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Identification of the Sites of *N*-Linked Glycosylation on the Human Calcium Receptor and Assessment of Their Role in Cell Surface Expression and Signal Transduction*

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The human calcium receptor (hCaR) is a G-protein-coupled receptor containing 11 potential *N*-linked glycosylation sites in the large extracellular domain. The number of potential *N*-linked glycosylation sites actually modified, and the effect on cell surface expression and signal transduction of blocking glycosylation at these sites, was examined by site-directed mutagenesis. Asparagine residues of the consensus sequences (Asn-Xaa-Ser/Thr) for *N*-linked glycosylation were mutated to glutamine individually and in various combinations to disrupt the potential *N*-linked glycosylation sites in the context of the full-length receptor. The cDNA constructs were transiently transfected into HEK-293 cells lacking endogeneous hCaR, and expressed receptors were analyzed by mobility differences on immunoblots, glycosidase digestion, intact cell enzyme-linked immunoassay, and extracellular calcium-stimulated phosphoinositide hydrolysis assay. Immunoblot analyses and glycosidase digestion studies of the wild type *versus* mutant receptors demonstrate that, of the 11 potential sites for *N*-linked glycosylation, eight sites (Asn-90, -130, -261, -287, -446, -468, -488, and -541) are glycosylated; the three remaining sites (Asn-386, -400, and -594) may not be efficiently glycosylated in the native receptor. Sequential mutagenesis of multiple *N*-linked glycosylation sites and analyses by immunoblotting, immunofluorescence, biotinylation of cell surface proteins, and intact cell enzyme-linked immunoassay indicated that disruption of as few as three glycosylation sites impairs proper processing and expression of the receptor at the cell surface. Disruption of five glycosylation sites reduced cell surface expression by 50–90% depending on which five sites were disrupted. Phosphoinositide hydrolysis assay results for various glycosylation-defective mutant receptors in general correlated well with the level of cell surface expression. Our results demonstrate that among 11 potential *N*-linked glycosylation sites on the hCaR, eight sites are actually utilized; glycosylation of at least three sites is critical for cell surface expression of the receptor, but glycosylation does not appear to be critical for signal transduction.

The calcium receptor (CaR)[1] is a G-protein-coupled-receptor (GPCR) involved in extracellular calcium homeostasis by controlling the rate of parathyroid hormone secretion from the parathyroid gland and the rate of calcium reabsorbtion by the kidney (1). Recent evidence suggests that the CaR is also involved in diverse cellular responses to extracellular calcium within microenvironments in other organs such as brain, skin, bone, and intestine (2). Extracellular calcium ion ($[Ca^{2+}]_o$) activates the CaR, leading to activation of phospholipase-C_β via the G_q subfamily of G-proteins; this increases phosphoinositide (PI) hydrolysis, which in turn causes release of calcium from intracellular stores (3).

The CaR, metabotropic glutamate receptors (4), and a subgroup of putative pheromone receptors in the vomeronasal organ (5–7) comprise a unique subset of the superfamily of GPCRs characterized by a relatively large (500–600-residue) amino-terminal extracellular domain (ECD). The ECD of the human CaR (hCaR) (8) contains 11 potential *N*-linked glycosylation sites (9), of which nine are highly conserved in bovine (3), rat (10, 11), rabbit (12), and chicken (13) CaRs. The remaining two sites are conserved among human, rabbit, and chicken CaRs, but only one of the two (Asn-386 or -400 in the hCaR sequence) is conserved in rat and bovine CaRs, respectively.

The carbohydrate moieties of glycoproteins in general are believed to be important for facilitating protein folding, protection from proteolysis, intracellular trafficking, secretion, and cell surface expression (14, 15). They may also be important for maintaining protein conformation, enzymatic activity, and other structural functions (15, 16). Among GPCRs, the role of carbohydrate moieties is somewhat less clear, with variable effects on ligand binding, signal transduction, and cell surface expression (17–23). The function of *N*-linked glycosylation for the CaR or other members of this unique GPCR subfamily with significantly larger ECDs and a greater number of putative *N*-linked glycosylation sites has not been studied extensively. Our previous study with tunicamycin indicated that inhibition of *N*-linked glycosylation blocks normal cell surface expression of the hCaR (24). However, this study did not permit determination of the precise number and location of *N*-linked glycosylation sites, nor did it allow us to define distinctive roles, if any, of specific sites in the processing, cell surface expression, and signal transduction of the hCaR. To address these questions, we mutated the potential *N*-linked glycosylation sites of the hCaR, substituting glutamine for asparagine to disrupt glycosylation, and transfected the mutant receptor cDNAs into

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[1] The abbreviations used are: CaR, calcium receptor; hCaR, human calcium receptor; GPCR, G-protein-coupled receptor; PI, phosphoinositide; ECD, extracellular domain; HEK-293 cells, human embryonic kidney-293 cells; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; PNGase F, peptide *N*-glycosidase F; Endo H, endo-β-N-acetylglucosaminidase H; BSA, bovine serum albumin; PIPES, 1,4-piperazinediethanesulfonic acid; Biotin-7-NHS, D-biotinoyl-ϵ-aminocaproic acid-*N*-hydroxysuccinimide ester; WT, wild type.

HEK-293 cells to analyze the effects on expression and function.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis of the hCaR—The hCaR cDNA construct in the eukaryotic expression vector pCR3.1 has been described (25). Site-directed mutagenesis to create the mutants listed in Table I was performed on hCaR cDNA in the pCR3.1 vector using a commercial kit (QuikChange™ site-directed mutagenesis kit, Stratagene Inc., La Jolla, CA), according to the manufacturer's instructions. Briefly, a pair of complementary primers with 25–35 bases was designed for each mutagenesis, and the mutation to change asparagine to glutamine was placed in the middle of the primers (sequences of all primers used available from the authors upon request). Parental hCaR inserted in pCR3.1 was amplified using *Pfu* DNA polymerase with these primers for 12 cycles in a DNA thermal cycler (Perkin-Elmer). After digestion of the parental DNA with *Dpn*I, the amplified DNA incorporated with the nucleotide substitution was transformed into *Escherichia coli* (DH-5α strain). The mutations were confirmed by automated DNA sequencing using a *Taq* DyeDeoxy Terminator Cycle Sequencing kit and ABI prizm-377 DNA sequencer (Applied Biosystems, Foster City, CA). Mutants with multiple *N*-linked glycosylation sites disrupted were created by changing asparagine to glutamine for a given *N*-linked glycosylation site and using this mutant DNA as template in sequential rounds of mutagenesis. For most mutants, we confirmed that two independent clones of the same mutant receptor cDNA showed identical properties.

Transient Transfection of Wild Type and Mutant hCaR Receptors in HEK-293 Cells—Receptor cDNAs in pCR 3.1 (Invitrogen, San Diego, CA) were prepared by a Qiagen maxiplasmid DNA preparation kit (Qiagen Inc., Chatsworth, CA) and were introduced into HEK-293 cells by Lipofectamine (Life Technologies Inc.) transfection method. The quality and quantity of plasmid DNA was electrophoretically assessed on an 0.8% agarose gel after measuring OD at 260 nm. For transfection, a given amount of the plasmid DNA was diluted in Dulbecco's modified Eagle's medium (DMEM) (BioFluids Inc., Rockville, MD) and mixed with diluted Lipofectamine, and the mixture was incubated at room temperature for 30 min. The DNA-Lipofectamine complex was further diluted in serum-free DMEM and added to 80–90% confluent HEK-293 cells plated in 75-cm^2 flasks using 10–15 μg of DNA. After 4–5 h of incubation, an equal volume of DMEM plus 20% fetal bovine serum (FBS) (BioFluids Inc., Rockville, MD) was added, and the media were replaced 24 h after transfection with fresh DMEM plus 10% FBS. Membrane protein extraction for immunoblotting, whole cell enzyme-linked immunoassay, or PI hydrolysis assay were performed 48 h after transfection.

Immunoblotting Analyses with Detergent-solubilized Cell Membrane Extracts—Confluent cells in 75-cm^2 flasks were rinsed with ice-cold phosphate-buffered saline (PBS), scraped on ice, and suspended in buffer A containing 5 mM Tris (pH 7.2), 2 mM EDTA, and Complete™ protease inhibitor mixture (Boehringer Mannheim) at 4 °C. The cells were forced through a 22-gauge needle 5–8 times, and the lysate was spun in a TLA-45 centrifuge at 45,000 rpm for 30 min at 4 °C to collect a crude membrane pellet. The pellet was resuspended in buffer B containing 20 mM Tris-HCl (pH 6.8), 150 mM NaCl, 10 mM EDTA, 1 mM EGTA, 1% Triton X-100 with freshly added protease inhibitor mixture. The protein content of each sample was determined by the modified Bradford method (Bio-Rad), and 40–60 μg of protein/lane was separated on 7.5% SDS-PAGE. The proteins on the gel were electrotransferred to nitrocellulose membrane and incubated with 0.1 μg/ml protein A-purified monoclonal anti-hCaR antibody, ADD (raised against a synthetic peptide corresponding to residues 214–235 of the hCaR protein) (26). Subsequently, the membrane was incubated with a secondary goat anti-mouse antibody conjugated to horseradish peroxidase (Kierkegaard and Perry Laboratories, Gaithersburg, MD) at a dilution of 1:5000. The hCaR protein was detected with an ECL system (Amersham Pharmacia Biotech).

Treatment of Detergent-solubilized Crude Cell Membrane Extracts with Glycosidases—To detergent-solubilized cell extracts, prepared for immunoblotting as described, we added the following concentrations of enzyme. For cleavage with *N*-glycosidase F (PNGase F), membrane preparations in buffer B (20 μl, approximately 40–60 μg of protein) were mixed with 1 μl of 10% SDS for denaturation. Samples were subsequently incubated with 0.8 unit of PNGase F (Boehringer Mannheim) for 2 h at 37 °C. For cleavage with endoglycosidase H (Endo H) (Boehringer Mannheim), the membrane preparation (20 μl) was mixed in 20 μl of 50 mM sodium acetate (pH 4.8). Samples were incubated with 0.5 milliunits of Endo H for 2 h at 37 °C.

Intact Cell Enzyme-linked Immunoassay to Determine Cell Surface Expression—To compare cell surface expression of receptors in transiently transfected HEK-293 cells, cells were plated and transfected with plasmid DNA in 75-cm^2 flasks as described above. 24 h after transfection, cells were released from the surface with trypsin. One half of the cells released were replated in another 75-cm^2 flask with complete DMEM plus 10% serum for intact cell enzyme-linked immunoassay, and the other half were plated on a 12-well plate, to be used for experiments involving PI hydrolysis assay. 48 h after transfection, transfected HEK-293 cells in 75-cm^2 flasks were detached with 1 mM EDTA in PBS containing 0.5% bovine serum albumin (BSA) and incubated with 0.5 ml of DMEM containing 10% FBS and 1 μg/ml monoclonal anti-hCaR antibody 7F8 or 7B10 (raised against purified extracellular domain of hCaR)[2] at 4 °C for 2 h. Following incubation, cells were pelleted by low speed centrifugation (1000 rpm, 4 °C) and washed three times with PBS. Cells were then incubated with 5 μg/ml peroxidase-conjugated goat anti-mouse IgG (γ) (Kierkegaard and Perry Laboratories) in DMEM containing 10% FBS for 1 h at 4 °C. After three washes with PBS, peroxidase substrate (2.5 mM each of H_2O_2 and *o*-phenylenediamine in 0.1 M phosphate-citrate buffer, pH 5.0) was added to each sample, and color reaction was followed for 5–10 min. Cells were precipitated by centrifugation, and absorbance of the supernatant was measured at 405 nm using a Thermo$_{max}$ microtiter plate reader (Molecular Devices, Sunnyvale, CA).

Biotinylation of the Cell Surface hCaR—HEK-293 cells were transfected in six-well plates using 1 μg of DNA and 25 μl of Lipofectamine per well as described above. 48 h after transfection, cell surface proteins of the intact HEK-293 cells were labeled with membrane-impermeant Biotin-7-NHS using the cellular labeling kit (Boehringer Mannheim). Briefly, adherent cells were washed twice with ice-cold PBS, treated with 50 μg/ml Biotin-7-NHS in biotinylation buffer (50 mM sodium borate, 150 mM NaCl) for 15 min at room temperature to biotinylate cell surface proteins. The reaction was stopped by adding 50 mM NH_4Cl for 15 min on ice. The cells were washed twice with ice-cold PBS and solubilized with 1 ml of buffer B/well containing 1% Triton X-100, 20 mM Tris-HCl (pH 6.8), 150 mM NaCl, 10 mM EDTA, 1 mM EGTA and with freshly added protease inhibitor mixture.

Immunoprecipitation of Biotinylated hCaR—300 μl of the whole cell lysate containing approximately 600 μg of total protein was further diluted with 300 μl of buffer B and incubated with 5 μl of 7F8 monoclonal hCaR-specific antibody (1 mg/ml stock) for 1 h at 4 °C. Subsequently, 25 μl of protein A/G-agarose (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added, and the incubation was continued for an additional 1 h. The protein A/G-agarose was washed three times with buffer B containing 1% SDS, and the immunoreactive proteins were eluted in 120 μl of 1× sample buffer at 70 °C for 5 min. 40 μl of sample was loaded per lane, and immunoblotting was performed as described above. Biotinylated forms were detected using peroxidase-conjugated Streptavidin followed by visualization of the biotinylated bands using a BM chemiluminescence kit (Boehringer Mannheim).

Immunofluorescence Microscopy—HEK-293 cells transiently transfected with WT and mutant hCaRs were grown on Permanox chamber slides (Nunc International Corp., Naperville, IL), washed twice with PBS, and fixed for 30 min with freshly prepared paraformaldehyde (4%, v/v) in PBS. After two rinses with PBS, cells were transferred either immediately or after permeabilization (in methanol at −20 °C for 3 min) into blocking solution (5% goat serum, 1% BSA in PBS) and incubated for 30 min. Cells were washed once in PBS plus 1% BSA and incubated in PBS plus 1% BSA containing monoclonal anti-hCaR antibody 7F8 (dilution of 1:100) for 2 h at 24 °C. Cells were washed in PBS plus 1% BSA three times prior to incubation with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Kierkegaard and Perry Laboratories) at a dilution of 1:200 for 1 h at 24 °C. After three final washes with PBS, cells were mounted with Fluoromont-G (Electron Microscopy Sciences, Ft. Washington, PA) and examined in a Zeiss Axiophot fluorescence microscope. Transfection efficiency as judged by counting the immunofluorescent cells after transfection and immunostaining is estimated to be approximately 30%.

Phosphoinositide Hydrolysis Assay—The PI hydrolysis assay has been described (24, 27). Briefly, 24 h after transfection, as described above, one half of the total transfected cells from a confluent 75-cm^2 flask were replated in a 12-well plate in medium containing 3.0 μCi/ml [^{3}H]myoinositol (NEN Life Science Products) in complete DMEM for another 24 h, followed by a 1-h preincubation with 1× PI buffer (120 mM NaCl, 0.5 mM $CaCl_2$, 5 mM KCl, 5.6 mM glucose, 0.4 mM $MgCl_2$, 20 mM

[2] P. K. Goldsmith and A. M. Spiegel, manuscript in preparation.

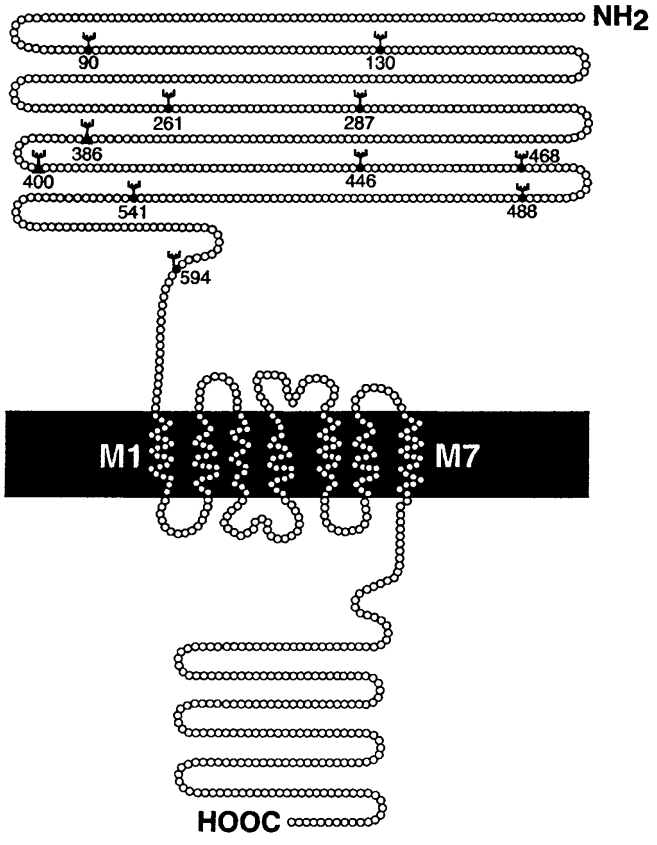

FIG. 1. **Schematic representation of the proposed topology of the hCaR, depicting locations of putative *N*-linked glycosylation sites.** Each *circle* represents a single amino acid residue. Putative *N*-linked glycosylation sites are indicated in the extracellular domain on asparagine residues at positions 90, 130, 261, 287, 386, 400, 446, 468, 488, 541, and 594. Nine highly conserved sites are marked as *solid black circles*, and two semiconserved sites are marked as *shaded triangles*. *M1* and *M7* represent the first and seventh of the seven transmembrane domains, with the *shaded region* denoting the plasma membrane. NH_2 and *COOH* represent the extracellular amino terminus and intracellular carboxyl terminus, respectively.

LiCl in 25 mM PIPES, pH 7.2). After removal of PI buffer, cells were incubated for an additional 30 min with different $[Ca^{2+}]_o$ in PI buffer. The reactions were terminated by the addition of 1 ml of acid-methanol (1:1000, v/v) per well. Total inositol phosphates were purified by chromatography on Dowex 1-X8 columns.

RESULTS

Identification of N-Linked Glycosylation Sites on the hCaR—Analysis of the hCaR cDNA identified 11 potential consensus sequences for *N*-linked glycosylation within the ECD of the receptor located at amino acid positions Asn-90, -130, -261, -287, -386, -400, -446, -468, -488, -541, and -594 (Fig. 1). Except for Asn-386 and -400, all other nine sites are highly conserved in human, bovine, rat, rabbit, and chicken CaRs and are henceforth referred to as highly conserved sites. Asn-386 is conserved in all above species except bovine, and Asn-400 is conserved in all above species except rat; these two sites are referred to here as semiconserved sites. To identify which sites are actually glycosylated, we first created a set of hCaR glycosylation-defective mutants (series I) listed in Table I. The mutant receptors comprising series I were constructed such that only one or two glycosylation consensus sites within the hCaR were disrupted by substitution of the Asn of the consensus site, Asn-Xaa-Ser/Thr, to Gln. The mutant receptors were expressed in HEK-293 cells, and their electrophoretic mobility compared with that of the wild type hCaR was assessed by immunoblot. The monoclonal antibody, ADD, used for immunoblots was made against a peptide epitope from the ECD, which does not encompass any of the potential glycosylation sites, and has been shown to bind strongly to the denatured hCaR (26).

As shown in Fig. 2, the wild type hCaR showed two characteristic immunoreactive bands of approximately 150 and 130 kDa (25, 28). Each of the series I mutants with one or two putative *N*-linked glycosylation consensus sites disrupted showed two distinct immunoreactive bands like the wild type hCaR. Direct comparison of the mobility of immunoreactive bands of the mutant *versus* wild type receptors revealed subtle but reproducible differences for certain of the glycosylation-defective mutants. Mutant receptors with a single site disrupted at Asn-90, -130, -261, -287, -446, -468, -488, or -541 all showed a small increase in mobility of both immunoreactive bands compared with the wild type hCaR. In contrast, single site mutants at Asn-594 and at either of the two semiconserved sites, Asn-386 or -400, showed no difference in mobility of their corresponding immunoreactive bands. Double mutants with two glycosylation sites disrupted at positions 90 and 130, 261 and 287, 446 and 468, and 468 and 488 all showed an even greater increase in mobility of immunoreactive bands compared with single site mutants. The immunoreactive bands of the double mutant at positions 541 and 594, however, showed identical mobility to that of the 541 single site mutant. The double mutant at positions 386 and 400 gave bands of the same size as wild type receptor. We interpret these data as indicating that sites Asn-386, -400, and -594 are not efficiently glycosylated in the hCaR expressed in transfected HEK-293 cells. Hence, disruption of the glycosylation consensus sequence at these sites does not alter receptor mass or mobility as measured on immunoblot of membranes from transfected cells. Asparagines at positions 90, 130, 261, 287, 446, 468, 488, and 541, in contrast, are glycosylated in the context of the native receptor expressed in transfected cells. Disruption of any of these sites causes a decrease in mass of the receptor, and disruption of two of these sites causes an additive decrease in glycosylation with corresponding changes in mobility of receptor glycoprotein on immunoblot.

To further explore the implications of the above findings, we created the series II and III hCaR mutants in which a larger number of glycosylation sites were disrupted (Table I). We first generated a mutant, N1–8Q, in which the eight sites determined above to be glycosylated were changed from Asn to Gln. Immunoblot of membranes from cells transfected with this mutant showed a single immunoreactive band (Fig. 3*A*), in contrast to the two bands typically seen for wild type and single and double site mutants studied above. This band was close to the 115-kDa size predicted for a nonglycosylated CaR protein, but upon digestion of membranes with either PNGase F or Endo H, there was a small but definite increase in mobility indicating that the N1–8Q mutant still undergoes some degree of glycosylation (Fig. 3*A*, *left*). We next studied a mutant with all 11 putative glycosylation sites disrupted, N1–11Q. Again, a single immunoreactive band was visualized in membranes from cells transfected with this mutant, but its size was smaller than that of the N1–8Q mutant, and it showed no change in mobility upon digestion of membranes with either enzyme, suggesting that the N1–11Q mutant in fact was not glycosylated (Fig. 3*A*, *right*).

We then studied a series of mutants (series II, Table I) in which one of the eight sites shown to be glycosylated in Fig. 2 was retained, while the other 10 potential glycosylation sites were disrupted. As seen in Fig. 3*B*, each of these mutants gave a single immunoreactive band like the nonglycosylated mutant N1–11Q, but the bands for each of the mutants retaining a single potential glycosylation site showed slightly decreased

TABLE I
hCaR mutants studied to determine sites of N-linked glycosylation

Series I receptor mutant	Consensus sequence(s) disrupted
Highly conserved sites	
hCaR(N90Q)	Asn-90
hCaR(N130Q)	Asn-130
hCaR(N261Q)	Asn-261
hCaR(N287Q)	Asn-287
hCaR(N446Q)	Asn-446
hCaR(N468Q)	Asn-468
hCaR(N488Q)	Asn-488
hCaR(N541Q)	Asn-541
hCaR(N594Q)	Asn-594
hCaR(N90Q/N130Q)	Asn-90 and Asn-130
hCaR(N261Q/N287Q)	Asn-261 and Asn-287
hCaR(N446Q/N468Q)	Asn-446 and Asn-468
hCaR(N468Q/N488Q)	Asn-468 and Asn-488
hCaR(N541Q/N594Q)	Asn-541 and Asn-594
Semiconserved sites	
hCaR(N386Q)	Asn-386
hCaR(N400Q)	Asn-400
hCaR(N386Q/N400Q)	Asn-386 and Asn-400
Series II receptor mutant	**Consensus sequence(s) maintained**
hCaR(N1–11Q)	None
hCaR(N1–11Q)$_{+90}$	Asn-90
hCaR(N1–11Q)$_{+130}$	Asn-130
hCaR(N1–11Q)$_{+261}$	Asn-261
hCaR(N1–11Q)$_{+287}$	Asn-287
hCaR(N1–11Q)$_{+446}$	Asn-446
hCaR(N1–11Q)$_{+468}$	Asn-468
hCaR(N1–11Q)$_{+488}$	Asn-488
hCaR(N1–11Q)$_{+541}$	Asn-541
Series III receptor mutant	**Consensus sequence(s) disrupted**
hCaR(N1–3Q)	Asn-90, Asn-130, and Asn-261
hCaR(N1–4Q)	Asn-90, Asn-130, Asn-261, and Asn-287
hCaR(N1–5Q)	Asn-90, Asn-130, Asn-261, Asn-287, and Asn-446
hCaR(N1–6Q)	Asn-90, Asn-130, Asn-261, Asn-287, Asn-446, and Asn-468
hCaR(N1–7Q)	Asn-90, Asn-130, Asn-261, Asn-287, Asn-446, Asn-468, and Asn-488
hCaR(N1–8Q)	Asn-90, Asn-130, Asn-261, Asn-287, Asn-446, Asn-468, Asn-488, and Asn-541
Series IV receptor mutant	**Consensus sequence(s) disrupted**
hCaR(N4–8Q)	Asn-287, Asn-446, Asn-468, Asn-488, and Asn-541
hCaR(N1,3,5,7,8)Q	Asn-90, Asn-261, Asn-446, Asn-488, and Asn-541
hCaR(N5–8Q)	Asn-446, Asn-468, Asn-488, and Asn-541

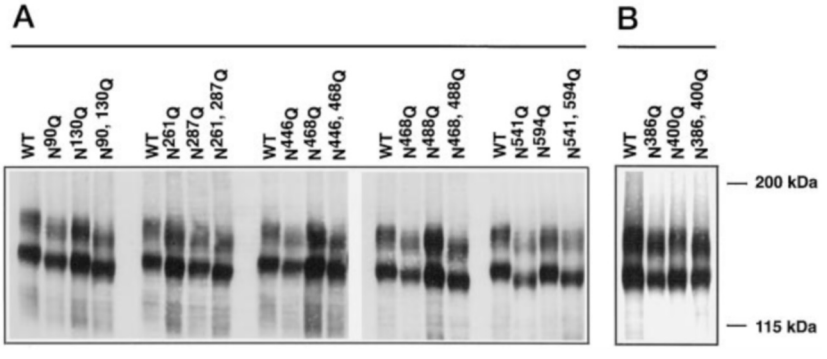

FIG. 2. **Immunoblots of membranes from HEK-293 cells transfected with hCaR series I mutants.** Crude membrane extracts were prepared from HEK-293 cells that were transiently transfected with wild type and mutant hCaR plasmid cDNAs as described under "Experimental Procedures." 40 μg of membrane protein from cells transfected with each cDNA were loaded in each lane (labeled at the *top*; mutants correspond to those listed in Table I, series I, highly conserved in *panel A* and semiconserved in *panel B*) and fractionated on a 7.5% gel by SDS-PAGE. Immunoblotting was performed with monoclonal anti-hCaR antibody ADD. Two major immunoreactive bands corresponding to ~150- and ~130-kDa *N*-linked glycosylated forms of the wild type hCaR are seen for each mutant. The blot shown here is representative of results seen in multiple independent transfections and immunoblots with series I mutants. The positions of molecular size standards are indicated on the *right*.

mobility compared with that of the N1–11Q mutant. After PNGase F or Endo H digestion, moreover, each of the bands corresponding to the series II mutants with one site retained showed an increase in mobility, unlike the N1–11Q mutant band, which was unchanged after enzymatic digestion (Fig. 3*B*). This indicated that each of the series II mutants had in fact undergone glycosylation at the site retained. Based on these observations, we conclude that Asn-386, -400, and -594, while not glycosylated in the context of the native hCaR in HEK-293 cells, can be glycosylated in the context of a mutant in which the eight sites that are ordinarily utilized have been disrupted.

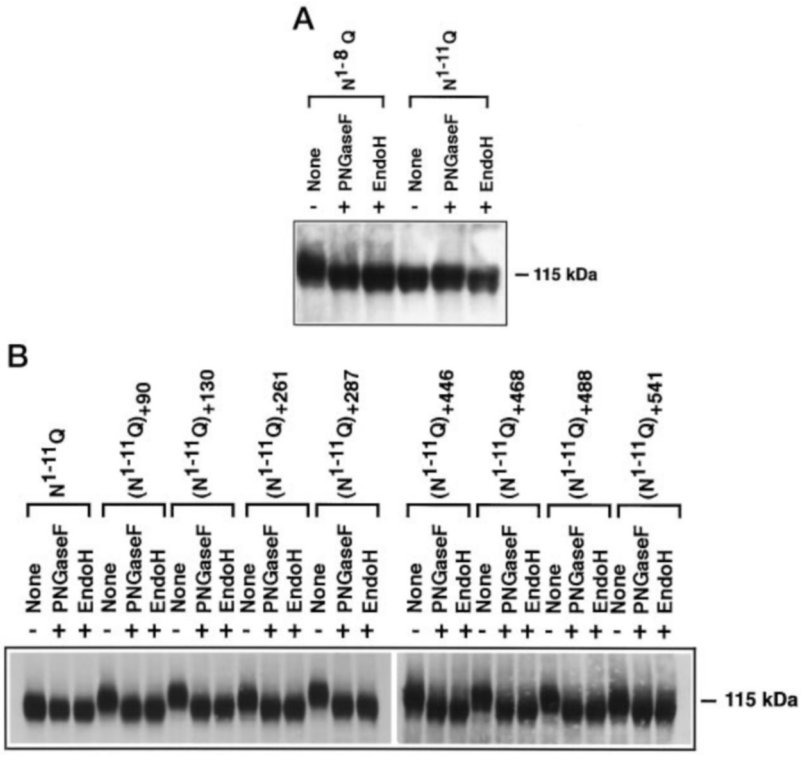

FIG. 3. **Enzymatic deglycosylation studies with PNGase F and Endo H to identify hCaR *N*-linked glycosylation sites using hCaR series II and III mutants.** 40–60 μg of crude membrane extracts of cells transfected with N1–8Q and N1–11Q mutant cDNAs (*A*) or with one of nine series II glycosylation mutant cDNAs (*B*) were incubated without (−) or with (+) PNGase F or with Endo H for 2 h at 37 °C as described under "Experimental Procedures." The reaction was stopped, and digested membrane proteins were analyzed by immunoblot as described in the legend to Fig. 2. The position of a 115-kDa molecular weight standard is shown on the *right*.

Mutation of Several N-Linked Glycosylation Sites Affects Processing and Cell Surface Expression of hCaR—The wild type hCaR has been shown to undergo *N*-linked glycosylation, and the glycosylation inhibitor, tunicamycin, has been shown to reduce cell surface expression of the receptor (24, 28). To evaluate the effect of *N*-linked glycosylation on the processing pattern of the hCaR, mutants with serially disrupted multiple *N*-linked glycosylation sites were constructed (series III mutants, Table I) and evaluated by transient expression in HEK-293 cells and immunoblotting. As shown in Fig. 4, immunoreactive bands corresponding to mutants in which the eight highly conserved sites shown to be glycosylated in Figs. 2 and 3 were serially disrupted showed a ladderlike increase in mobility compared with the wild type bands, consistent with the idea that all these sites are glycosylated. Mutants with one (N90Q), two (N90Q/N130Q), or three (N1–3Q) sites disrupted all showed a pattern with two distinct immunoreactive bands as for the wild type hCaR. The mutants with four (N1–4Q) or five (N1–5Q) sites disrupted showed a less discrete upper band, and mutants with six (N1–6Q), seven (N1–7Q), or eight (N1–8Q) sites disrupted all showed a single band on the immunoblot (Fig. 4).

Glycoproteins modified with complex, fully processed carbohydrates are resistant to Endo H digestion (29). The upper, ~150-kDa band of the wild type hCaR has been shown to be Endo H-resistant, while the lower, ~130-kDa band is Endo H-sensitive, indicating that it is modified with high mannose carbohydrates characteristic of proteins that have not yet trafficked from the endoplasmic reticulum to the Golgi (25, 28). For membranes expressing the glycosylation mutants N90Q, N90Q/N130Q, and N1–3Q, which displayed a doublet band pattern of immunoreactivity, digestion with Endo H caused a decrease only in the size of the lower band like the wild type hCaR (Fig. 5*A*). Mutant N1–4Q showed a very faint Endo H-resistant upper band. Unlike these mutant receptors, in membranes expressing the N1–5Q mutant, Endo H digestion reduced the size of the single expressed band, and no Endo H-resistant band was observed. This same digestion pattern is also seen for N1–6Q, N1–7Q, and N1–8Q mutants (data not shown).

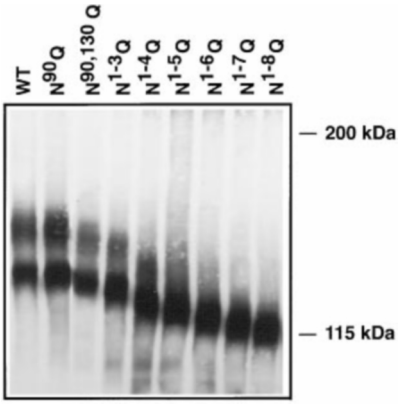

FIG. 4. **Determination of the effect of *N*-linked glycosylation on the processing patterns of the hCaR using series I and III mutants.** 40–60 μg of crude membrane extracts of cells transfected with wild type hCaR (*WT*), hCaR(N90Q), hCaR(N90Q/N130Q), and six different series III glycosylation mutant cDNAs with sequentially disrupted highly conserved sites (see Table I, series III for positions of disrupted sites) were subjected to 7.5% SDS-PAGE and transferred to nitrocellulose membrane for immunoblot analysis with monoclonal antibody ADD as described under "Experimental Procedures." The positions of molecular weight standards are shown on the *right*.

These results suggested that the N1–5Q mutant and glycosylation mutants with sequential additional glycosylation sites disrupted might show impaired trafficking and cell surface expression. Intact cell receptor immunoassay showed that disruption of even the first three glycosylation sites reduces hCaR cell surface expression and that disruption of additional sites causes progressively greater reduction in cell surface expression. N1–5Q mutant cell surface expression is barely above the value for vector-transfected control cells (Fig. 5*B*). Another monoclonal antibody, 7B10, gave similar results as the 7F8 antibody (data not shown), making it less likely that reduced cell surface immunoreactivity is due to lack of antibody reac-

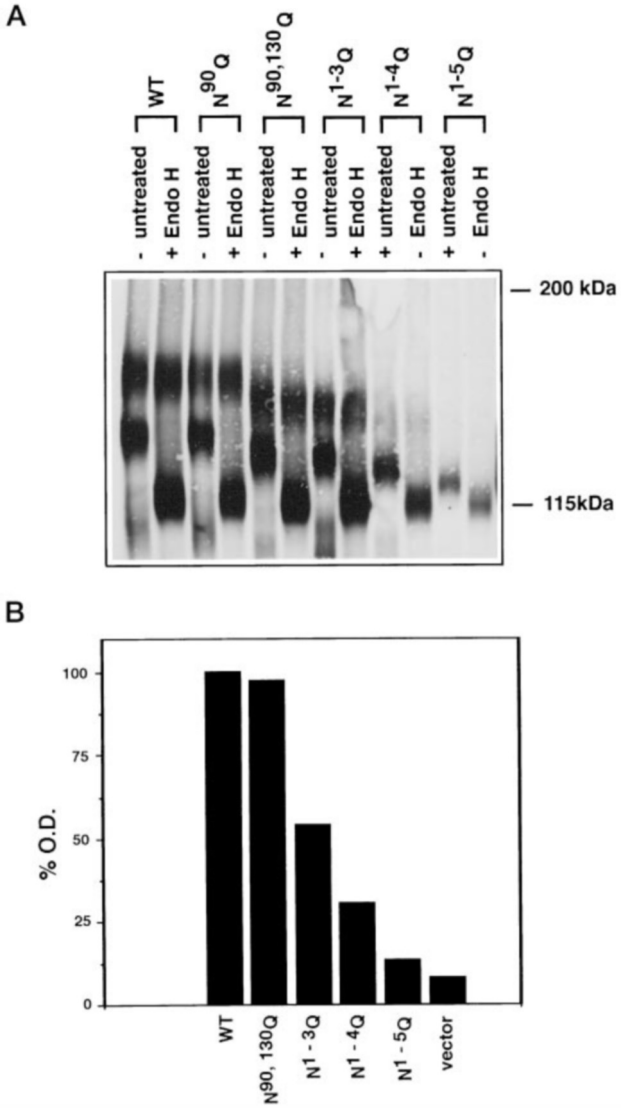

FIG. 5. *A*, enzymatic deglycosylation studies with Endo H to determine Endo H sensitivity of several series I and III mutants. 40 μg of crude membrane extracts of cells transfected with WT or mutant (N90Q, N90Q/N130Q, N1–3Q, N1–4Q, and N1–5Q) hCaR cDNAs were incubated without (−) or with (+) Endo H for 2 h at 37 °C as described under "Experimental Procedures." The reaction was stopped by the addition of an equal volume of buffer containing 0.25 M Tris-HCl, pH 6.8, 4% (w/v) SDS, 4% (v/v) 2-mercaptoethanol, and 20% (v/v) glycerol. The reaction mixtures were subjected to 7.5% SDS-PAGE and transferred to nitrocellulose membrane for immunoblot analysis with monoclonal antibody ADD as described under "Experimental Procedures." The positions of molecular weight standards are shown on the *right*. *B*, intact cell enzyme-linked immunoassay for quantitation of cell surface expression of wild type and mutant hCaRs. 75-cm^2 flasks of HEK-293 cells were transfected with 15 μg of wild type or mutant hCaR plasmid DNA or with 15 μg of vector plasmid DNA lacking hCaR insert. 48 h after transfection, cells were detached with 1 mM EDTA, and cells in suspension were incubated first with 7F8 monoclonal hCaR antibody and, after washing, with peroxidase-conjugated anti-mouse immunoglobulin secondary antibody as described under "Experimental Procedures." Peroxidase substrate was added, and OD was measured at 405 nm using a Thermo$_{max}$ (Molecular Devices) microtiter plate reader. The OD readings are expressed as a percentage of the value for the wild type hCaR (set at 100%). Data are the mean of triplicate determinations (S.E. values fell within the *symbols*) and are from one of two experiments with monoclonal 7F8. Similar results were obtained in an additional experiment with 7B10 monoclonal antibody (data not shown).

tivity with the mutant receptor.

To confirm the reduction in the cell surface expression of the glycosylation mutants observed using the intact cell immunoassay, we performed cell surface biotinylation and immunofluorescence cytochemistry experiments. Proteins on the cell surface of HEK-293 cells transiently transfected with WT, N1–3Q, N1–4Q, and N1–5Q mutant receptors were labeled with membrane-impermeant Biotin-7-NHS prior to lysing the cells. The hCaR was immunoprecipitated from the cell lysate with anti-hCaR 7F8 monoclonal antibody; the immunoprecipitate was then analyzed on immunoblots stained either with streptavidin to detect biotinylated cell surface proteins or with anti-hCaR monoclonal antibody ADD to detect total hCaR immunoreactive species. Streptavidin identified a single band at 150 kDa for WT and a faster migrating band for both N1–3Q and N1–4Q mutants, and no band was detected for N1–5Q mutant (Fig. 6*A*, *left*). Consistent with previous immunoblot and intact cell immunoassay results (Fig. 5, *A* and *B*), N1–3Q, N1–4Q, and N1–5Q mutants showed a progressive loss of cell surface expression as reflected in the staining of biotinylated protein by streptavidin. A duplicate blot of the same samples with anti-hCaR ADD monoclonal antibody detected both the cell surface and intracellular forms of the WT and mutant hCaRs (Fig. 6*A*, *right*). Thus, with ADD two bands are detected in the immunoprecipitate from cells transfected with WT hCaR: the 150-kDa band corresponding to the cell surface form also detected with streptavidin and the 130-kDa band corresponding to an intracellular form not detected by streptavidin. For the N1–5Q mutant for which streptavidin failed to detect a band, ADD detects a band corresponding to an intracellular, incompletely processed (Endo-H-sensitive) form of the receptor.

Immunofluorescence analysis of cells transfected with either WT or the same mutant receptors revealed that in nonpermeabilized cells, WT, N1–3Q, and N1–4Q mutants show distinct plasma membrane labeling, but N1–5Q did not (Fig. 6*B*, *parts A–D*). By contrast, in permeabilized cells, N1–5Q mutant showed a distinct perinuclear labeling pattern as did the WT hCaR (Fig. 6*B*, *parts E* and *F*). Nontransfected cells (*A–F*) or vector-transfected cells (*G*) incubated in 7F8 antibody showed no significant staining.

Additional Five-site Glycosylation Mutants of the hCaR to Assess Effects on Cell Surface Expression—To determine whether hCaR trafficking to the cell surface is dependent on a critical number of glycosylation sites and/or on the specific position of the sites, along with N1–5Q we constructed two additional five-site-disrupted glycosylation mutants, N4–8Q and N1,3,5,7,8Q, as depicted in Table I (series IV). Intact cell immunoassay indicated that the N4–8Q and N1,3,5,7,8Q mutants are expressed at the cell surface at a higher level compared with the N1–5Q mutant; however, their cell surface expression level is >50% reduced compared with the wild type hCaR (Fig. 7*A*). Next, we performed a PI hydrolysis assay to assess the functional response of these mutants to extracellular calcium (Fig. 7*B*). The N1–5Q mutant showed no detectable functional PI response, whereas the N4–8Q and N1,3,5,7,8Q mutants showed a 60–70% decrease in maximal response compared with wild type receptor and a significantly right-shifted concentration-dependent increase in $[Ca^{2+}]_o$ response. The PI hydrolysis assay results correlate with the observation that a significant fraction of the N4–8Q and N1,3,5,7,8Q mutant receptors expressed reach the cell surface and are functional. Failure of the N1–5Q mutant to reach the cell surface precludes its functional response.

Assessment of the Role of N-Linked Glycosylation of the hCaR in Signal Transduction Using N1–4Q and N5–8Q Glycosylation Mutants—Because mutant forms of the hCaR with five or more glycosylation sites disrupted fail to reach the cell surface, we are unable to test whether forms of the hCaR with such dramatic reduction in carbohydrate content are capable of sig-

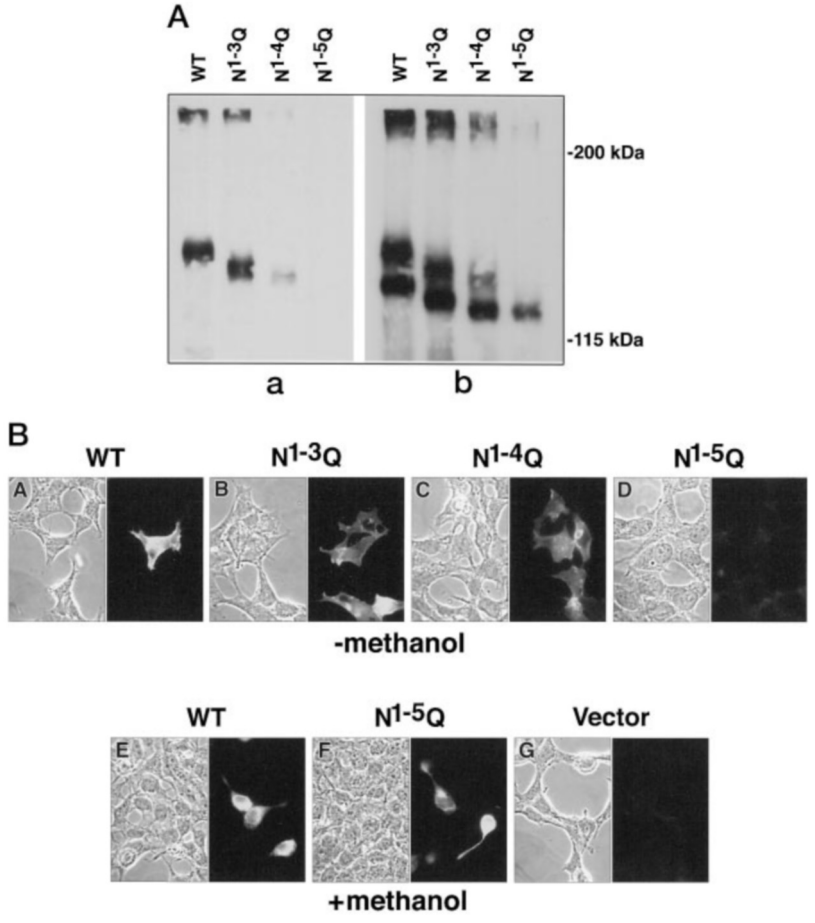

FIG. 6. *Panel A*, determination of cell surface expression level of the WT and series III glycosylation mutants by immunoprecipitation of biotinylated cell surface proteins. HEK-293 cells were transfected with either WT hCaR or with N1–3Q, N1–4Q, and N1–5Q mutants, and cell surface proteins were labeled with Biotin-7-NHS. The cells were washed, lysed, and immunoprecipitated with anti-hCaR 7F8 monoclonal antibody and subjected to SDS-PAGE. The cell surface expression of WT and mutant hCaRs was detected with peroxidase-conjugated streptavidin (*panel a*). Both cell surface (fully processed, Endo H-resistant) and intracellular (partially processed, Endo H-sensitive) forms were detected with anti-hCaR ADD monoclonal antibody (*panel b*) in a duplicate blot of the same samples. *Panel B*, cellular localization of the WT and series III glycosylation mutants by immunofluorescence staining with hCaR monoclonal antibody 7F8. The WT, N1–3Q, N1–4Q, and N1–5Q mutant hCaR receptors were analyzed by immunofluorescence microscopy after transfection of the corresponding cDNAs into HEK-293 cells. In each *panel*, the same microscopic field of cells is shown in phase contrast (*left*) and immunofluorescence (*right*). The cell surface expression of the WT and mutant receptors was compared using transfected unpermeabilized cells (*parts A–D*) and permeabilized cells (*parts E–G*) using 7F8 monoclonal antibody and fluorescein isothiocyanate-conjugated anti-mouse secondary antibody as described under "Experimental Procedures" (magnification × 600).

nal transduction using the intact cell PI hydrolysis assay. However, because the first four-site-disrupted mutant, N1–4Q, showed some level of cell surface expression as judged by intact cell immunoassay (Fig. 5*B*), we constructed another four-site-disrupted mutant, N5–8Q (Table I, series IV) and tested the signal transduction abilities of both mutants. In order to study the effect of reduced glycosylation of the hCaR on signal transduction rather than trafficking to the cell surface, we sought to study the signaling properties of these mutants at similar expression levels as the wild type receptor. We first determined the levels of cell surface expression by intact cell immunoassay for each mutant receptor after transfecting HEK-293 cells with equal amounts of plasmid DNA, and found that for a given amount of plasmid DNA transfected, both mutants show a reduced cell surface expression compared with wild type (data not shown). To achieve comparable levels of expression, HEK-293 cells were transfected with 10 μg of wild type hCaR cDNA and with 15 and 12 μg for N1–4Q and N5–8Q mutant cDNAs, respectively. Intact cell immunoassay confirmed that cell surface expression was comparable for wild type and the two mutant receptors with these DNA amounts transfected (Fig. 8, *inset*). Next, we compared $[Ca^{2+}]_o$ response of the N1–4Q and N5–8Q mutant receptors, at these comparable levels of cell surface expression, with the wild type hCaR in the PI hydrolysis assay (Fig. 8). The N1–4Q mutant showed some reduction in dose-dependent response compared with the wild type hCaR, whereas the N5–8Q mutant showed no significant dose-dependent difference compared with the wild type hCaR up to 12 mM $[Ca^{2+}]_o$ (Fig. 8).

DISCUSSION

The importance of *N*-linked glycosylation for expression and function has been extensively studied for many GPCRs but not for the unique subset to which the CaR belongs. Most GPCRs of the large rhodopsin subfamily that includes adrenergic receptors and a number of peptide receptors have relatively short extracellular amino termini with one or two glycosylation sites. In some (17, 18, 20) but not all (19) cases, glycosylation of at least one site is required for efficient cell surface expression. The glycoprotein hormone receptors have a large amino-terminal ECD, approximately two-thirds the size of the CaR, with three (follicle-stimulating hormone) or six (luteinizing hormone, thyroid-stimulating hormone) putative glycosylation sites (21, 23, 30). For both the follicle-stimulating hormone and thyroid-stimulating hormone receptors, glycosylation of a specific subset of sites (one of three for follicle-stimulating hormone and two of six for thyroid-stimulating hormone) was shown to be absolutely required for cell surface expression (21, 23). For the follicle-stimulating hormone receptor, enzymatic deglycosylation studies of wild type receptor showed that carbohydrate is not, however, essential for hormone binding (23). Results for the luteinizing hormone receptor are controversial. One study concluded that glycosylation at two of six sites is required for proper receptor folding but not for hormone binding *per se* (31). A different study employing tunicamycin to inhibit glycosylation suggested that carbohydrate is not required at all for proper receptor folding and hormone binding (30). Loss of function observed in other mutagenesis studies was attributed to differences in primary sequence rather than disruption of glycosylation.

Previous studies on the CaR indicated that it is relatively heavily glycosylated (3) and that inhibition of glycosylation with tunicamycin blocks receptor expression at the cell surface (24). The present studies were directed at defining which of the 11 putative glycosylation sites in the ECD are actually used

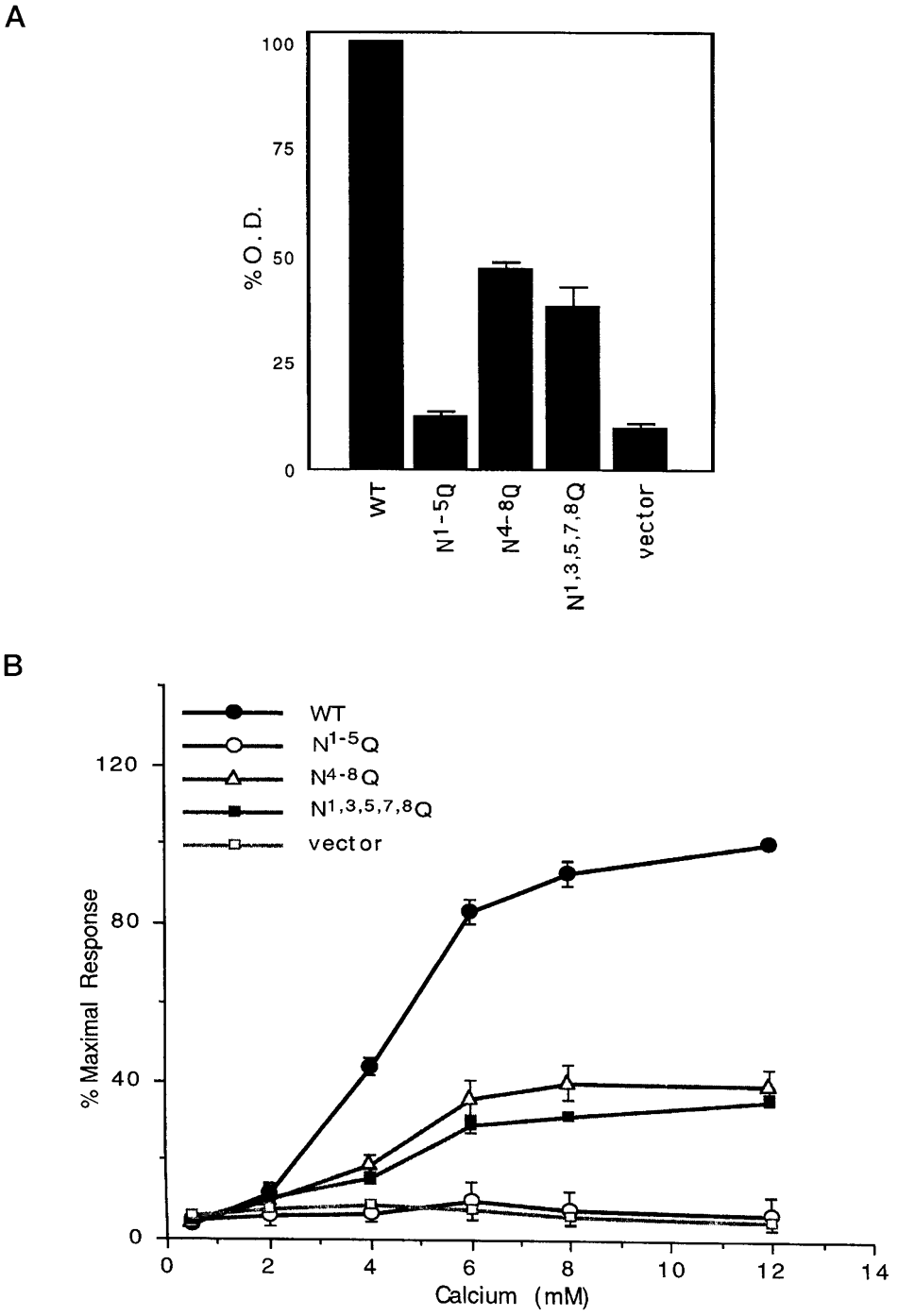

FIG. 7. *A*, comparison of cell surface expression of three different series III and IV mutants with five glycosylation sites disrupted using intact cell enzyme-linked immunoassay. 75-cm^2 flasks of HEK-293 cells were transfected with 15 μg of wild type or mutant (N1–5Q, N4–8Q, and N1,3,5,7,8Q) hCaR plasmid cDNAs or with 15 μg of vector plasmid DNA lacking hCaR insert. After 24 h, cells from each flask were divided into two aliquots; one half of the cells were replated on another 75-cm^2 flask and used for intact cell enzyme-linked assay. The other half were plated on a 12-well plate for PI hydrolysis assay as described in the legend to Fig. 6*B*. 48 h after transfection, cells from the 75-cm^2 flasks were detached with 1 mM EDTA, and cell surface expression of hCaR was measured by enzyme-linked immunoassay with 7F8 monoclonal antibody and peroxidase-conjugated anti-mouse immunoglobulin secondary antibody as described under "Experimental Procedures." OD was measured at 405 nm and is expressed as a percentage of the value for the wild type hCaR (set at 100%). Data shown here are the mean ± S.E. of triplicate determinations from one of three experiments. Similar results were obtained with 7B10 monoclonal antibody (data not shown). B, comparison of phosphoinositide hydrolysis response to calcium stimulation of mutants studied in Fig. 6*A*. One half of the HEK-293 cells from the same transfection experiment shown in Fig. 6*A* were plated on a 12-well plate 24 h after transfection, and complete DMEM containing [^{3}H]myoinositol was added to the 12-well plate. Cells were processed for a PI hydrolysis assay after an additional 24-h incubation in complete DMEM containing [^{3}H]myoinositol. Each value shown is the mean ± S.E. of three different experiments each done in duplicate at each calcium concentration. All results (cpm of labeled inositol phosphates generated) are expressed as a percentage of the maximal response of the wild type hCaR.

and the role of glycosylation in receptor expression and function. Based on studies of a series of mutants in which glycosylation consensus site asparagines were changed to glutamine singly and in various combinations, we identified eight of the 11 putative sites as containing *N*-linked sugar. Disruption of any of these eight sites alone caused a slight increase in mobility of the expressed receptor on SDS-PAGE; disruption of tandem sites caused additive changes in mobility. Glycosidase

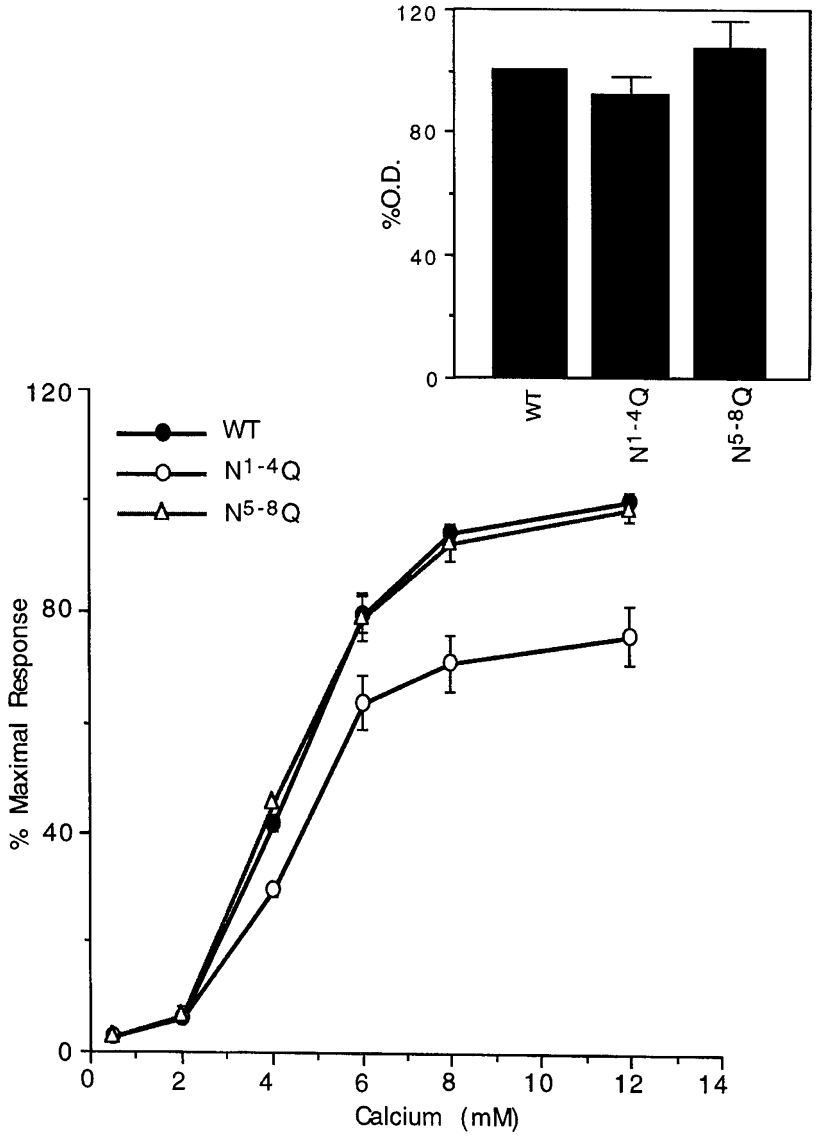

FIG. 8. **Concentration dependence for calcium stimulation of PI hydrolysis in transiently transfected HEK-293 cells expressing WT, N1–4Q, or N5–8Q mutant hCaR cDNAs.** Each 75-cm^2 flask of HEK-293 cells was transfected with 10 μg of wild type or 15 μg of N1–4Q or 12 μg of N5–8Q mutant hCaR plasmid DNA. After 24 h, cells from each flask were divided into two aliquots. One half of the cells were replated on another 75-cm^2 flask and used for intact cell enzyme-linked assay, and the other half were plated on a 12-well plate for a PI hydrolysis assay as described in the legend to Fig. 6, *A* and *B*. *Inset*, intact cell enzyme-linked immunoassay results with comparable levels of expression of WT and N1–4Q and N5–8Q mutant hCaRs using the indicated amounts of plasmid DNA for transfection. A calcium-stimulated PI hydrolysis assay was done using cells from the same transfection. Each data point is the mean value of three independent experiments. For each experiment, calcium concentrations were assayed in duplicate. Results (cpm of labeled inositol phosphates generated) are expressed as a percentage of the maximal response of the wild type hCaR.

digestion of mutants in which all but one of these eight sites were disrupted confirmed that each of these sites could be glycosylated. The remaining three sites in contrast did not appear to be glycosylated unless the other eight sites were disrupted, suggesting that they are far less efficiently modified. This overall pattern, obtained in HEK-293 cells transiently transfected, may not exactly reflect the pattern of glycosylation of CaRs expressed endogenously in tissues such as parathyroid and kidney, but the similarity in size of bands obtained in immunoblots of membranes from transfected 293 cells and from parathyroid (26, 28) suggests that the glycosylation pattern is likely to be quite similar.

The principal functional effect of disruption of CaR glycosylation sites we were able to define is loss of cell surface expression. Elimination of one or two of the employed glycosylation sites did not measurably impair expression. The disease familial hypocalciuric hypercalcemia is caused by loss of function mutations of the hCaR (1). Many single amino acid mutations have been identified that cause this disease (28) but none to date that involve mutation of a consensus glycosylation site, consistent with the lack of significant functional impairment of disruption of single glycosylation sites. Elimination of three or more glycosylation sites caused progressive reduction in cell surface expression. Elimination of five or more sites led to a form of the receptor characterized by a single, Endo H-sensitive band on immunoblots. Previous studies of other CaR mutants have indicated that this band probably represents a form of the CaR that is misfolded, retained in the endoplasmic reticulum, and unable to undergo normal processing in the Golgi needed for cell surface expression (25, 28). Immunofluorescence experiments and cell surface protein labeling with biotin confirmed that the Endo-H-sensitive band seen on immunoblots corresponds to an intracellular form of the hCaR.

We interpret the present studies to indicate a critical role for *N*-linked glycosylation of a minimum number of CaR ECD sites in normal folding of the receptor as it is being synthesized in the endoplasmic reticulum. Interestingly, the degree of functional impairment differed with the site of disruption of glycosylation. The N1–5Q mutant showed more severe loss of expression than did the N4–8Q mutant; although five sites are disrupted in both mutants, apparently the loss of more amino-terminal sites has a greater impact on protein folding and retention of the receptor in the endoplasmic reticulum. All of these results must be qualified by the caveat that effects of asparagine to glutamine substitution, although a conservative change, could be due to altered primary sequence rather than disruption of glycosylation. The fact that mutation at single and even double sites did not impair function makes this concern less likely. Also, the ability of tunicamycin to inhibit cell surface expression of the receptor supports a key role for gly-

cosylation in receptor folding and trafficking (24).

Any attempt to study the impact of disruption of glycosylation of the CaR on signal transduction is limited by the lack of a ligand binding assay and the requirement of cell surface expression for measurement of extracellular calcium activation of the receptor in the PI hydrolysis assay. Thus, our studies cannot exclude a role for carbohydrate in calcium binding to and activation of the receptor. Nonetheless, the ability of various glycosylation-deficient mutants to respond to calcium appeared to be impaired in proportion to their reduction in cell surface expression. Mutants such as N1–4Q and N5–8Q when expressed at levels comparable with wild type showed roughly equivalent extracellular calcium-stimulated PI hydrolysis to the wild type CaR, suggesting that a substantial lack of carbohydrate in the ECD does not impair receptor signaling. More definitive studies including enzymatic deglycosylation of purified CaR combined with direct measures of calcium activation will be required to elucidate the role, if any, of ECD carbohydrate in receptor function.

The present studies have defined an important role for specific glycosylation sites in CaR processing and cell surface expression. These studies have identified mutant forms of the receptor with multiple glycosylation sites disrupted that nonetheless are able to be expressed as functional receptors at the cell surface. Such mutants may facilitate expression and purification of a form of the CaR ECD useful for x-ray crystallographic studies needed to define the three-dimensional structure of this unique receptor domain.

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