 ± 0.08	3.1 ± 0.05[a]	0.40 ± 0.13	0.17 ± 0.08	0.16 ± 0.04	0.19 ± 0.05
Maximal cAMP response (% of WT) ± SE	100 ± 3	50 ± 2[a]	107 ± 4	87 ± 4	177 ± 4[a]	107 ± 4
ED_{50} of acidification rate (nM) ± SE	0.11 ± 0.02	0.51 ± 0.05[a]	0.07 ± 0.02	0.15 ± 0.2	0.13 ± 0.3	0.11 ± 0.5
Maximal acidification rate (% of WT) ± SE	100 ± 3	35 ± 3[a]	96 ± 13	101 ± 9	172 ± 10[a]	109 ± 12

[a] Statistically significant change (n = 6) in biological activity.

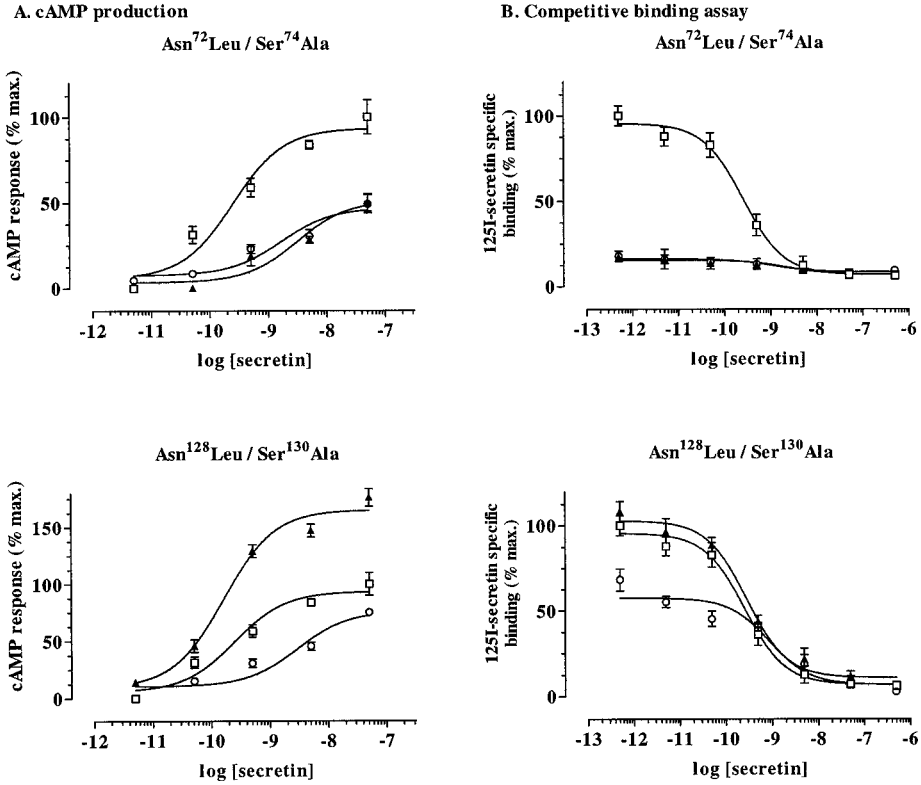

FIG. 6. Effects of mutations (*upper panels*, $Asn^{72}Leu$ and $Ser^{74}Ala$; *lower panels*, $Asn^{128}Leu$ and $Ser^{130}Ala$) on receptor functions (A, cAMP production; B, competitive binding assays). The basal and maximal cAMP responses of the wild-type receptor were 4.4 and 23.1 pmol/well, respectively. Total specific binding of the wild-type receptor was, on the average, 27% (27,000 cpm) of the total radioactivity added (100,000 cpm/well). The scale of the *y*-axis is different for the $Asn^{128}Leu$ mutant in the cAMP data due to the fact that this mutant had elevated cAMP responses. Each *data point* represents the mean ± SE of six measurements and is expressed as a percentage of the maximal response of the wild-type receptor. □, Wild-type receptor; ▲, Asn mutants; ○, Ser mutants.

TABLE 2. Biological activities of the wild-type, Asn, and Ser mutants in the transfected cells

	Mean ED_{50} ± SE (nM)	Maximal cAMP response (% to HSR)	K_d ± SE (nM)	Binding capacity ± SE (fmol/10^6 cells)
HSR	0.25 ± 0.08	100 ± 3	0.24 ± 0.05	606.5 ± 30
$Asn^{72}Leu$	3.10 ± 0.05[a]	50 ± 1[a]	2.2 ± 0.11[a]	104.9 ± 20[a]
$Ser^{74}Ala$	1.50 ± 0.09[a]	50 ± 4[a]	1.0 ± 0.06[a]	107.9 ± 21[a]
$Asn^{128}Leu$	0.16 ± 0.04	177 ± 4[a]	0.26 ± 0.05	656.1 ± 33
$Ser^{130}Ala$	2.90 ± 0.09[a]	70 ± 6[a]	0.80 ± 0.12[a]	413.6 ± 56[a]

[a] Statistically significant change (n = 6).

contrast, a second mutation of the same glycosylation consensus sequence ($Ser^{130}Ala$) led to reduced maximal responses and increased ED_{50} and K_d values in the cAMP and competitive binding assays (Fig. 6 and Table 2). Our results suggest that the mutation at this position from serine to alanine has a negative effect on the binding of the ligand with the receptor.

Immunostaining of the wild-type and mutant receptors

The variation in the functional activities of the mutant receptors may be caused by the changes in membrane trafficking or ligand binding (24, 25). To distinguish these possibilities, we tagged the c-Myc epitope to the C-termini of the wild-type and mutant receptors for immunostaining studies. In the control cAMP assays, the wild-type and the c-Myc-tagged receptor were found to have similar responses, indicating that the presence of a c-Myc tail does not alter receptor function (data not shown). By immunostaining of the c-Myc tail with the 9E10 mouse monoclonal antibody, the location of the receptors within the cells was detected. Confocal laser microscopy clearly showed that both the wild-type and mutant receptors were delivered and expressed on

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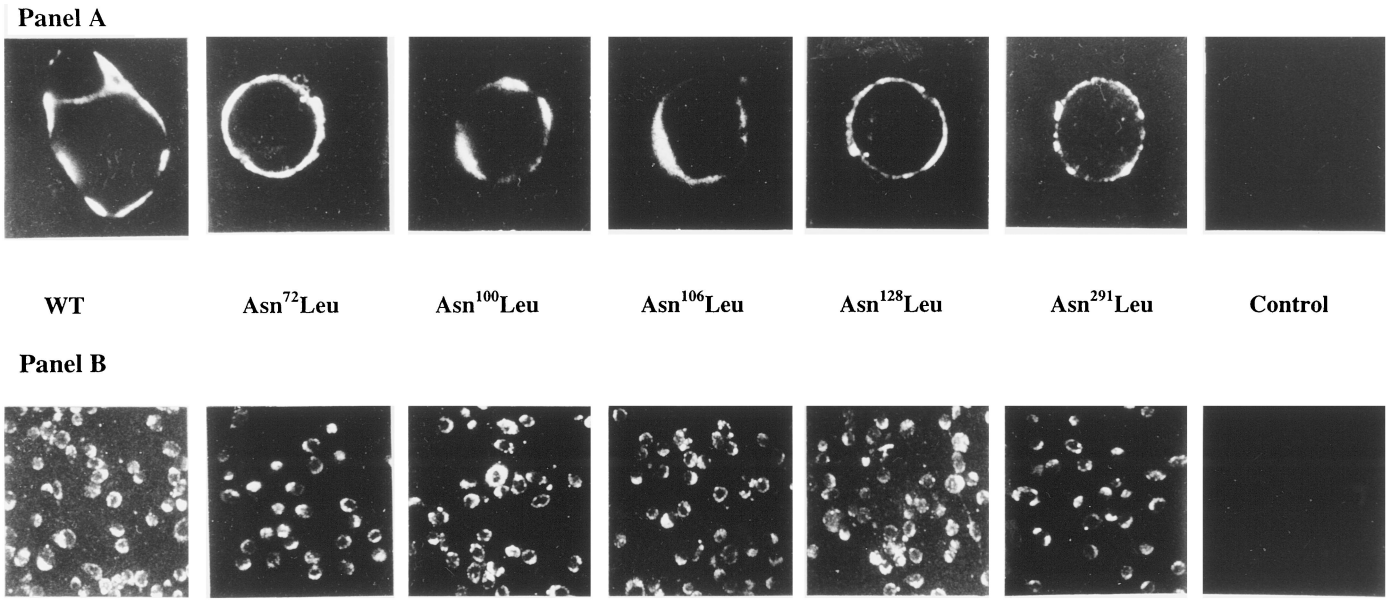

FIG. 7. Detection of the wild-type (WT) and mutant receptors tagged with the c-Myc epitope at the C-terminus using confocal microscopy. A shows a single cell view and B shows a wide angle view of the receptors. Control studies using cells transfected with vector alone gave no fluorescent signals.

the cell surface (Fig. 7, A and B), as the predominant fluorescent signals were detected as a ring-like structure at the plasma membrane.

Discussion

Glycosylation is important for cell surface proteins, aiding many processes. It aids protein maturation by protecting the protein from degradation, and it helps plasma membrane addressing and secretion by directing the protein to the proper Golgi compartment. It can also aid ligand recognition by 1) enhancing proper folding after translocation of the receptor into the ER lumen, 2) formation of disulfide linkages and 3) active participation in ligand interaction. The functional role of glycosylation in G protein-coupled receptors in these processes is less predictable (24–29). For example, the lack of carbohydrate moieties may cause a failure in ligand binding (38) or a defect in receptor conformation (39). The conservation of the *N*-linked glycosylation sites between the human and the rat secretin receptors with other members of the receptor family suggests a potential role for some of these sequences and hence the importance of carbohydrate moieties. To the best of our knowledge, this is the first study to investigate the functional contribution of glycosylation to the hSR.

To determine whether glycosylation is necessary for hSR function, we have used various glycosylation inhibitors to alter the *N*-linked glycosylation pattern in the transfected cells. There are three main types of *N*-linked glycosylation patterns: the complex, the hybrid, and the high mannose type. The glycosylation pattern of a protein can be studied by using inhibitors that can specifically interfere with the key enzymes or act as substrate analogs in the glycosylation pathway. Tunicamycin inhibits all kinds of *N*-linked glycosylation. Castanospermine prevents the formation of high mannose oligosaccharides by the inhibition of glucosidases I and II in the endoplasmic reticulum. Swainsonine alters the glycosylation pattern of a protein to the hybrid type by competitive inhibition of the enzyme α-mannosidase I. Deoxymannojirimycin is a mannose analog that can prevent hybrid chain formation by inhibiting the removal of mannose residues from high mannose glycans in the Golgi apparatus. Thus, by including these inhibitors in the culture medium, the glycosylation pattern of hSR expressed on the cell surface can be altered, and the effects of these inhibitors on hSR function can then be examined. Our studies showed that *N*-linked glycosylation, especially the formation of the pentasaccharide core and the high mannose side chain, is essential to hSR function. Our data are consistent with the findings in human VIP1 receptor (40). It was suggested that the folding process of receptor proteins had been completed after high mannose-type *N*-glycosylation, thus allowing the matured receptors to leave the endoplasmic reticulum (41).

To extend our study on the effect of glycosylation on hSR function, five Asn to Leu mutants and subsequently two Ser to Ala mutants corresponding to the consensus *N*-linked glycosylation sequence (Asn-X-Ser/Thr, where X can be any amino acid except proline) (42–44) were constructed. The Asn^{72}Leu and Ser^{74}Ala mutants consistently caused drastic impairment of receptor functions from ligand interaction, to cAMP production, to changes in extracellular acidification rates. Evidently, in addition to secretin receptors (18–20), the glycosylation consensus at this position is conserved among all members within this receptor family, including human, rat, mouse, and bovine PACAP type 1 receptors (45–48), human and rat VIP1 receptors (49, 50), and human VIP2 receptor (51). A similar mutagenesis approach in human VIP1 receptor also indicated the functional importance of this glycosylation site. Couvineau *et al.* (40) found that *N*-glycosylation at either Asn^{64} (not conserved in hSR) or Asn^{69} (corresponding position in hSR is Asn^{72}) is necessary and

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sufficient to ensure presentation of functional receptors to the cell surface. Taken together, this motif is probably a true glycosylation site for this subfamily of receptors, and glycosylation at this position is essential to the interaction of this family of receptors with their peptide ligands. However, at present, we do not know whether the apparent contribution of the carbohydrate side chain at this position to biological activity is due to its direct involvement in ligand binding. It is equally possible that the effect is secondary, and there is no direct contact between the carbohydrate moieties and human secretin during receptor activation. In other words, the lack of glycosylation at this position could have significantly changed the overall tertiary conformation of the receptor and, hence, altered its interaction with secretin.

One interesting but controversial finding of the present study was that the Asn to Leu mutation at position 128 enhanced receptor function in cAMP and Cytosensor assays. The maximal responses of this mutant in both measurements are significantly higher than those of the wild-type receptor. This enhancement effect is probably due to a postreceptor mechanism, as there is no change in ligand affinity as indicated by the K_d value in the competitive binding studies. By sequence comparison with other family members, this site is conserved with only the rat secretin receptor, suggesting that this effect is secretin receptor specific. In fact, the $Asn^{128}Leu$ mutant is of special interest because it represents the first example in this family of receptors where mutation of a single residue could lead to enhanced receptor function. In the second $Ser^{130}Ala$ mutant corresponding to the same *N*-glycosylation site, significant decreases in the maximal responses were observed in both cAMP and binding assays. The current work suggests that mutation of amino acid residue at position 128 or 130 may affect G protein coupling or presentation of critical regions of the extracellular portion of this receptor. This can have either a positive or a negative impact, with the elimination of a distinct site of glycosylation either augmenting or reducing the response to secretin stimulation. The present findings open new avenues suggesting that hSR function could be manipulated in either direction through site-specific mutagenesis.

Another aspect of the study was to investigate the functional impact of individual carbohydrate side-chains on cell surface targeting. The wild-type and mutant receptors were visualized by fluorescence laser confocal microscopy. In this study, the fluorescent signal was observed as a ring-like structure on the surface of the cells expressing the wild-type and mutant receptors, indicating that the receptors are properly addressed to the cell surface. In addition, although the morphology and the fluorescent signals of individual cells are highly variable, it was found that average fluorescent signals on the cells expressing the mutant and wild-type receptors were similar. Taken together, our data suggest that mutation of individual *N*-glycosylation sites of the hSR have little effect on the delivery of the receptor to the plasma membrane. This is consistent with the observation in human VIP1 receptor, in which *N*-glycosylation at either Asn^{58} or Asn^{69} is sufficient to ensure correct delivery to the cell surface (40). It was suggested that transfer of *N*-linked glycans from the oligosaccharide-lipid donor to the receptor in the endoplasmic reticulum is needed for subsequent transport of the receptor out of the endoplasmic reticulum (40, 52). Data from this experiment support the idea that functional changes in the mutants (position 72, 74, 128, and 130) are unlikely to be due to changes in the maturation or the delivery of the receptors to the cell surface, but are related to changes in the structure of the receptor. These conformational changes may lead to the reduction in binding affinities (mutants $Asn^{72}Leu$, $Ser^{74}Ala$, and $Ser^{130}Ala$) or augmentation of signal transduction mechanisms (mutant $Asn^{128}Leu$).

In summary, the present study supports the idea that *N*-glycosylation of peptidergic receptors is required to maintain high affinity binding states, as demonstrated in receptors for VIP1 (40), calcitonin (53), somatostatin (54), and cholecystokinin (55). Our study demonstrates for the first time that *N*-glycosylation, particularly at positions 72–74, is essential to hSR function. In addition, mutations at position 72, 74, 128, or 130 may affect receptor function by changing the structure of the receptor, rather than by changing the process of receptor delivery to the cell surface.

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