Mutagenesis of N-Glycosylation Sites in the Human Vasoactive Intestinal Peptide 1 Receptor. Evidence That Asparagine 58 or 69 Is Crucial for Correct Delivery of the Receptor to Plasma Membrane†

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Received August 24, 1995; Revised Manuscript Received November 17, 1995⊗

ABSTRACT: The functional role of N-linked carbohydrates in the human vasoactive intestinal peptide (VIP) 1 receptor was investigated by site-directed mutagenesis (Asn → Thr) of the four consensus N-glycosylation sites on Asn [58], Asn[69], Asn[100] (N-terminal extracellular domain) and Asn[293] (second extracellular loop). Mutated receptors were investigated after transient expression in Cos-7 cells, by ligand binding assay, affinity cross-linking, western blotting, and confocal laser microscopy of epitope-tagged receptor proteins. Mutations of each consensus site revealed that Asn[58], Asn[69], and Asn[100] were occupied by a 9-kDa N-linked carbohydrate whereas Asn[293] was not used for glycosylation. Each mutated receptor was expressed (western blot) and delivered at the plasma membrane (confocal microscopy) of Cos-7 cells. They displayed a dissociation constant similar to that of the wild-type receptor, i.e., 0.5−1 nM. In contrast, no VIP binding to Cos-7 cells could be observed with the mutant devoid of consensus N-glycosylation sites due to a strict sequestration of this mutant in the perinuclear endoplasmic reticulum. However, when solubilized with a zwitterionic detergent, this mutant bound [^{125}I]VIP specifically, indicating that it retained intrinsic binding activity. The construction of other mutants in which three out of four N-glycosylation sites were altered, demonstrated that N-glycosylation at either Asn[58] or Asn[69] is necessary and sufficient to ensure correct delivery of the receptor to the plasma membrane. Further pharmacological studies involving incubation of Cos-7 cells with castanospermine or deoxymannojirimycin immediately after transfection of mutated cDNAs encoding receptors with a single glycosylation site at Asn[58] or Asn[69] suggested that carbohydrate at Asn[58] was involved in a calnexin-dependent folding process of the receptor whereas carbohydrate at Asn[69] was not. These studies highlight the functional importance of the N-glycosylation of the human VIP 1 receptor which belongs to a new subfamily of seven membrane-spanning receptors.

A human VIP[1] receptor has been cloned recently from an intestinal cDNA library (Couvineau et al., 1994a). It encodes a seven transmembrane domain protein consisting of 460 amino acids with a large 144 amino acid N-terminal extracellular domain (Couvineau et al., 1994a). Like its counterpart cloned from a rat lung cDNA library (Ishihara et al., 1992), this receptor should be classified on a pharmacological basis as a VIP 1 receptor which differs from the VIP 2 receptor cloned in rat hypophysis (Lutz et al., 1993) and a human lymphoblast cell line (Svoboda et al., 1994). The VIP 1 receptor had been previously purified to homogeneity (Couvineau et al., 1990) and characterized as a glycoprotein (Nguyen et al., 1986; El Battari et al., 1987; Laburthe & Couvineau, 1988). The VIP 1 and VIP 2 receptors have 50% homology and belong to an emerging subfamily within the superfamily of G protein-coupled heptahelical receptors. This subfamily comprises receptors for secretin, VIP, pituitary adenylate cyclase-activating peptide, growth hormone-releasing factor, gastric inhibitory polypeptide, glucagon, glucagon-like peptide 1, parathyroid hormone, and calcitonin, with homologies ranging between 30% and 50% [reviewed in Couvineau et al. (1994b) and Segre and Goldring (1993)]. In contrast, it displays a low overall homology (<20%) with other receptors of the superfamily of G protein-coupled receptors (Couvineau et al., 1994b; Segre & Goldring, 1993; Strader et al., 1994; Savarese & Fraser, 1992) and has probably emerged early during evolution.

Despite the original structure of VIP receptors and other members of this subfamily, their stucture−function relationships are still largely unknown. We recently investigated the structure−function relationship of the human VIP 1 receptor by site-directed mutagenesis, showing that some highly conserved amino acids in the N-terminal extracellular domain of the receptor play a crucial role for ligand binding (Couvineau et al., 1995). In view of the presence of eight cysteine residues in the N-terminal extracellular domain of the human VIP 1 receptor (Couvineau et al., 1994a), most of which are highly conserved in the subfamily of VIP receptors (Couvineau et al., 1994b, Segre & Goldring, 1993), we also investigated their requirement for ligand binding and

† This work was supported by the Association pour la Recherche sur le Cancer (Grant ARC 6404/94) and Association Française de lutte contre la Mucoviscidose (Grant AFLM 94).

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§ On leave from CNRS URA 1924, Marseilles, France. Supported by a postdoctoral fellowship from ARC.

⊗ Abstract published in *Advance ACS Abstracts,* January 15, 1996.

[1] Abbreviations: VIP, vasoactive intestinal peptide; PBS, phosphate-buffered saline; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; CST, castanospermine; dMM, deoxymannojirimycin.

0006-2960/96/0435-1745$12.00/0

identified six crucial cysteine residues in this domain (Gaudin et al., 1995). Altogether, these data presented the first genetic evidence showing the importance of the N-terminal domain of VIP 1 receptor for ligand binding.

This domain also contains three consensus N-glycosylation sites on Asn^{58}, Asn^{69}, and Asn^{100} (Couvineau et al., 1994a), some of which are in the vicinity of or even adjacent to amino acid residues crucial for ligand binding (Couvineau et al., 1995). In this context and in view of the absence of data regarding the importance of the N-glycosylation in the subfamily of G protein-coupled heptahelical receptors to which belong VIP receptors, we have used site-directed mutagenesis to determine the role of each of the three consensus N-glycosylation sites in the N-terminal extracellular domain and a fourth one (Asn^{293}) in the second extracellular loop of the human VIP 1 receptor. After transfection of cDNAs into Cos cells, we investigated the effects of single or multiple mutations on ligand binding, receptor expression, molecular weight, and cellular localization. Our analysis demonstrates that N-glycosylation on Asn^{58} or Asn^{69} is necessary for correct delivery of the receptor to the plasma membrane while the receptor mutant of the N-glycosylation site is sequestered in the perinuclear endoplasmic reticulum but retains its affinity for VIP when solubilized with CHAPS.

EXPERIMENTAL PROCEDURES

Materials. Enzymes for cloning, sequencing, and oligonucleotide-directed mutagenesis were obtained from Promega or BRL. [α-^{35}S]dATP (1000 Ci/mmol) and other radioactive reagents were obtained from Amersham. Synthetic porcine VIP and peptide histidine methioninamide were purchased from Neosystem, disuccinimidyl suberate was from Pierce Chemical Co., culture medium was from Gibco, and synthetic oligonucleotides were from Eurogentec. [^{125}I]VIP was prepared and purified as described (Laburthe et al., 1978, 1987). PNGase F (EC 3.2.2.18) was purified from *Flavobacterium meningospeticum* as described (Tarentino et al., 1985). All other chemicals were purchased from Sigma.

Site-Directed Mutagenesis. The 1.4-kilobase *Eco*RI fragment containing the entire coding sequence of the human intestinal VIP receptor (Couvineau et al., 1994a) was subcloned into the *Eco*RI site of the plasmid p-Select-1 (Promega), and single-stranded DNA (+ strand) was produced in *Escherichia coli* JM109. Full-length VIP receptor mutants (see Figure 1) were generated by oligonucleotide-directed mutagenesis in the recognition signal for N-linked glycosylation of Asn-X-Ser/Thr (Asn → Thr). Specifically, [Thr^{58}]VIP receptor was made using the mismatched oligonucleotide 5′-GAGGCCCAGCTGGAGACTGAGACAATAGGCTGC-3′, [Thr^{69}]VIP receptor using 5′-AGCAAGATGTGGGACACCCTCACCTGCTGGCCA-3′, [Thr^{100}]VIP receptor using 5′-TCCATTCAAGGCCGCACTGTAAGCCGCAGCTGC-3′, and [Thr^{293}]VIP receptor using 5′-TGCTGGGACACCATCACCTCCTCACTGTGGTGG-3′. VIP receptor mutants in which three or four Asn were substituted for Thr (see Figure 1) were obtained using the above-described oligonucleotides. Identification of the desired mutations was obtained by direct sequencing. Inserts encoding mutant sequences were subcloned in the eucaryote expression vector pCDNA 1. The wild-type and mutant receptors were all tagged at the C-terminus with a marker dodecapeptide (Tag) as described (Couvineau et al., 1994a).

Transfection of Cos-7 Cells. Wild-type and mutant VIP receptors were transfected into Cos-7 cells by the electroporation method. Briefly, 4×10^6 cells were transfected with 20 μg of receptor DNA together with 20 μg of sperm salmon DNA used as a carrier, in 500 μL of cold phosphate-buffered saline. After electroporation (330 V, 500 μF, infinite resistance), cells were put on ice for 10 min and then transferred into Dulbecco's modified Eagle's medium containing 10% (v/v) heat-inactivated fetal bovine serum and 1% (v/v) penicillin−streptomycin before seeding. After 48 h, transfected Cos-7 cells were washed twice with cold phosphate-buffered saline, then harvested with a rubber policeman, centrifuged at 3000 rpm for 5 min at 4 °C, and resuspended in an appropriate buffer (see below) for subsequent assays.

Ligand Binding and Cyclic AMP Assays. The functional properties of wild-type and mutant VIP receptors were analyzed by [^{125}I]VIP binding to transfected cell membranes. The cell pellets (see above) were incubated for 30 min on ice in a hypotonic 5 mM HEPES buffer, pH 7.4. Thereafter, cells were homogenized as described (Couvineau et al., 1985), and the homogenate was centrifuged at 11 000 rpm for 15 min at 4 °C. The pellet was washed with 20 mM HEPES buffer and stored at −80 °C until use. This pellet was referred to as the membrane preparation. Membranes (200 μg of protein/mL) were incubated for 60 min at 30 °C in 20 mM HEPES buffer, pH 7.4, containing 2% (w/v) bovine serum albumin, 0.1% (w/v) bacitracin, and 0.05 nM [^{125}I]VIP in the presence of increasing concentrations of unlabeled VIP or other peptides, when necessary. The reaction was stopped as described (Couvineau et al., 1985). Specific binding was calculated as the difference between the amount of [^{125}]VIP bound in the absence and the presence of 1 μM unlabeled VIP. Binding data were analyzed using the LIGAND computer program (Munson & Rodbard, 1980). Cyclic AMP was assayed in transfected Cos-7 cells as previously described (Couvineau et al., 1994a).

Solubilization of VIP Receptor. Suspensions of transfected cell membranes were solubilized with the zwiterrionic detergent CHAPS (6 mM) as described in detail elsewhere (Couvineau et al., 1990). The ligand binding assay of solubilized VIP receptor was carried out as described (Couvineau et al., 1990), and receptor-bound [^{125}I]VIP was separated from free ligand by vacuum filtration through Whatman GF/C filters pretreated with 0.3% (v/v) poly(ethylenimine) (Couvineau et al., 1990).

Cross-Linking Experiments and PNGase F Treatment. Transfected cells were incubated for 4 h at 4 °C with 0.05 nM [^{125}I]VIP. The incubation medium was 2.5 mL of Dulbecco's modified Eagle's medium containing 15 mM HEPES (pH 7.5), 150 μM phenylmethanesulfonyl fluoride, 1% (w/v) bovine serum albumin, and 0.1% (w/v) bacitracin. At the end of the incubation, the cells were washed three times with ice-cold phosphate-buffered saline. The cross-linking step consisted then of incubating the cells for 30 min at 37 °C with 4 mL of 60 mM HEPES buffer (pH 8.0) containing 150 mM NaCl and 2 mM disuccinimidyl suberate as described (Fabre et al., 1987). The reaction was stopped and quenched by addition of 2 mL of 60 mM HEPES buffer containing 60 mM ammonium acetate. The cells were then solubilized with SDS−sample buffer, and proteins were analyzed by SDS−polyacrylamide gel electrophoresis as described (Couvineau et al., 1985). In some experiments

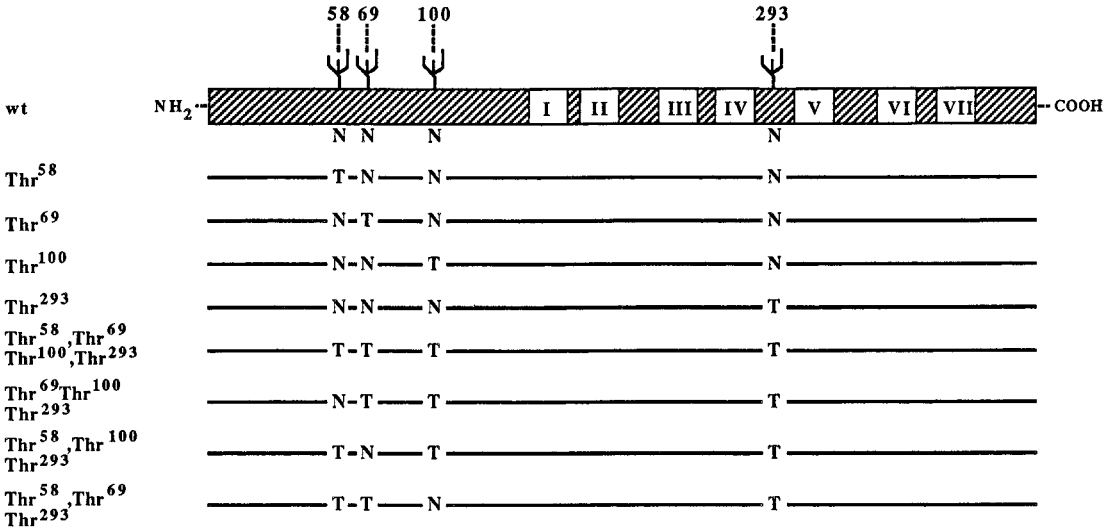

FIGURE 1: Schematic representation of the wild-type and mutant human VIP 1 receptors relevant to the present report. The receptor is symbolized by a hatched box extending from the NH_2 terminus (extracellular) to the COOH terminus (intracellular). Open boxes containing roman numbers identify the seven transmembrane domains. In wild-type receptors ψ shows the consensus N-glycosylation sites. For all mutants, the asparagine residue (N) of the Asn-X-Ser/Thr consensus glycosylation sites has been mutated into a threonine residue (T) at position(s) 58, 69, 100, and/or 293. See text for details.

cross-linked [^{125}I]VIP−receptor complexes were submitted to PNGase F treatment as follows: after cross-linking, cell proteins were solubilized in 100 mM Tris-HCl buffer, pH 8.6, containing 1% (v/v) Triton X100, 0.25% (w/v) SDS, 0.1% (v/v) β-mercaptoethanol, and 2 mM phenylmethanesulfonyl fluoride. PNGase F (2 units/ml) was then added for 1 h at 37 °C, and the incubation mixture was subsequently analyzed by SDS−polyacrylamide gel electrophoresis as described (Fabre et al., 1987). All gels were run and fixed as described (Couvineau et al., 1985; Fabre et al., 1987). They were dried and exposed for 5−10 days at −80 °C to a Trimax-type XM film (3M) with a Trimax intensifying screen.

Western Blot Analysis. Membranes (100 μg of protein) of transfected cells were solubilized in SDS−sample buffer, and proteins were separated on a 10% SDS−polyacrylamide gel and then transferred to nitrocellulose sheets as described (Couvineau et al., 1992). The nitrocellulose sheets were incubated for 2 h in 50 mM Tris buffer, pH 8, containing 2 mM $CaCl_2$, 80 mM NaCl, 5% (w/v) dry milk, and 0.2% (v/v) NP40. The same incubation was repeated with the mouse monoclonal anti-Tag antibody 9E10 (Evan et al., 1985) at the final dilution of 1:10. After three successive washings, the nitrocellulose sheets were incubated with ^{125}I-labeled F(ab′)2 sheep anti-mouse Ig. After three washings in phosphate-buffered saline, nitrocellulose sheets were dried and autoradiographed.

Confocal Laser Scanning Microscopy. Transfected cells were grown on 12-mm glass coverslips for 48 h as described above. After being washed with phosphate-buffered saline, cells were fixed with 2% (v/v) paraformaldehyde for 15 min and then permeabilized in phosphate-buffered saline containing 0.2% gelatin and 0.075% saponin (PBSGS). After being washed with PBSGS, permeabilized cells were incubated for 30 min at room temperature with the mouse monoclonal anti-Tag antibody 9E10 (Evan et al., 1985) diluted 1:250. The cells were then washed three times with PBSGS and exposed to the secondary antibody (FITC-sheep anti-mouse IgG at a 1:200 dilution). In double-labeling experiments, permeabilized cells were incubated with the mouse monoclonal anti-Tag antibody 9E10 (Evan et al., 1985) diluted 1:250 and a rabbit polyclonal anti-endoplasmic reticulum antibody (Louvard et al., 1982) diluted 1:100 for 30 min at room temperature. After being washed, the coverslips were incubated for 45 min with FITC-goat anti-rabbit and rhodamine-rabbit anti-mouse antibodies diluted 1:200. The coverslips were mounted in Glycergel, and selected fields were scanned using a True Confocal Scanner Leica TCS 4D composed of a Leica Diaplan inverted microscope equipped with an argon−crypton ion laser (488 nm) with an output power of 2−50 mW and a VME bus MC 68020/68881 computer system coupled to an optical disk for image storage (Leica Lasertchnik GmbH). The emitted light was collected through a log-pass filter on the target of the photomultiplier. Each sample was treated with a kalman filter to increase the ratio of signal vs background. All image generating and processing operations were carried out using the Leica CLSM software package. Screen images were taken on Kodak Ektachrome using a 35 mm camera.

Treatment of Transfected Cos-7 Cells with Castanospermine or Deoxymannojirimycin and Subsequent Assays. Following transfection of Cos-7 cells with wild-type or mutated receptor cDNAs (see Results), cells were aliquoted and then seeded in 12-well trays (for subsequent binding assay) or on 12-mm glass coverslips (for subsequent confocal microscopy) and grown for 2 days in culture medium (see above) containing 1 mM castanospermine or 1 mM deoxymannojirimycin or no drug. Binding assay on intact cells (see legend to Figure 7) and confocal laser microscopy were carried out as described above.

RESULTS

The amino acid sequence of the human intestinal VIP receptor contains four consensus N-linked glycosylation sites (Asn-X-Ser/Thr), three at the amino-terminal extracellular domain (Asn^{58}, Asn^{69}, and Asn^{100}) and one in the second extracellular loop (Asn^{293}) (Couvineau et al., 1994a). Figure 1 shows a schematic representation of the wild-type VIP receptor and all mutant receptors generated in this study. To determine the actual location of the glycosylation sites

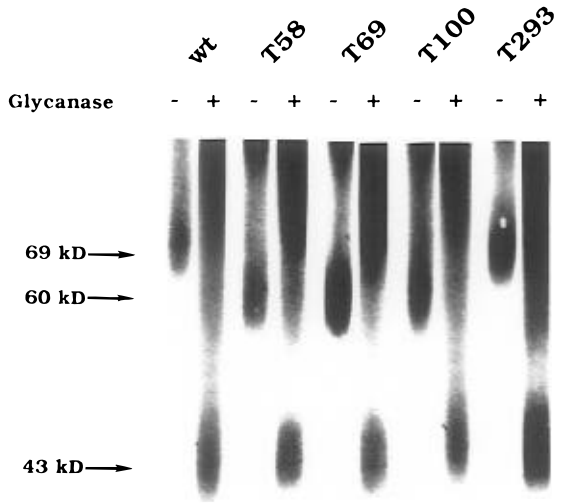

FIGURE 2: Cross-linking of [^{125}I]VIP to transfected Cos-7 cells and PNGase F treatment of labeled receptors. Cells transfected with wild-type receptor (wt), [Thr^{58}] receptor (T58), [Thr^{69}] receptor (T69), [Thr^{100}] receptor (T100), or [Thr^{293}] receptor (T293) were incubated with [^{125}I]VIP and then treated with 2 mM disuccinimidyl suberate as described under Materials and Methods. Labeled proteins were solubilized, treated (+) or not (−) with *N*-glycanase (PNGase F), and analyzed by SDS−polyacrylamide gel electrophoresis. The molecular mass of the labeled receptor forms is indicated by arrows on the left side. It has been verified that no labeled band was observed when cross-linking experiments were carried out with untransfected Cos-7 cells.

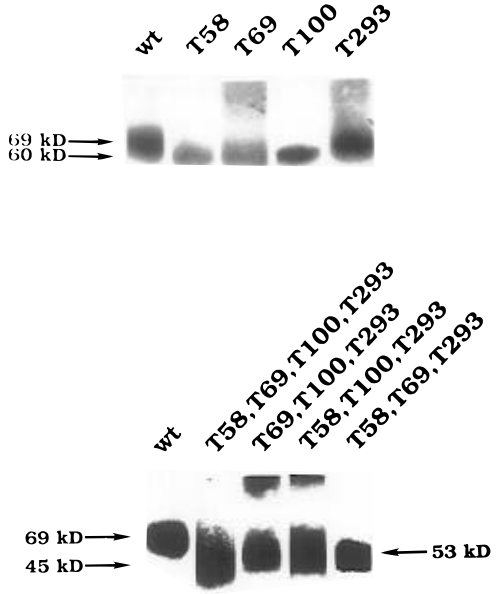

FIGURE 3: Western blot analysis of the wild-type and mutated human VIP 1 receptors after transfection into Cos-7 cells. (Top) The wild-type receptor (wt) and receptor proteins mutated at each of the four consensus glycosylation sites, i.e., [Thr^{58}] VIP receptor (T58), [Thr^{69}] VIP receptor (T69), [Thr^{100}] VIP receptor (T100), and [Thr^{293}] VIP receptor (T293), were run on the same gel. (Bottom) The wild-type receptor (wt) and receptor proteins mutated at the four consensus glycosylation sites, i.e., [Thr^{58},Thr^{69},Thr^{100}, Thr^{293}] VIP receptor (T58, T69, T100, T293), or at three out of four consensus glycosylation sites, i.e., [Thr^{69},Thr^{100},Thr^{293}] VIP receptor (T69, T100, T293), [Thr^{58},Thr^{100},Thr^{293}] VIP receptor (T58, T100, T293), and [Thr^{58},Thr^{69},Thr^{293}] VIP receptor (T58, T69, T293), were run on the same gel. Proteins from transfected cells (100 μg per lane) were separated by SDS−polyacrylamide gel electrophoresis, transferred onto nitrocellulose, and immunoblotted using the monoclonal anti-Tag antibody 9E10. The molecular mass of the labeled receptor forms is indicated by arrows. It has been verified that no labeled band was observed when Western blot analysis was carried out with untransfected Cos-7 cells. See Materials and Methods for details.

in the VIP receptor, substitution mutations were first introduced into the cDNA encoding the VIP receptor which altered each of the four consensus glycosylation sites. Figure 2 shows cross-linking experiments performed on the wild-type receptor and the four mutants. SDS−polyacrylamide gel electrophoresis analysis revealed a band at an approximate molecular weight of 69 000 for the wild-type receptor whereas a band at 60 000 was observed with the [Thr^{58}], [Thr^{69}], and [Thr^{100}] VIP receptor mutants. Similar shifts in molecular weight were observed by western blot experiments (Figure 3) using the anti-Tag antibody (see Experimental Procedures). These data strongly suggest that glycosylation sites on the corresponding Asn were occupied by a 9-kDa N-linked carbohydrate in the wild-type receptor although we cannot exclude the possibility that conformational change may play a role in migration through the gels. In contrast, affinity cross-linking did not reveal significant molecular weight shift with the [Thr^{293}] VIP receptor mutant, suggesting that the N-linked glycosylation site on Asn^{293} was not used, at least in Cos-7 cells. This was confirmed by western blotting; no significant shift in molecular weight for the [Thr^{293}] VIP receptor mutant was observed as compared to the wild-type receptor (Figure 3). Table 1 shows that all four mutants bound VIP with the same dissociation constants as the wild-type receptor, indicating that individual mutations did not alter the affinity of VIP receptor for its ligand. Nor was there any change in the specificity of mutated receptors as tested with the VIP-related peptide, peptide histidine methioninamide (Laburthe et al., 1986). Indeed, this peptide was 200 times less potent than VIP in inhibiting [^{125}I]VIP binding whatever the mutant considered as well as in the wild-type receptor (not shown). Finally, the [Thr^{58}], [Thr^{69}], [Thr^{100}], and [Thr^{293}] VIP receptor mutants were investigated for their ability to mediate stimulation of cAMP production by VIP in transfected Cos-7 cells. The dose−responses of VIP-stimulated cAMP were very similar for the wild-type receptor and the four mutants (not shown), suggesting that individual mutations of N-glycosylation sites do not affect the signal transduction pathway.

Table 1: Binding Parameters of Wild-Type and Mutant Human VIP 1 Receptors Expressed in Cos-7 Cells

constructs	dissociation constant (nM)	binding capacity (fmol/mg of protein)
wt[a]	0.61 ± 0.06[b]	5.31 ± 1.50
T58	1.18 ± 0.35	4.52 ± 0.11
T69	0.35 ± 0.04	3.53 ± 0.68
T100	0.87 ± 0.36	4.71 ± 0.88
T293	0.86 ± 0.11	9.20 ± 1.50
T58, T69, T100, T293	not detectable	not detectable
T69, T100, T293	0.54 ± 0.25	1.08 ± 0.04
T58, T100, T293	0.55 ± 0.11	5.18 ± 1.11
T58, T69, T293	not detectable	not detectable

[a] The receptor constructs were the following: wt, wild-type receptor; T58, [Thr^{58}] receptor; T69, [Thr^{69}] receptor; T100, [Thr^{100}] receptor; T293, [Thr^{293}] receptor; T58, T69, T100, T293, [Thr^{58},Thr^{69},Thr^{100},Thr^{293}] receptor; T69, T100, T293, [Thr^{69},Thr^{100},Thr^{293}] receptor; T58, T69, T293, [Thr^{58},Thr^{69},Thr^{293}] receptor; T58, T69, T293, [Thr^{58},Thr^{69},Thr^{293}] receptor. [b] Values are the means ± SEM of at least three experiments.

An additional mutant receptor cDNA was then constructed in which single amino acid subsitutions have been introduced within all consensus N-glycosylation sites, e.g., [Thr^{58},Thr^{69},

Thr^{100},Thr^{293}] VIP receptor (see Figure 1). After transfection into Cos-7 cells, this mutant did not bind [^{125}I]VIP (Table 1) though the mutant protein was expressed in transfected cells as demonstrated by western blotting using the anti-Tag antibody (Figure 3). Western blotting further indicated that this mutant protein migrated as a 45-kDa polypeptide (Figure 3). The size of this protein was identical to that of the PNGase F-treated form of the wild-type VIP receptor or of the [Thr^{58}], [Thr^{69}], [Thr^{100}], and [Thr^{293}] VIP receptor mutants treated with this enzyme (Figure 3). The absence of binding observed with the [Thr^{58},Thr^{69},Thr^{100},Thr^{293}] VIP receptor mutant which was devoid of carbohydrate could suggest that this mutant was not normally delivered at the plasma membrane of transfected cells and/or had no intrinsic binding activity. Confocal laser microscopy immunofluorescence studies were then conducted with the wild-type receptor and the five above-described mutant receptors in order to determine whether glycosylation of the VIP receptor does play a role in its subcellular localization as suggested for other membrane proteins (Lodish, 1988). Owing to the C-terminal Tag, the wild-type receptor as well as the [Thr^{58}] (Figure 4), [Thr^{69}], [Thr^{100}], and [Thr^{293}] (not shown) VIP receptor mutants could be detected on the plasma membrane of transfected Cos-7 cells and to a lesser extent within the cells. In contrast, the [Thr^{58},Thr^{69},Thr^{100},Thr^{293}] VIP receptor mutant was only located intracellularly in the perinuclear endoplasmic reticulum where it colocalized with a marker for the endoplasmic reticulum (Figure 4). Confocal laser microscopy clearly showed that this mutated VIP receptor remained strictly sequestered in the perinuclear zone and was not detected in the whole endoplasmic reticulum labeled by a specific rabbit polyclonal antibody (Louvard et al., 1982). Next, we tried to answer the question as to whether this [Thr^{58},Thr^{69},Thr^{100},Thr^{293}] VIP receptor mutant which was sequestered intracellularly still retained intrinsic binding affinity for VIP. For that purpose, transfected Cos-7 cell homogenates were solubilized with the zwitterionic detergent CHAPS under conditions which proved adequate for extracting active VIP receptors which were no longer associated with Gs proteins in solution and displayed a dissociation constant of 10−20 nM (Couvineau et al., 1990). The [Thr^{58}, Thr^{69},Thr^{100},Thr^{293}] VIP receptor mutant in solution bound specifically [^{125}I]VIP with a dissociation constant of 17 ± 5 nM (three experiments) as calculated by Scatchard analysis of binding data (not shown). This was very similar to the the dissociation constant of the wild-type receptor used as a control and solubilized under the same conditions, i.e., 13 ± 2 nM (three experiments). However, the amount of active [Thr^{58},Thr^{69},Thr^{100},Thr^{293}] VIP receptor mutant was eight times lower than that of the wild-type receptor. It has been verified that no specific [^{125}I]VIP binding could be observed in CHAPS extracts from Cos-7 cells transfected with vector alone, showing that the Cos cells do not have an unexpected intracellular VIP receptor. These experiments demonstrate that a significant population of glycosylation-defective VIP receptor mutant retained its intrinsic binding affinity for VIP but was not delivered to the plasma membrane of Cos-7 cells.

In order to determine whether glycosylation at one site out of the three sites [and which site(s)] which were used for N-glycosylation in Cos-7 cells, e.g., Asn^{58}, Asn^{69}, and Asn^{100}, was sufficient to ensure correct binding affinity and delivery of the VIP receptor to the plasma membrane, three additional mutant receptor cDNAs were generated in which all consensus N-linked glycosylation sites were mutated (Asn → Thr) but one (see Figure 1). Western blotting of mutated receptor proteins indicated that the remaining consensus N-glycosylation sites at Asn^{58}, Asn^{69}, or Asn^{100} were occupied by a 9-kDa carbohydrate (Figure 3). It appeared that [Thr^{69},Thr^{100},Thr^{293}] VIP receptor and [Thr^{58},Thr^{100},Thr^{293}] VIP receptor mutants were able to bind VIP with the same dissociation constants as the wild-type receptor whereas the [Thr^{58},Thr^{69},Thr^{293}] VIP receptor mutant did not generate any specific [^{125}I]VIP binding after transfection into Cos-7 cells (Table 1). Confocal laser scanning microscopy revealed that, like the [Thr^{58},Thr^{69},Thr^{100},Thr^{293}] VIP receptor mutant (Figure 4), the latter VIP receptor mutant having only one consensus N-glycosylation site at Asn^{100} was sequestered in the perinuclear endoplasmic reticulum (not shown). Finally, the [Thr^{58},Thr^{69},Thr^{100}] VIP receptor cDNA was also generated. After transfection in Cos-7 cells, western blotting indicated that this mutant migrated as a 45-kDa polypeptide and that its migration was not affected by PNGase F treatment (not shown). This further supports the absence of N-glycosylation on Asn^{293} after transfection of the human VIP 1 receptor in Cos-7 cells. As expected, this mutant does not bind VIP and is sequestered in the perinuclear endoplasmic reticulum (not shown).

We further investigated the role of glycosylation at Asn^{58} or Asn^{69} on the delivery of the receptor to the plasma membrane and receptor binding activity by using (i) castanospermine (CST), an inhibitor of glucosidases I and II involved in the removal of glucose from the 14 oligosaccharide N-linked core glycan in the endoplasmic reticulum (Hirschberg & Snider, 1987), (ii) deoxymannojirimycin (dMM), an inhibitor of α-mannosidase I involved in the removal of mannose residues from high-mannose glycans in the Golgi apparatus (Hirschberg & Snider, 1987). Cos-7 cells were transfected with the wild-type VIP receptor, [Thr^{69},Thr^{100},Thr^{293}] VIP receptor, or [Thr^{58},Thr^{100},Thr^{293}] VIP receptor mutant cDNAs and immediately grown in CST- or dMM-containing culture media. As evidenced by confocal laser microscopy immunofluorescence studies, incubation with CST resulted in the sequestration of the [Thr^{69},Thr^{100}, Thr^{293}] VIP receptor mutant in the perinuclear endoplasmic reticulum whereas it did not affect the delivery of the [Thr^{58}, Thr^{100},Thr^{293}] VIP receptor mutant to the plasma membrane (not shown). Incubation with dMM did not modify the delivery of either mutant to the plasma membrane. Consistent with these observations, the wild-type receptor which is glycosylated at Asn^{58} and Asn^{69} was correctly delivered to the plasma membrane in both CST- and dMM-treated cells (not shown). Further experiments were conducted in order to determine the specific binding of [^{125}I]VIP to intact cells under the above-described experimental conditions. It appeared that incubation with CST resulted in an important decrease in the specific binding of [^{125}I]VIP in cells transfected with the [Thr^{69},Thr^{100},Thr^{293}] VIP receptor mutant cDNA whereas no alteration of [^{125}I]VIP binding was observed for the [Thr^{58},Thr^{100},Thr^{293}] VIP receptor mutant or the wild-type receptor (Figure 5). Also consistent with confocal microscopy analysis was the fact that cell incubation with dMM did not modify the specific [^{125}I]VIP binding to cells transfected with either of the two mutant cDNAs or the wild-type receptor cDNA (Figure 5).

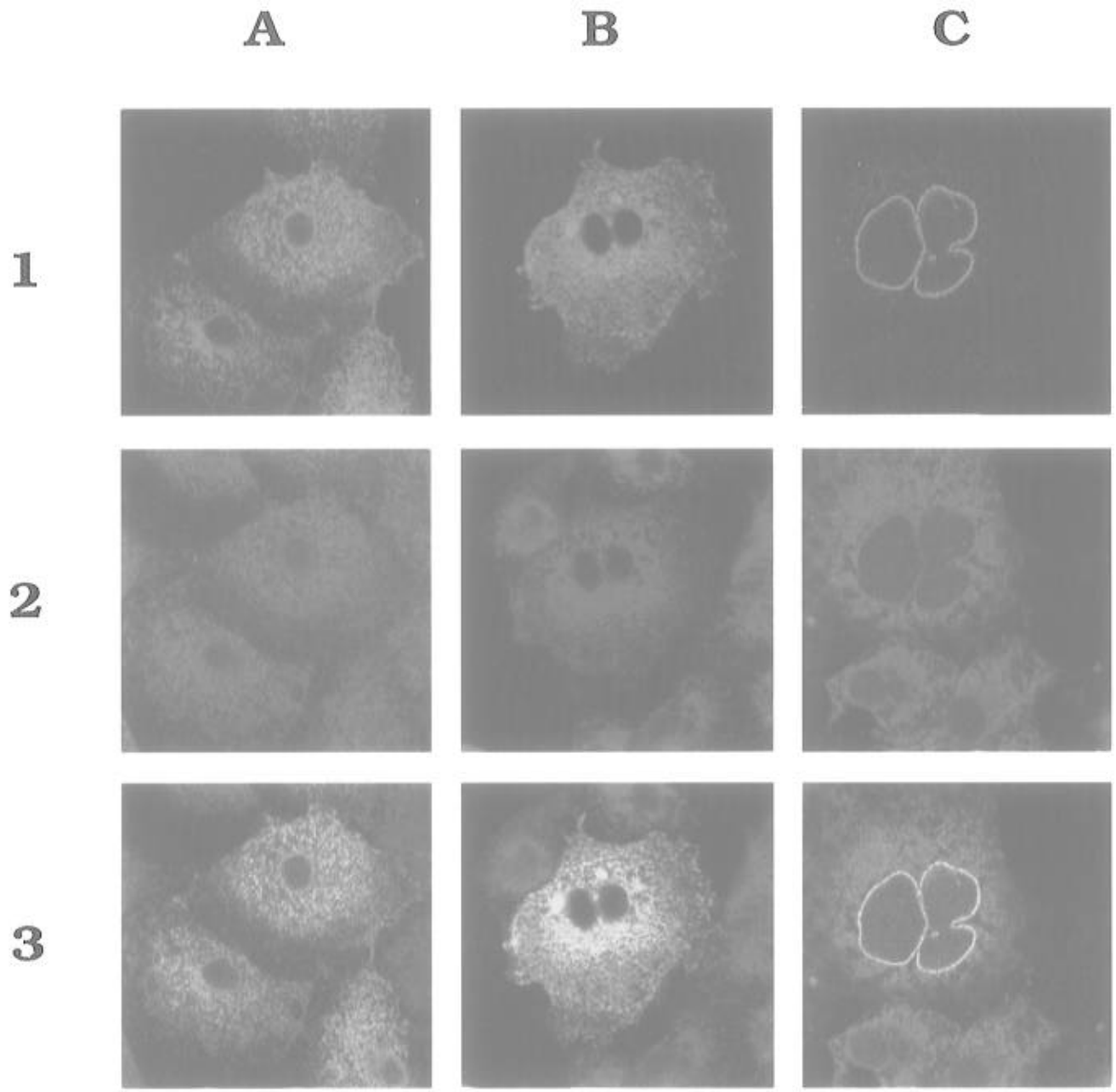

FIGURE 4: Confocal laser scanning microscopic detection of the wild-type receptor, [Thr^{58}] receptor, and [Thr^{58},Thr^{69},Thr^{100},Thr^{293}] receptor mutants after transfection into Cos-7 cells. The wild-type receptor (column A), the [Thr^{58}] VIP receptor (column B), and the [Thr^{58},Thr^{69}, Thr^{100},Thr^{293}] VIP receptor (column C) were analyzed by confocal laser scanning microscopy after transfection of the corresponding cDNAs into Cos-7. Transfected cells were incubated with the mouse monoclonal anti-Tag antibody 9E10 (row 1), a rabbit polyclonal anti-endoplasmic reticulum antibody (row 2), or both for double-labeling experiments (row 3) as described in detail under Materials and Methods. The wild-type receptor is expressed at the plasma membrane (A1) and also colocalizes (A3) with an endoplasmic reticulum marker (A2). A similar pattern is observed for the [Thr^{58}] VIP receptor mutant (column B). In contrast, the [Thr^{58},Thr^{69},Thr^{100},Thr^{293}] VIP receptor mutant is strictly detected in the perinuclear zone (C1) where it colocalizes with the endoplasmic reticulum marker (C3). However, this mutant cannot be detected in the whole endoplasmic reticulum (C2). It should be noted that column C shows the perinuclear localization of the [Thr^{58},Thr^{69},Thr^{100},Thr^{293}] VIP receptor mutant in a dividing cell but that identical perinuclear localization has been readily observed in nondividing cells after numerous transfections of Cos-7 cells. It has been verified that untransfected Cos-7 cells incubated with the mouse monoclonal anti-Tag antibody 9E10 gave no staining.

DISCUSSION

To the best of our knowledge, this work represents the first investigation of the role of consensus sequences for N-glycosylation in the human VIP 1 receptor and also in the new subfamily of heptahelical G protein-coupled receptors to which belong VIP receptors. Systematic site-directed mutagenesis of each of the four consensus N-glycosylation sites in the human VIP 1 receptor followed by expression of mutated proteins into Cos-7 cells and analysis of their molecular weight by western blot and affinity cross-linking suggested that the three sites in the N-terminal extracellular domain (Asn^{58}, Asn^{69}, and Asn^{100}) of the receptor are used in Cos-7 cells and are occupied by a carbohydrate moiety, the apparent molecular weight of which can be roughly estimated at 9000. In contrast, the site located in the second extracellular loop (Asn^{293}) appears to be free of N-linked carbohydrate. These data are in line with a previous biochemical study on VIP receptors in a human pancreatic

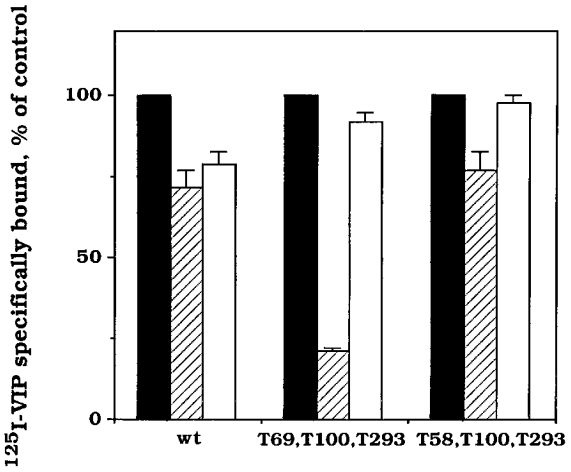

FIGURE 5: Specific [^{125}I]VIP binding to intact Cos-7 cells transfected with the wild-type receptor, [Thr^{58},Thr^{100},Thr^{293}] VIP receptor, or [Thr^{69},Thr^{100},Thr^{293}] VIP receptor mutant cDNAs: Effects of castanospermine and deoxymannojirimycin. After transfection with cDNAs encoding the wild-type receptor (wt), [Thr^{58},Thr^{100}, Thr^{293}] VIP receptor (T58, T100, T293), or [Thr^{69},Thr^{100},Thr^{293}] VIP receptor (T69, T100, T293) mutants, Cos-7 cells were grown for 2 days in standard culture medium (dark bars) or standard culture medium containing either 1 mM castanospermine (hatched bars) or 1 mM deoxymannojirimycin (empty bars). After the culture medium was removed and washed with 500 μL of PBS, the binding assay was carried out for 60 min at 30 °C with 0.05 nM [^{125}I]VIP in 400 μL of PBS containing 2%(w/v) BSA and 1 mg/mL bacitracin. At the end of the incubation, the medium was discarded and cells were gently washed with 400 μL of PBS before counting. Specific binding was calculated as the difference between the amount of [^{125}I]VIP bound in the absence and the presence of 1 μM unlabeled VIP. Results are expressed as percent of control, i.e., specific binding observed for cells grown in standard culture medium. They are the means ± SEM of three experiments.

cell line which showed that the kinetics of deglycosylation of VIP receptors by PNGase F was consistent with the presence of three complex-type carbohydrate chains in the receptor, each accounting for 9−10 kDa in its apparent molecular mass (Fabre et al., 1987). It can be assumed from the present study that these complex-type carbohydrate chains are N-linked at Asn^{58}, Asn^{69}, and Asn^{100} in the human VIP 1 receptor. Altogether, these observations are consistent with the optimal conditions for N-glycosylation of consensus sites within extracellular domains of multispan membrane proteins (Landolt-Marticorena & Reithmeier, 1994): (i) the acceptor site is well spaced (greater than 10 residues) from the transmembrane domain; this is true for the three sites in the N-terminal domain of the human VIP 1 receptor (Couvineau et al., 1994a); (ii) the loop should be greater than 30 residues in size. The second extracellular loop which contains Asn^{293} is 21 residues in size in the human VIP 1 receptor (Couvineau et al., 1994a); (iii) the N-glycosylated segment is the first in the protein to contain a suitable extracytosolic consensus site.

This work provides several major contributions over previous biochemical studies (Nguyen et al., 1986; El Battari et al., 1987, 1991; Fabre et al., 1987, 1993; Laburthe & Couvineau, 1988) regarding the role of N-glycosylation in the structure−function relationship of the human VIP 1 receptor. We have shown that mutations of each of the four individual consensus N-glycosylation sites do not alter the intrinsic binding affinity of the receptor. Nor do they modify its correct delivery to the plasma membrane of transfected Cos-7 cells as demonstrated by confocal laser scanningmicroscopy. In fact, glycosylation at Asn^{58} or Asn^{69} appears to be necessary and sufficient to ensure both complete intrinsic activity and delivery of the human VIP 1 receptor to the plasma membrane. However, these two properties are clearly not dependent on each other as demonstrated by the behavior of the VIP receptor mutant devoid of consensus N-glycosylation sites. Indeed, no [^{125}I]VIP binding can be detected on Cos-7 cells transfected with this mutant due to its intracellular sequestration in the perinuclear endoplasmic reticulum. However, when extracted by CHAPS from transfected Cos-7 cell homogenates, a significant proportion of this mutant protein retains its ability to bind VIP with a dissociation constant which is similar to that of the wild-type receptor. This observation suggests that the intrinsic binding activity of the receptor may be unrelated to the glycosylation of the receptor protein. Another interesting observation in this study is that all glycosylation sites are not equally relevant for correct delivery of the receptor to the plasma membrane. Indeed, when the consensus N-glycosylation site at Asn^{100} is left on its own by site-directed mutagenesis, the corresponding mutated receptor protein is sequestered in the perinuclear endoplasmic reticulum like the mutant devoid of N-glycosylation sites. This suggests that among the three N-glycosylation sites used by Cos-7 cells in the N-terminal extracellular domain of the receptor only Asn^{58} or Asn^{69} is relevant for delivery of the receptor to the plasma membrane. The reason why some of the above-described receptor mutants are sequestered in the endoplasmic reticulum but only at the perinuclear level is unknown but is in line with the current view of the endoplasmic reticulum as a dynamic patchwork of specialized subregions (Sitia & Meldolesi, 1992). Since transfer of oligosaccharide from oligosaccharide−lipid donor to protein occurs in the endoplasmic reticulum (Hirschberg & Snider, 1987), our results suggest that N-linked oligosaccharide chains on Asn^{58} or Asn^{69} are required for transport of the VIP receptor precursor out of the endoplasmic reticulum. It may be worth pointing out that the extracellular N-terminal tail of the receptor coding sequence is interrupted by four introns (Sreedharan et al., 1995) resulting in the presence of the crucial Asn^{58} and Asn^{69} on different exons, e.g., exons 2 and 3, respectively.

Our data also indicated that the sites of N-glycosylation at Asn^{58} or Asn^{69} which are crucial for correct delivery of the VIP receptor to the plasma membrane are not equivalent in terms of the mechanism whereby the cell machinery ensures the process of folding and quality control of the VIP receptor. Indeed, when the glycosylation site at Asn^{58} is left on its own by site-directed mutagenesis, the corresponding mutated receptor protein is sequestered in the perinuclear endoplasmic reticulum upon treatment of cells with castanospermine and a very low VIP binding activity can be detected on castanospermine-treated cells (see Figure 5). Since castanospermine inhibits glucosidases I and II, impeding glucose trimming from the 14 oligosaccharide N-linked core glycan in the endoplasmic reticulum and thereby calnexin binding to the monoglucosylated core glycans (Herbert et al., 1995), our data suggest that carbohydrate at Asn^{58} of the VIP receptor is involved in a calnexin-dependent process of folding and quality control of the receptor. In contrast,

castanospermine does not modify the expression of VIP binding activity or the delivery to the plasma membrane of the mutated VIP receptor that only retains an N-glycosylation site at Asn^{69} (see Figure 5). This suggests that the carbohydrate at Asn^{69} is involved in a calnexin-independent conformational maturation process of the VIP receptor. This is consistent with the current view indicating that the cell is not entirely dependent on the calnexin-mediated folding pathway since there are glycoproteins that can bypass this mechanism (Helenius, 1994). The existence of a calnexin-dependent mechanism of protein folding and control of quality for the glycosylation site at Asn^{58} and a calnexin-independent mechanism for the glycosylation site at Asn^{69} is consistent with the absence of an effect of castanospermine on the binding activity and delivery to the plasma membrane of the wild-type receptor which is glycosylated at both sites. Finally, deoxymannojirimycin, an inhibitor of α-mannosidase I involved in the removal of mannose residues from the high-mannose glycans in the Golgi apparatus (Hirschberg & Snider, 1987), does not modify the binding activity and delivery to the plasma membrane of the wild-type receptor or the mutated receptors with a single glycosylation site at Asn^{58} or Asn^{69} (see Figure 5). This observation is consistent with the fact that the folding process of receptor proteins has been completed, allowing them to leave the endoplasmic reticulum (Fielder & Simmons, 1995). It also indicates that complex N-linked glycosylation of the VIP 1 receptor is not necessary for ligand binding.

In conclusion, we have demonstrated that either of the two consensus N-glycosylation sites at Asn^{58} or Asn^{69} within the N-terminal extracellular domain of the human VIP 1 receptor is crucial for correct delivery of the receptor protein to the plasma membrane. However, glycosylation at Asn^{58} and Asn^{69} is involved in calnexin-dependent and -independent mechanisms of protein folding and quality control, respectively. The absence of these two sites results in the sequestration of the receptor protein in the perinuclear endoplasmic reticulum. However, even in the total absence of consensus N-glycosylation sites the receptor protein still binds VIP when extracted from its intracellular store with detergent. These data further document the crucial role of the N-terminal extracellular domain of the human VIP 1 receptor not only for ligand binding (Couvineau et al., 1995) but also for delivery to the plasma membrane. Whether this domain is also crucial in other members of the new family of seven membrane-spanning receptors to which VIP receptors belong remains to be established.

ACKNOWLEDGMENT

We thank Daniel Louvard (Institut Curie, Paris) for his kind gift of rabbit polyclonal anti-endoplasmic reticulum antibody and Patrice Codogno and Stuart Moore for helpful discussions. We also thank the IFR "Cellules Epithéliales" for confocal microscopy facilities.

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BI952022H