Glycosylation Is Important for Binding to Human Calcitonin Receptors†

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ABSTRACT: Human calcitonin receptor (hCTR) subtypes contain three or four potential Asn-linked glycosylation sites in their extracellular amino termini. The role of glycosylation in hCTR function has not been identified, but it has been suggested that inhibition of glycosylation does not affect binding or signaling. To determine the role of glycosylation in hCTR biology, we studied the effects of inhibition of glycosylation and of substitution of Asn residues that are potential glycosylation sites. Native and mutated hCTRs were studied after transient expression in monkey kidney COS-1 cells. Tunicamycin, administered as part of a treatment protocol that inhibited glycosylation of all expressed receptors, decreased salmon calcitonin (sCT) binding affinities and signaling potencies at hCTRs with three or four potential glycosylation sites. In hCTR3, which contains three potential glycosylation sites at positions 26, 78, and 83, site-specific substitution of Asn-26 by Ala had no effect on sCT binding affinity or potency, whereas substitution of Asn-78 or Asn-83 lowered sCT affinity and potency. A mutant hCTR3 in which all three Asn residues were substituted with Ala exhibited no high-affinity sCT binding and potencies of several calcitonin analogues that were more than 100-fold lower than that of native hCTR3. Our data show that glycosylation is important for high-affinity binding and potency of calcitonin analogues at hCTRs.

Calcitonin receptors (CTRs) are members of the subfamily of G protein-coupled receptors (GPCRs) that include receptors for parathyroid hormone/parathyroid hormone-related peptide (PTH/PTHrP), glucagon, vasoactive intestinal peptide (VIP), and several others (*1*). Although most GPCRs are glycoproteins (*2*), the effects of inhibiting glycosylation during synthesis of GPCRs or of enzymatic deglycosylation of expressed receptors on GPCR biology have varied. For example, within the CTR subfamily, nonglycosylated human PTH/PTHrP receptors, which were synthesized in HEK 293 cells treated with tunicamycin, bound PTH analogues with high affinity, signaled with high potency, and were expressed to the same extent as native receptors (*3*). In contrast, mutant human VIP receptors that are lacking potential Asn-linked glycosylation sites bind ligands with a high affinity but are not expressed on the surface of monkey kidney COS-7 cells (*4*). A mutant rat glucagon receptor in which all potential Asn-linked glycosylation sites were deleted exhibited a normal level of expression on the surface of COS-1 cells but no high-affinity binding (*5*). Thus, for other members of the CTR subfamily, unglycosylated receptors have displayed each of the following: (1) a phenotype indistinguishable from the native, fully glycosylated receptor (*3*), (2) decreases in levels of expression with high-affinity binding (*4*), and (3) normal levels of expression with decreases in binding affinity (*5*).

Although four potential Asn-linked glycosylation sites are conserved across species in CTRs (*6*), there are naturally occurring human (hCTR3) (*7*) and porcine (*8*) CTRs that are lacking the first of these potential sites. hCTR3 is produced by alternative RNA splicing (*9*) and is lacking the first 47 amino acids, which includes a potential Asn-linked glycosylation site, present in the other hCTR subtypes [hCTR1 (*10*, *11*) and hCTR2 (*11*−*13*)]. hCTR3 displays a biology that is indistinguishable from that of hCTR2 (*7*). [hCTR1, which contains 16 additional amino acid residues in the first putative intracellular loop, displays a lower binding affinity and potency than hCTR2 and hCTR3 (*11*, *14*).] A role for glycosylation in hCTR function has been sought previously (*15*, *16*). Inhibition of glycosylation of hCTRs in T47D breast cancer cells by tunicamycin was shown to decrease the total extent of binding but to have no effect on the binding affinity of those hCTRs expressed on the cell surface (*15*). These data were interpreted as showing that tunicamycin decreased the level of expression of cell surface hCTRs. However, an equally plausible interpretation is that tunicamycin decreased the affinity of newly synthesized hCTRs (to a level that was too low to be measured in a standard binding assay) and that the high-affinity sites were those synthesized prior to tunicamycin addition. High-affinity sites may still be present at the cell surface because the rate of turnover of receptors synthesized prior to administration of tunicamycin may be slow. The rate of cell surface hCTR turnover is not known (*17*). Recently, Quiza et al. (*16*) studied binding to CTRs from several species that were digested with endoglycosidase F. These investigators found that partially deglycosylated CTRs displayed binding affinities and specificities indistinguishable from those of fully glycosylated CTRs; however, complete deglycosylation could

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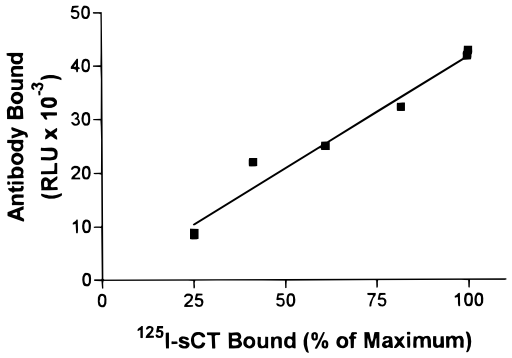

FIGURE 1: Comparison of M2 monoclonal antibody and salmon [^{125}I]calcitonin binding to COS-1 cells expressing FLAG-hCTR3. The levels of specific [^{125}I]sCT and anti-FLAG-M2 antibody binding were measured in wells containing various amounts of COS-1 cells expressing FLAG-hCTR3. The total cell number was 40 000 in all wells with addition of untransfected COS-1 cells. There was no specific signal from untransfected cells or from cells expressing CTRs that did not contain the FLAG epitope (data not shown). The FLAG epitope did not affect the binding of [^{125}I]sCT as the apparent affinity was the same for FLAG-hCTR3 and hCTR3. The data are the mean ± SE of two or three determinations from two experiments.

not be achieved under the nondenaturing conditions needed to study receptor function. Thus, the published data do not allow for a conclusion with regard to the role of glycosylation in CTR biology.

We studied the effects of tunicamycin and of elimination of potential glycosylation sites by specific mutation on binding and signaling of hCTRs in transiently transfected COS-1 cells. We show that lack of glycosylation leads to decreases in affinity of binding and potency of signaling by calcitonin (CT) analogues.

RESULTS

The following native and mutated hCTRs were studied: hCTR3, 427-amino acid hCTR; hCTR2, 474-amino acid hCTR that is identical to hCTR3 but contains 47 additional amino acids at the amino terminus, including an Asn residue that is a potential site for glycosylation; hCTR3/N26A, hCTR3 in which Asn at position 26 is substituted with Ala; hCTR3/N78A, hCTR3 in which Asn at position 78 is substituted with Ala; hCTR3/N83A, hCTR3 in which Asn at position 83 is substituted with Ala; and hCTR3/N26/78/83A, hCTR3 in which Asn residues at positions 26, 78, and 83 are substituted with Ala. Several of these hCTRs were constructed with a FLAG epitope placed at the amino terminus and are designated, for example, FLAG-hCTR3. We showed previously that there was no difference in binding, signaling, or expression between untagged and FLAG epitope-tagged hCTR2 (*17*), and there were no differences in these parameters in the mutant hCTRs studied here when they could be measured (data not shown).

Several hCTR mutants did not bind salmon [^{125}I]iodocalcitonin ([^{125}I]sCT) with sufficiently high affinity to be characterized in a binding assay. To estimate the level of expression of these hCTRs, we developed a cell-based antibody-directed chemiluminescent assay using FLAG epitope-tagged hCTRs and the monoclonal antibody M2. In Figure 1, we illustrate the direct correlation between the luminescent signal and the extent of binding of [^{125}I]sCT to FLAG-hCTR3. These data validate this assay for quantitation

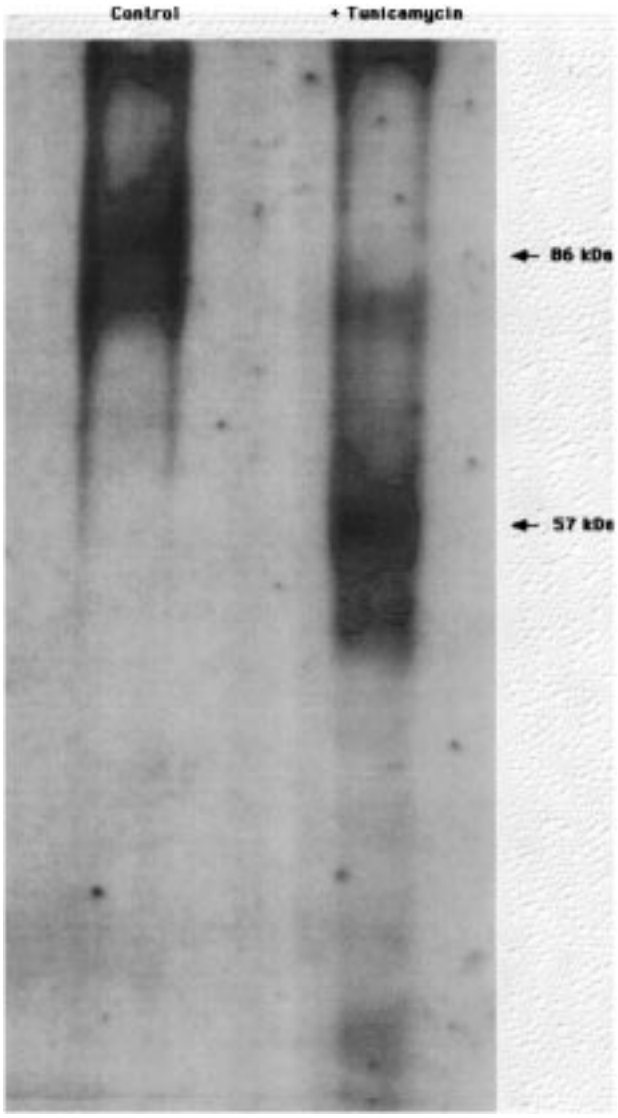

FIGURE 2: Effect of tunicamycin on hCTR.Western blot of $FLAG_2$-hCTR2 isolated from control or tunicamycin-treated (6.4 μg/mL for 48 h) COS-1 cells. Samples were analyzed by 10% (w/v) SDS−PAGE and luminescence using anti-FLAG-M2 antibody.

of expression of functional receptors. We cannot, however, provide a similar validation for unglycosylated or mutant receptors that do not bind [^{125}I]sCT (see below). As it is possible that unglycosylated or mutant receptors bind the M2 antibody with different affinities than hCTR3, perhaps because of differences in the accessibility of the FLAG epitope, we use antibody binding as a semiquantitative estimate of expression of these receptors.

To begin to study the effect of the absence of glycosylation on hCTR biology, we used transiently transfected COS-1 cells exposed for 48 h to a maximally effective dose of tunicamycin. We added the tunicamycin 5 h after transfection with plasmids encoding hCTR3 or hCTR2 to inhibit glycosylation of all hCTRs expressed on these cells. Doses of tunicamycin of <6.4 μg/mL did not inhibit glycosylation completely (data not shown). Tunicamycin-treated cells displayed a more rounded appearance and were more easily dislodged from the cell culture plate than untreated cells. In Figure 2, we show that tunicamycin treatment changed hCTR2 from a protein that migrated on SDS−PAGE as a diffuse band with an apparent size of 86 kDa to a band with

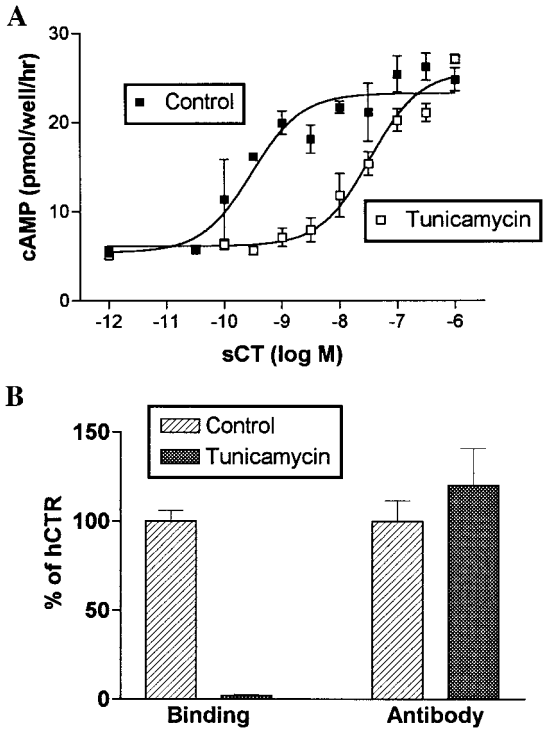

FIGURE 3: Effects of tunicamycin on hCTR signaling and expression. (A) Concentration dependence of salmon calcitonin stimulation of cyclic AMP formation in control and tunicamycin-treated COS-1 cells expressing hCTR3. sCT stimulation of cells was performed for 1 h at 37 °C in cells incubated in buffer containing 0.5 mM 3-isobutyl-1-methylxanthine in control and tunicamycin-treated (6.4 μg/mL for 48 h) COS-1 cells. The data are the mean ± range of duplicate determinations in a representative set of three experiments. (B) Salmon [^{125}I]calcitonin and antibody binding to control and tunicamycin-treated COS-1 cells expressing FLAG-hCTRs. The levels of specific [^{125}I]sCT and anti-FLAG-M2 antibody binding were measured in control and tunicamycin-treated (6.4 μg/mL for 48 h) COS-1 cells. The FLAG epitope did not affect the binding of [^{125}I]sCT. The data are the mean ± SE of two or three determinations from two experiments.

an apparent size of 57 kDa. We compared the dose response of sCT for stimulation of cyclic AMP formation in control and tunicamycin-treated intact cells expressing hCTR3 or hCTR2. The half-maximally effective dose of sCT in cells expressing hCTR3 was 0.30 nM (0.1−0.9 nM, 95% confidence limits) in control cells and 34 nM (19−59 nM) in tunicamycin-treated cells (Figure 3A). That is, the unglycosylated hCTR3 exhibited a potency with respect to sCT that was 100-fold lower than that of native hCTR3. In cells expressing hCTR2, tunicamycin caused a similar lowering of the sCT potency (data not shown). Because there is a good correlation between potency and affinity under these conditions (*14*), these data are consistent with the idea that the lack of glycosylation causes a decrease in binding affinity. We measured the level of binding to hCTRs on the surface of intact cells using [^{125}I]sCT and found no high-affinity specific binding to tunicamycin-treated cells (Figure 3B), whereas there was readily measurable binding to control cells expressing hCTR3 [equilibrium inhibitory constant (K_i) of sCT = 4.1 nM (2.7−6.1 nM)] (data not shown) or hCTR2 (K_i of sCT = 9.0 nM) (*14*). To attempt to confirm that the inability to bind to hCTRs in tunicamycin-treated cells was caused by lowering of the hCTR affinity, we estimated the level of hCTR expression using the antibody assay (Figure 1). In Figure 3B, we show that there was a similar level of antibody binding to FLAG epitope-tagged hCTRs in control and tunicamycin-treated cells. This is consistent with there being no effect of tunicamycin treatment on hCTR expression. Thus, tunicamycin treatment appeared to decrease the affinity of hCTR3 and hCTR2 but not the level of receptor expression.

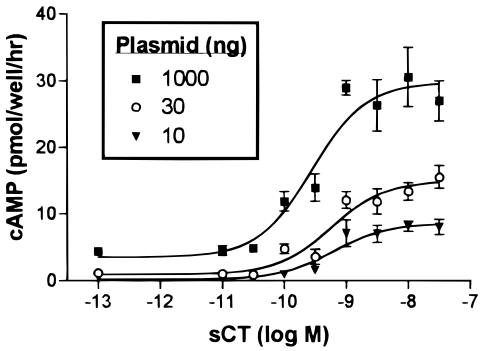

FIGURE 4: Concentration dependence of salmon calcitonin stimulation of cyclic AMP formation in COS-1 cells expressing increasing levels of hCTR3. sCT stimulation of cells was performed for 1 h at 37 °C in cells incubated in buffer containing 0.5 mM 3-isobutyl-1-methylxanthine. There was a plasmid amount-dependent increase in the level of [^{125}I]sCT binding in these cells that paralled the increase in the level of maximal stimulation (data not shown). The data are the mean ± SE of duplicate determinations from two experiments.

The similar maximal stimulation of cyclic AMP formation by sCT (Figure 3A) in control and tunicamycin-treated cells provides further evidence in support of the idea that tunicamycin does not affect hCTR expression. This is so because the extent of sCT stimulation of cyclic AMP formation is directly related to the number of hCTR3s expressed. In Figure 4, we illustrate the results of experiments in which cells were transfected with three different amounts of plasmid DNA encoding hCTR3. There was a direct relationship between the level of maximal stimulation by sCT of cyclic AMP formation and the amount of plasmid used in the transfection. Thus, it is likely that the similar maximal stimulation of cyclic AMP formation in control and tunicamycin-treated cells reflects the presence of similar numbers of receptors. There was no significant difference in the potency of sCT in cells transfected with increasing amounts of plasmid encoding hCTR3 under these conditions: 0.62 nM (0.25−1.6 nM) with 10 ng of plasmid DNA, 0.57 nM (0.28−1.2 nM) with 30 ng, and 0.28 nM (0.13−0.62 nM) with 1000 ng. This is noteworthy because it was possible that changes in hCTR mutant receptor expression could have caused changes in their apparent potencies (see below). We conclude, therefore, that tunicamycin pretreatment causes a decrease in binding affinity that is manifest as a decrease in potency, but does not appear to affect hCTR expression in these cells.

To assess the role of glycosylation in hCTR biology in a different way, we constructed a series of mutant receptors that were substituted with Ala at the potential Asn-linked glycosylation sites. Because hCTR3, which contains only three Asn residues in its amino terminus that are potential sites for glycosylation, is a naturally occurring receptor and exhibits affinity, potency, and a level of expression indistinguishable from those of hCTR2 (*7*), we used hCTR3 as the parent receptor for site-specific substitution. hCTR3/

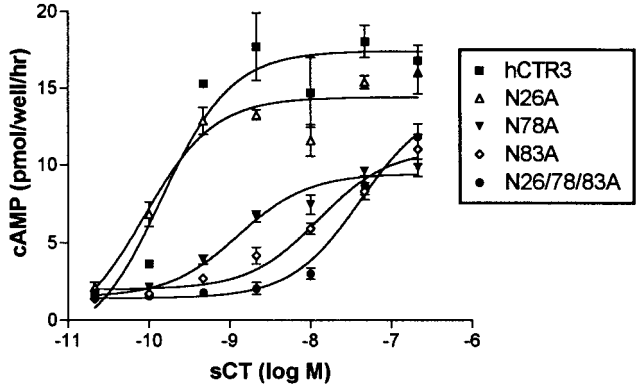

FIGURE 5: Concentration dependence of salmon calcitonin stimulation of cyclic AMP and formation in COS-1 cells expressing hCTR3, hCTR3/N26A, hCTR3/N78A, hCTR3/N83A, or hCTR3/N26/78/83A. sCT stimulation of cells was performed for 1 h at 37 °C in cells incubated in buffer containing 0.5 mM 3-isobutyl-1-methylxanthine. The data are the mean ± SE of duplicate or triplicate determinations from three experiments.

Table 1: Potencies (EC_{50}) and Levels of Maximal Stimulation of Cyclic AMP Production by Salmon Calcitonin at hCTR3 Mutant Receptors

	EC_{50} (nM)[a]	-fold stimulation[a]
hCTR3	0.17 (0.057−0.51)	11 (9.8−12)
hCTR3/N26A	0.12 (0.039−0.38)	9.5 (8.5−11)
hCTR3/N78A	1.7 (0.52−5.6)	6.0 (5.1−7.0)
hCTR3/N83A	6.3 (2.2−18)	7.1 (6.0−8.1)
hCTR3/N26/78/83A	15 (3.5−63)	5.8 (4.3−7.4)

[a] Mean (95% confidence interval). Values for hCTR3 and hCTR3/N26A are not different from each other but are statistically different from those of hCTR3/N78A, hCTR3/N83A, and hCTR3/N26/78/83A.

N26A exhibited high-affinity binding of [^{125}I]sCT similar to that of hCTR3, whereas hCTR3/N78A, hCTR3/N83A, and hCTR3/N26/78/83A exhibited no high-affinity binding (data not shown). Figure 5 and Table 1 contain data for the signaling characteristics of these hCTR3 mutants. hCTR3/N26A was indistinguishable from native hCTR3 in both sCT potency and the level of maximal stimulation of cyclic AMP formation. In contrast, hCTR3/N78A, hCTR3/N83A, and hCTR3/N26/78/83A exhibited potencies and maximal level of signaling by sCT that were reduced compared to those of native hCTR3. There was a decrease in the level of maximal signaling for hCTR3/N78A, hCTR3/N83A, and hCTR3/N26/78/83A compared to that of hCTR3. However, we are not certain whether the modest decrease in the level of maximal signaling of these mutant receptors was caused by a decrease in intrinsic receptor efficacy or whether it is due to a reduced level of mutant receptor expression. For example, although the antibody assay showed no difference in the levels of expression of FLAG epitope-tagged native hCTR3 and hCTR3/N26/78/83A (data not shown), we cannot exclude a modest decrease in the levels of expression that the antibody assay may not measure. More importantly, the effect on potency caused by the lack of glycosylation sites was clear. hCTR3/N78A, hCTR3/N83A, and hCTR3/N26/78/83A exhibited decreased potencies for sCT, whereas the potency for hCTR3N26A was the same as that of hCTR3. Indeed, the decrease in sCT potency exhibited by hCTR3/N26/78/83A compared to that of hCTR3 (90-fold) was the same reduction as that exhibited by unglycosylated hCTR3 in tunicamycin-treated cells (100-fold). Since potency appears to reflect affinity, and decreases in potency cannot be ascribed to differences in the level of receptor expression under these conditions (Figure 4), these data show that our inablility to measure the extent of high-affinity binding of [^{125}I]sCT to these mutant receptors exists because the lack of glycosylation decreases the affinity of sCT binding. Last, although there appeared to be a trend toward greater losses in sCT potency and the levels of maximal signaling for hCTR3/N26/78/83A, these characteristics were not different for the triply substituted mutant compared to hCTR3/N78A or hCTR3/N83A.

We showed previously that salmon iodocalcitonin (I-sCT) exhibits higher affinity and potency for hCTR2 than sCT and human calcitonin (hCT) (*18*). The order of affinities for binding and potencies for signaling is as follows: I-sCT > sCT > hCT. We measured the potencies of these three analogues with respect to hCTR3 and hCTR3/N26/78/83A and found that the order of potencies of I-sCT, sCT, and hCT with respect to hCTR3 and hCTR3/N26/78/83A were the same as for hCTR2 (data not shown). However, as expected, the potencies of I-sCT and hCT were proportionately lower for hCTR3/N26/78/83A than for hCTR3. Thus, the decreased potencies exhibited by the mutant receptors with decreased glycosylation levels was not peculiar for sCT but applied to I-sCT and hCT also.

DISCUSSION

Our studies of the role of glycosylation in hCTR biology differ from previous studies in two ways. First, we used a transient transfection cell system in which the synthesis of hCTRs was initiated under fully controlled conditions. This allowed us to perform experiments with tunicamycin to inhibit Asn-linked glycosylation of all hCTRs expressed. There was no potential for receptors that had been synthesized prior to tunicamycin addition to remain on the cell surface as is possible in studies with cell lines that constitutively express hCTRs (*15*). And second, we constructed hCTR mutants that contained one or three site-specific substitutions with Ala residues at all the potential Asn-linked glycosylation sites. hCTR3/N26/78/83A is a fully unglycosylated receptor. Thus, our characterizations of hCTRs in tunicamycin-treated cells and of hCTR3/N26/78/83A were of hCTRs that contained no carbohydrate moieties in their amino termini. Our data definitively show that hCTRs that are not glycosylated exhibit decreased binding affinities and potencies compared to native hCTRs. Our data do not, however, show that carbohydrate moieties in the amino termini of hCTRs directly bind calcitonins because the changes in affinity may have been caused by conformational changes rather than by the loss of direct carbohydrate contact sites. We cannot be certain whether changes in the level of glycosylation modestly affect expression of the mutant receptors, but it appears that inhibition of glycosylation of native hCTR3 by tunicamycin did not decrease its level of expression in COS-1 cells

Although it has been suggested that carbohydrate moieties are necessary for the proper targeting of glycoproteins to the cell surface membrane, this may not be the case for hCTRs in COS-1 cells as functional, unglycosylated hCTRs were expressed on the cell surface at levels similar to that of native hCTRs. We cannot exclude the possibility that

changes in folding, transit through the intracellular organelles, and retention in the surface membrane occurred with the unglycosylated hCTRs. We were not able to study whether unglycosylated hCTRs were trapped intracellularly because even native hCTRs are found intracellularly in COS-1 cells (*17*). We think, however, that hCTRs in tunicamycin-treated cells and the Asn-to-Ala mutant hCTRs on the cell surface represented hCTRs that exhibited decreased binding affinities and potencies because they were unglycosylated.

It is noteworthy that all potential Asn-linked glycosylation sites in hCTR are in the amino terminus as it has been shown that the majority of the binding energy for binding of calcitonin to receptors involves interactions at the amino terminus. For example, chimeric receptors that contain the amino terminus of hCTR engrafted on the glucagon (*19*) or thyrotropin-releasing hormone (our unpublished observations) receptors bind sCT with high affinity. In contrast, the hCTR amino terminus does not mediate signaling as these same two receptor chimeras do not signal when calcitonin analogues bind. Indeed, as expected, it is the transmembrane bundle of the hCTR that is needed for signaling. A chimeric receptor that contained the amino terminus of the PTH/PTHrP receptor engrafted on the body of hCTR signaled, albeit with a lower potency than native hCTR, when bound by calcitonin (*20*).

Although we have shown that the affinity of interaction between calcitonin and hCTR3 is decreased in the absence of glycosylation, the changes that account for the lowered affinity are not clear. It is possible that carbohydrate moieties interact directly with calcitonin as part of the "binding pocket". An alternative possibility is that sugar moieties are needed to constrain the amino acid residues of the amino terminus in a conformation that is optimal for calcitonin binding. Distinguishing between these two possibilities may be approached indirectly with experiments in which one can attempt to delineate the specific amino acid residues in hCTR and calcitonin that bind one another. For example, one could use molecular biological and chemical synthetic approaches to introduce complementary changes into the ligand and receptor such as we have done for the thyrotropin-releasing hormone receptor (*21*). However, definitive resolution of the structure of the calcitonin−hCTR complex and the role of the sugar moieties will require physical determination by nuclear magnetic resonance spectroscopy or crystallography.

In conclusion, fully unglycosylated forms of native hCTRs, that is, hCTR receptors in tunicamycin-treated cells and an hCTR3 mutant in which the three potential Asn-linked glycosylation were substituted with Ala, exhibit decreased affinity for calcitonin analogues and concomitant decreased potencies for signaling. And, unglycosylated receptors synthesized from genes encoding native hCTRs in tunicamycin-treated COS-1 cells appear to be expressed on the cell surface at levels similar to that of native hCTRs.

EXPERIMENTAL PROCEDURES

Generation of I-sCT and [^{125}I]sCT. Monoiodinated sCTs, [^{125}I]sCT and nonradioactive I-sCT, were prepared using chloramine T and purified with high-performance liquid chromatography as described previously (*18*). There was a 1.5 min difference in the chromatographic retention times of I-sCT and sCT that allowed for purification of monoiodinated sCTs, and therefore, the maximal theoretical specific radioactivity of [^{125}I]sCT (2175 Ci/mmol) was achieved. To accurately determine the concentration of nonradioactive I-sCT, a trace quantity of Na^{125}I (diluted with nonradioactive NaI to 2 Ci/mmol) was included in the iodination reaction mixture.

DEAE-Dextran-Mediated Transfection of COS-1 Cells. COS-1 cells (1×10^6) in Dulbecco's modified Eagle's medium (DMEM, GIBCO-BRL) with 5% NuSerum (Becton Dickinson) were seeded in 100 mm dishes on the day prior to transfection. On the day of transfection, a mixture was made in which supercoiled plasmid DNA was resuspended in 2.85 mL of Hank's balanced salt solution with 10 mM HEPES at pH 7.4 (HBSS, GIBCO-BRL), at a final DNA concentration of 2 μg/mL. After the solution was vortexed vigorously, 0.15 mL of DEAE-dextran [10 mg/mL in phosphate-buffered saline (PBS)] was added and the solution mixed. The tissue culture medium in the dish was aspirated, and the cells were rinsed twice with 8 mL of HBSS warmed to 37 °C. The transfection mixture (3.0 mL) was added gently to the side of the dish, distributed evenly by tilting, and the plate was incubated at 37 °C. After 30 min, 7 mL of DMEM containing 10% NuSerum and 80 μM chloroquine was added, and incubation at 37 °C was continued. After 2.5 h, the medium was aspirated and replaced with 3 mL of DMEM containing 10% NuSerum and 10% dimethyl sulfoxide for 2.5 min. Finally, the medium was aspirated, and 10 mL of fresh DMEM containing 10% NuSerum was added. Cells were incubated at 37 °C for 24−48 h before reseeding into 12-well plates for experiments 1 day later.

Lipofectamine Transfection with Tunicamycin. In a 6- or 12-well plate, $1-2 \times 10^5$ COS-1 cells were seeded per well in 1−2 mL of DMEM with 5% NuSerum and incubated at 37 °C for 24 h. On the day of transfection, 2 μg of plasmid DNA/well was diluted in 190 μL of serum-free OPTI-MEM medium (GIBCO-BRL) and the mixture combined with 10 μL of Lipofectamine reagent (GIBCO-BRL), gently mixed, and incubated at room temperature for 15−45 min to allow DNA−liposome complexes to form. OPTI-MEM (0.8 mL) was added to the DNA−liposome complexes, and they were gently mixed and overlaid onto cells that had been rinsed with 2 mL of OPTI-MEM. The cells were then incubated for 5 h at 37 °C; 1 mL of DMEM containing 10% NuSerum and 12.8 μg/mL tunicamycin was added without removing the transfection mixture (no tunicamycin was added in controls), and the cells were placed back into the incubator. After 18−24 h, the medium was replaced with 2 mL of fresh DMEM with 5% NuSerum without or with tunicamycin (6.4 μg/mL). Experiments were performed 48 h after transfection.

Binding with [^{125}I]sCT and sCT. HBSS solution containing 1 mM phenylmethanesulfonyl fluoride (Sigma), 1 mg/mL bacitracin, and 1 mg/mL bovine serum albumin (BSA) was used to dilute [^{125}I]sCT to a final concentration of approximately 300 pM. The cells were washed twice with ice-cold HBSS. Then 300 μL of the binding buffer was added to each well without or with various concentrations of sCT. The plates were incubated at 4 °C for 3−4 h or overnight. After incubation, the binding buffer was aspirated and the cells were washed three times with cold HBSS. Finally, 1 mL of 0.4 N NaOH was added to each well, and the lysates were transferred to test tubes and counted in a γ-counter.

Cyclic AMP Radioimmunoassay. Twenty-four to forty-eight hours after transfection, cells were reseeded into 12-well plates at 100 000 cells/well in 1 mL of DMEM with 5% NuSerum. On the day of experiments, cells were washed with prewarmed (37 °C) HBSS twice; 0.3 mL of HBSS containing 0.5 mM 3-isobutyl-1-methylxanthine (SIGMA) and various concentrations of sCT were added, and the mixture was incubated at 37 °C. After 1 h, the medium was aspirated and 0.9 mL of a mixture of 40 mL of MeOH, 0.4 mL of concentrated HCl, and 24 mL of 10 mM EDTA was added, and the cells were scraped and transferred to glass tubes to which 0.67 mL of chloroform was added. The tubes were centrifuged at 3000 rpm for 3−5 min, and 50 μL of the aqueous phase from each was transferred to a 1.5 mL Eppendorf tube. The samples were then placed under an air stream to remove the MeOH, and 1 mL of 50 mM sodium acetate (NaAc) (pH 6.2) was added to each tube, followed by 50 μL of acetylation mixture (consisting of 2:1 triethylamine/acetic anhydride). The samples were then vortexed and incubated at room temperature for 15 min. Anti-cyclic AMP Ab (Calbiochem) was diluted 1:18000 in 50 mM NaAc (solution A), and a solution containing 3% normal rabbit serum and [^{125}I]cAMP (0.035 μCi/mL, Biomedical Technologies Inc.) in 50 mM NaAc was prepared (solution B). After acetylation, 5 μL of each sample was transferred to a test tube to which 95 μL of 50 mM NaAc was added. Then 100 μL of solution A and B were added to each test tube, and the mixtures were incubated at 4 °C overnight. On day 2, anti-rabbit γ-globulin Ab (ARNEL) was diluted 1:10 in 50 mM NaAc and 100 μL was added to each test tube, and the mixtures were incubated for 4 h at 4 °C. One milliliter of cold 50 mM NaAc was added to each tube, and the mixtures were centrifuged at 3000 rpm for 15 min. The supernatant was discarded, and the immunoprecipitates were counted in a γ-counter. The amount of radioactivity in the samples was compared to that of the standard cyclic AMP (Calbiochem), and the absolute amounts of cyclic AMP were calculated.

Construction of hCTR Mutants. Mutant hCTRs were created with PCR techniques using the hCTR expression plasmid pMT4ProlacFLAGShCTR (*14*), which encoded a synthetic hCTR gene (*14*), as the template. hCTR3 was constructed as follows. (1) pMT4ProlacFLAGShCTR was digested with *Eco*RI and *Not*I, and the two fragments (1.5 and 5.1 kb) were purified using GENECLEAN II (BIO 101). (2) The 1.5 kb fragment was digested with *Fsp*I, and the 1.3 kb fragment was isolated and purified. (3) The 5.1 kb DNA, 1.3 kb DNA, and a linker DNA of 20 bp were ligated using T4 DNA ligase and screened with restriction enzymes *Eco*RI and *Bsp*EI that generated a 350 bp fragment with Prolac-FLAG-hCTR but a 180 bp fragment with hCTR3. (4) hCTR3 was confirmed by sequencing. FLAG-hCTR3 was constructed via PCR. (1) The sense primer (5′CAAGAATTC-CACCATGGACTACAAGGACGACGACGACAAGATG-GATGCGCAGTACAAATGCTAT3′) containing the sequence for encoding the FLAG epitope (DYKDDDDK) ("primer A") and the antisense primer (5′GTTACCCTTTG-GCAGCCTAACGTCGAAAAAACACGAAAATCCCGA-GGGAT3′) ("primer B") were used for PCR and generated a PCR product of ∼440 bp. (2) The PCR fragment and pMT4ProlacFLAGShCTR were digested with *Eco*RI and *Cla*I and ligated. (3) The construct was screened by length differentiation and confirmed by sequencing. FLAG-hCTR/N78/83A was constructed via overlapping PCR. (1) The sense primer (5′TGA GTG ACA ATG ACA TCC AC3′) ("primer C") and antisense primer B and two overlapping primers 5′CCTGAAAACGCACGCACGTGGTCCGCATA-TACTATGTGCAATGCTTT3′ and 5′CATAGTATATGCG-GACCACGTGCGTGCGTTTTCAGGATGTTTAAACC3′ were used for PCR. (2) The PCR fragment was ligated to the pMT4ProlacFLAGShCTR using *Eco*RI and *Cla*I sites. hCTR3/N78/83A was generated by deleting a fragment from FLAG-hCTR/N78/83A in a way similar to the generation of hCTR3 from pMT4ProlacFLAGShCTR described above. FLAG-hCTR3/N26/78/83A was constructed via PCR using sense primer A and antisense 5′CCACGTACGTGCG-CAATATGGACCTTCTCCTTGG3′ as a primer; *Eco*RI and *Bsiw*I restriction sites were used for ligation into FLAG-hCTR/N78/83A, and the sequence was confirmed. $FLAG_2$-hCTR2, which contains two FLAG epitopes with a Gly between them in the amino terminus, was constructed from FLAGhCTR2 (22).

Chemiluminescent Anti-M2 Assay in COS-1 Cells. Cells were transfected as described above and seeded up to 40 000 cells/well in a 96-well plate. On day 2, cells were washed twice with PBS containing 2 mM $CaCl_2$ and $MgCl_2$ (PBS/$CaCl_2$/$MgCl_2$) and incubated for 1 h at room temperature with a "blocking solution". Cells were then washed twice with PBS/$CaCl_2$/$MgCl_2$ and incubated for 1 h at room temperature with Anti-FLAG-M2 (1:300 dilution in PBS/0.5% BSA/0.1% sodium azide) (Eastman Kodak Co.). Cells were then washed twice with PBS/$CaCl_2$/$MgCl_2$ and incubated with secondary β-galactosidase-conjugated antibody (1:5000 dilution in PBS/0.5% BSA/0.1% sodium azide) (TROPIX) for 1 h at room temperature. After the incubation, the cells were washed twice with PBS/$CaCl_2$/$MgCl_2$. Finally, 100 μL of Galacton-Star/Sapphire-II substrate/enhancer formulation (TROPIX) was added to each well; the mixtures were incubated for 5 min, and the luminescence was measured for 10 s.

Membrane Preparation and Western Blotting. COS-1 cells were transfected in 10 cm dishes (1 million cells/dish) using the DEAE-dextran method as described. Forty-eight to seventy-two hours after transfection, the growth media from 10 dishes were transferred to conicals and centrifuged at 1000*g* for 10 min to collect detached cells. Thereafter, 2 mL of suspension buffer [25 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 5 μg/mL aprotinin, 5 μg/mL leupeptin, and 50 μg/mL bacitracin] was added to each dish, and cells were scraped using a rubber policeman. The scraped cells were combined with those from the media and centrifuged at 1000*g* for 10 min, and the pellets were frozen at −80 °C. The cells were resuspended in 14 mL of the suspension buffer and homogenized with 15 strokes of a Dounce homogenizer. Homogenized cells were centrifuged at 500*g* for 15 min, and the supernatants were transferred to ultracentrifuge tubes, centrifuged at 100000*g* for 60 min at 4 °C, and resuspended in suspension buffer. An aliquot of each membrane preparation was taken, and an equal volume of 2× Laemmli buffer with 40 mM TCEP-HCl (PIERCE) was added. After mixing, the samples were heated at 65 °C for 30 min, 10× NEM in DMSO (500 mM) was added to each sample to a final concentration of 50 mM, and the mixtures were heated at 50 °C for 45 min. Twenty micrograms of protein were

electrophoresed on a 10% SDS−PAGE gel; the proteins were transferred onto nitrocellulose in transfer buffer (14.4 g/L glycine and 3.03 g/L Tris-Base in 20% MeOH) at 25 V and 4 °C overnight. The Galact-Immune kit from TROPIX was used (primary anti-FLAG M2 antibody was from Sigma) for immunodetection, and experiments were carried out according to the manufacturer's recommendations.

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