The Asn-420-linked Sugar Chain in Human Epidermal Growth Factor Receptor Suppresses Ligand-independent Spontaneous Oligomerization

POSSIBLE ROLE OF A SPECIFIC SUGAR CHAIN IN CONTROLLABLE RECEPTOR ACTIVATION*

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To elucidate a role(s) of Asn-linked sugar chain(s) in the function of epidermal growth factor receptor (EGFR), a series of the EGFR mutants were prepared in which potential glycosylation sites in the domain III were eliminated by site-directed mutagenesis. Although the wild-type and mutants of Asn-328, Asn-337, and Asn-389 underwent autoprophosphorylation in response to epidermal growth factor (EGF), the Asn-420 → Gln mutant was found to be constitutively tyrosine-phosphorylated. This abnormal ligand-independent phosphorylation of the mutant appears to be due to a ligand-independent spontaneous oligomer formation, as shown by a cross-linking experiment using the purified soluble extracellular domain (sEGFR). As revealed by the dissociation of the Asn-420 → Gln sEGFR oligomer by simple dilution, it seems likely that the equilibrium is shifted toward oligomer formation to an unusual degree. Furthermore, it was also found that the mutation caused a loss of the ability to bind EGF. These findings suggest that the sugar chain linked to Asn-420 plays a crucial role in EGF binding and prevents spontaneous oligomerization of the EGFR, which may otherwise lead to uncontrollable receptor activation, and support the view of a specific role of an Asn-linked sugar chain in the function of a glycoprotein.

Epidermal growth factor receptor (EGFR),1 a 170-kDa glycoprotein with a single transmembrane span, mediates cellular response to epidermal growth factor (EGF) and transforming growth factor-α. The binding of ligands to the extracellular domain of the EGFR induces dimerization of the receptor and activation of its intrinsic tyrosine kinase activity, leading to the receptor autoprophosphorylation and the phosphorylation of tyrosine residues in various cellular substrates, many of which serve as intracellular signal molecules (1–3). The phosphorylated tyrosine residues in the EGFR molecule provide high affinity binding sites for proteins that contain the Src homology region 2 domain, thus allowing the modulation of the intracellular signaling pathway (4, 5). It is generally thought that one of the initial events leading to the receptor activation involves ligand-induced conformational changes in the extracellular domain of the receptor, followed by receptor dimerization (6, 7).

The extracellular region of human EGFR contains 12 potential sites for N-glycosylation (8), and roles of the N-linked oligosaccharides in the receptor functions have been extensively investigated (9–14). It has been indicated that the binding of EGF to the EGFR was remarkably reduced in the case of A431 cells that had been treated with N-glycosylation inhibitors, suggesting that N-linked sugar chains appear to be required for receptor function (10). It has also been reported that the interaction of certain lectins with receptor oligosaccharides leads to an alteration in ligand binding capacity as well as tyrosine kinase activity (11–13). It has also been found that modulation of N-glycan biosynthesis by N-acetylglucosaminyltransferase III, an enzyme that plays a major role in the biosynthesis of the hybrid and complex types of N-linked oligosaccharides (15), inhibits EGF binding as well as receptor autoprophosphorylation (14). Thus, N-linked oligosaccharides on EGFR appear to play important roles in receptor function, particularly regarding ligand binding. However, a specific N-linked oligosaccharide(s) responsible for the expression of the functional receptor has not yet been identified, and, as a result, the molecular basis for the requirement of the sugar chain in receptor function remains obscure.

On the basis of amino acid sequence conservation, the extracellular domain of the EGFR is divided into four subdomains, namely domains I–IV (8). Of the four domains, domain III is believed to play a critical role in ligand binding such as the binding of EGF and transforming growth factor-α, as suggested by the studies using a recombinant soluble extracellular domain of the EGFR (sEGFR) (16–19) and its limited proteolytic fragment (20). The isolated fragment, which contained domain III, retains the capacity to bind the ligand, a finding that is consistent with evidence that domain III is specifically cross-linked with the EGF in affinity cross-linking experiments (21). The importance of the domain III in ligand binding is also supported by an analysis using a chimeric chicken/human EGFR (22). Since the isolated fragment does not form an oligomer, even on ligand binding, it is more likely that some other subdomain in the extracellular domain is directly associated with the dimerization of the receptor. The above findings suggest that ligand binding is a primary role of domain III of EGFR. These and aforementioned suggestions concerning the involvement of the sugar chains have led us to hypothesize that sugar chains in domain III may play a role in the function of this receptor, possibly in ligand binding.
To investigate and elucidate the significance of the sugar chain in the function of the EGFR, identification of the responsible glycosylation site would be a highly desirable first step, and it follows that the most probable candidates to examine would be the sugar chains in domain III. In this study, we prepared and characterized mutant EGFRs in which potential N-glycosylation sites have been replaced by site-directed mutagenesis, in order to eliminate sugar chains being attached to domain III. These mutants were transiently expressed in COS-1 cells to examine the effect of the loss of the sugar chain on receptor function. The role of the specific sugar chain identified as being important was further investigated using a purified soluble extracellular domain of the mutant. Such a detailed characterization of the mutant receptor in which this important sugar chain is absent might lead to an understanding of the function of this sugar chain in signaling by growth factor receptors.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction endonucleases and DNA modifying enzymes were purchased from Takara, Toyobo, and New England Biolabs. Oligonucleotide primers were synthesized by Greiner Japan. Human EGF was purchased from Wakunaga. 125I-EGF was purchased from Amersham Pharmacia Biotech. Other common chemicals were from Wako Pure Chemicals or Nacalai Tesque.

**Plasmid Construction and Site-directed Mutagenesis**—Human EGFR cDNA (23), kindly provided by Dr. Masabumi Shibuya (Institute of Medical Science, University of Tokyo, Tokyo, Japan), was subcloned into pCDNA3.1 vector (Invitrogen, San Diego, CA) for the transient expression in COS-1 cells. Mutations were introduced according to the method of Kunkel (24), as described previously (25). The single-stranded uracil-substituted DNA templates were prepared from Escherichia coli strain C6326 transformed by pBluescript(Stratagene) plasmids containing a 1.2-kb BamHI-BamHI and a 0.97-kb BamHI-EcoRI fragment of the EGFR cDNA. To delete the potential N-linked glycosylation sites in EGFR domain III (Asn-328, Asn-337, Asn-389, and Asn-420), mutations to substitute glutamine for asparagine were performed with the uracil template for the 1.2- or 0.97-kb fragment and the following primers: Asn-328 → Gln, 5'-ATT GGT GGC GTG TAT GGA GAG-3'; Asn-337 → Gln, 5'-GGG GGA GCA TTG TTT GAA GTG-3'; Asn-389 → Gln, TGG CTC AGA ACC AGG GAC-3'; Asn-420 → Gln, TGG CAC AGT CAG GAC-3'. When a His-tagged sEGFR was prepared, the transmembrane and cytoplasmic regions were deleted, and (Gly)4-(His)6-STOP was introduced at the position following Ile-619 in the juxtamembrane region of the EGFR. These alterations were also made in the 0.97-kb fragment by site-directed mutagenesis using a primer 5'-GGG CCT AAG ATC GGC GGC GGC CAC CAT CAT CAT CAT TAA GAA TTC TGT CTC GTG-3'. All the mutated sequences were verified by automated DNA sequencing using a Dye Terminator Cycle Sequencing Kit and ABI Prism 310 Genetic Analyzer (Perkin-Elmer, Applied Biosystems).

**Transient Transfection of Wild-type and Mutant EGFRs into COS-1 Cells**—COS-1 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 μg/ml kanamycin, and 5 μl/gliter glucose under 5% CO2, 95% air at 37 °C. The transfected COS-1 cells were maintained in 10-cm dishes for 2 days in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 10 μg/ml kanamycin, and 5 μl/gliter glucose, and then starved for 18 h in fetal calf serum-free Dulbecco’s modified Eagle’s medium. The cells were then stimulated with or without 100 ng/ml human EGF for 10 min at 37 °C. The resulting cells were washed twice with ice-cold phosphate-buffered saline, and lysed by sonication in 50 mM Hepes-NaOH (pH 7.4), 150 mM NaCl, 1 mM MgCl2, 1.5 mM EGTA, 100 μM NaF, 200 μM sodium orthovanadate, 1% Nonidet P-40, and 10% glycerol. After removal of insoluble materials by centrifugation at 10,000 g for 10 min at 4 °C, the samples were analyzed by SDS-PAGE using a 7.5% gel and immunoblotting. Expression of the EGFRs and tyrosine phosphorylation were examined by probing with an anti-human EGFR and an anti-phosphotyrosine, respectively.

**Insect Cell Culture and General Manipulation of Viruses**—Spodoptera frugiperda (SF) 21 cells were maintained at 27 °C in Grace’s insect medium (Life Technologies, Inc.), supplemented with 10% fetal bovine serum, 3.33 g/liter yeastolate, 3.33 g/liter lactalbumin hydrolysate, and 100 μg/ml kanamycin. Recombinant viruses were manipulated as described (28).

**Preparation of Transfer Plasmids and Recombinant Viruses**—cDNAs for the His-tagged sEGFRs were ligated into BamHI and EcoRI sites of a baculovirus transfer vector, pVL1395 (Invitrogen), and the resulting plasmids were purified using Qiagen Plasmid Minikit. A baculovirus transfer plasmid (5 μg) was co-transfected into 5 × 105 Sf21 cells with 10 ng of BaculoGold DNA (Pharmingen). Transfection experiments were carried out by the Lipofectin (Life Technologies, Inc.) method (29), as described previously (30). Media containing the recombinant viruses generated were collected 5 days after transfection. The titers of recombinant viruses were further amplified by several rounds of infection prior to use.

**Expression and Purification of Recombinant sEGFRs**—Sf21 cells, at 70% confluence, were infected with the recombinant baculoviruses carrying the wild-type and mutant sEGFR cDNAs, and the culture medium was harvested 5 days after infection in order to purify the recombinant proteins that had been secreted by the infected cells. Cell debris in the culture medium was removed by centrifugation at 20,000 × g for 30 min at 4 °C. The secreted sEGFR was precipitated by saturated ammonium sulphate and pelleted by centrifugation. The pellet was dissolved in and dialyzed against 20 mM Hepes-NaOH (pH 7.4) and 500 mM NaCl. The dialyzed materials were applied to a N125-chelating Sepharose Fast Flow column (Amersham Pharmacia Biotech) equilibrated with 20 mM Hepes-NaOH buffer (pH 7.4) containing 40 mM NaCl. The column was then washed thoroughly with 20 mM Hepes-NaOH buffer (pH 7.4) containing 500 mM NaCl and 100 mM imidazole. The sEGFR was eluted from the column with 20 mM Hepes-NaOH buffer (pH 7.4) containing 200 mM imidazole. Aliquots of fractions were analyzed by SDS-PAGE, followed by silver staining to monitor elution of the sEGFR and its purity. The purified sEGFR was concentrated using a Centriprep-30 concentrator (Amicon) to a final concentration of approximately 0.7 mg/ml. The mutant sEGFRs were also purified, as was the wild-type protein. The purified sEGFRs were stored at 4 °C until used.

**Cowbell Cross-linking Experiment**—The purified wild-type and mutant sEGFRs (0.2 mg/ml) were incubated with or without 20 μM human EGF in 20 mM Hepes-NaOH buffer (pH 7.4) containing 100 mM NaCl for 1 h at room temperature. The mixtures were allowed to react with 10 μl disuccinimidyl suberate (DSS; Nacalai Tesque), a covalent chemical cross-linking reagent, for 1 h at room temperature, and the reactions were then terminated by the addition of Tris-HCl (pH 7.4) to a final concentration of 500 mM. The resulting cross-linked products were analyzed by SDS-PAGE and immunoblot analysis using the anti-His tag antibody.

**125I-EGF Binding Assay**—Fifty ng of the purified wild-type sEGFR were incubated with 50, 100, 150, and 250, and 500 ng 125I-EGF (310 cpm/pmol) in 50 μl of 20 mM Hepes-NaOH buffer (pH 7.4) containing 40 mM imidazole and 0.1% bovine serum albumin in microtubes for 1 h at room temperature. Five μl of 1125I-chelating Sepharose Fast Flow resin were added to the reaction mixture, which was then mixed by rotation of the tube for 10 min. Materials were then washed twice by precipitation by centrifugation at 14,000 × g for 10 min at 4 °C and washed twice with the same ice-cold buffer as was used for binding. The pellets were resuspended in 500 μl imidazole HCl (pH 7.4) to elute the ligand-receptor complex from the resin, and the radioactivities of the supernatants were measured on a γ-counter. Nonspecific binding was determined in duplicate experiments in the presence of 100-fold excess amounts of nonradioactive EGF. The dissociation constant for the binding.
Role of N-Glycan in the EGFR Function

EGF-induced Tyrosine Phosphorylation of the Mutant EGFRs Expressed in COS-1 Cells—To explore the issue of whether the mutations at the glycosylation sites affect EGF-induced tyrosine autophosphorylation, the wild-type and mutant EGFRs expressed in COS-1 cells were stimulated by EGF. The Asn-328 → Gln, Asn-337 → Gln, and Asn-389 → Gln mutant EGFRs as well as the wild-type were tyrosine-phosphorylated in an EGF-dependent manner, suggesting that these mutants were indistinguishable from the wild-type in terms of the cell surface expression and the receptor function such as the ligand-induced activation (Fig. 2B). On the other hand, the Asn-420 → Gln mutant EGFR was phosphorylated even in the absence of EGF, to an extent similar to that found in the wild-type, which had been stimulated with EGF (Fig. 2B). These results were very reproducible, as obtained by four independent experiments, and clearly show that the EGFR is converted to a constitutively active form by disruption of the sugar chain at Asn-420. It therefore appears that the sugar chain at Asn-420 is involved in the controllable ligand-induced activation of the EGFR.

Furthermore, we also created the additional mutant EGFR, Asn-328/337/389 → Gln, in which only Asn-420 was intact in domain III and transiently transfected the triple mutant into COS-1 cells, in order to determine whether glycosylation at Asn-420 is sufficient for the receptor function. However, immunoblotting analysis with anti-EGFR antibody showed that this mutant protein was not expressed in COS-1 cells, similar to the case of the Asn-all → Gln mutant (data not shown).

Preparation of the His-tagged sEGFR—It seems unlikely that a loss of the sugar chain in the extracellular region would directly affect a function involving the cytosolic domain, which is located across the membrane, without any primary effect on the structure or function of the extracellular portion. Therefore, it would be more reasonable to hypothesize that the consequence of the mutation at Asn-420 is associated with an alteration in a function that is intrinsic to the extracellular portion, rather than the cytosolic domain. Thus, we prepared and characterized the recombinant His-tagged sEGFR for the Asn-420 → Gln mutant in order to investigate the more detailed role of the Asn-420-linked sugar chain. Fig. 3A shows the schematic representation of the His-tagged sEGFR, in which an entire extracellular domain is followed by a (Gly)3 and (His)6 tag at the C terminus. The protein would be predicted to consist of 628 amino acids after the cleavage of the signal peptide.

The sEGFRs for the wild-type, Asn-328 → Gln, Asn-337 → Gln, and Asn-420 → Gln were successfully expressed in the SF-21 cells infected with the recombinant baculoviruses and were efficiently secreted into the culture medium. The recombinant sEGFRs were purified from the culture medium using a Ni2+-chelating Sepharose FF column. The Asn-389 → Gln sEGFR was not produced in the infected insect cells. SDS-PAGE and immunoblot analyses of the purified recombinant sEGFRs demonstrated bands at 90 and 88 kDa for the wild-type and the mutants, respectively, as shown in Fig. 3 (B and C). The difference of 2 kDa appears to be consistent with the loss of a single sugar chain, suggesting that Asn-328, Asn-337, and Asn-420 are actually glycosylated in the insect cells.

Covalent Cross-linking of Asn-420 → Gln sEGFR—It may reasonably be considered that the impairment of the extracellular domain function, which is associated with a dimer formation, may lead to the EGF-independent activation in the Asn-420 → Gln mutant EGFR because the receptor dimerization, involving the extracellular domain, is thought to be a critical step in the mechanism of receptor activation (6, 7). Thus, the purified sEGFRs were subjected to chemical cross-linking experiments, in order to examine whether the Asn-420 → Gln
The wild-type and Asn-328 → Gln sEGFRs were incubated in the presence or absence of EGF and subsequently treated with DSS, a covalent cross-linking reagent. The resulting cross-linked products were analyzed by SDS-PAGE and immunoblotting. The wild-type sEGFR remained in a monomeric form in the absence of EGF, whereas the sEGFR formed a dimer in response to the addition of the ligand (Fig. 4A). This suggests that the dimerization of the wild type is properly regulated by the binding of EGF. In addition, the band at approximately 100 kDa that was found in the presence of both EGF and DSS indicates that the monomer sEGFR complexed with EGF. The same was true in the case of the Asn-328 → Gln and Asn-337 → Gln mutant sEGFRs, as shown in Fig. 4B. These data suggest that the His-tagged sEGFRs for the wild-type and these mutants are functionally active in terms of binding to EGF and ligand-induced dimerization. On the other hand, no band corresponding to the monomeric form was observed in the Asn-420 → Gln mutant sEGFR, but rather it was found that the Asn-420 → Gln sEGFR is highly oligomerized even in the absence of EGF, as shown by the much larger cross-linked products. These results clearly indicate that the disruption of the sugar chain linked to Asn-420 induces the spontaneous oligomerization of the extracellular domain of the receptor, in a manner that is independent of EGF stimulation. It therefore appears that the receptor dimerization, which is regulated by the EGF-binding, involves the sugar chain at Asn-420.

**SDS-PAGE Separation of sEGFR under Non-reducing Conditions**—Since domains II and IV in the extracellular portion of the receptor are cysteine-rich regions (8), the possibility that spontaneous oligomerization results from the aggregation by abnormal intermolecular disulfide linkages cannot be excluded. To test this possibility, SDS-PAGE analysis of the purified wild-type and Asn-420 → Gln sEGFRs was carried out under non-reducing conditions. The non-reduced proteins exhibited essentially the same profiles as the reduced ones, and therefore it seems unlikely that the oligomerization is due to the intermolecular covalent interactions (data not shown).
Dissociation of Asn-420 → Gln sEGFR Oligomers—To further explore the nature of the oligomerization in the Asn-420 → Gln mutant, it is necessary to ascertain the involvement of non-covalent interaction in spontaneous oligomerization. The transition between monomeric and oligomeric states was investigated by monitoring their fractions at various protein concentrations. It was found that the oligomer derived from Asn-420 → Gln sEGFR is dissociated into monomer and dimer at sufficiently low concentrations (Fig. 5). Thus, the results suggest that the loss of the Asn-420-linked sugar chain facilitates oligomer formation via non-covalent interactions, and it appears that the equilibrium for the transition is shifted greatly toward oligomer formation in the mutant.

EGF Binding to sEGFR—To examine the effect of the elimination of the Asn-420-linked sugar chain on the binding of EGF, the wild-type and Asn-420 → Gln mutant sEGFRs were subjected to a binding assay using 125I-EGF. The dissociation constant $K_d$ of EGF for the wild-type sEGFR was found to be approximately $1.0 \times 10^{-7}$ M, as determined by Scatchard analysis (Fig. 6, A and B). This value obtained for the tagged sEGFR was in good agreement with the value reported for a non-tagged form (16–19). In the Asn-420 → Gln mutant sEGFR, however, essentially no binding was observed, even at the highest concentration of the ligand. Thus, it appears that the disruption of the sugar chain at Asn-420 in the sEGFR impairs ligand binding. The results are consistent with the suggestion that the sugar chain also plays an essential role in the binding of EGF, even though it is not clear, at present, whether its involvement is direct or indirect.

DISCUSSION

In this study, several domain III-N-glycosylation mutants of the EGFR were prepared and characterized, in order to identify a sugar chain that is important or essential in the receptor function such as ligand binding, regulated dimerization, or ligand-induced autophosphorylation. The results showed that the loss of the sugar chain linked to Asn-420 leads to spontaneous oligomerization, which results in the ligand independent activation of the receptor. Thus, it could be demonstrated that the Asn-420-linked sugar chain plays an essential role in the properly regulated dimerization of the EGFR by the ligand. The sugar chain identified in this study appears to “prevent” the spontaneous activation of the receptor, and the findings therefore suggest that the sugar chain is absolutely required to maintain the controllable properties of the receptor by ligand. It was also found that the Asn-420 → Gln mutant of the sEGFR does not bind EGF, suggesting that the Asn-420-linked sugar chain is also required for the interaction of the receptor with the ligand.

Since the oligomer form of the Asn-420 → Gln mutant sEGFR is dissociated at sufficiently low concentrations of the protein (Fig. 5), oligomer formation seems to be reversible. On the other hand, although the equilibrium of dimerization or oligomerization in the wild-type sEGFR is in favor of the monomer state in the absence of the ligands, the binding of the ligands and the subsequent conformational changes of the receptor would shift the equilibrium in the direction of oligomer
formation. In this respect, it is likely that the equilibrium in the Asn-420 → Gln mutant would intrinsically be shifted toward a higher level of oligomerization. Domain III, which contains an Asn-420-linked sugar chain, is capable of binding ligands but not dimer formation even in the presence of the ligand, as indicated by a study using an isolated limited-proteolytic fragment that includes this domain (20). It seems more likely that oligomerization of the receptor involves other domain such as domain IV, which may play a role in the dimerization of the receptor. Therefore, it is entirely possible that the mutated domain III that lacks the Asn-420-linked sugar chain could mimic the ligand-bound state of the wild-type domain III and thereby induce oligomerization dependently on other domain(s), thus conferring receptor-receptor interaction.

The binding affinity of sEGFR toward EGF appears to be dramatically increased on dimerization (or oligomerization), even if the dimer is formed by covalent cross-linking (16–19). Thus, it is reasonably likely that the affinity is significantly higher in the dimerized or oligomerized state, as opposed to the monomer state. However, although the Asn-420 → Gln sEGFR appears to be oligomerized, essentially no binding is observed at an EGF concentration of 500 nM in the binding assay. The replacements at other N-glycosylation sites in domain III had no effect on EGF-induced receptor autophosphorylation (Fig. 2B), and thus it seems certain that these mutants bind EGF. These findings indicate the unique importance of the Asn-420-linked sugar chain in the binding of EGF as well as the controllable dimerization because, of four sugar chains in the domain, only the Asn-420-linked sugar chain plays an essential role in the binding of EGF.

It is known that the $K_B$ value for the binding of mammalian EGF to chicken EGFR, which lacks the sugar chain corresponding to the human Asn-420-linked chain in domain III, is 100 times higher than that in human EGFR (31). The analyses, which used a series of chimeric chicken/human EGFRs, indicated that domain III of the chicken receptor is responsible for this lower affinity to EGF (22). Although structural determinants for the distinct binding properties of the chicken receptor have been investigated in terms of the difference in the primary structure of domain III (32–34), this could, in part, be explained by the lack of the equivalent sugar chain whose loss leads to the impairment of EGF binding in human EGFR. Nevertheless, the issue of whether the sugar chain is directly associated with the binding currently remains unclear, and it is also possible that the sugar chain may be involved in the correct folding of the domain and/or is required to maintain the conformation of domain III, so that efficient EGF binding can take place.

Because of the defect in the ligand-regulated activation of the receptor, the Asn-420 → Gln EGFR appears to have been converted to a constitutive active form. Spontaneous oligomerization accompanied by tyrosine phosphorylation has been reported for oncogenic mutants of EGFR and its related growth factor receptors, the ErbB family (35, 36), and therefore these abnormal properties would be closely associated with their transforming activities. The controllable properties of growth factor receptors such as EGF must be absolutely required for an appropriate response of cells to stimulation by growth factors, since the impairment of the regulated transmembrane signaling could lead to uncontrolled cell growth, e.g. transformation, or other abnormalities, such as altered intracellular signal transduction and defects in the growth factor-stimulated cell growth. Our present study clearly revealed the significance and involvement of a specific N-linked sugar chain in the regulated activation of growth factor receptor and also provide new insights into the mechanism of ligand-induced receptor activation. Furthermore, the findings here would support the view of a specific role of an Asn-linked sugar chain in the function of a glycoprotein.

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