Overexpression of N-Acetylglucosaminyltransferase III Enhances the Epidermal Growth Factor-induced Phosphorylation of ERK in HeLaS3 Cells by Up-regulation of the Internalization Rate of the Receptors*

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N-Acetylglucosaminyltransferase III (GnT-III) is a key enzyme that inhibits the extension of N-glycans by introducing a bisecting N-acetylglucosamine residue. In this study we investigated the effect of GnT-III on epidermal growth factor (EGF) signaling in HeLaS3 cells. Although the binding of EGF to the epidermal growth factor receptor (EGFR) was decreased in GnT-III transfectants to a level of about 60% of control cells, the EGF-induced activation of extracellular signal-regulated kinase (ERK) in GnT-III transfectants was enhanced to 1.4-fold that of the control cells. A binding analysis revealed that only low affinity binding of EGF was decreased in the GnT-III transfectants, whereas high affinity binding, which is considered to be responsible for the downstream signaling, was not altered. EGF-induced autophosphorylation and dimerization of the EGFR in the GnT-III transfectants were the same levels as found in the controls. The internalization rate of EGFR was, however, enhanced in the GnT-III transfectants as judged by the uptake of 125I-EGF and Oregon Green-labeled EGF. When the EGFR internalization was delayed by dansylcadaverine, the up-regulation of ERK phosphorylation in GnT-III transfectants was completely suppressed to the same level as control cells. These results suggest that GnT-III overexpression in HeLaS3 cells resulted in an enhancement of EGF-induced ERK phosphorylation at least in part by the up-regulation of the endocytosis of EGFR.

It is a generally accepted fact that N-glycans play an important role in the folding, stability, and sorting of glycoproteins (1, 2). They have a common core structure, and their branching patterns are determined by glycosyltransferases (3–6). β1–4 N-Acetylglucosaminyltransferase III (GnT-III) catalyzes the addition of N-acetylglucosamine (GlcNAc) to the β-mannoside of the tri-mannose core, to produce a bisecting GlcNAc (7, 8). The introduction of a bisecting GlcNAc results in the suppression of further processing and elongation of N-glycans, since other glycosyltransferases are not able to act on the resulting biantennary sugar chains. To elucidate the biological function of bisecting GlcNAc, GnT-III-overexpressing transfectants have been examined from many aspects (9–15). For example, we previously reported that the introduction of GnT-III in B16 mouse melanoma cells suppresses lung metastasis (9), increases E-cadherin-mediated homotypic adhesion (10), and enhances cell adhesion to hyaluronate via the modulation of N-glycans of CD44 (11). GnT-III transfection also reduces the susceptibility of human leukemia K562 cells to the cytotoxicity of natural killer cells (12) and suppresses the expression of hepatitis B virus in a hepatoma cell line (13). It has been reported that GnT-III overexpression affects signal transduction such as nerve growth factor signaling by suppressing the dimerization of Trk A on PC12 cells (14).

Epidermal growth factor receptor (EGFR) is one of membrane glycoproteins and the oligosaccharide side chains linked to the extracellular domain are essential for its function (16–18). However, the relevance of the composition and structure of N-glycans to epidermal growth factor (EGF) signaling is not fully understood. It was previously reported that the EGF binding to its receptor is significantly decreased in GnT-III-transfected human glioma cells U373 MG (15). In this paper, we report on a study involving the downstream EGF signaling in GnT-III-transfected HeLaS3 cells to further elucidate the mechanism by which this enzyme affects EGF signaling. Our findings show that the internalization rate of EGFR was up-regulated and that EGF-induced extracellular signal-regulated kinase (ERK) phosphorylation was enhanced in the GnT-III transfectants.

EXPERIMENTAL PROCEDURES

Cell Lines, Culture, and Transfection—HeLaS3 cells were obtained from the American Type Culture Collection (Rockville, MD) and were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum. Transfection was carried out using LipofectAMINE (Life Technologies, Grand Island, NY). For the EGF-stimulated kinase activity assay, HeLaS3 cells were incubated for 16 h with DMEM supplemented with 10% dialyzed fetal bovine serum. This medium was then replaced with serum-free DMEM and incubated for an additional 2 h. Then, EGF or antibody to EGF receptor was added to the medium and incubated for 15 min at 37 °C.

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memented with 10% fetal bovine serum. A GnT-III expression vector was constructed by inserting the cDNA, which encodes for the open reading frame of human GnT-III into a mammalian expression vector pCXN2, which was regulated by the β-actin promoter. HeLaS3 cells were transfected with pCXN2/GnT-III or pCXN2 using LipofectAMINE reagent (Life Technologies, Inc.) following the manufacturer’s instructions. Selection was performed in the medium, which contained 1.4 mg/ml neomycin and after a 2-week incubation, neomycin-resistant colonies were isolated and recloned by serial dilution to ensure clonality. Positive clones were selected by Western blotting. CHO cells were transfected with pTHuman EGFR (19), which was kindly provided by Dr. Masaharu Shibuya (Institute of Medical Science, University of Tokyo, Tokyo, Japan) to produce CHO-HER cells and cloned. CHO-HER cells were then transfected with pCXN2/GnT-III or pCXN2 as described above.

**Immunoprecipitation and Western Blotting—**Cell cultures (60–80% confluent) were rinsed twice with ice-cold phosphate-buffered saline (PBS), harvested in lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% (w/v) Nonidet P-40, 10% (w/v) glycero, 5 mM sodium pyrophosphate, 10 mM NaF, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 5 μg/ml leupeptin, and 1 mM dithiothreitol). Cell lysates were centrifuged at 15,000 × g for 10 min at 4 °C, the supernatants were collected, and the protein concentrations were determined using a protein assay CBB kit (Bio-Rad Laboratories, Inc.). For the immunoprecipitation, whole cell lysates (500 μg) were incubated with 4 μg of sheep anti-human EGFR antibody (06-129, Upstate Biotechnology, Lake Placid, NY) for 2 h at 4 °C, and then with 15 μl of Protein G-Sepharose 4 Fast Flow (Amersham Pharmacia Biotech) for 2 h at 4 °C. For Western blot analysis, whole cell lysates or immunoprecipitates were subjected to 10% SDS-polyacrylamide gel electrophoresis (PAGE), and the resulting proteins were transferred to nitrocellulose membranes (Schleicher & Schuell). The blots were probed with anti-GnT-III antibody (Fujirebio Inc., Japan), sheep anti-EGFR antibody (06-129, Upstate Biotechnology) or anti-phosphotyrosine antibody (4G10, Upstate Biotechnology). For ERK1/2 activation analysis, the blots were probed with anti-ACTIVE MAPK polyclonal antibody (Promega, Madison, WI) and anti-MAPK (ERK2) monoclonal antibody (05-157, Upstate Biotechnology). After the blots were incubated with peroxidase-conjugated secondary antibody, immunoreactive bands were visualized using an ECL kit (Amersham Pharmacia Biotech).

**GnT-III Enzyme Assay—**GnT-III activity was determined by high liquid performance chromatography using the fluorescence-labeled sugar chain, GlcNAc-1,2-Man-1,6-GlcNAc-1,2-Man-1,3-3Manβ-1,4-GlNAcβ-1,4-GlNAc-pyridylamino as a substrate, as described previously (20).

**Cell Surface Biotinylation and Immunoprecipitation of EGFR—**Cell surface biotinylation was performed as described previously (21). Briefly, cells were rinsed twice with PBS supplemented with 0.1 mM CaCl2 and 1 mM MgCl2 and then incubated with freshly prepared sulfo-succinimidobiotin (s-NHS-biotin; Pierce) diluted in the same solution (1 mg/ml) for 30 min on ice. The reaction was quenched with 50 mM NH4Cl. The resulting cell lysate was immunoprecipitated with anti-EGFR antibody as described above and subjected to 10% SDS-PAGE and then transferred to nitrocellulose membranes. The membranes were probed with anti-EGFR antibody as described above.

**Internalization of EGFR and Transferrin Receptor—**To monitor EGFR and transferrin receptor internalization, a previously described protocol (23, 24) was followed. Cells were seeded at a density of 8 × 104 cells/well in a 12-well plate and then cultured overnight. 125I-EGF (final concentration 1 ng/ml) or 125I-transferrin (final concentration, 1 μg/ml) in M-BSA was added to each well and incubated at 37 °C for 1.5, 3.0, 4.5, and 6.0 min. At the indicated times, cells were rapidly rinsed three times with ice-cold M-BSA to remove unbound ligand. Subsequently, the cells were incubated for 5 min with 0.2 μM acetic acid (pH 2.8), which contained 0.5 mM sodium chloride at 4 °C. The acid wash was combined with another short rinse with the same acidic solution to determine the amount of surface-bound 125I-ligand. Finally, the cells were solubilized in 1 mM sodium hydroxide for the quantitation of the internalized 125I-ligand. Nonspecific binding was measured in the presence of a 200-fold molar excess of unlabeled ligand and was not more than 5% of the total cell-associated radioactivity. Specific binding is calculated for all data points. For Oregon Green-labeled EGF internalization assay, GnT-III transfectants and mock transfectants cultured on glass coverslips were serum-starved for 2 h at 37 °C. These transfectants were then chilled to 4 °C and incubated with 200 ng/ml of Oregon Green 514-conjugated EGF (Molecular Probes, Eugene, OR) for 1 h at 4 °C. Cells were warmed to 37 °C for 10 min, transferred to ice, and stripped of the cell surface-bound ligand by incubation in ice-cold 0.2 M acetic acid (pH 2.5), which contained 0.5 mM NaCl for 5 min. They were fixed in 4% paraformaldehyde (buffered at pH 7.4 with 0.1 M phosphate buffer) for 10 min at 4 °C, and mounted with Permafluor aqueous mounting medium (Immunon, Pittsburgh, PA), and observed on a fluorescent microscope (Provis AX80) (Olympus, Japan). Nuclei were visualized with 1:5000 diluting of 4’,6-diamidino-2-phenylindole (Molecular Probes).

**RESULTS**

**Establishment of HeLaS3 Clones Stably Expressing GnT-III—**HeLaS3 cells were transfected with pCXN2/GnT-III or pCXN2 alone and neomycin-resistant clones were selected as described under “Experimental Procedures.” Transfection was verified by Western blotting using an anti-GnT-III antibody in conjunction with an enzyme activity assay. The selected clones showed high expression of GnT-III (Fig. 1) and an elevated...
enzymatic activity (Table I). We consider the bands seen near 220 kDa in Fig. 1 are nonspecific, since these are observed in mock transfectants. All other bands were considered to be derived from GnT-III. Following experiments were performed with both clones and similar results were observed for all data. The data with clone 1 are shown in subsequent figures.

**Analysis of EGF Receptor Internalization in GnT-III Transfectants**—Changes in EGFR levels on GnT-III-transfected cells were then determined. We examined the issue of whether the transfection affected the cell surface expression of EGFR. Using cell surface biotinylation and precipitation, it was shown that the two cell types expressed nearly the same amount of EGFR on cell surface (Fig. 2A). Subsequently, lectin blotting was performed to analyze the alterations in the carbohydrate structures associated with EGFR. E-PHA binds preferentially to bisecting GlcNAc residues in N-glycans. The upper panel of Fig. 2B shows that bisecting GlcNAc increased significantly on EGFR in the case of the GnT-III transfectants. As a control, the same blot was probed with anti-EGFR antibody, the results of which confirmed that the amount of immunoprecipitated EGFR was nearly the same (lower panel of Fig. 2B). From this result, it was also indicated that the molecular mass of immunoprecipitated EGFR in the GnT-III transfectants was lower than that of the mock transfectants. These data suggest that the transfection of GnT-III into HeLaS3 cells increased the amount of bisecting oligosaccharide structures and shortened the N-glycans associated with EGFR without affecting their cell surface expression.

**Enhancement of ERK Activation in GnT-III Transfectants**—To determine the effect of GnT-III transfection on EGF signaling, EGF-induced phosphorylation of ERK was investigated. GnT-III transfectants and mock transfectants were treated with 50 ng/ml EGF, and cell lysate samples were analyzed by Western blotting. Although no difference in the level of tyrosine phosphorylation between whole cell lysates of GnT-III transfectants and mock transfectants was observed (Fig. 3A), ERK phosphorylation was up-regulated in the case of the GnT-III transfectants by 140% over that of the mock transfectants (upper panel of Fig. 3B). As a control, the same blot was probed with anti-ERK2 antibody, thus confirming that the amount of ERK2 was nearly the same (lower panel of Fig. 3B).

**Examination of Subtype of EGFR Receptors**—To elucidate the mechanism by which GnT-III transfection affected EGF signaling, a binding analysis of 125I-EGF to EGFR in GnT-III transfectants and mock transfectants was performed. As shown in Fig. 4A, the binding of 125I-EGF to EGFR was significantly reduced in the GnT-III transfectants, compared with the mock transfectants. A Scatchard analysis revealed that both high and low affinity EGFR were present in GnT-III and mock transfectants, and $K_{d}$ or the number of EGF binding sites per cell of the high affinity EGFR did not change significantly, but the binding sites of the low affinity class were decreased by about 40% in GnT-III transfectants (Fig. 4B, Table II). These data suggest that modulation of N-glycans with the bisecting GlcNAc by GnT-III on EGFR may disturb the function of the low affinity of EGFR but not that of the high affinity of EGFR.

**Examination of EGFR Dimerization and Autophosphorylation**—To further clarify EGFR function in GnT-III transfectants, EGF-induced EGFR dimerization and autophosphorylation were examined. After cells were treated with 50 ng/ml EGF, cell surface proteins were cross-linked by using EDAC, and EGFR was then immunoprecipitated as described under “Experimental Procedures.” In the presence of EGF and EDAC, the approximate molecular mass of the 340-kDa proteins were detected that corresponded to a dimer of EGFR (Fig. 5A). The dimerization level of EGFR was not significantly different between GnT-III transfectants and the mock transfectants. After the cells were treated with 50 ng/ml EGF, EGFR was immunoprecipitated with the anti-EGFR antibody and subjected to blotting analysis with anti-phosphotyrosine antibody. As shown in Fig. 5B, the autophosphorylation of EGFR was not significantly changed in GnT-III transfectants compared with mock transfectants. Collectively, these results indicate that the

| Cell type | GnT-III activity (pmol/h/mg protein) |
|-----------|-------------------------------------|
| GnT-III-1 | 470.3 ± 50.8*                      |
| GnT-III-2 | 472.5 ± 102.8*                     |
| Mock      | Not detected                       |

**Fig. 2. Analysis of EGFR of GnT-III transfectants.** A, cell surface biotinylation and immunoprecipitation (IP) of EGFR from the GnT-III transfectants and mock transfectants. Cells were biotinylated, and whole cell lysates were immunoprecipitated with an anti-EGFR antibody. The samples were subjected to 10% SDS-PAGE and transferred to a nitrocellulose membrane, and the biotinylated proteins were detected as described under “Experimental Procedures.” B, lectin blot analysis of the immunoprecipitated EGFR from the GnT-III transfectants and the mock transfectants. EGFR was immunoprecipitated from 500 μg of proteins of a whole cell lysate and transferred to nitrocellulose membranes, which were probed by E-PHA (upper panel) or anti-EGFR antibody (lower panel). WB, Western blot.

**Fig. 3. The time course of tyrosine phosphorylation and ERK phosphorylation in mock- and GnT-III-transfected HeLaS3 cells.** Cells were stimulated with 50 ng/ml EGF and harvested at the indicated times. Whole cell lysates of mock and GnT-III transfectants were subjected to 10% SDS-PAGE and transferred to a nitrocellulose membrane. The blots were probed with anti-phosphotyrosine (4G10), anti-ERK2 and anti-ERK1 (panel A), anti-phosho ERK (panel B, upper) and anti-ERK2 (panel B, lower) as described under “Experimental Procedures.” WB, Western blot; α-pTyr, anti-phosphotyrosine.
levels of dimerization and autophosphorylation of EGFR were not changed significantly in the two cell types.

Enhancement of Internalization Rate of EGFR in GnT-III Transfectants—To understand how ERK phosphorylation is up-regulated in GnT-III transfectants, we performed an internalization assay, since endocytosis is generally thought to affect ERK activation (25, 26). As shown in Fig. 6A, the rate of \(^{125}\text{I}\)-EGF induced EGFR internalization was increased by about 40% in the GnT-III transfectants compared with mock transfectants. In Fig. 6B, the internalized Oregon Green-labeled EGF in the cytoplasm was clearly detected showing dot-like fluorescence. EGFR endocytosis in the GnT-III transfectants (upper left panel) was appeared to be increased compared with mock transfectants (upper right panel). GnT-III transfections and mock transfectants were then treated with the internalization chemical inhibitor, dansylcadaverine and hypertonic medium. Dansylcadaverine delays receptor internalization at steps that are proximal to the formation of early endosomes. Cells were serum-starved for 24 h and incubated with dansylcadaverine, and the EGFR internalization rate and ERK phosphorylation were examined. When the internalization of EGFR in two cell types was suppressed to the same level by dansylcadaverine (upper panel of Fig. 7A), the difference in ERK phosphorylation diminished (lower panel of Fig. 7A). A hypertonic medium, which contained 0.45 M sucrose, also inhibits clathrin-mediated endocytosis. The internalization of EGFR in the two cell types was completely inhibited in the hypertonic medium (upper panel of Fig. 7B) and ERK phosphorylation in two cell types nearly vanished (lower panel of Fig. 7B). These data, therefore, suggest that the up-regulation of EGFR internalization could account for the increase in ERK activation in the GnT-III transfectants. We performed internalization assay by using CHO-HER cells, and it was observed that endocytosis of EGFR was also up-regulated in CHO-HER cells, which had been transfected with GnT-III (Fig. 8). To see the specificity of effects of GnT-III overexpression, internalization rate of transferrin receptor was examined. As shown in Fig. 9, there was no difference in internalization rate of transferrin receptor between GnT-III- and mock-transfected HeLaS3 cells.

**DISCUSSION**

In this paper, we report on a study of the effect of GnT-III transfection on EGF signaling in HeLaS3 cells. Consistent with our previous report (15), EGF binding to its receptor was significantly decreased in GnT-III transfectants. However, our findings showed that EGF-induced ERK phosphorylation was up-regulated in the GnT-III transfectants. To know the mechanism by which ERK phosphorylation is up-regulated, we first examined EGF binding to EGFR in GnT-III transfectants. A Scatchard analysis of the binding assay data revealed that only low affinity binding was decreased in the GnT-III transfectants, whereas the high affinity binding was not changed. The fact that the cell surface expression of EGFR was not altered in GnT-III transfectants suggests that about 40% of the low affinity receptors lost their function, whereas high affinity receptors remained intact. 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 5–10% of the EGFR population, is required, and sufficient for EGF-induced responses (27, 28).

The difference between high affinity and low affinity EGFR has not yet been fully elucidated. It has been shown that the intracellular part of the EGFR regulates its affinity for EGF. It was observed that the activation of PKC converted high affinity EGFR to low affinity EGFR (29), a deletion of the entire intracellular domain (30) or domain from amino acids 921 to 940 (31) diminished the high affinity EGFR, and HeLa cells that express a mutant dynamin (K44A) lost the ability for high affinity binding of EGFR (32). It has been also demonstrated that high affinity EGFR represents the cytoskeleton-associated population (33–36). One possible mechanism by which GnT-III overexpression affected the EGF binding of low affinity receptor is that GnT-III transfection alters the status of the intracellular domain of EGFR. We observed that GnT-III overexpression in CHO-HER cells changed the reactivity of EGFR toward the antibody which recognized a part of intracellular domain of EGFR (residues 996–1022).\(^2\) Therefore, the introduction of GnT-III might result in some modification of the intracellular domain of EGFR. Another possible mechanism is that GnT-III affects an affinity-modulating protein, the presence of which has been suggested by van der Heyden et al. (31).

In this study, we demonstrated that the overexpression of GnT-III affected the internalization rate of EGFR. This effect seems specific to EGFR, since internalization rate of transferrin receptor was not affected, which is internalized by different mechanisms (24). The internalization of the EGFR is generally thought to be essential for ERK activation (25, 26), as well as the internalization of other receptors such as insulin receptor (37), insulin-like growth factor receptor (38), G protein-coupled receptor (39), and serotonin 5-HT<sub>1A</sub> receptor (40). Several studies have also demonstrated that downstream signaling molecules locate in endosomes with activated EGFR in response to EGF stimulation and support a role for membrane trafficking in EGF signaling (41–43). Thus, the up-regulation of the internalization rate of EGFR observed in GnT-III transfectants could be involved in the enhancement of downstream

\(^{2}\) M. Takahashi, Y. Ikeda, and N. Taniguchi, unpublished observation.
signaling. Quite recently, Tong et al. (44) demonstrated that endocytosis of EGFR affects downstream of Ras. We observed that activation levels of Ras were not changed in GnT-III transfectants compared with mock transfectants (data not shown). This observation also supports the hypothesis that the enhancement of EGF-stimulated ERK activation in GnT-III transfectants is due to the increase of internalization of EGFR. The mechanisms by which GnT-III overexpression affects endocytosis is now under investigation. Recently, Altschuler et al. (45) reported that the internalization of MUC1 is affected by its O-glycosylation state and that MUC1, which is expressed in glycosylation-defective cells, accumulates in intracellular compartments. They hypothesized that more of the underglycosylated MUC1 can fit into a clathrin-coated pit because of less steric hindrance and that this enhances the recruitment of cytoplasmic proteins regulating endocytosis such as dynamin. Our results are consistent with this hypothesis since GnT-III overexpression suppresses the processing and elongation of N-glycans. Another potential cause of enhancement of endocytosis is the molecular changes observed in the intracellular domain of EGFR (residues 996–1022) as stated above. The residues 996–1022 are located in the CaIn domain, so-called because it mediates the EGF-induced calcium response and internalization (46), and modification within or near the domain could cause a change of status or the physicochemical

### Table II

**Analysis of ²²¹²EGF binding**

| Cell type     | High affinity type | Low affinity type |
|---------------|--------------------|-------------------|
|               | $K_d$ (pM)  | $125I$-EGF binding sites/10⁶ cells | $K_d$ (pM)  | $125I$-EGF binding sites/10⁶ cells |
| GnT-III       | 3.99 ± 1.14 | 2.32 ± 0.29       | 450 ± 120   | 4.44 ± 1.04             |
| Mock          | 4.09 ± 1.10 | 2.55 ± 0.24       | 711 ± 216   | 6.93 ± 0.92             |

**FIG. 5.** Dimerization and autophosphorylation of EGFR from mock- and GnT-III-transfected HeLaS3 cells. **A**, dimerization analysis of EGFR from mock- and GnT-III-transfected cells. Cells were treated with 50 ng/ml EGF for 2 h at 4 ºC and then incubated with 15 mM EDAC for 20 min at room temperature. Cells were harvested and subjected to 7% SDS-PAGE and transferred to a nitrocellulose membrane. EGFR were detected with anti-EGFR antibody. IP, immunoprecipitation; WB, Western blot. **B**, autophosphorylation of EGFR from mock- and GnT-III-transfected cells. Cells were stimulated with 50 ng/ml EGF and harvested at the indicated times. Whole cell lysates were immunoprecipitated with anti-EGFR. They were subjected to 10% SDS-PAGE and the blots were probed with anti-phosphotyrosine (α-pTyr, upper panel) and anti-EGFR antibody (lower panel) as described under “Experimental Procedures.”

**FIG. 6.** EGF-induced internalization of EGFR in mock- and GnT-III-transfected HeLaS3 cells. **A**, mock- and GnT-III-transfected HeLaS3 cells cultured in 12-well dishes were serum-starved for 24 h, followed by a incubation with M-BSA for 30 min at 37 ºC. Cells were subsequently incubated with ²²¹²EGF in M-BSA and after the indicated times (1.5, 3.0, 4.5, and 6.0 min), surface-bound ligand was extracted with acidic binding buffer and the internalized radioactivity was determined by alkaline lysis as described under “Experimental Procedures.” Data were expressed as the rate of internalized radioactivity divided surface-bound radioactivity. Data represent the averages ± S.E. of three independent experiments. **B**, mock- and GnT-III-transfected HeLaS3 cells were serum-starved and exposed to 200 ng/ml Oregon Green 514-conjugated EGF (Molecular Probes) at 4 ºC. Cells were then warmed for 10 min at 37 ºC, after which the uninternalized cell surface ligand was stripped by an acid wash, and these transfectants were fixed in 4% paraformaldehyde. The distribution of Oregon Green-labeled EGF was visualized by fluorescence microscopy (upper panels). Lower panels show the nuclei of these cells stained with 4',6-diaminido-2-phenylindole (scale bar, 10 μm).
constitution of the molecule such as charges or steric hindrance, which could, in turn, affect the receptor internalization rate.

It was reported that the progression of hepatic neoplasms is retarded in GnT-III/−/− mice (47, 48). They suggested that GnT-III plays an important role in tumor progression in liver. We offer evidence here that ERK phosphorylation is up-regulated in GnT-III transfectants, and it might account for the tumor progression activity of GnT-III. Further investigations are now under way with a goal of identifying and characterizing the molecules that are involved in changes of EGF signaling in GnT-III transfectants.

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FIG. 7. Effects of dansylcadaverine and hypertonic medium on EGFR internalization and ERK phosphorylation in mock- and GnT-III-transfected HeLaS3 cells. A, mock- and GnT-III-transfected HeLaS3 cells were serum-starved for 24 h, followed by a 30-min incubation with or without dansylcadaverine (500 μM) at 37 °C. B, cells were serum-starved for 24 h followed by a 60-min incubation with sucrose (0.45 M) at 37 °C. Upper panel of A and B, the internalization rate of EGFR was determined as described in the figure legend of Fig. 6A and under "Experimental Procedures." Lower panel of A and B, cells were incubated with 50 ng/ml EGF and harvested at the indicated times (0, 2, and 10 min). Each cell lysate sample was analyzed by Western blotting as described under "Experimental Procedures." pERK, phospho-ERK; WB, Western blot.

FIG. 8. EGF-induced internalization of EGFR in mock- and GnT-III-transfected CHO-HER cells. Internalization of EGFR in mock- and GnT-III-transfected CHO-HER cells was determined as described for Fig. 6A, and under "Experimental Procedures." Data represent the averages ± S.E. of three independent experiments.

FIG. 9. Ligand-induced internalization of transferrin receptor in mock- and GnT-III-transfected HeLaS3 cells. Internalization of transferrin receptor in mock- and GnT-III-transfected HeLaS3 cells was determined as described under "Experimental Procedures." Data were expressed as the rate of internalized radioactivity divided surface-bound radioactivity. Data represent the averages ± S.E. of three independent experiments.
