Identification of the N-glycosylation sites on glutamate carboxypeptidase II necessary for proteolytic activity

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Abstract

Glutamate carboxypeptidase II (GCPII) is a membrane peptidase expressed in the prostate, central and peripheral nervous system, kidney, small intestine, and tumor-associated neovasculature. The GCPII form expressed in the central nervous system, termed NAALADase, is responsible for the cleavage of N-acetyl-L-aspartyl-L-glutamate (NAAG) yielding free glutamate in the synaptic cleft, and is implicated in various pathologic conditions associated with glutamate excitotoxicity. The prostate form of GCPII, termed prostate-specific membrane antigen (PSMA), is up-regulated in cancer and used as an effective prostate cancer marker. Little is known about the structure of this important pharmaceutical target. As a type II membrane protein, GCPII is heavily glycosylated. In this paper we show that N-glycosylation is vital for proper folding and subsequent secretion of human GCPII. Analysis of the predicted N-glycosylation sites also provides evidence that these sites are critical for GCPII carboxypeptidase activity. We confirm that all predicted N-glycosylation sites are occupied by an oligosaccharide moiety and show that glycosylation at sites distant from the putative catalytic domain is critical for the NAAG-hydrolyzing activity of GCPII calling the validity of previously described structural models of GCPII into question.

Keywords: NAALADase; GCPII; PSMA; glycosylation; proteolytic activity; enzyme kinetics

The covalent addition of sugars to proteins is one of the major modifications that occur in the endoplasmic reticulum and the Golgi system. Clarification of the importance of N-glycosylation for subcellular transport, folding, and/or attaining of enzymatic activity is a major interest of contemporary glycobiology (Helenius and Aebi 2001; Rudd et al. 2001), and several well-studied examples of the role of N-linked carbohydrates in these processes have been reported (Kadowaki et al. 2000; Muhlenhoff et al. 2001; Trotti et al. 2001).

Glutamate carboxypeptidase II (GCPII, EC 3.4.17.21) is a membrane peptidase expressed in a number of tissues such as prostate, central and peripheral nervous system, kidney, small intestine, and tumor-associated neovasculature (Berger et al. 1995; Chang et al. 1999). The GCPII form expressed in the central nervous system is responsible for the cleavage of N-acetyl-L-aspartyl-L-glutamate (NAAG) yielding free glutamate in the synaptic cleft (Robinson et al. 1987), and is thought to be implicated in various pathologic conditions associated with altered glutamatergic signal transmission (Doble 1999; Danbolt 2001). Inhibition of the enzyme was shown to protect against ischemic injury in a neuronal culture model of stroke and in rats after transient middle cerebral artery occlusion (Slusher et al. 1999). The physiological significance of the GCPII expression in other tissues, but jejunum (Halsted et al. 1998), is still unknown.

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Abbreviations: NAAG, N-acetyl-L-aspartyl-L-glutamate; NAALADase, N-acetylated-α-linked-acidic dipeptidase; GCPII, glutamate carboxypeptidase II; rhGCPII, recombinant human glutamate carboxypeptidase II; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PSMA, prostate-specific membrane antigen; PBS, phosphate-buffered saline; FBS, fetal bovine serum; PNGase F, peptide-N^4-(*N*-acetyl-β-D-glucosaminyl) asparagine amidase F; MALDI-TOF, matrix-assisted laser desorption/ionization with time-of-flight detection; ER, endoplasmic reticulum; CD, catalytic domain; HPAEC-PAD, high-performance anion exchange chromatography with pulsed amperometric detection; GlcNAc, N-acetylglucosamine; Man, mannose; Fuc, fucose.

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The prostate form, termed prostate-specific membrane antigen (PSMA), is up-regulated in cancer, and is used as an effective prostate cancer marker (for review, see Gregorakis et al. 1998).

GCPII is a type II transmembrane protein and, like many other membrane-bound polypeptides, it is glycosylated. Human GCPII consists of 750 amino acids and the molecular weight of the nonmodified polypeptide calculated from known amino acid composition is 84 kD, but electrophoretic mobilities of GCPIIs derived from various tissues and/or species strongly indicate the possibility of posttranslational modifications representing 10%–30% of the total molecular weight of the enzyme (Holmes et al. 1996; Tiffany et al. 1999). Ten potential N-glycosylation sites were identified in human GCPII, and the same number of the N-glycosylation sequons with similar distribution throughout the polypeptide can be found in the rat and mouse GCPII orthologs (Bzdega et al. 1997; Bacich et al. 2001). Very little is known about nature of GCPII glycosylation, and data concerning the relationship between GCPII glycosylation and its carboxypeptidase activity are sparse and sometimes equivocal. Holmes et al. (1996) performed analysis of the glycosylation of GCPII derived from multiple sources using a variety of exo- and endoglycosidase treatments. The results indicate that the carbohydrates are primarily N-linked, and it was estimated that 20%–25% of the native GCPII molecular weight is composed of carbohydrates. Tiffany et al. (1999) reported hydrolyzing activities against NAAG in both membrane and cytosolic fractions of eight normal prostate tissues from different species. The existence of cytosolic, and therefore arguably nonglycosylated GCPII, is further supported by findings of Lapidus et al. (2000). To the contrary, we have previously shown that N-glycosylation is essential for GCPII hydrolytic activity, and that the recombinant construct expressed intracellularly lacks carboxypeptidase activity (Barinka et al. 2002).

In this paper, we have examined the influence of N-glycosylation on secretion and folding of the recombinant human GCPII (rhGCPII) expressed in insect cells. We have analyzed each of the N-glycosylation consensus sequences with respect to their occupancy by carbohydrates. Finally, to clarify the role of individual N-linked oligosaccharides for GCPII carboxypeptidase activity, we have generated a series of constructs in which each of the N-glycosylation consensus sequences was deleted, and examined the catalytic activity of each mutant.

Results

Each of the predicted N-glycosylation sites is occupied in S2 cells

There are several reports in the body of scientific literature indicating the presence of a hydrolytically active cytosolic form of human GCPII in the prostate and cell lines of prostatic origin (Su et al. 1995; Grauer et al. 1998; Tiffany et al. 1999; Lapidus et al. 2000; Schmittgen et al. 2003). For a cytosolic species, it is not conceivable that it is modified by the addition of N-linked oligosaccharides. However, unlike previous reports, our laboratory's data indicate that N-terminally truncated GCPII species expressed in the cytosol is enzymatically inactive (Barinka et al. 2002). Therefore, we decided to investigate the catalytic role of N-glycosylation in GCPII activity in depth.

Mass spectrometry analysis revealed that the molecular weight of glycosylated rhGCPII is approximately 89.5 kD (data not shown). Because the mass of the polypeptide backbone calculated from known amino acid composition is 79.8 kD, posttranslational modifications represent 9.7 kD, that is, less then 1 kDa per the predicted N-glycosylation site. Due to the inherent variability of individual N-linked sugar chains and the minimal changes in molecular weight of the protein upon deletion of the single N-glycosylation acceptor site, we were not able to assess the actual presence of sugar chains from the mobility shift by SDS-PAGE (data not shown). To discuss the profound effect of N-glycosylation site elimination on the rhGCPII carboxypeptidase activity, it was important to analyze whether the individual sites are indeed modified by an oligosaccharide.

To map occupancy of individual predicted N-glycosylation sites, 10 μg of the purified wild-type rhGCPII was processed as described in Materials and Methods. The results, as exemplified by analysis of the predicted N638 N-glycosylation sequon, clearly demonstrated that sugar chains are attached to each of the ten predicted N-glycosylation acceptor sites in rhGCPII expressed in S2 cells (Fig. 1). Overall, nine sites of N-glycosylation were identified in *in gel* digests, and the site containing N476 was identified using the isolated glycopeptides.

Blocking N-glycosylation prevents rhGCPII secretion

Tunicamycin is an inhibitor of the first step in the N-glycosylation pathway (Tkacz and Lampen 1975), and, consequently, this compound is widely used to analyze the effects of glycosylation on glycoprotein activities in cell cultures.

S2 cells expressing wild-type rhGCPII were grown in medium with or without tunicamycin (10 μg/mL). Thirty-six hours postinduction, the cells were harvested and rhGCPII expression analyzed (Fig. 2). Wild-type glycosylated rhGCPII has a molecular mass of approximately 89.5 kD (mass spectrometry analysis; data not shown), and immunoreactive bands of this molecular weight were observed both in S2 cells stably transfected with rhGCPII and in the conditioned medium (Fig. 2, lanes 2,8). When cultured in the presence of tunicamycin, the predominant rhGCPII form detected in S2 cell lysates migrates at the same position as rhGCPII from samples with N-linked sugar chains cleaved off by

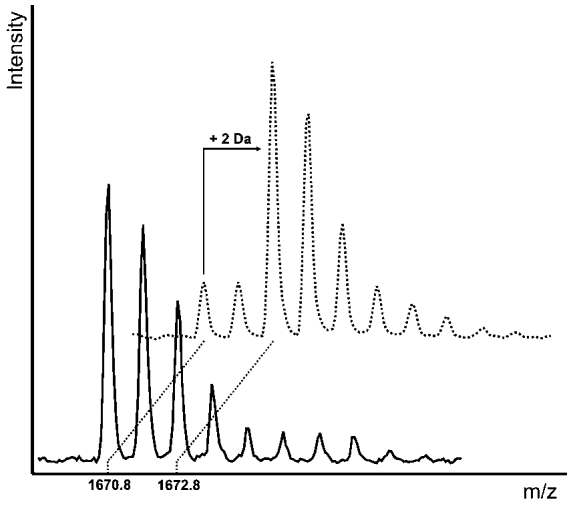

Figure 1. An example of identification of a glycosylation site using deglycosylation in normal and isotopic water ($H_2{}^{18}O$). Detailed view of the isotopic cluster of the peak corresponding to the DFTEIASKFSERLQ peptide covering the N638 site is provided. The peptide deglycosylated in normal water has a typical isotopic distribution, but the mass of the peptide deglycosylated in presence of ^{18}O is shows a 2 D mass shift. The incomplete mass shift is because deglycosylation was done in a mixture of normal and isotopic water (83% of $H_2{}^{18}O$). During the hydrolysis of the N-glycosidic bond, the mass of the deglycosylated peptide is increased by 1 mass unit, thus yielding a teoretical monoisotopic mass $[M + H]^+$ of 1670.83.

PNGase F treatment, which indicates the lack of N-glycosylation (Fig. 2, cf. lanes 3 and 4). Additionally, very weak and diffuse immunoreactive species of higher molecular weights can be visualized in lysates from tunicamycin-treated cells. These species can be removed by the addition of PNGase F (Fig. 2, lanes 4,5). This observation suggests that N-linked glycosylation in S2 cells was not completely blocked by the presence of tunicamycin and some residual glycosylation remained.

Only the rhGCPII form corresponding to the glycosylated enzyme was detected in the growth medium of tunicamycin-treated cells (Fig. 2, lane 6), and no nonglycosylated rhGCPII was secreted. The presence of N-linked oligosaccharide chains on rhGCPII is thus necessary for efficient secretion of rhGCPII from S2 cells while nonglycosylated enzyme is retained intracellularly.

Construction of N-glycosylation mutants of rhGCPII

Analysis of the reported human GCPII sequence (Israeli et al. 1993) reveals the presence of 10 sequons for N-linked glycosylation. In a previous study, we showed that N-glycosylation is indispensable for hydrolytic activity of rhGCPII (Barinka et al. 2002). Consequently, we decided to examine the relative contributions of oligosaccharide chains at these predicted N-glycosylation sites to carboxypeptidase activity of rhGCPII. Eleven mutants were constructed (see Fig. 3), in which asparagine (or, in one case, threonine) residues in the N-glycosylation consensus sequences were mutated to alanines. Unlike the naturally occurring GCPII, which is a membrane-bound glycoprotein, all recombinant mutants as well as the parental, wild-type rhGCPII, lack the intracellular and membrane-spanning domains. Consequently, as the coding sequences are cloned in frame with a BiP leader peptide present in the pMT/BiP/V5-His A vector, the proteins are supposed to be secreted into the growth medium. Identities of all constructs were confirmed by dideoxynucleotide-terminated sequencing and the corresponding plasmids, together with the pCoHygro hygromycin-resistance coding plasmid, were used for the production of stable transfectants of Schneider's S2 cells.

Each of the N-glycosylation mutants is functionally expressed and secreted into the medium

Stably transfected S2 cells were transferred into the SF900II serum-free medium and the protein expression was induced with 500 μM $CuSO_4$. Three days postinduction, the conditioned media were separated from the cells, dialyzed, and rhGCPII expression and carboxypeptidase activities determined (Fig. 4). Expression levels of individual mutants, as determined by Western blotting followed by image densitometry, are comparable to or more often slightly lower than the wild-type enzyme, but their respective NAAG-hydrolyzing activities differ markedly (see below). Upon induction, each of the 12 constructs (11 mutants and wild-type rhGCPII) is efficiently secreted into a medium. These re-

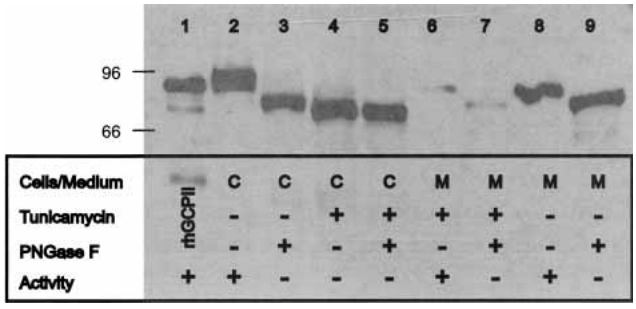

Figure 2. Expression and secretion of wild-type recombinant GCPII in the presence of tunicamycin. S2 cells stably transfected with cDNA coding for wild-type rhGCPII were grown in the presence or absence of tunicamycin, an inhibitor of N-glycosylation. Thirty-six hours postinduction, the cells and conditioned media were harvested, resolved by 9% SDS-PAGE, electroblotted on a nitrocellulose membrane, and immunostained. Portion of cell lysates and the conditioned media were subjected to PNGase F treatment to assess degree of N-glycosylation of the individual rhGCPII forms. rhGCPII, purified rhGCPII (100 ng); cells/medium, C—cell lysates, M—conditioned media; Tunicamycin +/–, cells grown in presence/absence of tunicamycin; PNGase F +/–, samples treated/not treated with PNGase F; Activity +/–, NAAG-hydrolyzing activity detectable/not detectable.

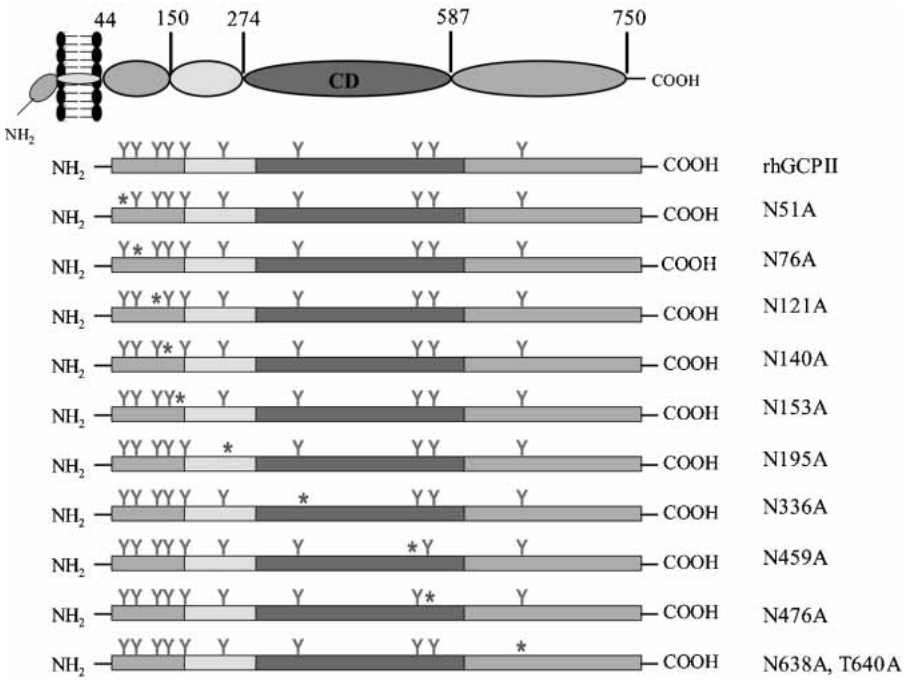

Figure 3. Schematic representation of the GCPII domain structure and glycosylation mutants used in this study. The diagram shows wild-type human GCPII and mutants with potential N-glycosylation sites mutated. Individual domains according to Rawlings and Barrett (1997): CD, putative catalytic domain; polypeptides spanning amino acids 44–150, 151–274, and 587–750, domains of unknown function. *Y* symbols are potential N-glycosylation sites. Asterisks indicate Asn or Thr residues mutated to Ala.

sults clearly indicate that none of the individual N-linked oligosaccharide chains is necessary for rhGCPII secretion, and correct overall folding.

The N76A, N336A, and N459A mutants retain more than 50% activity compared to wild-type rhGCPII

Out of the 11 constructs, three mutants, namely N76A, N336A, and N459A, exhibit activities exceeding 50% of the activity of wild-type rhGCPII. The most active species is the N76A mutant, retaining as much as 70% of the wild-type activity. Interestingly, the N336A and N459A glycosylation sites are situated within the predicted catalytic domain of GCPII, and these mutations were, therefore, supposed to introduce major changes to proteolytic activity of rhGCPII.

Exopeptidase activities of the N121A, N140A, N153A, N195A, N638A, and T640A rhGCPII mutants are profoundly compromised

A greater than 10-fold drop in GCPII carboxypeptidase activities were observed for the N121A, N140A, N153A, N195A, and N638A mutants; the N121A, N195A, and N638A constructs are virtually inactive (see Fig. 4). To verify that the observed decrease in NAAG-hydrolyzing activity of the N638A mutant (the least active mutant) resulted from the absence of the sugar chain and not from the substitution of the asparagine residue in the protein sequence, we constructed the T640A mutant, in which the N-glycosylation recognition sequence was deleted by the threonine-to-alanine mutation. The specific NAAG-hydrolyzing activity of this mutant is very low and identical to that of the N638A (see Fig. 4), suggesting that the observed activity changes can be attributed to the loss of this oligosaccharide. The complete profile of the oligosaccharide structures residing at this glycosylation site is shown in Table 1. It would appear from these data that structures of the corresponding oligosaccharides may be rather small (such as the predominant Fuc-Man_3-$GlcNAc_2$ hexasaccharide; see Table 1), and does not need to be of the type found in higher eukaryotes (Holmes et al. 1996).

Surprisingly, each of the above-mentioned glycosylation sites is located outside of the putative catalytic domain, indicating that even more distant segments of the rhGCPII polypeptide chain influence the active site of the enzyme.

Absence of individual carbohydrate chains predominantly influences the V_{max} value

To analyze whether the observed decrease in catalytic activities of the majority of the mutants in this study can be attributed to a substrate binding defect (K_M) or lower turn-

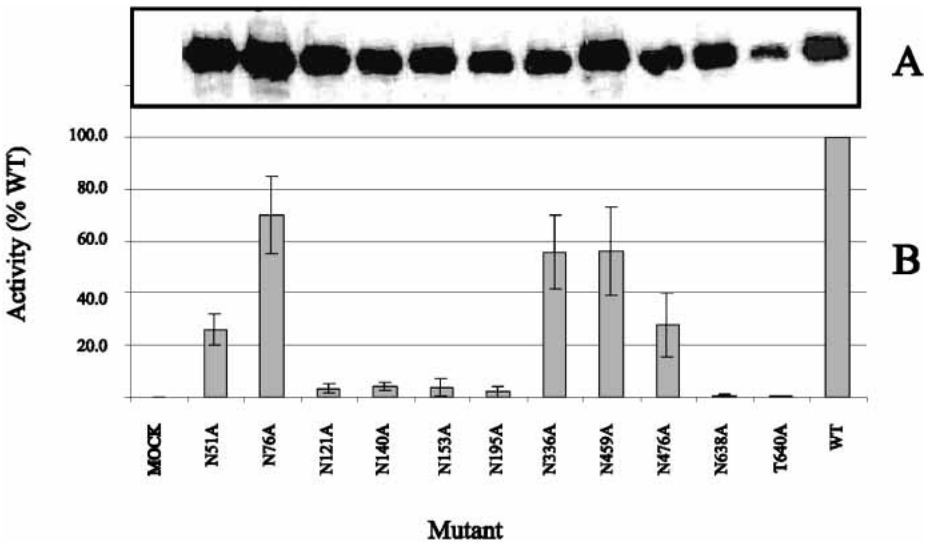

Figure 4. Expression and hydrolytic activities of wild-type and mutated rhGCPII. S2 cells stably transfected with cDNA for rhGCPII or N-glycosylation mutants thereof were grown in SF900II serum-free medium. Protein expression was induced with 500 μM $CuSO_4$ and the conditioned media harvested three days later. The media were dialyzed and assayed for rhGCPII expression and carboxypeptidase activities. (*A*) Immunoblot analysis of rhGCPII expression. Fifteen microliters (5 μL in the case of the rhGCPII) of the conditioned medium mixed with a denaturing loading buffer was loaded into a single lane, proteins resolved by 9% SDS-PAGE, electroblotted on a nitrocellulose membrane, and immunostained using anti-GCPII antibody as described in Materials and Methods. Relative band intensities were recorded with a CCD-camera and rhGCPII amount in each sample calculated from a calibration curve of known rhGCPII concentrations. (*B*) Carboxypeptidase activities of the individual rhGCPII mutants. Dialyzed conditioned media were mixed with the reaction buffer (the final volume of 180 μL), and the reactions were started by addition of 20 μL of 1 μM NAAG. Following 20-min incubation, 200 μL of 200 mM sodium phosphate was added, reaction products were separated on an anion-exchange column, and eluted labeled glutamate was quantified by liquid scintillation. Measured activities were related to the amount of each mutant in the conditioned medium, as determined by Western blot densitometry. The results are shown in the format of mean ± standard deviation.

over rate (V_{max}), Michaelis-Menten kinetic data were obtained for the three mutants with the lowest activities observed. The results, as summarized in Table 2, clearly show that the observed activity changes result primarily from changes in V_{max}. The specific activity of the N638A construct is less than 0.6% of the wild-type rhGCPII, and a similar decrease in specific activity was observed for the T640A mutant. The K_M values of these mutants are much less affected and are comparable to that of wild-type rhGCPII.

Table 1. *Glycosylation profile of the C-terminal glycopeptide*

m/z	Structure	%
1078.9	Fuc Man3 GlcNAc2	46
933.2	Man3 GlcNAc2	10
1281.9	Fuc Man3 GlcNAc3	1
1136.0	Man3 GlcNAc3	8
1241.0	Fuc Man4 GlcNAc2	12
1095.0	Man4 GlcNAc2	3
1257.2	Man5 GlcNAc2	1
1419.2	Man6 GlcNAc2	13
1581.2	Man7 GlcNAc2	3
1742.9	Man8 GlcNAc2	3

Values in the *m/z* column represent experimental monoisotopic masses of MNa+ ions. All the isolated glycans were degradable by α-mannosidase or δ-fucosidase digestion, confirming the proposed structures (data not shown). The glycans are ordered by their corresponding retention times and the percentage is calculated directly from the HPAEC-PAD analysis.

Discussion

Carbohydrate modifications of proteins play a major role in modulating their structure and/or function, and the scientific literature describing this phenomenon has become more numerous in recent years (for reviews, see Helenius and Aebi 2001; Rudd et al. 2001). We have previously shown that N-glycosylation is indispensable for GCPII enzymatic activity. In an apparent contradiction, however, an N-terminally truncated and enzymatically active cytosolic GCPII-like species in prostatic LNCAP cells has been described in several reports (Tiffany et al. 1999; Lapidus et al. 2000). Because N-glycosylation of such a species is not plausible, we decided to analyze the role of N-glycosylation for GCPII activity in more detail using site-directed mutagenesis and N-glycosylation manipulations in vivo.

We report that secretion of nonglycosylated rhGCPII was abolished by tunicamycin treatment, that is, by inhibiting

Table 2. *Kinetic characterization of wild-type and mutated recombinant human GCPII*

GCPII mutant	K_M (nM)	V_{max} (pmole · mg^{-1} · sec^{-1})
wild-type GCPII (rhGCPII)	321 ± 131	15,700 ± 1700
N121A	100 ± 22	511 ± 11
N195A	66 ± 2	311 ± 33
N638A	334 ± 24	89 ± 2

The kinetic parameters for NAAG were determined by saturation kinetics for the wild-type and mutated recombinant enzymes (see Materials and Methods). Concentrations of individual rhGCPIIs were determined using Western Blot densitometry together with a calibration curve of known rhGCPII concentrations.

the first step in the N-glycosylation pathway. Only glycosylated protein is secreted into the medium while the nonglycosylated form is retained intracellularly. It is well established that one of the indirect effects of glycans on protein folding is their interaction with the calnexin–calreticulin system found in the endoplasmic reticulum (ER) of nearly all eukaryotes (Zhang et al. 1997). This system, together with glucosidases I and II and glucosyltransferase, is thought to provide a mechanism for promoting folding and maturation of proteins within the ER. It is very likely that nonglycosylated rhGCPII fails to fold correctly, and is consequently retained within the ER. N-glycosylation is therefore vital for acquiring/maintaining the overall 3D conformation of rhGCPII. This assumption is further corroborated by the fact that following deglycosylation with PNGase F, the protein readily precipitates out of the solution (data not shown). Additionally, Ghosh and Heston recently reported that a PSMA-transfected PC3 cell line cultured in the presence of tunicamycin fails to display the protein on the outer membrane, but retains it in the ER (Ghosh and Heston 2003).

Although the tunicamycin treatment completely abolished protein secretion, deletion of a single N-glycosylation site did not impair expression or secretion. These data suggest that none of the individual N-glycosylation sites are essential for the correct overall rhGCPII folding. However, preventing the attachment of N-linked oligosaccharide chains to a particular site within rhGCPII might alter the local structure of the protein, and consequently, kinetic parameters of the NAAG-hydrolyzing activity (see below).

There are 10 potential consensus N-glycosylation sites (sequons) in human glutamate carboxypeptidase II. It is estimated that of all the N-glycosylation sequons in proteins, 90% are glycosylated (Gavel and von Heijne 1990). Even though this piece of information can be understood as that "nearly all acceptor sites are glycosylated," we chose to analyze the actual occupation of individual sites by an N-linked sugar attachment. Our analysis shows that each of the N-glycosylation sequons is indeed modified by an oligosaccharide chain. Three of these sequons, namely N336, N459, and N476, are situated in the proposed catalytic domain spanning amino acids 275 to 586 (Rawlings and Barrett 1997). Surprisingly, Asn to Ala mutations in these sites resulted in only mild changes in NAAG-hydrolyzing activities, and these mutants were, in fact, with the exception of the N76A construct, the most active species analyzed. Because they are situated within the predicted catalytic domain, it could be speculated that these amino acid substitutions could directly disturb the GCPII active-site architecture. For example, N459 lies in the vicinity of the GCPII active site, as D453 is believed to be one of the five zinc-binding amino acids and S454 and R463 are thought to comprise part of the substrate-binding site similar to their corresponding residues, M180 or I193, respectively, in the *Vibrio* aminopeptidase (Chevrier et al. 1994).

Surprisingly, the most profound effects on NAAG-hydrolyzing activity were observed upon deletion of the N-glycosylation acceptor sites outside the predicted catalytic domain. For example, the hydrolytic activities of the N121A, N195A, N638A, and T640A constructs were more than 30-fold lower than those of the wild-type protein. In the case of N638A, the results agree with the recently published finding that membrane preparations from cells transfected with a similar construct (the N638A mutant of full-length PSMA) were devoid of both NAAG and folylpolygammaglutamate activities (Ghosh and Heston 2003). Obviously, regions far outside of the putative catalytic domain can influence the hydrolytic activity of GCPII. Taken together with our additional data analyzing the association of N- and C-terminally truncated GCPII variants and their NAAG-hydrolyzing activities (the C terminus seems to be critical for GCPII carboxypeptidase activity; C. Barinka, P. Mlcochova, P. Šácha, I. Hilgert, P. Majer, B.S. Slusher, V. Hořejší, and J. Konvalinka, unpubl.), these results question the validity of currently used structural models (Rawlings and Barrett 1997; Rong et al. 2002).

In an attempt to reestablish wild-type like NAAG-hydrolyzing activity in the N638A and T640A mutants, we introduced a new N-glycosylation recognition sequon in the sequence close to the original acceptor site. This was accomplished by introducing a K645N point mutation by which the -N645-F646-S647- sequence was created. However, the carboxypeptidase activities of the two newly constructed double mutants (N638A/K645N and T640A/K645N) remained very low and did not significantly differ from parental mutants (data not shown).

In conclusion, we show that N-glycosylation is indispensable for the correct folding and subsequent secretion of human GCPII and that N-glycosylation sites are critical for GCPII carboxypeptidase activity. We confirm that all predicted N-glycosylation sites are occupied by an oligosaccharide moiety, and show that glycosylation at sites distant from the putative catalytic domain are critical for the NAAG-hydrolyzing activity of GCPII, suggesting that cur-

rently used structural models of GCPII may not be valid. Final interpretation of these results will only be possible after the elucidation of the 3D structure of GCPII.

Materials and methods

Site-directed mutagenesis

For each mutant, the asparagine in the Asn-X-Ser/Thr consensus sequence was changed to alanine using a QuickChange Site-Directed Mutagenesis Kit (Stratagen). The pMNAEXST plasmid described previously (Barinka et al. 2002) was used as the template, and nucleotide changes were introduced by two complementary oligonucleotide primers harboring a desired mutation. The presence of each mutation was verified by dideoxynucleotide-terminated sequencing. An identical approach was used to construct the T640A (threonine 640 changed to alanine) mutant. Nucleotide sequences of the primers used are summarized in Table 3.

Transfection of insect cells and stable cell line generation

Schneider's S2 cells (Invitrogen) were maintained in the SF900II medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS, Gibco) at 22°–24°C. Stable cell lines expressing individual mutants were generated by cotransfection with 19 μg of the expression plasmid and 1 μg of a pCoHYGRO selection vector (Invitrogen) using a kit for calcium phosphate-mediated transfection (Invitrogen). Stable transfectants were selected by cultivation of the cells in the complete medium (SF900II + 10% FBS) containing 400 μg/mL Hygromycin B (Invitrogen).

Expression of wild-type and mutated rhGCPIIs

Stably transfected S2 cells were transferred into the SF900II serum-free medium and protein expression was induced with 500 μM $CuSO_4$ (Sigma). Three days postinduction, the cells were harvested by centrifugation. To remove phosphate anions that could interfere with NAAG-hydrolyzing activity measurements, the conditioned media were dialyzed against 50 mM Tris-HCl, 10 mM NaCl (pH 7.4), overnight at 4°C. The samples (both cells and conditioned media) were frozen and kept at −70°C until needed.

Large-scale rhGCPII expression and purification

The protocol for large-scale expression and purification of rhGCPII was identical to that described previously (Barinka et al. 2002).

Inhibition of glycosylation pathways

S2 cells stably transfected with wild-type rhGCPII were grown in 60-mm tissue culture plates in the SF900II medium supplemented with 10% FBS. At a density of 5×10^6 cells/mL, tunicamycin (Sigma, 1 mg/mL stock solution in DMSO) was added to a final concentration 10 μg/mL, and rhGCPII expression was induced with 500 μM $CuSO_4$. These cells were incubated for an additional 36 h and harvested by centrifugation.

Table 3. *Nucleotide sequences of the primers used*

Mutant	Nucleotide Sequences (5′ → 3′)
N51A	GCTTTGGAGTAATGGCAGTAGCTTCATTGGAGG CCTCCAATGAAGCTACTGCCATTACTCCAAAGC
N76A	GGTATCTGTGTAAAAGCATATAAGAACTTCTTG CAAGAAGTTCTTATATGCTTTTACACAGATACC
N121A	GGGATGAGTCTTAGCTGGGTAGGACAACAGG CCTGTTGTCCTACCCAGCTAAGACTCATCCC
N140A	GGTTCAAATAATGATGTGGCGAAAATCTCATTTCCATC GATGGAAATGAGATTTTCGCCACATCATTATTTGAACC
N153A	GGTACAATATCCGAAACAGCTTCATATCCTGGAGGAGG CCTCCTCCAGGATATGAAGCTGTTTCGGATATTGTACC
N195A	CAATTTTCCCAGAGCAAGCGATTTTCATGTCCCGTTCC GGAACGGGACATGAAAATCGCTTGCTCTGGGAAAATTG
N336A	GACTTTTTGTGTAGAAAAGGCTCCAGTAAAGCCAGGTCC GGACCTGGCTTTACTGGAGCCTTTTCTACACAAAAAGTC
N459A	CAACTCTCAGAGTGTAGGCTCCTTCTATAGATGAGTCAGC GCTGACTCATCTATAGAAGGAGCCTACACTCTGAGAGTTG
N476A	GCTTTTCAGCTCTTTTGTTAGGGCGTGTACCAAGCTGTAC GTACAGCTTGGTACACGCCCTAACAAAAGAGCTGAAAAGC
N638A	GCAATTTCTGTAAAAGCCTTTACTGCAGAAAAAAGTG CACTTTTTTCTGCAGTAAAGGCTTTTACAGAAATTGC
T640A	CTGAACTTGGAAGCAATTTCTGCAAAATTCTTTACTGC GCAGTAAAGAATTTTGCAGAAATTGCTTCCAAGTTCAG

Activity measurements

Radioenzymatic assays using ^{3}H-NAAG (radiolabeled at the C-terminal glutamate) were performed as described previously (Barinka et al. 2002).

Kinetic analysis

Michaelis-Menten (saturation) kinetics were measured in a reaction setup similar to that used for the activity measurements with substrate concentrations ranging from 0.025 to 50 μM NAAG. Initial velocity measurements for each concentration point were carried out in triplicate. Typical turnover of the substrate did not exceed 25%. K_M and V_{max} were determined by a nonlinear least-squares fit of the initial velocity versus substrate concentration using a GraFit software package (Erithacus Software Limited).

Antibodies

Hybridomas secreting monoclonal antibodies (mAbs) were prepared by standard methods from mice (F1 hybrids of BALB/c and B10.A strains) immunized with the recombinant human GCPII, prepared as described previously (Barinka et al. 2002).

Western blotting and protein quantification

Proteins were resolved by reducing, 0.1% SDS, 9% polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotting on a nitrocellulose membrane. The membrane was probed with the GCP-04 anti-rhGCPII mouse monoclonal antibody (1 mg/mL) at a 1:5000 dilution, followed by incubation with 1:20,000 horseradish peroxidase conjugated goat antimouse antibody (Pierce) for 2 h. The blot was developed using a West Dura chemiluminescence substrate (Pierce) according to the manufacturer's protocol. The relative intensities of immunoblot bands were quantified by recording the image with a CCD-camera followed by analysis using AIDA image analyzing software (Raytest Isotopenmessgerate).

Analysis of individual sites of N-glycosylation

Occupancy of the N-glycosylation sites was determined as described previously (Gonzalez et al. 1992) by comparison of measured masses of peptides corresponding to the sites of glycosylation. The peptides were generated following SDS-PAGE separation by means of *in-gel* digestion with sequencing grade trypsin (Promega), or a sequencing grade Asp-A protease (Roche) in either normal or isotopic water $H_2{}^{18}O$ (Fluka). The peptides were extracted from the gel, desalted, concentrated using a C-18 microcolumn (ZipTip C18, Millipore) and analyzed by mass spectrometry (Volf et al. 2002).

Isolation of glycopeptides

rhGCPII (90 μg) was digested with trypsin, and the glycopeptides were captured on a concanavalin A–Sepharose resin (Amersham), washed, and eluted with 0.01 M Tris-HCl (pH 8.0), 0.15 M NaCl, and 0.3 M D-mannose (Sigma). Individual glycopeptides were separated by reverse-phase chromatography on a Vydac C-18 column (5 μm, 2 × 150 mm, Dionex), equilibrated with 0.1% trifluoroacetic acid, and eluted by an acetonitrile gradient to 70% over 120 min. The glycopeptides were identified by MALDI-MS in their native and/or deglycosylated forms.

High-performance anion exchange chromatography of oligosaccharides

Glycans released from glycopeptides or from the intact protein by a treatment with PNGase F (New England BioLabs) in 50 mM sodium bicarbonate (pH 8.0) were desalted on minicolumns (10 μL bead volume) filled with nonporous graphitized carbon (Packer et al. 1998), and separated by HPAEC-PAD (DX 500, Dionex) on a Carbopac PA100 column. Elution was performed with 5-min isocratic steps using 0.1 M NaOH (solvent A) followed by a linear gradient from 0% to 35% of 0.1 M NaOH, 0.6 M NaOAc (solvent B) over 48 min and another linear gradient to 100% solvent B within 10 min at the flow rate 1 mL/min.The collected peaks were desalted (as above) and analyzed by MALDI-MS. To further characterize the isolated oligosaccharides, digestion with α-mannosidase and/or α-fucosidase was performed using 5 U/mL or 0.8 U/mL of the enzyme in 50 mM sodium citrate buffer, pH 5.0, respectively (Harvey 1999).

Mass spectrometry

Positive ion MALDI mass spectra were recorded with the BIFLEX II reflectron time-of-flight mass spectrometer (Bruker-Franzen). Saturated solution of α-cyano-4-hydroxy-cinnamic acid (Sigma) or ferulic acid (Sigma) in 30% acetonitrile/0.2% TFA or 2,5-dihydrobenzoic acid (Sigma) in 50% methanol were used as matrices. Spectra were calibrated externally using the monoisotopic $[M + H]^+$ ion of peptide standard somatostatin (Sigma). Postsource decay MALDI-MS spectra were acquired as described previously (Bezouska et al. 1999).

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