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Role of N-glycans in growth factor signaling

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Secreted proteins and membrane proteins are frequently post-translationally modified by oligosaccharides. Therefore, many glycoproteins are involved in signal transduction. One example is growth factor receptors, which are membrane proteins that often contain oligosaccharides. The oligosaccharides in those growth factor receptors play crucial roles in receptor functions. An analysis of glycosyltransferase-transfectants revealed that the branching structures of oligosaccharide also serve as important determinants. For example, N-glycans of epidermal growth factor receptor (EGFR) are involved in receptor sorting, ligand binding and dimerization. The addition of a bisecting GlcNAc to N-glycans increases the endocytosis of EGFR. N-glycans of Trk, a high affinity nerve growth factor receptor, also affect its function. Thus, oligosaccharides play an important role in growth factor signaling.
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Introduction

Changes in oligosaccharide structure are associated with many physiological and pathological events, including tumor invasion, as recently reviewed by Hakomori *et al.* [1]. The biochemical role of the oligosaccharides in glycoproteins has been examined from many points of view including intracellular trafficking, proteinase susceptibility, molecular conformation, protein stability, and protein-protein interactions. A number of membrane bound receptors for growth factors and cytokines are glycosylated, and evidence has been presented to show that oligosaccharide moieties are crucial for the functions for some of those receptors.

Strategies for examining the function of oligosaccharides in glycoproteins in culture cells are somewhat limited. Of the two major strategies, one involves eliminating the oligosaccharide moiety from the target proteins by introducing a mutation in the oligosaccharide binding sites of the protein. By the introducing of mutations in target proteins overexpressed in culture cells, the effect of deglycosylation can be clearly indicated. However, information on the effect of processing of oligosaccharides cannot be obtained by this strategy. To examine the role of each structure, it is necessary to manipulate the oligosaccharides via either the introduction or inactivation of glycosyltransferase genes; both genetic mutation methods and interference RNA (RNAi) have been used to inactivate genes in cultured cells. The drawback of this strategy is that it is difficult to determine the key target protein(s) since the oligosaccharides in a variety of proteins could be altered. Therefore, interpretation of the results requires care in this case. However, this is a method of choice for altering the structure of oligosaccharides in glycoproteins of culture cells at the present time.

Our interest has been focused on the physiological role of the glycosyltransferases which are involved in biosynthesis of N-glycans in glycoproteins, especially the branching enzymes such as *N*-acetylglucosaminyltransferase III (GnT-III) [2], GnT-V [3,4] and α1-6 fucosyltransferase (a1-6FucT) [5]. Studies of these glycosyltransferases indicate that the structure of N-glycans on growth factor receptors is involved in the regulation of their functions. Here we present an overview of the role of oligosaccharides on the growth factor receptor, with emphasis on the N-glycan on the EGF receptor and NGF receptor.

EGF signaling and N-glycans

Human EGF receptor (EGFR) is a 170 kDa type I membrane protein which consists of 1186 amino acid residues and contains 12 typical N-glycosylation consensus sites [6] (Figure 1). It has a single transmembrane domain, which separates an extracellular ligand-binding domain from a cytoplasmic domain, which encodes a tyrosine kinase, a common structure for

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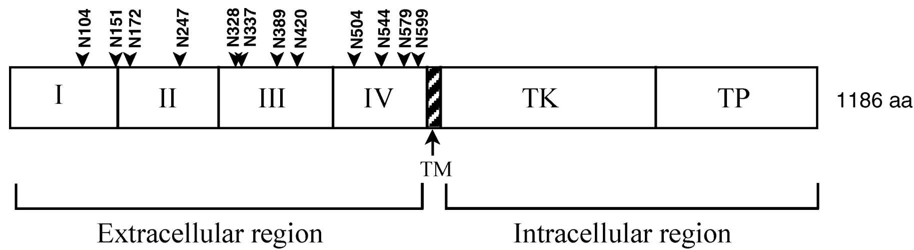

Figure 1. Structural feature and potential N-glycosylation sites in EGFR. Twelve potential sites for N-glycosylation, Asn-X-Ser/Thr, in EGFR are schematically represented. I, II, III, and IV denote the four subdomains in the extracellular region: TM: transmembrane region; TK: tyrosine kinase domain; TP: tyrosine phosphorylation domain.

receptor tyrosine kinases. EGFR can be activated by EGF and TGFα; the binding of ligands to the extracellular domain of EGFR induces dimerization of the receptor and activation of its intrinsic tyrosine kinase activity, leading to receptor autophosphorylation and the phosphorylation of tyrosine residues in various signaling molecules. For example, the activated EGFR forms receptor complexes comprised of Shc, Grb2, and Sos, which in turn trigger the activation of the Ras-ERK cascade. EGFR tyrosine kinase also stimulates phosphatidylinositol 3-kinase (PI3K) leading to the activation of a downstream signaling kinase Akt. EGF signaling also includes the PLCγ to CMK/PKC pathway and the Stat1/p91 pathway. The activation of these signaling systems triggers cellular responses such as cell growth, differentiation, and cell survival.

The large number of glycosylation sites suggests that EGFR exists as many different forms in terms of glycosylation, and the glycosylation patterns have been reported by several groups. In CHO cells, nine out of 12 typical N-glycosylation consensus sequences are actually glycosylated, as is one of the four atypical N-glycosylation sites (Asn-X-Cys) [7]. The glycoform was predicted to be different, depending on the sites [8]. Mature EGFR in A431 cells was also reported to contain both complex-type and high mannose-type N-glycans [9,10]. The roles of these N-glycans in EGFR functions have been extensively investigated [11–14]. It has been reported, by using glycosylation inhibitors such as tunicamycin, that an initial N-glycosylation is required for the proper sorting of EGFR to the membrane as well as for ligand binding [11,14]. Once the EGFR is N-glycosylated, it acquires ligand binding ability, and the processing of oligosaccharides in which the high mannose-type is altered to the complex-type was reported not to be necessary [12,13]. It has also been suggested that the interaction of certain lectins with receptor oligosaccharides leads to an alteration in ligand binding capacity as well as tyrosine kinase activity [15–18]. Thus, N-glycans on EGFR appear to play important roles in receptor function. We introduced mutations in the glycosylation sites in the extracellular domain of EGFR in an attempt to determine the N-glycosylation site(s) responsible for its function [19]. The extracellular portion of the EGFR can be subdivided into four domains, I–IV, also known as the L1, S1, L2, and S2 domains, respectively. Domain I (amino acid residues 1–165) and III (residues 310–481) have a 37% sequence identity whereas domains II (residues 166–309) and IV (residues 482–621) are both rich in cysteine. It has been suggested that domains I and III in human EGFR play a critical role in ligand binding [20]. We prepared EGFR mutants in which the Ser/Thr in each of the potential N-glycosylation sites in domain III were replaced by glutamine: Asn-328-Gln, Asn-337-Gln, Asn-389-Gln, Asn-420-Gln. Interestingly, among these mutants, the Asn-420-Gln mutant EGFR was observed to spontaneously undergo oligomerization, resulting in the phosphorylation of the receptor in the absence of ligand (Figure 2). Therefore, the N-glycan linked to Asn-420 appears to 'prevent' the spontaneous dimerization and activation of EGFR. It is also found that the Asn-420-Gln mutant of EGFR does not bind EGF, suggesting that the Asn-420-linked N-glycan is also required for the binding of EGF to EGFR. Since there is no evidence to suggest the Asn-420-linked N-glycan is directly associated with ligand binding, it is also possible the sugar chain may be involved in the maintaining of proper structure for ligand binding. In any case, it is likely that the binding of EGF to EGFR induces

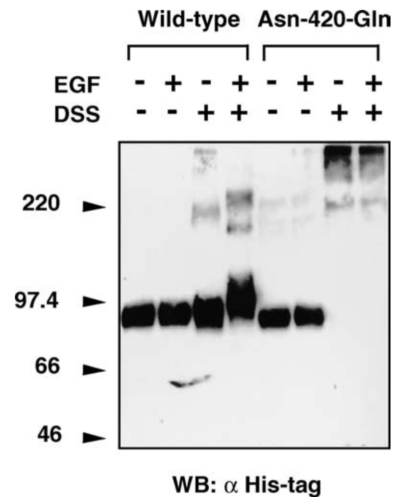

Figure 2. Analysis of EGF-induced oligomerization of sEGFR mutants by covalent cross-linking. Purified His-tagged sEGFRs (0.2 mg/ml) for the wild-type and Asn-420-Gln mutant produced in Sf-21 cells were cross-linked by using DSS (disuccinimidyl suberate) in the presence or absence or 20 μM EGF. The cross-linked products were separated by SDS-PAGE and detected by means of an anti-His tag antibody.

conformational changes which cancel the inhibitory effect of the N-glycan on Asn-420 toward the dimerization of EGFR. Fernandes *et al.* also suggested that the N-glycans in domain III are required for a conformation needed for EGF binding [21]. Recent studies of X-ray crystal structures demonstrated that the binding of a ligand to EGFR alters the relative angle between domain I and III, which is allosterically linked to receptor dimerization [22,23, reviewed in 24]. The structure shows that the ligand comes into contact with a single EGFR molecule through specific regions in both domain I and domain III; the B loop of EGF interacts with domain I while the A loop and the C-terminal region of EGF interact with domain III of EGFR. It has also been suggested that ligand binding alters the relative conformation of domains I and III, followed by a change in conformation of the dimerization loop projecting from each of the domain II that enables receptor dimerization. The issue of whether the conformation of spontaneous dimerization of Asn-420-Gln mutant EGFR is similar to that of ligand-induced dimerization is not known at present. Our results, however, strongly suggest that the N-glycan on Asn-420 in EGFR regulates dimerization by determining the relative orientation of domains I and III in the unliganded EGFR. Further study will be needed to identify the precise mechanisms by which the dimerization of EGFR is regulated by N-glycan(s).

The question arises as to the specific role of the processing of N-glycans in EGF signaling. Although it has been reported that the terminal processing, in which the high mannose-type is altered to the complex-type, is not essential for ligand binding as stated above, we were interested in the effect of branching structure(s) in N-glycans on EGF signaling. GnT-III is an enzyme which catalyzes the addition of GlcNAc to the β-mannoside of the tri-mannose core, producing a bisecting GlcNAc [2]. The introduction of a bisecting GlcNAc results in the suppression of further processing and elongation of the N-glycans, since other glycosyltransferases are not able to act on the resulting triantennary sugar chain. Rebbaa *et al.* reported that the binding of EGF to EGFR is significantly decreased in GnT-III transfected U373 MG glioma cells which originally contained bisecting oligosaccharide structures [25]. When the down-stream EGF signaling in the GnT-III transfected HeLaS3 cells was investigated, it was found that EGF-induced ERK phosphorylation was up-regulated in the GnT-III transfectants in spite of the decreased ligand binding to the EGFR [26]. A Scatchard analysis of the binding assay indicated that only the low affinity binding was decreased in the GnT-III transfectants whereas the high affinity binding was not changed. EGFR dimerization and autophosphorylation were not down-regulated in the GnT-III transfectants, which is consistent with the fact that the high affinity class, which constitutes 2–5% of the EGFR population, is required and sufficient for EGF signaling. When the endocytosis of EGFR was examined, it was revealed that the rate of EGF induced EGFR endocytosis was increased by about 40% in the GnT-III transfectants, leading to the up-regulation of downstream ERK phosphorylation (Figure 3). Thus, it can

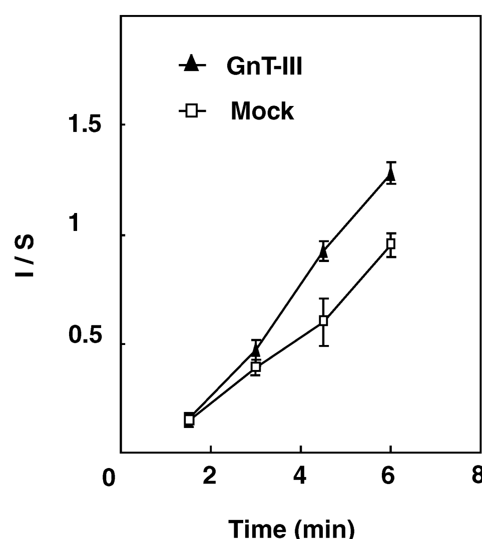

Figure 3. EGF-induced internalization of EGFR in mock- and GnT-III-transfected HeLa-S3 cells. Mock- and GnT-III-transfected HeLaS3 cells were serum-starved and treated with ^{125}I-EGF for the indicated times. The surface-bound ligand was extracted with acidic binding buffer, and the internalized one was determined by alkaline lysis. Data are expressed as the rate of internalized radioactivity divided by the surface-bound radioactivity.

be concluded that the overexpression of GnT-III in HeLaS3 cells, which introduces a bisecting GlcNAc in the N-glycan on EGFR, (1) decreases low affinity binding to about 60% of control cells without affecting high affinity binding and (2) up-regulates EGF-induced EGFR endocytosis resulting in an enhancement in downstream signaling. Although the changes in N-glycans of EGFR were confirmed by lectin blotting using E-PHA, the issue of whether the changes in the N-glycans are actually involved in biological changes observed in GnT-III transfectants is not certain. However, since binding affinity is thought to be regulated by the relative position of domains I and III, shortening the N-glycans by introducing bisecting GlcNAc might alter EGF binding affinity. The mechanisms by which GnT-III overexpression affects endocytosis has also not been elucidated at this time. Altschuler *et al.* reported that the endocytosis of MUC1 is affected by its O-glycosylation state and MUC1, when expressed in glycosylation-defective cells, accumulates in intracellular compartments by enhanced endocytosis [27]. They hypothesized that more of the underglycosylated MUC1 can fit into a clathrin-coated pit because of a decrease in steric hindrance and that this enhances the recruitment of cytoplasmic proteins that regulate endocytosis, such as dynamin. The same scenario could explain the case of EGFR in GnT-III transfectants, *i.e.*, the shortening effects of the bisecting GlcNAc on N-glycans. Another potential cause of the enhancement of endocytosis is the molecular changes observed in the intracellular domain of EGFR (residues 996–1022). Further investigations will be needed to elucidate this issue.

Ganglioside is also important for the regulation of EGF signaling, and it might be related to the hypothesis that the population of EGFR located on a lipid raft is involved in downstream signaling. The interaction of ganglioside and EGFR will be discussed in the section written by Dr. Bremer in this issue.

Since EGF signaling is becoming a target for anticancer therapeutics, the manipulation of the N-glycan could be the candidate of anticancer strategy.

NGF-signaling and N-glycan

Nerve growth factor (NGF) belongs to a family of neurotrophic factors important for the growth and survival of diverse peripheral and central neurons [28]. The NGF signaling pathway is initiated by the direct binding of NGF to the high affinity NGF receptor (Trk). Trk is a 145 kDa type I membrane protein consisting of 790 amino acid residues with 13 potential sites for N-glycosylation, four of which are highly conserved within the Trk family of neurotrophin receptors and nine less well conserved. It contains a single transmembrane domain which separates the extracellular ligand-binding domain from a cytoplasmic domain encoding a tyrosine kinase. The binding of ligands to the extracellular domain of the Trk induces dimerization of the receptor and the activation of its intrinsic tyrosine kinase activity, leading to receptor autophosphorylation. The activated receptors phosphorylate tyrosine residues in various signaling molecules and exert down-stream signaling. For example, activated Trk triggers activation of the Ras-ERK, PI3K-Akt, PLCγ-CMK/PKC pathway cascades. The activation of these signaling systems triggers cellular responses such as cell differentiation and cell survival.

The role of N-glycan in Trk has been studied by several groups. Using tunicamycin, Watson *et al.* reported that N-glycan is critical for the function of Trk [29]. They treated PC12 cells, a rat pheochromocytoma cell line, with tunicamycin and found that unglycosylated Trk is constitutively phosphorylated in the absence of NGF. However, the constitutively active unglycosylated Trk was found to localize intracellularly, not to the cell surface, and was unable to transduce a signal via the Ras-ERK pathway. Mutoh *et al.* also proposed that the N-glycan in Trk is important for its localization and subsequent signaling [30]. They reported the treatment of PC12 cells with tunicamycin resulted in the different localization of Trk with its activator GM1 ganglioside and inhibited their association. They also reported that unglycosylated Trk lost its ability to form a complex with GM1. The binding of Trk to GM1 might be required for the normal trafficking of Trk, and therefore, the binding site of GM1 in Trk might act as an important determinant. The relationship of sugar structures of glycolipid GM1 and Trk in the mechanism of the NGF signaling pathway has not been clarified but appears to be related to the existence of the novel regulation of Trk activation.

What, then, is the effect of the processing of N-glycan on Trk signaling? We reported on an intriguing effect of GnT-III on Trk signaling [31]. GnT-III is highly expressed in rat kidney and brain tissues. To examine the intracellular role of GnT-III and its product in neural cells, the GnT-III gene was overexpressed in PC-12, which has very low GnT-III activities despite their neural origin. Following treatment with NGF, the mock transfected control cells differentiated into sympathetic neurons, but the GnT-III transfectants did not respond to NGF as indicated by the rate of cell growth and morphological changes. In addition, tyrosine phosphorylation of Trk by NGF could not be detected in the GnT-III transfectants although the degree of NGF binding remained unaltered. Moreover, the dimerization of Trk was not induced in the GnT-III transfectants under NGF treatment despite the intact binding of NGF to the cells. The above study shows that the modification of Trk N-glycan by the overexpression of GnT-III results in a disturbance of the dimerization and disrupts its signal transduction under NGF treatment. Therefore, it is possible that the processing of N-glycan on Trk could be involved in the mechanisms responsible for regulating NGF signaling.

Insulin receptor and N-glycan

The insulin receptor (IR) is a membrane-bound glycoprotein belonging to the tyrosine kinase receptor family [32]. The mature form of IR is a heterodimer composed of two α-subunits (135 kDa) and two β-subunits (95 kDa). From the primary structure, it was revealed that the α-subunit has 14 potential N-glycosylation sites and β-subunit has 4. The β-subunit has also been shown to contain an O-linked carbohydrate. The participation of N-glycans on IR in the binding of insulin was demonstrated by using tunicamycin [33–38]. Functionally distinct roles for glycosylation can be assigned to the α- and β-subunits of IR via the use of the site-directed mutagenesis of individual sites. In the α-subunit, a mutation of the first four N-glycosylation sites [39] or a mutation of the first or second pair of sites [40] leads to the accumulation of the proreceptor in the ER and resistance to proteolytic cleavage, indicating that these four N-glycans are necessary for the proper processing and intracellular transport of IR. Glycosylation at either Asn397 or Asn418 is also required for normal receptor biosynthesis and function [41]. In contrast, the mutation of the four N-glycosylation sites on the β-receptor subunit have no effect on the biosynthesis of the receptor, the transport of the receptor to the cell surface, or the ligand binding but inhibits signaling because of its defective autophosphorylation and tyrosine kinase activity [42]. It was shown that only the third and fourth N-glycans regulate the functional status of IR. Thus, it has been proposed that specific N-glycan(s) differentially affect the function of IR.

Future prospective

The role of oligosaccharides in glycoproteins is now beginning to be elucidated. Although some examples exist, such as PDGF, which do not require N-glycosylation for their function [43],

many membrane-bound receptors appear to require glycosylation for their full activity. For the receptor membrane protein, oligosaccharides appear to be especially important for intracellular trafficking and for maintaining conformation, including determining the flexibility of the protein. As stated in the section on EGFR, it is possible that oligosaccharides play a role in determining the relative orientation of the domain or restricting the area of the movement of the domain. Elucidation of receptor function on structural basis is the major premise for such a discussion.

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