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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Kausik Ray‡, Peter Clapp, Paul K. Goldsmith, and Allen M. Spiegel§

From the Metabolic Diseases Branch, NIDDK, National Institutes of Health, Bethesda, Maryland 20892

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 immunosassay, 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, biontinyl conjugation of cell surface proteins, and intact cell enzyme-linked immunosassay 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.

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§ To whom correspondence should be addressed: National Institutes of Health, Bldg. 10, Rm. 9N-222, Bethesda, MD 20892. Tel.: 301-496-4128; Fax: 301-496-9943; E-mail: Allen@AMB.NIDDK.NIH.gov.

* 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; PBS, 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-piperazineethanesulfonic acid; Biotin-7-NHS, N-biotinyl-L-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 pcDNA3.1 has been described (25). Site-directed mutagenesis to create the mutants listed in Table I was performed on hCaR cDNA in the pcDNA3.1 vector using a commercial kit (QuickChange site-directed mutagenesis kit, Stratagene Inc., La Jolla, CA), according to the manufacturer’s instructions. Briefly, a pair of complementary primers with 25–55 bases was designed for each mutation, 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 pcDNA3.1 was amplified using PCR DNA polymerase with these primers for 12 cycles in a DNA thermal cycler (Perkin-Elmer). After digestion of the parental DNA with DpnI, the amplified DNA incorporated with the nucleotide substitution was transformed into Escherichia coli (DH5α strain). The mutations were confirmed by automated DNA sequencing using a Taq DyeDeoxy Terminator Cycle Sequencing kit and ABI prism-377 DNA sequencer (Applied Biosystems, Foster City, CA). Mutants with multiple N-linked glycosylation sites disrupted were created by changing asparagines to glutamines for 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 pcDNA3.1 (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) (BioFluidics 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-cm² flasks using 10–15 μg of DNA. For cleavage with Endo H, 0.5 milliunits of Endo H for 2–5 h at 37°C. For immunoblotting, whole cell enzyme-linked immunosorbent assay (ELISA) was performed 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 μM sodium borate, 150 μM NaCl) for 15 min at room temperature to biotinylate cell surface proteins. The reaction was stopped by adding 50 μg NH₄Cl 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 7.2), 2 mM EDTA, and Complete protease- and phosphatase-inhibitor mixture. The PI hydrolysis assay has been described (24, 27). Briefly, 24 h after transfection, as described above, the intact HEK-293 cells, cells were plated on 12-well plate, to be used for experiments involving PI hydrolysis assay. 48 h after transfection, transfected HEK-293 cells in 75-cm² flasks were detached with 1 ml EDTA in PBS containing 0.5% bovine serum albumin (BSA) and incubated with 0.5 ml DMEM containing 10% FBS and 1 μg/ml monoclonal anti-hCaR antibody 7F8 or 7B10 (raised against purified extracellular domain of hCaR) 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 (Kirkegaard and Perry Laboratories) in DMEM containing 10% PBS for 1 h at 4°C. After three washes with PBS, peroxidase substrate (2.5 mM each of H₂O₂ and o-phenylenediamine in 0.1% 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 ThermoMax microtiter plate reader ( Molecular Devices, Sunnyvale, CA).

Calcium Receptor Glycosylation Sites

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-cm² flasks as described above. 24 h after transfection, cells were released from the surface with trypsin. One half of the cell suspension was used to count the number of adherent cells in another half of the cell suspension. 24 h after transfection, transfected HEK-293 cells in 75-cm² flasks were detached with 1 ml EDTA in PBS containing 0.5% bovine serum albumin (BSA) and incubated with 0.5 ml DMEM containing 10% FBS and 1 μg/ml monoclonal anti-hCaR antibody 7F8 or 7B10 (raised against purified extracellular domain of hCaR) 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 (Kirkegaard and Perry Laboratories) in DMEM containing 10% PBS for 1 h at 4°C. After three washes with PBS, peroxidase substrate (2.5 mM each of H₂O₂ and o-phenylenediamine in 0.1% 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 ThermoMax microtiter plate reader ( Molecular Devices, Sunnyvale, CA).

Immunofluorescence Microscopy—HEK-293 cells transiently transfected with WT and mutant hCaR cDNAs 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 transfected 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 (Kirkegaard 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 Fluoromount-G (Electron Microscopy Sciences, Ft. Washington, PA) and examined in a Zeiss Axioskop 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, the intact HEK-293 cells, cells were plated on a 24-well plate in medium containing 3.0 μCi/ml [[3H]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 CaCl₂, 5 mM KCl, 5.6 mM glucose, 0.4 mM MgCl₂, 20 mM

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Calcium Receptor Glycosylation Sites

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. 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. NH₂ and COOH represent the extracellular amino terminus and intracellular carboxyl terminus, respectively.

RESULTS

Identification of N-Linked Glycosylation Sites on the hCaR—

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. Glycosylation-defective mutants (series II) 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).

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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. 3B). 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.

TABLE I

Calcium Receptor Glycosylation Sites

| Series I receptor mutant                  | Highly conserved sites         | Consensus sequence(s) disrupted |
|------------------------------------------|--------------------------------|--------------------------------|
| 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            |                                |

| Series II receptor mutant                 | Semiconserved sites            | Consensus sequence(s) maintained |
|------------------------------------------|--------------------------------|---------------------------------|
| 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                        |                                |

| Series III receptor mutant                | Serine conserved sites         | Consensus sequence(s) disrupted |
|------------------------------------------|--------------------------------|--------------------------------|
| hCaR(N1–11Q)                             | None                           |                                |
| hCaR(N1–11Q,N90)                         | Asn-90                         |                                |
| hCaR(N1–11Q,N130)                        | Asn-130                        |                                |
| hCaR(N1–11Q,N261)                        | Asn-261                        |                                |
| hCaR(N1–11Q,N287)                        | Asn-287                        |                                |
| hCaR(N1–11Q,N446)                        | Asn-446                        |                                |
| hCaR(N1–11Q,N468)                        | Asn-468                        |                                |
| hCaR(N1–11Q,N488)                        | Asn-488                        |                                |
| hCaR(N1–11Q,N541)                        | Asn-541                        |                                |

| Series IV receptor mutant                 | Consensus sequence(s) disrupted |
|------------------------------------------|--------------------------------|
| hCaR(N4–8Q)                              | Asn-261, Asn-287, Asn-446, Asn-468, Asn-488, and Asn-541 |
| hCaR(N1,3,5,7,8)Q                        | Asn-90, Asn-130, and Asn-261, Asn-287, Asn-446, and Asn-468 |
| hCaR(N3–8Q)                              | Asn-261, Asn-287, Asn-446, Asn-468, and Asn-488 |

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.
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 double 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. 5A). 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).

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 (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.
assay, 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. 6A, 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. 6A, 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. 6B, parts A–D). By contrast, in permeabilized cells, N1–5Q mutant showed a distinct perinuclear labeling pattern as did the WT hCaR (Fig. 6B, 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. 7A). Next, we performed a PI hydrolysis assay to assess the functional response of these mutants to extracellular calcium (Fig. 7B). 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 \([\text{Ca}^2+]_o\) 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 hCaR 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).

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-termin al 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). 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
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 glutamines 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...
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-
glycosylation in receptor folding and trafficking (24).

Any attempt to study the impact of disruption of glycosylation 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 none-the-less 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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Kausik Ray, Peter Clapp, Paul K. Goldsmith and Allen M. Spiegel

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