N-glycosylation of CRF receptor type 1 is important for its ligand-specific interaction

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Assil, Iman Q., and Abdul B. Abou-Samra. N-glycosylation of CRF receptor type 1 is important for its ligand-specific interaction. Am J Physiol Endocrinol Metab 281: E1015–E1021, 2001.—The corticotropin-releasing factor (CRF) receptor type 1 (CRFR1) contains five potential N-glycosylation sites: N38, N45, N78, N90, and N98. Cells expressing CRFR1 were treated with tunicamycin to block receptor glycosylation. The nonglycosylated receptor did not bind the radioligand and had a decreased cAMP stimulation potency in response to CRF. To determine which of the polysaccharide chain(s) is/are involved in ligand interaction, the polysaccharide chains were deleted using site-directed mutagenesis of the glycosylation consensus, N-X/S/T. Two sets of mutations were performed for each glycosylation site: N to Q and S/T to A, respectively. The single mutants Q38, Q45, Q78, Q90, Q98, A40, A47, A80, A92, and A100 and the double mutants A40/A47 and A80/A100 were well expressed, bound CRF, sauvinagine (SVG), and urotensin-I (UTS-I) with a normal affinity, and increased cAMP accumulation with a high efficiency. In contrast, the combined mutations A80/A92/A100, A40/A80/A92/A100, and A40/A47/A80/A92/A100 had low levels of expression, did not bind the radioligand, and had a decreased cAMP stimulation. These data indicate the requirement for three or more polysaccharide chains for normal CRFR1 function.

corticotropic-releasing factor receptors; tunicamycin

CORTICOTROPIN-REleasing factor (CRF), a 41-amino acid polypeptide isolated from hypophyseal extracts (31), has a potent ACTH-releasing activity in vivo and in vitro (30). Urotensin-I (UTS-I), a 41-amino acid peptide isolated from the caudal neurosecretory organ of the teleost fish (15), and sauvinagine (SVG), a 40-residue peptide isolated from the skin of the amphibian species *Pyllophone sauvagei* (18), have high sequence homology (~50%) to CRF and are equipotent to CRF in releasing ACTH from rat anterior pituitary cells in vitro and in vivo (27). Urocortin (UCN), a UTS-I homolog cloned from mammalian brain (10, 17, 19, 28, 32, 36), is also a potent ACTH secretagogue in vitro and in vivo.

The CRF-related peptides exert their effects through specific G protein-coupled receptors encoded for by two distinct genes in mammalian, avian, and xenopus species: CRFR1 (5, 23, 34, 35) and CRFR2 (3, 16, 29). In catfish, a third CRF receptor gene, CRFR3, has been recently characterized with a unique distribution in the central nervous system and the pituitary (2). The amino termini of the CRF receptors, which are thought to contain important domains for ligand binding (6, 24), have several potential glycosylation sites and six conserved cysteines.

Earlier studies have shown that the CRF receptor is differently glycosylated in different regions of the central nervous system (12). The molecular cloning of CRFR1 revealed that this receptor, which has five potential N-linked glycosylation sites, has a similar affinity for the different CRF-like ligands: CRF, SVG, UTS-I, and UCN. Therefore, we examined the hypothesis that glycosylation of CRFR1 influences ligand binding affinity and/or the EC50 of cAMP stimulation by the different CRF-like ligands.

The literature presents conflicting views regarding the role of N-glycosylation in the function of G protein-coupled receptors. Treatment with tunicamycin of cell lines stably expressing the parathyroid hormone (PTH)/PTH-related peptide (PTHrP) receptor had no effect on ligand binding and cAMP stimulation (4, 37); however, a similar treatment of transiently transfected cells dramatically decreased cAMP stimulation potency (37). Tunicamycin greatly reduced cell surface expression, binding, and signaling abilities of many cell surface receptors such as the secretin receptor (22), the thyrotropin receptor (21), and the Ca2+ receptor (11). However, tunicamycin disrupts the glycosylation of all the N-glycosylation sites of the receptor and of other cellular proteins. To determine which polysaccharide chain(s) is/are important for function, we performed site-directed mutagenesis on the glycosylation consensus sites. Because the hydroxyl groups of S or T within the N-glycosylation consensus, N-X-S or T, serve as hydrogen bond donor during glycosylation of the N residue, substitution of S or T within a glycosylation consensus prevents glycosylation of the N residues. Therefore, we targeted each glycosylation site with two independent mutations: N to Q or S/T to A, respectively. Comparison of the functional properties of the two sets of mutations helps to distinguish the function of the poly-

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saccharide chain from the effects of amino acid substitu-

tions.

MATERIALS AND METHODS

Materials. Unless otherwise indicated, all chemicals were purchased from Sigma Chemicals (St. Louis, MO). Na$^{125}$I was purchased from Du Pont-New England Nuclear (Boston, MA). The peptides human CRF-(1–41) (CRF), [Tyr$^0$,Gln$^1$,Leu$^16$]SVG (YQLS), and UTSl-I were synthesized in the New England General Hospital (MGH) Biopolymer Facility, HPLC purified, and analyzed by mass spectroscopy, amino-terminal sequenc-
ing, and acid hydrolysis. SVG was obtained from Bachem (King of Prussia, PA); disuccinimidyl suberate (DSS) was purchased from Pierce (Rockford, IL). Tissue culture media were from MGH Media Facilities (Boston, MA). Anti-CAMP antibody was purchased from Dr. A. Baukal (Dr. K. Catt's Laboratory, National Institute of Child Health and Human Development, National Institutes of Health).

Radiodiodation. The oxidation-resistant SVG analog YQLS, in which the methionine at position 17 was replaced by a leucine, was labeled with $^{125}$I by use of the chloramine-T method. Excess nonincorporated $^{125}$I was removed by adsorp-
tion of the peptide on a $C_{18}$ Sep-Pak cartridge (Waters). The labeled peptide was eluted from the Sep-Pak cartridge with a 60% acetonitrile (ACN)-0.1% trifluoroacetic acid (TFA), and the eluate was diluted 1:1 with 0.1% TFA and purified on a $C_{18}$ reverse-phase column by means of an HPLC system that was equilibrated with 0.1% TFA and eluted with a gradient of 30–70% ACN in 0.1% TFA (35).

Cell culture and tunicamycin treatment. LLCPK-1 cells, stably transfected with the mouse CRFR1 cDNA (20), were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 200 U/ml penicillin and 20 μg/ml streptomycin sulfate. The cells were cultured at 37°C in a humidified atmosphere in 5% CO$_2$. COS-7 cells were transiently transfected with the mouse CRFR1 and the various CRFR1 glyco-
sylation mutants at 90% confluence by use of the DEAE-dextran method (34). LLCPK-1 cells were treated with 6 μg/ml tunicamycin for 48–72 h in tunicamycin before functional assays were performed.

Site-directed mutagenesis. Site-directed mutagenesis (14) was used to introduce N to Q or S/T to A as follows: Q38, Q45, Q78, Q90, Q98, A40, A47, A80, A92, and A100. A40 and A80 were used to make two double mutants, A40/A47 and A80/ A100, and A80/A100 was used to make other multiple muta-
tions as appropriate. A unique restriction site, PstI, was introduced in both A80/A92/A100 and A40/A47; this site was used to construct the A40/A47/A80/A92/A100 (ALL S/T-A). All mutations were confirmed by sequencing.

Measurement of receptor expression on the cell surface. The c-myC epitope tag (QKLSEEDL) was included within the amino-terminal domain of the mouse CRFR1 between Glu$^31$ and Ser$^32$ (26). Ascites fluid was developed in the peritoneal cavity of pristane-primed Balb-c mice (Charles River Laborat-
ories, Wilmington, MA) by injecting 9E10 hybridoma cells, obtained from American Type Tissue Culture. The 9E10 monoclonal antibody specifically recognizes the c-myC epiti-
lope. Stably transfected LLCPK-1 cells (90–95% confluent) and transiently transfected COS-7 cells in 24-well plates (72 h after transfection) were rinsed with phosphate-buffered saline (PBS) containing 5% heat-inactivated FBS and incubated with the monoclonal antibody 9E10 monoclonal antibody at 1:1,000 dilution. The cells were incubated for 2 h at room temperature, rinsed with PBS, and incubated for another 2 h with $^{125}$I-labeled sheep anti-mouse immunoglobulin G diluted in PBS-5% FBS (200,000 cpm/well). The superna-
tant was removed, and the cells were washed and lysed with 1 N NaOH. The cell lysates were collected and counted in a Micromedic gamma counter.

Radioligand binding to intact LLCPK-1 and COS-7 cells. Binding assays were performed as described previously (34). Intact LLCPK-1 cells stably transfected with CRFR1 or in-
tact COS-7 cells transiently transfected with CRFR1 and CRFR1 glycosylation mutants were plated into 24-well plates, and binding was done when cells reached 90–95% confluence. The cells were rinsed with a Tris-based binding buffer (50 mM Tris-HCl, pH 7.6, 100 mM NaCl, 2 mM CaCl$_2$, 5 mM KCl, 5% heat-inactivated horse serum, 0.5% heat-inactivated FBS), and $^{125}$I-YQLS (100,000 cpm/well) was added in the presence of increasing concentrations of the competing peptide for 2–4 h at room temperature. The cells were then rinsed (3×) with binding buffer and lysed with 1 N NaOH (0.25 ml × 3). The cell lysates were collected, and the radioactivity was counted in an automated gamma counter (Micromedic Systems, Horsham, PA).

cAMP stimulation assay. LLCPK-1 cells or COS-7 cells were seeded in 24-well plates and were allowed to reach 90–95% confluence. The cells were chilled on ice and rinsed with ice-cold PBS. The cells were then challenged with the CRFR ligands at different concentrations in DMEM containing 2 mM 3-isobutyl-1-methyoxanthine (IBMX), 1 mg/ml BSA, and 35 mM HEPES, pH 7.4, at 37°C for 15 min. The media were then removed, and the cells were rapidly frozen on dry ice. Intracellular cAMP was extracted by thawing the cells in 1 ml of 50 mM HCl, and cAMP was then measured using RIA (1).

Chemical cross-linking of $^{125}$I-YQLS to CRFR1 and CRFR1 glycosylation mutants. Cells expressing wild-type or mutant receptors were plated in 24-well plates and allowed to reach 90–95% confluence. Cells were then rinsed with PBS. $^{125}$I-YQLS (1,000,000 cpm/well) was added in HEPES binding buffer (25 mM HEPES, pH 7.6, 125 mM NaCl, 5 mM KCl, 5% heat-inactivated horse serum, 0.5% heat-inactivated FBS) for 2–4 h at room temperature. The buffer was removed, the cells were rinsed with PBS to remove bound tracer, and DSS (0.5 mM) was added to the cells in PBS (pH 8.2) for 20–30 min. The cells were then rinsed with PBS, lysed with SDS sample buffer, and analyzed on a 5–20% SDS-polyacrylamide gel electrophoresis. CRFR1 or CRFR1 glycosylation mutants cross-linked to the radioiodinated ligand were visualized by autoradiography.

Western blot. Stably transfected LLCPK-I cells expressing the c-myC-CRFR1 receptor were allowed to reach confluence in a 10-cm dish. The cells were then lysed with 0.5 ml of 1% SDS in Tris-HCl (62.5 mM, pH 6.2). The lysates were then forced through a 25-gauge needle 5–10 times and centrifuged for 10 min at 12,000 rpm at 4°C. The supernatant was analyzed on 5–20% SDS-polyacrylamide gel gradient. The proteins were transferred onto a nitrocellulose membrane and incubated with the anti-c-myC monoclonal antibody (9E10) at 1:2,000 dilution, followed by a peroxidase-labeled antiamoine antiserum at 1:3,000 dilution. The signal was developed using an NEN kit (Chemiluminescence Reagent Plus) and visualized on Kodak X-ray film.

RESULTS

Effects of tunicamycin on CRFR1 functions. The effects of tunicamycin treatment were examined in LLCPK-1 cells stably expressing the murine c-myC-CRFR1. Treatment of the cells with 1–6 μg/ml tunicamycin decreased receptor glycosylation (Fig. 1A). In cells treated with 6 μg/ml tunicamycin, glycosylation was suppressed completely (Fig. 1A). Therefore, a concentration
of 6 μg/ml tunicamycin was used for the subsequent experiments. Cell surface receptor expression in tunicamycin-treated cells was 25% that of control (data not shown). However, tunicamycin-treated cells did not show any detectable radioligand binding (Fig. 1B). To examine the role of glycosylation in signal transduction, we measured intracellular cAMP accumulation in cells challenged with increasing concentrations (0.1–1,000 nM) of CRF. The EC50 of CRF for cAMP simulation increased ~100-fold in tunicamycin-treated cells compared with that in control cells (Fig. 1C).

Cell surface expression and radioligand binding of CRFR1 glycosylation mutants. To determine which polysaccharide chain(s) is/are important for ligand binding and signal transduction, we performed single N-to-Q or S/T-to-A mutations at each of the potential glycosylation sites. The two sets of mutations allow us to distinguish the functional impact of deletion of a single polysaccharide chain from that resulting from residue substitution. To measure cell surface expression of the mutants, we used c-myc-CRFR1 as a template to construct the glycosylation-deficient mutants. Cell surface expression of the N-to-Q mutants Q38, Q45, Q78, Q90, and Q98 was 108, 140, 51, 47, and 37% that of c-myc-CRFR1 (Table 1). Cell surface expression of the S/T-to-A mutants A40, A47, A80, A92, and A100 was 123, 174, 94, 148, and 85% that of c-myc-CRFR1 (Table 1). Specific binding of 125I-YQLS to Q38, Q45, Q78, Q90, and Q98 was 105, 82, 17, 19, and 18% that of c-myc-CRFR1 (Table 1). Specific binding of 125I-YQLS to A40, A47, A80, A92, and A100 was 101, 93, 129, 91, and 28% of c-myc-CRFR1 binding (Table 1).

Analysis of the competition curves showed that only two mutants, Q38 and Q78, exhibited a binding preference for SVG relative to CRF and UTS-I; the apparent dissociation constant (Kd) for SVG decreased 5- to 11-fold (Fig. 2). However, the SVG apparent Kd in the corresponding S-to-A mutants A40 and A80 was similar to that for CRF and UTS-I (Fig. 2); this indicates that the observed ligand selectivity of the N-to-Q mutants is not related to lack of a polysaccharide chain; rather, it reflects the effects of residue substitution (N to Q) at positions 38 and 78, respectively.

Effects of the mutations on CRF-, SVG-, and UTS-I-stimulated cAMP accumulation. To further investigate the role of glycosylation in ligand interaction, COS-7 cells expressing mutant receptors were incubated with increasing concentrations of the three peptides CRF, SVG, or UTS-I, and intracellular cAMP accumulation in presence of IBMX was measured. Some of the single-glycosylation mutants showed an enhanced stimulation by one of the CRF-related peptides. For instance, UTS-I was

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Expression</th>
<th>Specific Binding</th>
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<tbody>
<tr>
<td>c-myc-CRFR1</td>
<td>100 ± 6</td>
<td>100 ± 7</td>
</tr>
<tr>
<td>Q38</td>
<td>108 ± 5</td>
<td>105 ± 5</td>
</tr>
<tr>
<td>Q45</td>
<td>140 ± 15</td>
<td>82 ± 9</td>
</tr>
<tr>
<td>Q78</td>
<td>51 ± 5</td>
<td>17 ± 1</td>
</tr>
<tr>
<td>Q90</td>
<td>47 ± 5</td>
<td>19 ± 3</td>
</tr>
<tr>
<td>Q98</td>
<td>37 ± 7</td>
<td>18 ± 2</td>
</tr>
<tr>
<td>A40</td>
<td>123 ± 5</td>
<td>101 ± 13</td>
</tr>
<tr>
<td>A47</td>
<td>174 ± 3</td>
<td>93 ± 7</td>
</tr>
<tr>
<td>A80</td>
<td>94 ± 4</td>
<td>129 ± 6</td>
</tr>
<tr>
<td>A92</td>
<td>148 ± 9</td>
<td>91 ± 4</td>
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<tr>
<td>A100</td>
<td>85 ± 2</td>
<td>28 ± 4</td>
</tr>
<tr>
<td>A40/A47</td>
<td>105 ± 4</td>
<td>94 ± 18</td>
</tr>
<tr>
<td>A80/A100</td>
<td>46 ± 5</td>
<td>67 ± 1</td>
</tr>
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</table>

Table 1. Expression and radioligand binding of the c-myc-CRFR1 and the glycosylation mutants

Data are means ± SD of 3 experiments. Results are presented as percent c-myc-corticotropin-releasing factor (CRF) receptor-1 (CRFR1) cell surface expression or binding. Cell surface expression and 125I-labeled [Tyr6, Gln3, Leu16] sauvagine (SVG) (YQLS) specific binding were measured with 9E10 anti-c-myc antibody and 125I-YQLS binding to intact COS-7 cells expressing the c-myc-CRFR1 and the glycosylation mutants. Nonspecific binding was subtracted.

Fig. 1. Effects of tunicamycin on corticotropin-releasing factor (CRF) receptor-1 (CRFR1) functions. A: Western blot analysis: c-myc epitope-tagged CRFR1 (c-myc-CRFR1) stably expressed in LLCPK-1 cells grown in the presence of increasing concentrations of tunicamycin. Cell extracts were analyzed by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with the 9E10 monoclonal antibody, followed by a peroxidase-labeled anti-mouse IgG. The bands were visualized using chemiluminescence. B: effects of tunicamycin on ligand binding to CRFR1. Binding of 125I-labeled [Tyr6, Gln3, Leu16]SAV (125I-YQLS) to LLCPK-I cells expressing c-myc-CRFR1 in the presence of increasing concentrations of CRF. The cells were incubated without or with 6 μg/ml tunicamycin (CRFR1 + T). Data are means ± SD calculated from triplicate wells in 1 of 2 similar experiments. C: effects of tunicamycin on CRF-stimulated cAMP accumulation. Tunicamycin-treated (6 μg/ml) CRFR1 glycosylation mutants. Nonspecific binding was subtracted.

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slightly more potent than CRF in Q38 and A40, Q45 and A47, and Q90 and A92 (Table 2); these data suggest that a polysaccharide chain at these sites decreases UTS-I interaction with CRFR1. In contrast, the enhanced SVG potency in cAMP simulation in Q45 reflects a residue effect, because this enhancement did not occur in the corresponding S-to-A mutant A47 (Table 2). Similarly, the decreased SVG potency in Q78 was not paralleled by a similar decrease in the A80 mutant (Table 2); this also indicates that the observed effect results from the substituted residue, not from deletion of the polysaccharide chain, at this site. The EC$_{50}$ of CRF was slightly increased in both the Q38 and A40 mutants (Table 2).

Effects of combined mutations on receptor function. Because deletion of one N-linked polysaccharide chain did not have a dramatic effect on ligand binding, we examined whether deletion of more than one N-linked polysaccharide chain influences binding and signal transduction. The data from single-residue mutations indicated that expression of the S/T-to-A mutants was higher than that of the N-to-Q mutants. Therefore, combined mutations were constructed using two or more S/T-to-A mutations. Cell surface expression of the double mutants A40/A47 and A80/A100 was 105 and 46% of control, respectively (Table 1). These two mutants showed a radioligand binding of 94 and 67% of control with an apparent $K_d$ that was not different from that of the wild type (Fig. 3). The EC$_{50}$s of CRF, SVG, and UTS-I on cAMP accumulation in COS-7 cells expressing the different glycosylation mutants

### Table 2. Effects of CRF, SVG, and UTS-I on cAMP accumulation in COS-7 cells expressing the different glycosylation mutants

<table>
<thead>
<tr>
<th>Receptor Expressed</th>
<th>CRF EC$_{50}$</th>
<th>SVG EC$_{50}$</th>
<th>UTS-I EC$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-myc-CRFR1</td>
<td>3.2 ± 0.4</td>
<td>1.0 ± 0.4</td>
<td>2.1 ± 0.3</td>
</tr>
<tr>
<td>Q38</td>
<td>5.6 ± 3.5</td>
<td>1.6 ± 0.4</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>Q45</td>
<td>1.0 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>Q78</td>
<td>3.3 ± 0.6</td>
<td>10.0 ± 0.4</td>
<td>4.3 ± 2.9</td>
</tr>
<tr>
<td>Q90</td>
<td>1.5 ± 0.6</td>
<td>1.8 ± 0.3</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>Q98</td>
<td>1.7 ± 0.2</td>
<td>2.0 ± 0.5</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>A40</td>
<td>5.2 ± 3.3</td>
<td>4.3 ± 3.8</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>A47</td>
<td>1.7 ± 0.3</td>
<td>3.5 ± 0.5</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>A80</td>
<td>1.3 ± 1.1</td>
<td>2.3 ± 1.1</td>
<td>1.0 ± 0.6</td>
</tr>
<tr>
<td>A92</td>
<td>3.5 ± 0.6</td>
<td>0.8 ± 0.1</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>A100</td>
<td>1.6 ± 0.4</td>
<td>1.5 ± 0.5</td>
<td>1.7 ± 0.4</td>
</tr>
<tr>
<td>A40/A47</td>
<td>0.4 ± 0.2</td>
<td>1.5 ± 0.7</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>A80/A100</td>
<td>0.6 ± 0.3</td>
<td>0.4 ± 0.1</td>
<td>0.7 ± 0.4</td>
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</table>

Values are means ± SE. EC$_{50}$, expressed as nanomoles, were calculated from 3 experiments. UTS-I, urotensin I. Intracellular cAMP accumulation was measured after a challenge with 0.1–1,000 nM of each peptide in presence of 2 mM 3-isobutyl-1-methylxanthine for 20 min at 37°C.

Fig. 3. Functional properties of the A40/A47 and A80/A100 double mutants. Top: binding of CRF, SVG, and UTS-I to COS-7 cells expressing c-myc-CRFR1 and the double-glycosylation mutants. In-cells were incubated with the radioiodinated SVG analog $^{125}$I-YQLS in presence of increasing concentrations of CRF, SVG, and UTS-I. Data are means ± SD of triplicates in 1 of 2 similar binding experiments.

Fig. 2. Binding of CRF, sauvanine (SVG), and urotensin (UTS)-I to COS-7 cells expressing CRFR1 mutants lacking glycosylation at positions 38 and 78. COS-7 cells were transfected with plasmid DNA encoding the mutant Q38, Q78, A40, or A80. The next day, the cells were seeded in 24-well plates and allowed to grow for 2 additional days. The cells were incubated with the radioiodinated SVG analog $^{125}$I-YQLS in presence of increasing concentrations of CRF, SVG, and UTS-I. Data are means ± SD of triplicates in 1 of 3 similar binding experiments.
SVG, and UTS-I were 0.4, 1.0, and 0.4 nM in the A40/A47 mutant and 0.6, 0.4, and 0.7 nM in the A80/A100 mutant, respectively (Table 2). Expression of A80/A92/A100, A40/A80/A92/A100, and A40/A47/A80/A92/A100 was dramatically decreased to 3.6, 14, and 15% that of c-myc CRFR1, respectively. None of these mutants showed a detectable radioligand binding (data not shown). Stimulation of cAMP accumulation was detectable in both the triple and quadruple mutants (A80/A92/A100, A40/A80/A92/A100); however, maximal stimulation was dramatically decreased (15–20% of the wild-type response), and the EC₅₀s for CRF-stimulated cAMP accumulation were increased 10- to 20-fold (Fig. 4). No cAMP stimulation was detected when all of the polysaccharide chains were deleted (data not shown).

SDS-PAGE analysis of the receptor mutants. To identify which position is N-glycosylated, ¹²⁵I-YQLS-cross-linked mutant receptors were analyzed on a 5–20% SDS-PAGE to examine for changes in their apparent molecular weight. Loss of a glycosylation moiety is predicted to reduce the apparent molecular weight. Most single-receptor mutants did not show a reduction in their apparent molecular weight (data not shown). This finding is similar to that observed with single PTH/PTHrP receptor glycosylation mutants (37). Deletion of the potential polysaccharide chain at position 38 decreased the apparent molecular weight (Fig. 5A); this indicates that position N38 is glycosylated. The double mutant A40/A47 had a lower molecular weight than either A40 or A47 (Fig. 5A); this indicates that both N38 and N45 are N-glycosylated. Similarly, the apparent molecular weight of the double mutant A80/A100 was lower than either A80 or A100 (Fig. 5A), indicating that both N78 and N98 positions are N-glycosylated. The apparent molecular weight of the triple mutant A80/A92/A100 is lower than that of the double mutant A80/A100 (Fig. 5B); this indicates that position N90 is also N-glycosylated.

DISCUSSION

The presence of highly conserved N-linked glycosylation sites in the CRF receptor family suggests an important role for receptor glycosylation in receptor functions. Our data that the CRF receptor in tunicamycin-treated cells did not bind its radioligand and exhibited a decreased sensitivity for agonist stimulation indicate that glycosylation is important for these functions. However, CRFR1 has five potential N-linked glycosylation sites. The dramatic effects of tunicamycin on the ligand binding and signal transduction of CRFR1 raised the possibility that one of the N-linked polysaccharide chains might be critical for ligand binding and/or signal transduction. Some of the single N-to-Q mutations, Q78, Q90, and Q98, had decreased expression, which was paralleled by decreased total binding. However, these mutants had normal apparent Kᵦₜ and normal Ec₅₀s for cAMP stimulation. Furthermore, the corresponding S/T-to-A mutants had normal expression, binding, and signaling. These data indicate that no single polysaccharide chain is essential for binding or cAMP stimulation. Additionally, the double-mutant receptors had full functional properties; this indicates that deletion of two polysaccharide chains is also well tolerated. Our results agree with the finding that mutation of one or two N-glycosylation sites did not affect cell surface expression and function of receptors such as those of PTH and the follicle-stimulating hormone (8, 9, 11). In contrast, our data showed that...
deletion of three or more polysaccharide chains severely impairs ligand binding and signal transduction. Taken together, the data suggest a functional redundancy in the requirement of CRFR1 for N-glycosylation where the presence of at least three out of five polysaccharide chains is sufficient for the expression of full functional properties of CRFR1.

It is interesting to notice that some N-to-Q and S/T-to-A mutations targeting the same glycosylation consensus site did not result in the same phenotype. For instance, the Q45 and A47 mutants, both of which result in the deletion of the polysaccharide chain at position 45, did not result in the same phenotype: Q45 had a 10-fold lower EC50 for SVG, whereas A47 had a 3.5-fold increase in the SVG EC50. These opposing effects could not have resulted from the deletion of the polysaccharide chain at position 45. The opposing effects resulting from Q45 and A47 mutation may indicate that residues at positions 45 and 47 are important for the interaction of CRFR1 with SVG; the polysaccharide chain at position 45 may be involved as well. However, the net effect cannot be determined from the mutation approach at this site.

The multiple receptors and ligands and the overlapping and sometimes unique tissue distribution of CRF ligands and receptors make it difficult to determine the specificity of the biological response to each of the ligands in different physiological conditions. The fact that the CRF receptor is differentially glycosylated in different regions of the brain (12) raises the possibility that differential glycosylation may regulate CRF receptor specificity. The data from single-point mutations suggest that deletion of a polysaccharide chain at positions 38, 45, and 90 enhances the receptor sensitivity for UTS-I stimulation about two- to threefold. Furthermore, the combined deletion of two polysaccharide chains at positions 38 and 45 or at positions 78 and 98 enhances the responsiveness to UTS-I and CRF more than to SVG. These data suggest that differential glycosylation may influence CRFR1 ligand specificity.

N-linked glycosylation of G protein-coupled receptors may be required for proper folding, intracellular stability (33), and delivery of the receptor to the cell surface (11, 21, 25). Our data suggest that deletion of one or two of the polysaccharide side chains of CRFR1 does not disturb receptor expression and cell surface targeting. Therefore, different receptors may have different requirements for folding and cell surface expression in terms of N-glycosylation.

Our data are also consistent with those observed in the rat PTH/PTHrP receptor, showing that mutations to eliminate glycosylation in one or two N-glycosylation sites did not affect cell surface expression and function, whereas mutations that eliminate all potential glycosylation sites dramatically impaired PTH/PTHrP receptor functions (37). A similar finding was also observed in the calcitonin receptor (7, 13). These data indicate an important role for N-linked glycosylation in the functioning of the CRF/PTH/calcitonin family of G protein-coupled receptors.

In conclusion, CRFR1 glycosylation plays an important role in ligand interaction. The data, however, indicate a functional redundancy regarding the role of an individual polysaccharide chain and suggest that some polysaccharide chains may selectively influence the EC50 of CRF, SVG, or UTS-I.

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REFERENCES


