Glycosylation of the receptor guanylate cyclase C: role in ligand binding and catalytic activity

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GC-C (guanylate cyclase C) is the receptor for heat-stable enterotoxins, guanylin and uroguanylin peptides. Ligand binding to the extracellular domain of GC-C activates the guanylate cyclase domain leading to accumulation of cGMP. GC-C is expressed as differentially glycosylated forms in HEK-293 cells (human embryonic kidney-293 cells). In the present study, we show that the 145 kDa form of GC-C contains sialic acid and galactose residues and is present on the PM (plasma membrane) of cells, whereas the 130 kDa form is a high mannose form that is resident in the endoplasmic reticulum and serves as the precursor for the PM-associated form. Ligand-binding affinities of the differentially glycosylated forms are similar, indicating that glycosylation of GC-C does not play a role in direct ligand interaction. However, ligand-stimulated guanylate cyclase activity was observed only for the fully mature form of the receptor present on the PM, suggesting that glycosylation had a role to play in imparting a conformation to the receptor that allows ligand stimulation. Treatment of cells at 20 °C led to intracellular accumulation of

a mature glycosylated form of GC-C that now showed ligand-stimulated guanylate cyclase activity, indicating that localization of GC-C was not critical for its catalytic activity. To determine if complex glycosylation was required for ligand-stimulated activation of GC-C, the receptor was expressed in HEK-293 cells that were deficient in *N*-acetylglucosaminyltransferase 1. This minimally glycosylated form of the receptor was expressed on the cell surface and could bind a ligand with an affinity comparable with the 145 kDa form of the receptor. However, this form of the receptor was poorly activated by the ligand. Therefore our studies indicate a novel role for glycosidic modification of GC-C during its biosynthesis, in imparting subtle conformational changes in the receptor that allow for ligand-mediated activation and perhaps regulation of basal activity.

Key words: glycosylation, guanylate cyclase C, human embryonic kidney-293 cells (HEK-293 cells), *N*-acetylglucosaminyltransferase, surface localization.

INTRODUCTION

Membrane-bound guanylate cyclases are glycoprotein receptors consisting of a ligand-binding ECD (extracellular domain) with several N-linked glycosylation sites, a single transmembrane domain, followed by a PKLD (protein kinase-like domain) and a guanylate cyclase domain [1]. Binding of ligands to the ECD leads to a conformational change that is transmitted to and activates the catalytic domain to produce cGMP, which in turn acts as a second messenger and regulates a number of physiological processes. GC-C (guanylate cyclase C) is a receptor for the peptide hormones guanylin and uroguanylin as well as a family of bacterial ST (heat-stable enterotoxin) peptides [2]. GC-C is expressed to high levels in the gastrointestinal tract [3,4], but expression is also detected in a number of extraintestinal tissues [5–7]. In the intestine, the role of GC-C appears to be in regulating the fluid and ion balance [8], but more recent evidence suggests that activation of the receptor by its ligands also regulates colonic cell proliferation [9,10] and also modulates a cGMP-gated channel, thereby inhibiting DNA synthesis in colonic cells [11]. It has been hypothesized that the prevalence of ST peptide-mediated diarrhoeas in developing countries could account for the low incidence of colon cancer in these regions of the world, as a consequence of sustained activation of GC-C. Therefore an understanding of the regulation of GC-C signalling at the protein level could provide novel approaches to exploit its activation potential in disease states.

GC-C activity is regulated at a number of levels, which include binding of ATP to the kinase-like domain [12], phosphorylation by protein kinase C [13,14], regulation of transcription of GC-C mRNA [15] and ligand binding to the ECD, leading to desensitization of the receptor in intestinal cells [16,17]. Ligand binding does not require glycosylation of the ECD of GC-C, since bacterially expressed protein can bind the ST peptide with an affinity similar to the full-length receptor expressed in mammalian cells [18]. However, mutational analysis of glycosylation sites of porcine GC-C has suggested that glycosylation of asparagine residues at 172 and 379 is essential for proper folding of the ECD to allow ligand-binding, since some mutant receptor is expressed in a form that binds a ligand with an affinity comparable with the wild-type receptor, while a large amount is inactive [19].

Studies with different members of the receptor guanylate cyclase family, such as the atrial natriuretic peptide receptors, GC-A and GC-B, have shown that glycosylation is essential for proper folding of the ECDs of these receptors to allow ligand binding [20–22]. For example, GC-A is present as two differentially glycosylated forms of M_w equal to 135 and 125 kDa in HEK-293 cells (human embryonic kidney-293 cells), but only the fully mature 135 kDa form binds atrial natriuretic peptide [20]. However, deglycosylation of mature GC-A with endoglycosidase F2 and endo H (endo- β -*N*-acetylglucosaminidase H) does not subsequently alter the affinity for the ligand, suggesting that glycosylation of GC-A might be important for the initial folding of

Abbreviations used: DIG, digoxigenin; ECD, extracellular domain; endo H, endo- β -*N*-acetylglucosaminidase H; ER, endoplasmic reticulum; GC-C, guanylate cyclase C; GNA, *Galanthus nivalis* agglutinin; GnT1, *N*-acetylglucosaminyltransferase 1; HEK-293 cells, human embryonic kidney-293 cells; NHS, *N*-hydroxysuccinimido; PKLD, protein kinase-like domain; PM, plasma membrane; PNGase F, peptide *N*-glycosidase F; ST, heat-stable enterotoxin.

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the receptor, but once folded, GC-A does not require the presence of sugar residues for ligand binding [21]. Similar observations have been made for GC-B which is also expressed as two differentially glycosylated forms with only the fully glycosylated form of the receptor capable of binding a C-type natriuretic peptide [22,23]. Thus glycosylation is important for imparting a conformation required for ligand binding in this class of receptors but whether glycosylation plays a role in the actual signalling process has not been studied yet.

The available crystal structure of the ECD of natriuretic peptide receptor C in unliganded form and in complex with its ligand C-type natriuretic peptide has indicated an additional role for N-linked sugars in the conformational change that takes place after ligand binding [24]. The structure consists of a dimer of the ECD with each monomer consisting of two interconnected subdomains. The unliganded natriuretic peptide receptor C dimer is stabilized by nine H bonds formed by the linker peptide between the two domains, five of which are formed with high mannose residues on Asn-248, suggesting that the sugars are involved in maintaining the receptor in closed conformation. After ligand binding, four of these bonds are broken, the sugar becomes disordered and conformational changes lead to a decrease in the distance between the two C-terminal ends of the two polypeptide chains comprising the dimer, thereby leading to receptor activation. Although sugar residues are not involved in actual ligand binding to the ECD, they are thus important for the conformational change that takes place after ligand binding leading to receptor activation.

Recently, we have shown that colonic cells, that harbour high levels of GC-C, become refractory to ST peptide stimulation following prior exposure of the cells to the ligand [25]. Interestingly, this cellular refractoriness was associated with reduced expression of the hyperglycosylated, 145 kDa form of GC-C, suggesting that glycosylation of GC-C appears to determine its ability to be activated by its ligand. This appeared to be a novel means of regulation of this receptor, and warranted further study as detailed in the present paper.

GC-C exists in two differentially glycosylated forms of 145 and 130 kDa. In the rat intestine, the hypoglycosylated form of GC-C of M_w 130 kDa was found to be the high mannose form of GC-C present in the ER (endoplasmic reticulum) [26]. Using inhibitors of glycosylation in HEK-293 cells engineered to express rat GC-C, it was suggested that the 130 kDa form could be the precursor of the 145 kDa form [27]. However, no functional difference between the two differentially glycosylated forms of GC-C, in terms of ligand binding or cGMP production, has been reported yet, despite their presence and changes in their relative ratios in a number of tissues and cell lines [26,28].

In view of the role of N-linked glycans in the activation of GC-A that has been suggested by the crystal structure, we investigated the role of glycosylation in signal transduction by GC-C using biochemical approaches. The unglycosylated 120 kDa form of GC-C is unable to bind the ligand and does not possess guanylate cyclase activity. The 130 kDa form is the precursor of the 145 kDa form, with the 130 kDa form present in the ER and 145 kDa form on the PM (plasma membrane). Although these two forms bind ST peptide with similar affinity and are catalytically active, only the mature 145 kDa form responds in terms of increased cGMP production on ligand binding. Expression of GC-C in a cell line that prevents complex glycosylation presents a hypoglycosylated form of GC-C on the surface of cells that is poorly stimulated by ligand. Since deglycosylation of completely glycosylated GC-C does not abolish ligand-mediated activation, it appears that the alterations in glycosylation that occur during the transit of GC-C to the PM confers a conformation on GC-C that initially allows ligand binding and then ligand stimulability, suggesting a novel

role for complex sugars in the signal transduction of receptor guanylate cyclases.

EXPERIMENTAL

Cell line generation and maintenance

HEK-293 cells stably expressing full-length GC-C, HEK-293-GCC, was available in the laboratory [17]. HEK-293S GnT1⁻ cells (where GnT1 stands for *N*-acetylglucosaminyltransferase 1) [29] were obtained from Dr H. G. Khorana and Dr P. J. Reeves (Massachusetts Institute of Technology, U.S.A.), and were cultured in DMEM:F12 (where DMEM stands for Dulbecco's modified Eagle's medium) containing 10% (v/v) foetal calf serum, 120 mg/l penicillin and 270 mg/l streptomycin. A stable cell line expressing GC-C (HEK-293S GnT1⁻-GCC) was generated by procedures reported earlier [17], and was routinely cultured in a medium containing 100 µg/ml G418. Cells were plated in 24-well culture dishes and stimulated with the ST peptide at confluence or the membrane prepared as described previously [12] for ligand-binding and deglycosylation experiments. Protein was estimated by a modified Bradford assay [30].

Deglycosylation of GC-C

For complete deglycosylation, 20 µg of total membrane from HEK-293-GCC or HEK-293S GnT1⁻-GCC cells was prepared and resuspended in 50 mM PBS (phosphate buffer containing 0.9% NaCl, pH 7.2), 0.1% SDS and 50 mM 2-mercaptoethanol. The tube was boiled for 5 min, cooled to room temperature (25 °C) and Nonidet P40 was added to a final concentration of 1%. PNGase F (peptide *N*-glycosidase F, 20 m-units; Roche, Indianapolis, IN, U.S.A.) was added and the reaction incubated at 37 °C for 8 h. For endo H treatment (Sigma), 20 µg of total membrane prepared from HEK-293-GCC cells was resuspended in 100 mM sodium citrate (pH 5.5), 0.1% SDS and boiled for 5 min. The reaction was cooled to room temperature and Triton X-100 was added at a concentration of 0.1%. The denatured membrane was then treated with 2 m-units of endo H at 37 °C for 8 h. The reaction was stopped by adding Laemmli buffer and samples were resolved by SDS/PAGE (6% gel), transferred on to PVDF membrane and subjected to Western-blot analysis with GC-C:C8 monoclonal antibody raised to the PKLD of GC-C [17].

For selective deglycosylation, membranes prepared from HEK-293-GCC cells were incubated with 2 units of β -galactosidase (Glyko, San Leandro, CA, U.S.A.) or 10 m-units of neuraminidase III (Glyko) or both in 50 mM phosphate buffer along with protease inhibitors at 4 °C for 12–13 h followed by 15 min at 37 °C. Glycosidase-treated membranes were then subjected to Western-blot analysis and *in vitro* guanylate cyclase assay as described below.

Detection of sugars present on GC-C

Membranes (3–4 mg) prepared from HEK-293-GCC cells were solubilized at a concentration of 2 mg/ml in immunoprecipitation buffer (20 mM Tris/HCl, pH 7.5/150 mM NaCl/2 mM EDTA/1% Triton X-100/protease and phosphatase inhibitors) for 1 h at 4 °C. The soluble fraction was precleared with 20 µg/ml normal rabbit antibody and then incubated overnight at 4 °C with a polyclonal antibody raised to the C-terminal domain antibody of GC-C [12] at a concentration of 20 µg/ml. The immunoprecipitate was separated by SDS/PAGE (7.5% gel), transferred on to PVDF membrane and sugars were identified

using the DIG (digoxigenin) Glycan Differentiation kit (Roche), according to the manufacturer's instructions.

Surface biotinylation of cells

Cells were washed with PBS (pH 8.0) containing 1 mM CaCl_2 and 0.5 mM MgCl_2 (PBS-CM), and then incubated in PBS-CM containing 500 $\mu\text{g/ml}$ sulpho-NHS-biotin (where NHS stands for *N*-hydroxysuccinimido; Sigma) for 30 min at room temperature. Excess biotin was quenched by incubation with 50 mM Tris/HCl (pH 7.5) for 5 min. Membranes were prepared from these cells and solubilized in immunoprecipitation buffer. The soluble fraction was incubated with 30 μl of streptavidin-agarose for 12 h at 4 °C, biotinylated proteins were resolved by SDS/PAGE (7.5 % gel), transferred on to PVDF membrane and subjected to Western-blot analysis with GC-C:4D7 antibody [12].

Immunostaining of HEK-293-GCC cells

Immunostaining was performed as described earlier [31]. HEK-293-GCC cells cultured on coverslips for 2–3 days were fixed in PBS containing 4 % (w/v) paraformaldehyde for 30 min. Cells were then washed and incubated in 2 % BSA and 0.1 % Triton X-100 in PBS for 1 h at room temperature to block non-specific sites and permeabilize cells. After washing again in PBS, cells were incubated overnight with 10 $\mu\text{g/ml}$ GC-C:4D7 in blocking buffer at 4 °C. Cells were washed and incubated with FITC-conjugated anti-mouse antibody (Invitrogen) diluted to 1:100 in blocking buffer for 1 h at room temperature. Cells were again washed and mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, U.S.A.) and visualized under a Zeiss fluorescence microscope using standard filters for FITC at $\times 63$ magnification.

Labelling of GC-C with ^{35}S -methionine

Confluent monolayer cultures of HEK-293-GCC cells were washed with cysteine-methionine-free medium, and then incubated with ^{35}S -methionine (100 $\mu\text{Ci/ml}$; PerkinElmer, Indianapolis, IN, U.S.A.) for 1 h at 37 °C in a CO_2 incubator. Cells were washed twice and harvested either immediately or following further culturing in serum-containing medium for different time periods (chase period). Cells were lysed and membranes were prepared as described above. Membrane protein was solubilized and subjected to immunoprecipitation with the C-terminal domain antibody. Following immunoprecipitation, samples were subjected to SDS/PAGE (7.5 % gel), followed by autoradiography using a BioMax Transcreen-LE and Fuji X-ray film. Intensity of the bands was quantified using an Alpha Imager Gel documentation system.

Subcellular fractionation of HEK-293-GCC cells

Subcellular fractionation of HEK-293-GCC cells was performed using sucrose density gradient centrifugation [32]. Cells (2.5×10^8) were scraped in 10 ml of homogenization buffer (250 mM sucrose/10 mM Hepes, pH 7.5/5 mM EDTA/1 mM dithiothreitol/protease and phosphatase inhibitors). Cells were homogenized using a glass homogenizer with a Teflon pestle and the homogenate was centrifuged at 1000 *g* for 10 min at 4 °C to remove intact cells. The supernatant was centrifuged at 10 000 *g* for 10 min at 4 °C. The post-mitochondrial supernatant was then loaded on to a 20–38 % sucrose density gradient [1.5 ml of 38 % sucrose, 1.0 ml of 35 % sucrose, 1.0 ml of 30 % sucrose and

0.8 ml of 20 % sucrose, with all solutions prepared in 10 mM Hepes (pH 7.5) and 1 mM dithiothreitol]. The gradient was centrifuged at 100 000 *g* in a Ti 50 rotor in Beckman ultracentrifuge for 2.5 h at 4 °C. The PM fraction, present at the interface between the 20 and 30 % sucrose solutions and the ER fraction, present in the 38 % sucrose solution, were collected and centrifuged at 100 000 *g* in Ti 50 rotor for 1 h at 4 °C. The pellets obtained were suspended in resuspension buffer and used for Scatchard analysis, *in vitro* guanylate cyclase assays and Western-blot analysis.

Receptor-binding analysis

Membrane fractions were incubated with radiolabelled ST peptide and used for binding analysis essentially as described earlier [33]. To monitor the affinity of the surface-localized GC-C for the ST peptide, confluent monolayers of HEK-293-GCC cells were incubated with increasing concentrations of ^{125}I -labelled ST_{Y72F} peptide for 1 h at 37 °C in DMEM:F12 with 0.2 % BSA. Cells were washed quickly with ice-cold PBS and lysed in 0.1 M HCl, and radioactivity in the lysates was monitored. To estimate GC-C content in the ER and PM fractions, receptor-binding analysis was performed using 10 μg of protein and 100 000 c.p.m. of ^{125}I -labelled ST_{Y72F} peptide in the presence or absence of 100 nM unlabelled ST peptide. Receptor-binding analysis was also performed on membranes prepared from HEK-293 GnT1-GCC cells as described above.

In vitro guanylate cyclase assays

Assays were performed as described earlier [12] with 5–20 μg of membrane protein, using MgGTP (4 mM MgCl_2 and 1 mM GTP) as a substrate in the presence or absence of 100 nM ST peptide. To measure the manganese-mediated activation of GC-C, 4 mM Mn and 1 mM GTP was used as substrate. In experiments performed to check Lubrol-PX-mediated activation, membranes were incubated with 0.3 % Lubrol-PX and 4 mM Mg and 1 mM GTP as substrate. Intracellular cGMP levels were measured by radioimmunoassay as described earlier [34].

Surface trypsinization of HEK-293-GCC cells

Cells were detached from the surface by incubating in DPBS (Dulbecco's PBS) containing 5 mM EDTA for 5 min and collected by centrifugation at 500 *g* for 2 min. Cells were resuspended in DPBS containing 0.1 % trypsin and 0.03 % EDTA and incubated at room temperature for 5 min. Trypsinization was stopped by adding 2 mM PMSF and 400 $\mu\text{g/ml}$ soya bean trypsin inhibitor, and the reaction was diluted in DPBS. Cells were collected by centrifugation at 2000 rev./min for 2 min, resuspended in homogenization buffer, sonicated and membranes were prepared as described above.

RESULTS

Glycosylation of GC-C in HEK-293-GCC cells

There are ten potential N-linked glycosylation sites in human GC-C and eight of them are conserved in GC-Cs present in different mammals (Figure 1). Western-blot analysis of membranes prepared from HEK-293-GCC cells showed the presence of two immunoreactive bands, of M_w 145 and 130 kDa, representing differentially glycosylated forms (Figure 2A). Membranes were treated with PNGase F, which cleaves N-linked glycans from asparagine residues, and endo H, which cleaves only high

Human	MKTLLDLALWS---LLFQPGWLSFSSQVSQ-NCHNGSYEISVLMMGNSAFAEPLKNLED
Pig	MKTPLLALALWS---LLQLGLTFWPSSVSQ-NCHNGSYEISVLMNNSAFPELDNLKA
Rat	MTS-LGLAVR---LLLQPTLMFWASQVRQ-KCHNGTYEISVLMMDNSAYKEPLQNLRD
Guinea Pig	MKSPLLGLVWVSLLLQLLPGLAFWNSQISQ-NCHNGSYEITVLMNNYAFQESLES LKT
Xenopus	MRKEHTWLC LQ-----IIL---WLTDLLEANCMGSLTMNVIMLNDSTMEWNKAVQE
Medaka	MSTFNWLSVLC-----AFG-VSASCVQDTG-QCMDG-ITVNVILLEDEESPWLSKYVGG
Human	AVNEGLEIVRGRQLNAGLNVTVNATFMYSDGLIHNSGDCRSSTCEGLDLLRKISNAQRMG
Pig	VVNEGVNIVRQRLLEAGLTVTVNATFVYSEGVYKSSDCRSSTCEGLDLLRTISSEKRMG
Rat	AVEEGLDIVRKRLREAELNVTVNATFIYSDGLIHKSGDCRSSTCEGLDLLREITDRKMG
Guinea Pig	AVNKGDIVKQRLQEAALYVTVNATFIHSDGLIHKSGDCRSSTCEGLDLLREIARQKRMG
Xenopus	AVSIGMHVVTKDLEREGIKVTINADFQTFNTDLYATPGCVSSGCEGVEKLNLRHTRRLG
Medaka	QILEAIEKDAAINAEEGMEFNLTVNFEFGFNTTLYRQGCITSACEGAEKLNKLMTVTELG
Human	CVLIGPSTYSTFQMY---LDTELSYPMISAGS FGLSCDYKETLTRLMS PARKLMYFLVN
Pig	CVLLGPSTYSTFQMY---LDTDLNYPMISAGS FGLSCDYKETLTRLMS PARKLMYFLVD
Rat	CVLMGPSTYSTFQMY---LDTELNYPMISAGS FGLSCDYKETLTRILPPARKLMYFLVD
Guinea Pig	CALMGPSCTYSTYQMY---LDTELNYPMISAGS FGLSCDHKETLTRMMS PARKLMYFLVD
Xenopus	CVILGPTCTYATYQMLS--LKNTFGVPLISAGS FGLSCDYHRS LARMLLPARKITYFFKE
Medaka	CAVLGPTCTYATFAIADVEKGFNLSTPIISAGS FGSSCDYAMNLQRLLPARKISDFFIN
Human	FWKTNDLPFKTYSWSTS YVYKNGTETEDCFWYLNAL EASVS YFSHELGFKVLRQDKEFQ
Pig	FWKVNNFPFKPFSWNTAYVFNKSTES EDCFWYLNAL EAGVS YFSQKLSFKEMLRGNEEFQ
Rat	FWKVNNAPFKTFSWNS YVYKNGSEPEDCFWYLNAL EAGVS YFSEVLSFKDVLRRSEQFQ
Guinea Pig	FWKASNLFFKFSWNTS YVFKNGTESEHC FWYINAL EAGVS YFSQVLGFKEMLRGNEELQ
Xenopus	FWQYEDF-IKPKKQSVYIYKWDGNTESC FWYINAL ESGVS YFNNA LKFKEILRTEGELM
Medaka	FWKEFT--IKPKWRTAYVYKRQPNTE DCLWYIGAL EADGRFLVNVS--RTILRHPGDLK
Human	DILMDH-NRKS NVIMCGGPEFLYKLG DRAVAE--DIVIILVDLFNDQYLEDNV TAPDY
Pig	NILMDQ-NRKS NVIMCGAPETVHTLKG GRAVAE--DTVIIILVDLFNDHYFMLNV TAPDY
Rat	EILMGR-NRKS NVIMCGT PETFYNVKGLKVAD--DTVVIILVDLFNSHYFEDDTRAPEY
Guinea Pig	KILKDP-NRRS NVIMCGT PQTMES LKIDWATE--DTVIIILVDLFNNYYLEENATAPDY
Xenopus	KVLQEN-NHKS NVILMCGT PNDIWNLHNKVAIPQ--DKVLILLDIFNTVY--DNKSSPY
Medaka	DVLDSQENRTSNLFI LCGSPD LKEVKNI SDAADNLDILFLIDLYNDVY--TNTSMPE
Human	MKNVLVLT LSPG-NSLLNSSFSRNLSPTKRDFRLPYLNGILVFGHMLK-IFLENGENIT
Pig	MKNVLVLT LPP E-NSVSNSSFSKDL SLVKNDFTLAYMNGVLLFGHMLK-IFLEKREDVTT
Rat	MDNVLVLT LPP E-KFIANASVSGRFPSE RDFS LAYLEG TLLFGHMLQ-TFLENGESVTT
Guinea Pig	MKNVLVLT LPPG-NSTINTSLSKESLQEFSDFALAYLDGILLFGHMLK-TFLRNGENTTA
Xenopus	MENVLVVTQ RPS-NMSKISNOTGI AKLLEDNYAAGYLDGVLLFGHILK-KFLGSDVINQT
Medaka	MRNVLVLTMPDTRTYTIKPDLTGN--DTMNDYMAAYHDAVLLVGQVMRDIAIRNPAEMQG
Human	PKFAHA--FRNLTFEGYDGPVTLDDWGDVDSTMVLLYTSVDTKKYKVL LTYDTHVNKTY
Pig	SKFAHA--FRNITFEGHMGPTVTLDCGDI DNTMFLLYTSVDTSKYKVL LTYDTRKNYTNP
Rat	PKFARA--FRNLTFQGLEGPVTLDDSGDIDNIMCLLYVSLDTRKYKVL MAYDTHKNQTI
Guinea Pig	HKFAHA--FRNLTFEGSTGPVTLDDSGDIDNTM VLLYTSVDTKKFKPLLFYDTRINQTT
Xenopus	FSFIDQ--FRNIS IIGALGPLILDAAGDRELNTLLYSSTATNNY TELIQDFTSTNQTTV
Medaka	MEYVNTNYFRNVSFNGIGGHYKLDSYGD RDVNFSVIYTSTDN-KYKILFSFDTENRTKQ
Human	VDMSPTFTWKNSKLPNDITGRGPQILM
Pig	VDKSPFTIWKNHKLPNDIPGRGPQILM
Rat	VATSPNFIWKNHRLPNDVPG LGPQILM
Guinea Pig	IDTHPTFIWKNHRLPHDIPGLGPHILL
Xenopus	MDTSPNFIWKNHRLPSDVPQSGPHILT
Medaka	MDPSPTFIWTK-ALPDDKPGSELETQD

Figure 1 Alignment of the amino acid sequences of the ECDs of human (S57551), porcine (D17513), rat (M55636), guinea pig (Z74734), *Xenopus* (D49837) and Medaka fish (AB016081) GC-Cs

Potential N-linked glycosylation sites were identified using the ScanProsite tool from Prosite database (<http://www.expasy.ch/prosite/>) and are shown as shaded areas. The region in porcine GC-C, which is reported to be involved in ST peptide binding is underlined [39].

mannose structures. Subsequent Western-blot analysis indicated that complete deglycosylation of the two forms of GC-C after PNGase F treatment resulted in the generation of a protein of M_w 120 kDa, the mass of the protein predicted from its cDNA sequence (Figure 2A). Digestion with endo H resulted in a reduction of the molecular mass of the 130 kDa form to a mass of the completely deglycosylated receptor, indicating that all the residues on the 130 kDa form of GC-C that were glycosylated contained high mannose. Interestingly, there was also a reduction in the mass of the 145 kDa form, but not to the mass of the completely deglycosylated receptor (Figure 2A). This indicated

that the completely glycosylated form of GC-C could consist of hybrid and complex sugars, allowing removal of only the high mannose residues on hybrid sugars by endo H, leaving the complex sugars intact.

Identification of sugars present on the two differentially glycosylated forms of GC-C

GC-C was immunoprecipitated from membranes prepared from HEK-293-GCC and sugars present in the two glycosylated forms were identified using DIG-labelled lectins. The 130 kDa form was

Figure 2 GC-C is differentially glycosylated in HEK-293-GCC cells

(A) Membranes prepared from HEK-293-GCC cells were incubated with *N*-glycosidase F or endo H. Proteins were subjected to Western-blot analysis with GCC:C8, a monoclonal antibody raised to the PKLD of GC-C. (B) Identification of sugars present on the two forms of GC-C. GC-C was immunoprecipitated from HEK-293-GCC cells and immunoprecipitates were resolved by PAGE, transferred on to PVDF membranes and incubated with DIG-conjugated lectins; GNA recognizes the high mannose form of glycoproteins, MAA (*Maackia amurensis* agglutinin) recognizes sialic acid linked $\alpha(2-3)$ to galactose, SNA (*Sambucus nigra* agglutinin) recognizes sialic acid linked $\alpha(2-6)$ to galactose and DSA (*Datura stramonium* agglutinin) recognizes galactose linked $\beta(1-4)$ to *N*-acetyl glucosamine. Blots were washed and then incubated with anti-DIG antibody conjugated to alkaline phosphatase and developed using NBT/X-phosphate.

recognized by GNA (*Galanthus nivalis* agglutinin) that recognizes the high mannose form of glycoproteins, suggesting that this form of GC-C could be localized in the ER (Figure 2B). The 145 kDa form was not recognized by GNA but was recognized by *Maackia amurensis* agglutinin, which recognizes sialic-acid-linked $\alpha(2-3)$ to galactose, and by *Sambucus nigra* agglutinin, which recognizes sialic-acid-linked $\alpha(2-6)$ to galactose. The 145 kDa form of GC-C also interacted with *Datura stramonium* agglutinin that recognizes galactose-linked- $\beta(1-4)$ to *N*-acetylglucosamine in complex and hybrid N-glycans. *Arachis hypogea* (Peanut) agglutinin did not recognize either the 130 or 145 kDa form, suggesting that GC-C does not have O-linked sugars (results not shown).

The 130 kDa form is the precursor for 145 kDa form

To confirm that the 130 kDa form of GC-C is a precursor for the 145 kDa protein, HEK-293-GCC cells were treated with 100 μ g/ml cycloheximide for different time periods (6 to 18 h), and membranes made from these cells were subjected to Western-blot analysis. As shown in Figure 3(A), there was a progressive decrease in the amount of the 130 kDa form with a concomitant increase in the 145 kDa protein after cycloheximide treatment, indicating that the hyperglycosylated form was derived from the 130 kDa precursor.

Surface biotinylation of HEK-293-GCC cells followed by pull down of biotinylated proteins with streptavidin-agarose and Western-blot analysis, indicated that only the 145 kDa form of GC-C was associated with the streptavidin beads (Figure 3B). Therefore the 145 kDa form of GC-C was on the surface of cells and the 130 kDa form was intracellular, probably in the ER. Immunocytochemical localization of GC-C in HEK-293-GCC cells showed that a large fraction of GC-C was indeed intracellular (Figure 3C).

Half-life of differentially glycosylated forms of GC-C

Cells were incubated with 35 S-methionine for 1 h, during which the 130 kDa form of GC-C was labelled almost exclusively

Figure 3 The 130 kDa form of GC-C is a precursor for the 145 kDa mature protein

(A) HEK-293-GCC cells were treated with cycloheximide and membranes were prepared. Membrane protein (30 μ g) was subjected to Western-blot analysis using GCC:D7. The signal obtained on Western blot was quantified by densitometric analysis to obtain the fraction of GC-C present in each form and this was plotted against the time of cycloheximide treatment as shown in the graph. Results shown are representative of experiments performed twice. (B) HEK-293-GCC cells were surface-biotinylated and the membrane was prepared. Biotinylated proteins were separated by interacting with avidin beads and then subjected to Western-blot analysis with GCC:D7. (C) HEK-293-GCC cells were cultured and processed for immunocytochemical localization using GCC:D7 monoclonal antibody and FITC-conjugated anti-mouse antibody. Results shown are representative of an experiment performed three times.

(Figure 4A). The radiolabel was then chased by addition of the complete tissue culture medium, and the extent of radioactivity in 130 and 145 kDa forms for different time periods was estimated by autoradiography. As seen in Figure 4(A), a significant amount of radioactivity was detected in the 145 kDa protein only after 3–6 h of chase, indicating that the maturation of GC-C was a slow process. By measuring the radioactivity in the lower band, we were able to estimate that the half-life of the 130 kDa precursor form of GC-C was approx. 12 h. This suggests that processing of the 130 kDa form of GC-C is a slow process, and a significant amount of synthesized protein is retained within the ER.

To estimate the half-life of the 145 kDa form of GC-C, we surface-labelled cells with sulpho-NHS-biotin and then replaced the biotinylation medium with the complete tissue culture medium. At different time periods after surface biotinylation, solubilized proteins were interacted with streptavidin-agarose beads, and proteins bound to the beads were subjected to gel electrophoresis and Western-blot analysis with a monoclonal antibody to GC-C [12]. As shown in Figure 4(B), the amount of the 145 kDa protein decreased with time, with an estimated half-life of approx. 4 h. Presumably, this represented degradation through proteolysis, since no fragment of lower M_w was detected on Western-blot analysis.

Glycosylation of GC-C is not important for ligand binding, but regulates catalytic activity

Given that a fraction of GC-C was present in the PM and a larger amount in the ER, we prepared purified PM and ER fractions by sucrose density gradient centrifugation. Analysis of the membrane

Figure 4 Half-life of 130 and 145 kDa forms of GC-C

(A) Cells were labelled with ^{35}S -methionine for 1 h and the medium is replaced with complete tissue culture medium. Monolayers were harvested at the indicated times, membrane protein solubilized and GC-C-immunoprecipitated. Immunoprecipitates were subjected to SDS/PAGE and phosphorimager analysis to quantify radioactivity in the bands (inset). Results shown are representative of experiments repeated twice. (B) Cells were surface-biotinylated and cultured further for the indicated times. Membrane protein was prepared from the cells and solubilized, followed by interaction with streptavidin-agarose beads. Protein bound to the beads was subjected to Western-blot analysis with GC-C:C8 monoclonal antibody. The intensities of the bands were measured by densitometric analysis as shown in the inset. Results shown are representative of experiments repeated twice.

fractions showed that the ER-associated protein, calnexin, was enriched in the ER fraction when compared with the PM fraction (Figure 5A). In agreement with our earlier results, the 145 kDa form of GC-C was present in the PM fraction and the 130 kDa form exclusively in the ER fraction. Scatchard analysis of the binding of radiolabelled ST peptide to GC-C present in the ER indicated that the 130 kDa form of GC-C was capable of interacting with the ligand with an affinity comparable with the receptor present in the PM (Figure 5B). First, this suggested that the complex sugars present in the 145 kDa form do not change the affinity of ST peptide for GC-C. More importantly, the conformation attained by the high mannose ER form is equally competent in binding ST peptide as is the 145 kDa mature protein at the surface, indicating that complex glycosylation that occurs in the Golgi complex is not required for the ECD of GC-C to adopt a conformation that allows ligand interaction. However, when membranes were prepared from cells that had been treated with tunicamycin, thereby allowing the accumulation of completely unglycosylated GC-C, the receptor fraction did not bind radiolabelled ST peptide, indicating that initial glycosylation was essential for the receptor to attain a conformation suitable for ligand binding (results not shown).

We performed *in vitro* guanylate cyclase assays with the PM and the ER fractions, using membrane proteins normalized for equal ST peptide-binding sites and Western-blot reactivity, thus ensuring that equal amounts of functional receptor were used for assays. Results are shown in Figure 5(C). PM-associated GC-C showed significant ligand-stimulated guanylate cyclase activity, nearly 3-fold higher than the basal activity. However, the ER-associated form showed an increase in basal guanylate cyclase activity with no further stimulation on ST peptide addition. To normalize for total GC-C content, we performed assays in the presence of MnGTP, to monitor guanylate cyclase activity independently of the presence of ligand. The guanylate cyclase activity was similar in both the PM and ER-associated fractions of GC-C [17.2 ± 2.4 cGMP pmol $\cdot$ (fmol of GC-C) $^{-1}$ $\cdot$ min $^{-1}$ in the ER fraction versus 18.4 ± 3.8 cGMP pmol $\cdot$ (fmol of GC-C) $^{-1}$ $\cdot$ min $^{-1}$

in the PM fraction]. Therefore it appears that maturation of GC-C during the transit from the ER to the PM confers on it an ability to be stimulated in the presence of its ligand.

It is possible that the localization of GC-C, either in the ER or the PM fractions, could determine the extent of its ligand-stimulated catalytic activity. Incubation of cells at 20 °C inhibits the transport of fully processed proteins from the Golgi complex to the PM but does not inhibit the changes in glycosylation that occur in the Golgi [35]. We incubated HEK-293-GCC cells at 20 °C for 20 h and treated cells were trypsinized to remove the PM localized form of GC-C. Membranes were prepared from control cells, incubated at 20 °C and subjected to *in vitro* guanylate cyclase assays and Western-blot analysis. As shown in Figure 6(A), trypsin treatment of control cells removed the 145 kDa form of GC-C, releasing a fragment corresponding to the intracellular domain alone, which was recognized by the monoclonal antibody. However, there was a significant amount of the trypsin-resistant 145 kDa form in cells that were incubated at 20 °C for 20 h, indicating that some fully glycosylated, mature GC-C accumulated intracellularly. Control cells which were incubated at 37 °C showed a high basal guanylate cyclase activity after trypsin treatment, which was contributed by the fraction of GC-C that was in the ER, as well as the released intracellular domain (Figure 6B). In agreement with our earlier results, this membrane fraction did not show significant ST peptide-mediated GC-C activation after surface trypsinization. Membranes prepared from cells that were incubated at 20 °C showed a 3–4-fold stimulation of guanylate cyclase activity in the presence of ST peptide, similar to cells that had been incubated at 37 °C. Importantly, this ST peptide-mediated activation was retained even after surface trypsinization, indicating that the fraction of GC-C that contributed to ST peptide-mediated activation was localized intracellularly and accumulated there as a mature form of GC-C. These results indicate that GC-C does not require the specific lipid or protein environment provided by the PM for ligand-mediated activation, but acquires ST peptide stimulability during its processing in the Golgi complex.

Figure 5 Subcellular fractionation of HEK-293-GCC cells

HEK-293-GCC cells were homogenized, fractionated on 20–38% sucrose gradient and the ER and PM fractions were collected. **(A)** Fractions (5 μ g) of ER and PM were subjected to Western-blot analysis using GCC:C8 monoclonal antibody. The blot was then stripped and probed with calnexin antibody, an ER-specific marker, to check the purity of the fractions. Inset: Western blot performed with total membrane protein before fractionation. **(B)** Scatchard analysis of 130 and 145 kDa forms of GC-C. To check the affinity of the 130 kDa form of GC-C, Scatchard analysis was performed using the ER membrane fractions. Binding was performed on monolayer cultures of cells to measure the affinity of the PM associated, 145 kDa form of GC-C. The data were analysed using Graph Pad Prism. Experiments were performed at least twice and values shown represent results from a single experiment. **(C)** *In vitro* guanylate cyclase assays were performed using 3–5 μ g of protein from ER and PM using MgGTP (in the ratio 4:1) as the substrate. The assay was performed in the absence (basal) and presence of 100 nM ST peptide (+ST). Values shown are means $\pm$ S.E.M. and represent results from two independent experiments, with each assay performed in duplicate.

Complex glycosylation is required for ligand-mediated activation of GC-C

To confirm that complex glycosylation and not the protein and/or lipid environment of the PM is essential for ligand-mediated GC-C activation, we investigated ST peptide activation of GC-C expressed in HEK-293 cells, that lack GnT1 (HEK-293S GnT1⁻). These cells have been described recently and used for the production of a uniformly glycosylated rhodopsin receptor [29]. GnT1 is required to initiate the addition of complex sugars in the Golgi complex and in its absence, no complex sugars are added to glycoproteins. Western-blot analysis of membranes prepared from these cells transfected with GC-C cDNA (HEK-293S GnT1⁻-GCC cells) showed that a single band of M_w 130 kDa was detected in these cells and endo H treatment lowered the M_w to 120 kDa, thereby showing that the 130 kDa form contained a high mannose structure (Figure 7A).

Cells were subjected to surface biotinylation and as shown in Figure 7(A), a significant fraction of the 130 kDa form was localized to the PM, indicating that complex glycosylation is not required for translocation of GC-C to the cell surface. The

Figure 6 Intracellular accumulation of GC-C by treatment of cells at 20 °C

HEK-293-GCC cells were incubated at 20 °C for 20 h to block protein transport from the *trans*-Golgi network to the PM. Control and cells treated at 20 °C were subjected to surface trypsinization to remove surface-localized GC-C and membranes were prepared. Results shown are representative of experiments performed at least twice. **(A)** Membrane protein (20 μ g) from cells treated at 20 °C and control cells, with or without surface trypsinization, was subjected to Western-blot analysis using GCC:C8 monoclonal antibody. **(B)** *In vitro* guanylate cyclase assays were performed with 10 μ g of membrane protein from control and trypsinized cells using MgGTP as the substrate. The membranes were incubated in the absence (basal) or presence of 100 nM ST peptide. Values shown are means $\pm$ S.E.M. and represent results from two experiments.

affinity of ST peptide binding to the cells was measured and found to be similar to that seen in HEK-293 cells, emphasizing again that glycosylation did not alter ligand interaction (Figure 7B). However, when monolayer cultures of HEK-293S GnT1⁻ cells were stimulated with ST peptide, only a marginal accumulation of intracellular cGMP accumulation was observed (Figure 7C). Moreover, when membranes were prepared from cells and *in vitro* guanylate cyclase activity monitored in the presence of ST peptide, activation was increased only 2-fold when compared with the > 10-fold activation seen on ST peptide-addition to GC-C expressed in control HEK-293 cells (Figure 7D). However, the activity measured in the presence of MnGTP was similar at equivalent receptor levels to that seen in control HEK-293 cells, indicating that only ligand-stimulated guanylate cyclase activity was compromised in HEK-293S GnT1⁻-GCC cells. Thus despite being surface-localized and catalytically active, GC-C devoid of complex sugars was poorly activated by the ligand suggesting the importance of the addition of complex sugar residues to the ECD of GC-C in allowing an efficient conformational change leading to guanylate cyclase activation.

Effect of enzymic deglycosylation on ligand-mediated GC-C activation

Results from experiments performed at 20 °C confirmed that the glycosylation of GC-C determined its basal and ligand-stimulated activity. An analysis of the sugars present in GC-C (Figure 2) had shown that sialic acid and galactose residues were

Figure 7 Properties of GC-C expressed in HEK-293 cells deficient in GnT1

GC-C cDNA was transfected in HEK-293 or HEK-293S GnT1⁻ cells and experiments were performed 48 h post-transfection. **(A)** Membrane protein was prepared from transfected cells and subjected to Western-blot analysis either directly or after treatment with endo H. Monolayer cultures of cells were subjected to surface biotinylation using sulphy-NHS-biotin. Biotinylated proteins were separated using avidin agarose and subjected to Western-blot analysis using GCC:C8 monoclonal antibody. Results shown are from a single experiment, with the experiment repeated twice. **(B)** Membranes prepared from transfected HEK-293 cells and HEK-293S GnT1⁻ cells were subjected to ST peptide-binding analysis by incubation with radiolabelled ST peptide in the presence or absence of unlabelled ST peptide. Results shown are the means $\pm$ S.E.M. for experiments repeated twice. Inset: representative Western-blot analysis (using GCC:C8 antibody) of membrane protein prepared from a set of transfected cells used for ST peptide-binding analysis. **(C)** Monolayers of transfected cells were incubated with 500 μ M IBMX (isobutylmethylxanthine) for 30 min and then incubated with or without 100 nM ST peptide for 30 min. The cells were lysed in 0.1 M HCl and the amount of cGMP synthesized was measured by radioimmunoassay. Results shown are the means $\pm$ S.E.M. for experiments repeated twice, with each assay performed in duplicate. **(D)** Membranes were prepared from HEK-293 or HEK-293S GnT1⁻ cells transfected with GC-C cDNA and subjected to *in vitro* guanylate cyclase assay in the presence of MnGTP or 0.3% Lubrol-PX and MgGTP as substrate. Results shown are representative of at least two independent experiments, and represent the means $\pm$ S.E.M.

present in the mature form of the receptor. We treated membranes prepared from HEK-293-GCC cells with terminal β -galactosidase, which cleaves terminal non-reducing β 1-4-linked galactose from oligosaccharides, and *N*-acetyl-neuraminidase III, which releases α 2-3-, α 2-6-, α 2-8- and α 2-9-linked sialic acid from oligosaccharides, either individually or together. Deglycosylated membranes were then subjected to Western-blot analysis and guanylate cyclase assays. As shown in Figure 8(A), after sialidase treatment, there was a significant increase in the mobility of the fully glycosylated form of GC-C, suggesting that the PM form of GC-C harboured several sialic acid residues after complete processing. There was a slight shift in the mobility of GC-C after treatment with terminal β -galactosidase alone, perhaps due to inaccessibility of many of the galactose residues to the enzyme when sialic acid residues are present. However, treatment of membranes with sialidase and terminal β -galactosidase further increased the mobility of GC-C and this form of GC-C co-migrated with the 130 kDa form of GC-C. Interestingly, there was no increase in the basal guanylate cyclase activity or no decrease in the ST peptide-mediated activation of GC-C after sialidase and/or galactosidase treatment (Figure 8B). These results indicate that the addition of sialic acid and galactose residues during the transit from the Golgi to the PM, is important for achieving the correct folding of the ECD of GC-C, to allow repression of basal activity and a conformation that is required for ligand stimulation. However, once this conformation is attained, removal of sialic acid or galactose residues does not reverse this process, and these sugars are no longer required for the maintenance of low basal activity of the receptor or affect ligand-stimulated activity.

DISCUSSION

The results suggest a novel role for glycosylation in GC-C signal transduction, and perhaps in general structural features of the receptor guanylate cyclase family. The differentially glycosylated forms of GC-C are localized in different compartments of the cell, the 130 kDa high mannose form in the ER and the 145 kDa form in the PM. Both the forms have similar affinities for ST peptide but only the 145 kDa form is ligand-stimulated, suggesting that the property of ligand stimulability is acquired in the Golgi complex during the transport of GC-C from the ER to the PM. The ligand stimulability was not imparted by the specific protein environment of the PM since the fully glycosylated GC-C, trapped intracellularly in the Golgi complex, was also activated by the ligand, as seen in the treatment of cells at 20 °C. In addition, expression of GC-C in HEK-293S GnT1⁻ cells resulted in the transport of the receptor to the surface, but a decrease in ST peptide-stimulation was observed, indicating that it was glycosylation and not localization of GC-C that determined its ligand stimulability.

On the basis of evidence collected from a number of previous studies, GC-C glycosylation appears to be regulated in a tissue-specific manner. The hypoglycosylated form is expressed throughout the rat intestine but the hyperglycosylated form is detected only in the duodenum and the colon [28]. Interestingly, the duodenum and the colon are also the regions of intestine that synthesize cGMP in response to ST peptide treatment, despite the presence of high-affinity ST peptide-binding sites throughout the intestine [4]. These earlier results can now be interpreted based on the results presented in this study, which indicate that

Figure 8 Deglycosylation of mature GC-C does not modify its catalytic activity

Membranes prepared from HEK-293-GCC cells were subjected to deglycosylation using terminal β -galactosidase or neuraminidase III. (A) The deglycosylated membrane (10 μ g) was subjected to Western-blot analysis using GCC:C8 monoclonal antibody. (B) The membrane (10 μ g) was subjected to *in vitro* guanylate cyclase assay using MgGTP as substrate. The membranes were incubated in the absence (basal) or presence of 100 nM ST peptide. Experiments were performed at least twice and the results shown represent a single experiment, with assays performed in duplicate.

the hyperglycosylated form of GC-C is the ligand-activable form of GC-C.

Recently, we have reported the importance of glycosylation in regulating the responsiveness of colonic cells to the ST peptide [25]. It appears that in Caco2 cells, application of ST peptide for prolonged periods of time decreases the subsequent response of the cells to further ST peptide application. This cellular refractoriness was correlated with a loss of the 145 kDa form of the receptor from the cell surface, with no decrease in levels of the 130 kDa form of GC-C. Cells were recovered from this 'desensitization' process after the removal of ST peptide and this was concomitant with the reappearance of the 145 kDa form of GC-C in cells. Therefore, our current study provides a molecular explanation for these results by showing that it is only the 145 kDa form of GC-C that responds to the ST peptide, and complex glycosylation that occurs during receptor maturation confers a conformation to the receptor that allows ST peptide-mediated activation.

As discussed earlier, mutational analysis of glycosylation sites of porcine GC-C has suggested that glycosylation is essential for the correct folding of the ECD [19]. In these mutant proteins, the affinity of the receptor for ST peptide remained similar, but there was a decrease in B_{max} value, suggesting that the amount of functional and ST peptide-bindable form was reduced as a consequence of misfolding of the receptor. Indeed, these mutant proteins showed a lower degree of stability in the presence of urea, indicating their poorly folded structure. These results, as interpreted by the authors, suggest that modification of these asparagine residues by glycosylation allows the ECD to adopt

a conformation that is initially required for ligand binding. This is in agreement with our observations made in this study with tunicamycin, where the 120 kDa form of GC-C does not bind ST peptide (results not shown).

Our studies suggest that binding of ligand to the ECD of GC-C does not always lead to activation of the intracellular cyclase domain. The high mannose form of GC-C expressed in HEK-293S GnT1⁻ cells, or in the ER, can bind ST peptide with high affinity but is functionally deregulated with increased basal activity and no further stimulation of catalytic activity after ligand binding. Therefore carbohydrates play an additional role in the regulation of ligand stimulatibility of GC-C. They are essential to impart the correct conformation that could down-regulate the basal activity of GC-C, as well as allow the activation of the catalytic domain on ligand binding. The sugar residues that are involved in the two processes are different: those responsible for ligand binding being added in the ER, whereas those required for ligand-mediated activation are added in the Golgi complex. Removal of complex sugars with sialidase or β -galactosidase after complete maturation of GC-C did not alter ligand-mediated activation or the basal activity of GC-C. This indicates that once the conformation amenable for ligand-mediated activation is attained, the sugars may not be necessary for the actual conformational changes that take place after ligand binding to the receptor.

The nature of the conformation that is conferred by the sugar residues and the critical asparagine residue(s) that carries these sugars is difficult to predict at this stage. GC-C exists in different oligomeric states under different activation states [12,36]. In the presence of non-ionic detergents, GC-C exists as a high-molecular-mass oligomeric form that is converted into a lower molecular mass form on ATP binding. Our preliminary results suggest that differential glycosylation may not alter the oligomeric state of GC-C, since both the 130 and 145 kDa forms of GC-C form the higher and lower oligomer respectively (results not shown). Moreover, the 130 kDa form exhibits activity with MnGTP as a substrate, suggesting that it can form the catalytically active dimer required for the catalytic centre in type III nucleotide cyclases [37]. Therefore the sugar residues may not be involved in the regulation of oligomerization and addition of complex sugar residues probably generates a conformation of the receptor that exerts a negative constraint on the receptor in its basal state, which is removed after the binding of the ligand.

In the absence of the crystal structure of the ECD of GC-C, the asparagine residues that are critical for the generation of a conformation amenable for ligand-mediated activation can only be suggested. Residues that are important for ST peptide binding in porcine GC-C [38], and a stretch of amino acids from Ser-387 to Lys-393 that has been shown to bind to ST peptide [39] lie in the C-terminal region of the ECD of GC-C, close to the transmembrane region of GC-C. This suggests that conformational changes could occur in the vicinity of this region and hence glycosylation sites near the transmembrane region could be important in ST peptide-mediated activation (Figure 1). A sequence comparison of the ECDs of GC-Cs cloned from different species shows that the GC-Cs possess several putative glycosylation sites. Most of these sites are conserved across various mammalian GC-Cs, but a comparison with *Xenopus* and Medaka fish GC-Cs showed that only two glycosylation sites, Asn-334 and Asn-379, are conserved in these genera as well (Figure 1). Interestingly, these two sites are close to the region involved in ST peptide binding, and sugar residues at one or both of these sites could be involved in the signalling events. Interestingly, ST peptide-mediated intestinal fluid accumulation in a suckling mouse model could be inhibited by oligosaccharides from human milk. The oligosaccharides blocked the binding to and activity of the ST

peptide in T84 human intestinal colonic cells [40]. Although the oligosaccharides inhibited cGMP production up to 70 %, they inhibited ligand binding only by 27 %. These studies are worth re-investigating in the light of the current observations.

There have been several reports on the regulation of signal transduction pathways by changes in the glycosylation of receptors. Altered glycosylation of notch receptors modulates their interaction with different ligands [41,42]. Mutation of the EGF receptor at Asn-420 in the ECD leads to spontaneous oligomerization and constitutive activation of the receptor [43]. It has been shown for the Trk receptor for nerve growth factor that glycosylation is essential to prevent ligand-independent activation of the receptor, again possibly by preventing receptor dimerization [44]. The present study gives a new insight into the role of glycosylation in ligand-mediated activation of GC-C. Considering the similarity in the glycosylation states, role of glycosylation and the mechanism of ligand binding that have been proposed for the guanylate cyclase class of receptors, it is possible that the activation of other members of this family of receptors could also depend on their glycosylation state.

Financial support from the Department of Biotechnology, Government of India is acknowledged. The HEK-293S GnT1⁻ cells were kindly provided by Dr H. G. Khorana and Dr P. J. Reeves. We acknowledge A. R. Shenoy for useful discussions. Y. G. is supported by the Indian Council of Medical Research.

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Received 5 January 2004; accepted 28 January 2004

Published as BJ Immediate Publication 28 January 2004, DOI 10.1042/BJ20040001