Tyrosine Kinase Activity of Epidermal Growth Factor Receptor Is Regulated by GM3 Binding through Carbohydrate to Carbohydrate Interactions*

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Epidermal growth factor receptor (EGFR), an N-glycosylated transmembrane protein with an intracellular kinase domain, undergoes dimerization by ligand binding resulting in activation of the kinase domain and phosphorylation. Ganglioside GM3 containing sialylactose inhibits the tyrosine kinase activity of EGFR through carbohydrate to carbohydrate interactions (CCI) between N-glycans with GlcNAc termini on EGFR and oligosaccharides on GM3. In this study, we provide further evidence for CCI between EGFR and GM3. (i) In vitro and in situ, the inhibitory effect of GM3 on EGFR tyrosine kinase was much higher in A431 cells upon exposure of the GlcNAc termini of the N-glycans to glycosidase treatment (neuraminidase and β-galactosidase) than in untreated A431 cells. Furthermore, the GM3-mediated inhibition was abrogated by co-incubation with N-glycan containing terminal GlcNAc. (ii) In situ, inhibition of EGFR phosphorylation by GM3 was not observed in α-mannosidase IB (ManIB)-knocked down A431 cells that accumulate high mannose-type N-glycans. (iii) EGFR binding to GM3 was enhanced in glycosidase-treated cells that accumulated GlcNAc termini, whereas GM3 did not bind to EGFR from ManIB-knocked down cells that accumulated high mannose-type N-glycans. These results indicate that GM3-mediated inhibition of EGFR phosphorylation is caused by interaction of GM3 with GlcNAc-terminated N-glycan on EGFR.

Epidermal growth factor receptor (EGFR) is a 170-kDa transmembrane glycoprotein with an intracellular kinase domain (1). EGFR is expressed in epithelial cells, especially in basement membrane of stratified epithelium and squamous epithelium, and it is overexpressed in solid cancers such as carcinoma cells. EGFR belongs to the erbB transmembrane receptor family, and plays a role in cell proliferation and differentiation of the epithelial surface. Ligand (EGF or α-cellulin) binding leads to dimerization of EGFR and tyrosine phosphorylation, which then activates intracellular signal transducers such as mitogen-activated protein kinase (MAPK), signal transducers and activators of transcription (STAT), Akt, etc. (2, 3).

The involvement of oligosaccharides on EGFR function has been widely studied. Twelve N-linked oligosaccharides (N-glycans) are attached to EGFR (1), and loss of these N-glycans reduced EGFR activation (4). Moreover, Taniguchi’s group (5–8) also reported that EGFR function was changed in an N-glycan structure-dependent manner. They demonstrated the biological significance of the core fucosylation of N-glycans on EGFR in intracellular signaling. Mice lacking fucosyltransferase 8 (Fut8−/− mice) have provided insight into the molecular mechanism of core fucosylation-regulated cell growth and cell differentiation. Studies using these mice demonstrated that a decrease in core fucose on EGFR caused a reduction in EGFR phosphorylation, which in turn reduced the affinity of EGF for EGFR (8). A biecting N-acetylgalactosamine (β-1,4-GlcNAc) modification of N-glycans also affects EGFR function (5). The addition of bisecting GlcNAc influences the structure of N-glycans by inhibiting transfer by N-acetylgalcosaminyltransferase IV and N-acetylgalcosaminyltransferase V (9). Bisecting GlcNAc-modified EGFR did not differ in its phosphorylation ratio, but caused an increase in extracellular signal-regulated kinase (ERK) phosphorylation due to the increase in EGFR internalization.

EGFR regulation by glycolipids was also studied. Cell growth delay of the human epidermoid carcinoma cell line A431 is caused by GM3 (NeuAcα3Galβ4Glcβ1Cer)-dependent inhibition of EGFR tyrosine kinase activity (10). GM3 is a ganglioside that binds to the extracellular domain of EGFR and inhibits its dimerization (11) without inhibiting ligand binding (10). Wang et al. (12) reported that GM3 mediated binding of EGFR with caveolin-1. However, the GM3 binding site on the EGFR extracellular domain and the structurally bound form of EGFR are unknown.

Hakomori (13–15) described the significance of carbohydrate-to-carbohydrate interactions on cell surface signaling and...
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cell adhesion. It is commonly known that glycosphingolipids at the cell surface membrane are associated or complexed with signal transducers (small G-proteins, cSrc, Src family kinases), tetraspanins, growth factor receptors, and integrins. Microdomains containing glycosphingolipids complexed with growth factor receptors modulate and regulate cell growth through inhibition or activation of signal transduction.

In addition to the above, interaction between GM3 and N-glycans with GlcNAc termini was recently reported (16). These data may be consistent with the hypothesis that GM3 interacts directly with glycans on EGFR to modulate EGFR activity on the cell surface. Therefore, we designed experiments to elucidate the relationship between carbohydrate to carbohydrate interactions and EGFR function. The N-glycans on EGFR were remodeled by glycosidase digestion or by knockdown of the gene controlling N-glycan synthesis by using antisense oligonucleotides to study GM3-mediated inhibition of ligand-induced EGFR phosphorylation. In this study, we report that regulation of EGFR phosphorylation by GM3 was dependent on the structure of the N-glycans attached to EGFR.

EXPERIMENTAL PROCEDURES

Cell Culture—Human ovarian epidermoid carcinoma A431 cells, which are known to overexpress EGFR in the membrane, were used in this study. A431 cells were cultured in DMEM (Sigma, number D5796) plus 10% heat-inactivated fetal bovine serum (Japan Bio Serum, number 82225), 100 μg/ml penicillin, 50 μg/ml streptomycin, and 110 μg/ml sodium pyruvate in 5% CO₂ at 37 °C.

Preparation of Membrane Fraction from A431 Cells—For preparation of membrane fractions, A431 cells were cultured, scraped, washed with lysis buffer (20 mM HEPES, 1 mM Na₂VO₄, 150 mM NaCl, 1 mM phenylmethylsulfonfyl fluoride, 1% aprotinin), and centrifuged at 5,800 × g for 5 min. Cell pellets were resuspended in lysis buffer and disrupted by a Dounce homogenizer (this step was repeated twice). The cell lysate was centrifuged at 1,750 × g for 5 min, and the supernatant was ultracentrifuged at 100,000 × g for 1 h at 4 °C. The resulting precipitate was collected as the membrane fraction. The membrane fraction was resuspended in an adequate amount of lysis buffer for determination of protein concentration or for use in further experiments.

Glycosidase (Neuraminidase F and β-Galactosidase) Digestion—A431 cell membrane fraction (150 μg of protein) was centrifuged at 1,000,000 × g for 10 min at 4 °C, and the precipitate was washed and resuspended in 0.25 M HEPES buffer (pH 6.5). Glycosidase treatment was performed as follows: the membrane fraction suspended in 100 μl of HEPES buffer (pH 6.5) was mixed with 50 μl of 50 mM CaCl₂, 100 μl of neuraminidase F (Seikagaku Kogyo Corp., number 120052, 100 millunits in 0.1% bovine serum albumin), and 100 μl of β-galactosidase (Seikagaku Kogyo Corp., number 100573, 10 millunits in 0.1% bovine serum albumin). The mixed solution was incubated for 1 h at 30 °C and subsequently centrifuged at 1,000,000 × g for 10 min at 4 °C. The reaction was terminated by aspirating the supernatant.

SDS-PAGE, Western Blot, and Lectin Blot—Membrane fraction was resuspended in 5× SDS-PAGE sample buffer, boiled for 5 min at 95 °C, resolved by SDS-PAGE (5–20% gradient gel), and transferred onto a polyvinylidene difluoride membrane (Millipore, Immobilon-P transfer membrane, number IPV/H00010). After blocking with 3% bovine serum albumin solution, a lectin blot was performed with GS-II-horseradish peroxidase (HRP) (EY Laboratories, number H-2402-2) to detect terminal GlcNAc, RCA120-HRP (Seikagaku Kogyo Co., number 300309) to detect terminal galactose, and ConA-HRP (Seikagaku Kogyo Co., number 300281) to detect high mannose-type oligosaccharides. A Western blot was performed with anti-phospho-EGFR (Tyr1068) antibody (Cell Signaling, number 2232) and anti-EGFR antibody (Santa Cruz, number sc-03), followed by HRP-conjugated anti-rabbit IgG (H+L) antibody. Detection was by enhanced chemiluminescence using SuperSignal West Dura (Pierce, number 34076).

In Vitro Inhibitory Effect of GM3 on EGF-induced EGFR Phosphorylation—An in vitro EGFR phosphorylation assay was performed as described by Zhou et al. (17) and Yoon et al. (18). Aliquots of solution containing 28 nmol (35 μg) of GM3 (Calbiochem number 345733) or 46 μg of GM1 (Seikagaku Co., number 400142) in chloroform:methanol (2:1) were dried under vacuum. Ethanol was added to these GM3 or GM1 samples that were dried again. Water (10 μl) was added to the completely dried GM3 or GM1 samples followed by sonication for 10 min. GlcNAc terminal penta-antennary oligosaccharide (Os Fr. B, 14 or 28 nmol), which was a isolated N-linked oligosaccharide from ovalbumin as described by Yoon et al. (16), was added to the GM3 suspension, and preincubated for 16 h at 37 °C. The GM3, GM1, or a preincubated mixture of GM3 and penta-antennary GlcNAc terminal N-glycan Os Fr. B was added to 10 μl of membrane fraction (25 μg each) and incubated for 15 min at room temperature. EGF solution (5 μl of 20 g/ml, recombinant Human EGF, R&D Systems) was added to each tube and incubated for 45 min at room temperature. After adding 5 μl of 200 mM HEPES, pH 7.0, 5 μl of 10 mM MnCl₂, 5 μl of 100 μM ZnCl₂, and 5 μl of 300 μM Na₂VO₄, the reaction mixture was cooled on ice. Then, 5 μl of 50 μM ATP were added and incubated for 10 min on ice. SDS-PAGE sample buffer was added, and the sample was boiled for 3 min at 95 °C. Triton X-100 was added to a final concentration of 0.2% and samples were boiled again for 5 min at 95 °C. Each sample was resolved by SDS-PAGE (5–20 gradient gel) followed by Western blot using anti-EGFR and anti-phospho-EGFR (Tyr1068) antibodies. The Western blot was visualized by fluorescence and quantified by densitometry using LAS3000mini (Fujifilm).

In Situ GM3-mediated Inhibition of EGFR Phosphorylation—An in situ EGFR phosphorylation assay was performed as described by Zhou et al. (17) and Yoon et al. (18). Aliquots of solution containing 125 or 250 μg GM3, or 250 μg GM1 in chloroform:methanol were dried under vacuum. Ethanol was added and the sample was dried again. Serum-free DMEM (200 μl) was added to the completely dried GM3 or GM1 and sonicated for 10 min. A total of 250 μM penta-antennary GlcNAc terminal N-glycan Os Fr. B were added to the GM3 suspension, and preincubated for 16 h at 37 °C. Separately, A431 cells cultured in 24-well plates in DMEM containing 10% fetal bovine serum were starved in serum-free DMEM for 24 h prior to confluence, were washed with 0.25 M HEPES buffer (pH 6.5),
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Exposing Terminal GlcNAc on N-Glycans of EGFR by Glycosidase Treatment—To preferentially expose GlcNAc termini on N-glycans of EGFR, the A431 cell membrane fraction was digested with glycosidase (neuraminidase F and β-galactosidase) and analyzed by SDS-PAGE followed by Western blotting with anti-EGFR antibody, or by lectin blotting with HRP-labeled Griffonia simplicifolia II lectin (GS-II) to detect GlcNAc termini or HRP labeled-caster bean lectin (RCA120) to detect galactose termini. Undigested EGFR from control cells gave a strong RCA120 signal, whereas that from glycosidase-treated cells was strongly reactive (Fig. 1A). This suggested that terminal galactose residues were cleaved from the N-glycan on EGFR by the glycosidase treatment. On the other hand, EGFR from cells not treated with glycosidase was weakly reactive with the GS-II lectin, whereas EGFR from glycosidase-treated cells was strongly reactive (Fig. 1B). This result suggests that GlcNAc termini were more abundant on EGFR glycan from glycosidase-treated cells.

Modulation of GM3-mediated Inhibition of EGFR Phosphorylation by Glycosidase Treatment of A431 Membrane Fraction

and treated with neuraminidase F and β-galactosidase as described under “Glycosidase Digestion.” After incubation for 1 h at 37 °C, cells were washed with serum-free DMEM and incubated in serum-free DMEM containing GM3, GM1, or a preincubitated mixture of GM3 and penta-antennary GlcNAc terminal N-glycan Os Fr. B for 3 h at 37 °C. Cells were washed with phosphate-buffered saline, phosphorylation was induced by incubation with 200 μl of EGF (0.1 μg/ml), and cells were incubated for 1 h at 4 °C, washed, and lysed in 30 ml of lysis buffer (1% SDS, 5 mM EDTA, 5 mM EGTA, 1 mM Na3VO4) for 30 min at 4 °C. The cell lysate was subjected to SDS-PAGE followed by Western blotting using anti-EGFR and anti-phospho-EGFR (Tyr1068) antibodies.

Knockdown of Gene Encoding α-Mannosidase IB with Antisense Oligonucleotides—To enrich for high-mannose type N-glycans in A431 cells, the α-mannosidase IB gene was knocked down using the antisense oligonucleotide 5′-TGG GGT AGT CAT CGT TTA CGC-3′. A431 cells (30% confluency) were treated with 200 nM antisense oligonucleotide and Oligofectamine (Invitrogen, number 12252-011) in serum-free DMEM for 6 h. After incubation for 1 h at 4 °C, washed, and lysed in 30 ml of lysis buffer (1% SDS, 5 mM EDTA, 5 mM EGTA, 1 mM Na3VO4) for 30 min at 4 °C. The cell lysate was subjected to SDS-PAGE followed by Western blotting using anti-EGFR and anti-phospho-EGFR (Tyr1068) antibodies.

EGFR Purification—EGFR was purified using anion exchange chromatography and gel filtration. The A431 cell membrane fraction solubilized in 1% Triton X-100 for 1 h at 4 °C was applied to the Mono Q 5/50 GL column (GE Healthcare, number 17-5166-01) equilibrated with 20 mM Tris-HCl (pH 8.0), 0.1% CHAPS. When absorbance at 280 nm was at a baseline level, the ratio of 20 mM Tris-HCl (pH 8.0), 2.0 M NaCl, 0.1% CHAPS was increased linearly up to 50% for 30 min at a flow rate of 1 ml/min. Each 1-ml fraction was resolved by SDS-PAGE and analyzed by silver staining and Western blotting.

Analysis of N-Glycan Structures of EGFR—Dried EGFR samples purified as described above were hydrolyzed using hydrazine (Hydracurl Hydrazin Degradation Reagent C, Honen Co.) at 100 °C for 2 h. After hydrazinolysis, oligosaccharides were purified on a cellulose column (Takara Shuzo Co., Ltd.), and subjected to pyridylamination (PA) using a pyridylamination kit (Takara Shuzo Co., Ltd.). PA-labeled samples were further purified by using an anion exchange column. PA-labeled oligosaccharides were analyzed by anion exchange chromatography, size fractionation, and reverse phase HPLC. Anion exchange HPLC was performed using Asahipak NH2P-50 (0.46 × 25 cm) (Showa Denko Co., Tokyo) at a rate of 1.0 ml/min with solvent A (acetonitrile, 50 mM acetic acid/triethylamine, pH 7.2, 60:40) and solvent B (acetonitrile, 1 mM acetic acid/triethylamine, pH 7.2, 50:50). After sample injection onto a column equilibrated with solvent A, the proportion of B was increased linearly up to 55% for 110 min. Size fractionation by HPLC was performed using TSKgel Amide-80 (0.46 × 25 cm) (TOSOH Co., Tokyo) at a rate of 1 ml/min with solvent C (acetonitrile, 200 mM acetic acid/triethylamine, pH 7.2, 65:35) and solvent D (acetonitrile, 200 mM acetic acid/triethylamine, pH 7.2, 50:50) (19). The solvent D gradient was increased linearly up to 100% for 50 min. Reverse phase HPLC was performed using Cosmosil 5C18-P (0.46 × 15 cm) (Nakarai Tesque) at a rate of 1.0 ml/min with solvent E (20 mM acetic acid/NH3, pH 4.0) and solvent F (20 mM acetic acid/NH3, pH 4.0, 1% butanol) (20). The column initially was equilibrated with solvent E/solvent F (90:10). After sample injection, the proportion of E was increased linearly up to 25% for 60 min. The individually PA-labeled oligosaccharides were identified by comparison with standards.

Interaction of GM3-coated Polystyrene Beads with EGFR—Experimental methods were described previously by Yoon et al. (18). A431 cell membrane fractions (30 μg of protein) were solubilized in 1% SDS by tumbling for 1 h at 4 °C, and centrifuged at 26,800 × g for 20 min. The supernatant was then used for the assay. Dried GM3 (16 nmol) was dissolved in 1 ml of ethanol/water solution (9:1). Polystyrene beads (6.85 × 107, Polystyrene Uniform Latex particles, 1-μm diameter, Sigma number 461733) were washed with phosphate-buffered saline several times and then washed with the ethanol solution. Subsequently, GM3 solution was mixed with the polystyrene beads and incubated with tumbling for 1 h at room temperature. Polystyrene beads were washed three times with 0.5 ml of phosphate-buffered saline, incubated with 300 μl of 0.1% gelatin for 1 h with tumbling at room temperature, and centrifuged at 1,500 × g for 5 min. After washing with phosphate-buffered saline, the GM3-coated beads were resuspended in 500 μl of Tris-buffered saline containing the membrane fraction, incubated overnight at 4 °C with tumbling, and then washed with Tris-buffered saline. The GM3-coated and bead-bound fraction was solubilized by boiling in SDS-PAGE sample buffer and analyzed by Western blotting with anti-EGFR antibody. Control experiments using GM1-coated beads were carried out as described above.

RESULTS

Exposing Terminal GlcNAc on N-Glycans of EGFR by Glycosidase Treatment—To preferentially expose GlcNAc termini on N-glycans of EGFR, the A431 cell membrane fraction was digested with glycosidase (neuraminidase F and β-galactosidase) and analyzed by SDS-PAGE followed by Western blotting with anti-EGFR antibody, or by lectin blotting with HRP-labeled Griffonia simplicifolia II lectin (GS-II) to detect GlcNAc termini or HRP labeled-caster bean lectin (RCA120) to detect galactose termini. Undigested EGFR from control cells gave a strong RCA120 signal, whereas that from glycosidase-treated cells was not reactive with RCA120 (Fig. 1A). This suggested that terminal galactose residues were cleaved from the N-glycan on EGFR by the glycosidase treatment. On the other hand, EGFR from cells not treated with glycosidase was weakly reactive with the GS-II lectin, whereas EGFR from glycosidase-treated cells was strongly reactive (Fig. 1B). This result suggests that GlcNAc termini were more abundant on EGFR glycan from glycosidase-treated cells.

Modulation of GM3-mediated Inhibition of EGFR Phosphorylation by Glycosidase Treatment of A431 Membrane Fraction
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**FIGURE 1.** Lectin blot of EGFR from glycosidase-treated cells. A membrane fraction from A431 cells (control) was prepared, and digested with glycosidases (neuraminidase F and β-galactosidase) to expose GlcNAc termini of N-linked glycans on EGFR as described under “Experimental Procedures.” Membrane fractions from control A431 cells and glycosidase-treated A431 cells were immunoblotted using anti-EGFR to detect total EGFR, and a lectin blot using GS-II-HRP (to detect GlcNAc termini of N-glycans) and RCA120-HRP (to detect galactose termini of N-glycans) as described under “Experimental Procedures.” A, expression of galactose termini of N glycans on EGFR; EGFR was detected by anti-EGFR antibody (upper panel). Galactose termini of N-linked glycans were detected by RCA120 lectin (lower panel). B, expression of GlcNAc termini of N-glycans on EGFR. EGFR was detected using anti-EGFR (upper panel). GlcNAc termini of N-linked glycans were detected by GS-II lectin (lower panel). Control, membrane fraction from control A431 cells; glycosidase-treated, membrane fraction from glycosidase-treated A431 cells.

**FIGURE 2.** In vitro effect of glycosidase treatment on GM3-mediated inhibition of EGFR phosphorylation. The inhibitory effect of GM3 on EGFR phosphorylation and its abrogation by penta-antennary GlcNAc terminal N-glycan Os Fr. B using a membrane fraction from glycosidase-treated and untreated A431 cells was analyzed as described under “Experimental Procedures.” Phosphorylation was analyzed by Western blot with anti-phospho-EGFR (Y1068) and anti-EGFR. Band densities were quantified and the amount of phosphorylated EGFR was calculated as the density using anti-PY1068 divided by the density using anti-EGFR and is shown as the percentage of the control (without GM3) in the lower panel (B). Control, A431 cell membrane fraction from glycosidase-untreated cells (lanes 1–6); glycosidase-treated, A431 membrane fraction from glycosidase-treated cells (lanes 7–12); lanes 1 and 7, none; lanes 2 and 8, 100 ng of EGF; lanes 3 and 9, 100 ng of EGF, 28 nmol of GM3; lanes 4 and 10, 100 ng of EGF, 28 nmol of GM3, 14 nmol of Os Fr. B; lanes 5 and 11, 100 ng of EGF, 28 nmol of GM3, 28 nmol of Os Fr. B; lanes 6 and 12, 100 ng of EGF, 28 nmol of GM1. Lanes 4, 5, 10, and 11, GM3 was preincubated with Os Fr. B at 37 °C for 16 h before adding to membrane fractions followed by EGF-induced phosphorylation. C, structure of Os Fr. B.

in Vitro or A431 Cells in Situ—Using glycosidase-treated or untreated A431 membrane fraction, GM3-mediated inhibition of EGFR phosphorylation in vitro was monitored by Western blotting with anti-phospho-EGFR (Y1068) antibody and anti-EGFR antibody in the presence or absence of EGF, GM3, GM1 or GlcNAc terminal penta-antennary N-glycan (Os Fr. B, Fig. 2C) (16). The density ratio of anti-PY1068 bands (phosphorylated EGFR, Fig. 2A, upper) to anti-EGFR (total EGFR, Fig. 2A, lower) is shown in the bar graph (Fig. 2B). EGF-induced EGFR phosphorylation in the glycosidase-treated membrane fraction was significantly reduced (to about 30% of control level) by addition of 28 nmol of GM3 (Fig. 2, lane 9), whereas EGFR phosphorylation in the untreated membrane fraction was about 60% of the control (without GM3) under the same conditions (100 ng of EGF and 28 nmol of GM3) (Fig. 2, lane 3). Because N-glycan with terminal GlcNAc and GM3 bind strongly (16), the increase in terminal GlcNAc on EGFR from glycosidase-treated membrane fractions may have increased binding to GM3.

An abrogating effect of penta-antennary GlcNAc terminal N-glycan Os Fr. B on GM3-dependent inhibition of phosphorylated EGFR was observed in vitro. The inhibitory effect of GM3 on EGFR phosphorylation (Fig. 2, lanes 3 and 8) was reduced by incubation with penta-antennary GlcNAc terminal N-glycan Os Fr. B (14 nmol) for 16 h (Fig. 2, lanes 4 and 10), and further reduced by 28 nmol of Os Fr. B (Fig. 2, lanes 5 and 11) in the presence of the same amounts of EGF (100 ng) and GM3 (28 nmol). These results indicate that GM3 binding to Os Fr. B with GlcNAc termini reduced the inhibitory effect of GM3 on EGFR phosphorylation and suggest that GM3 binds to GlcNAc termini in EGFR.

A similar effect on GM3-dependent inhibition of phosphorylated EGFR was observed in cultured A431 cells treated with glycosidases (neuraminidase F and β-galactosidase) in situ. Cells in culture were treated with glycosidases, incubated with GM3 or a mixture of GM3 and penta-antennary GlcNAc terminal N-glycan Os Fr. B. EGF phosphorylation was induced by addition of 20 ng of EGF, and cell extracts were subjected to SDS-PAGE and Western blotting. GM3-mediated inhibition of EGFR phosphorylation in glycosidase-treated and untreated A431 cells were then determined. EGFR phosphorylation was significantly reduced (to about 40% of the control) by addition of 250 μM GM3 in glycosidase-treated A431 cells (Fig.

**A**

**B**

**C**

GlcNAcB6

GalB4GlcNAcB4GlcNAcB4

ManB4GlcNAcB4GlcNAcB4

**A431 membrane fraction from glycosidase-treated or untreated A431 cells in situ—Using glycosidase-treated or untreated A431 membrane fraction, GM3-mediated inhibition of EGFR phosphorylation in vitro was monitored by Western blotting with anti-phospho-EGFR (Y1068) antibody and anti-EGFR antibody in the presence or absence of EGF, GM3, GM1 or GlcNAc terminal penta-antennary N-glycan (Os Fr. B, Fig. 2C) (16). The density ratio of anti-PY1068 bands (phosphorylated EGFR, Fig. 2A, upper) to anti-EGFR (total EGFR, Fig. 2A, lower) is shown in the bar graph (Fig. 2B). EGF-induced EGFR phosphorylation in the glycosidase-treated membrane fraction was significantly reduced (to about 30% of control level) by addition of 28 nmol of GM3 (Fig. 2, lane 9), whereas EGFR phosphorylation in the untreated membrane fraction was about 60% of the control (without GM3) under the same conditions (100 ng of EGF and 28 nmol of GM3) (Fig. 2, lane 3). Because N-glycan with terminal GlcNAc and GM3 bind strongly (16), the increase in terminal GlcNAc on EGFR from glycosidase-treated membrane fractions may have increased binding to GM3.

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3, lane 11), whereas EGFR phosphorylation in untreated cells was about 60% of the control (without GM3) under the same conditions (20 ng of EGF and 250 μM of GM3) (Fig. 3, lane 4).

An abrogating effect of penta-antennary GlcNAc terminal N-glycan Os Fr. B on GM3-dependent inhibition of phospho-

FIGURE 3. In situ GM3-mediated inhibition of EGFR phosphorylation in glycosidase-treated A431 cells. A431 cells were treated with glycosidase (neuraminidase F and β-galactosidase), and GM3-mediated inhibition and the abrogating effect of penta-antennary GlcNAc terminal N-glycan Os Fr. B on the inhibition were assayed as described under “Experimental Procedures.” Phosphorylation was analyzed as described in the legend to Fig. 2. Control, glycosidase-untreated A431 cells (lanes 1–7); glycosidase-treated, glycosidase-treated A431 cells (lanes 8–14); lanes 1 and 8, none; lanes 2 and 9, 20 ng of EGF; lanes 3 and 10, 20 ng of EGF, 125 μM GM3; lanes 4 and 11, 20 ng of EGF, 250 μM GM3; lanes 5 and 12, 20 ng of EGF, 125 μM GM3, 280 μM Os Fr. B with no preincubation; lanes 6 and 13, 20 ng of EGF, 125 μM GM3, 280 μM Os Fr. B with a 16-h preincubation; lanes 7 and 14, 20 ng of EGF, 250 μM GM3. Preincubations were performed by mixing GM3 with Os Fr. B followed by 16 h at 37 °C before addition to A431 cells.

FIGURE 4. Lectin blot of EGFR from ManIB-knocked down cells. The gene encoding ManIB was knocked down by treating cells with antisense oligonucleotide to cause accumulation of high mannose-type N-linked glycans on EGFR, and the membrane fraction was prepared as described under “Experimental Procedures.” Membrane fractions from control cells and ManIB-knocked down cells were subjected to Western analysis using anti-EGFR antibody, and to lectin analysis using ConA-HRP as described under “Experimental Procedures.” EGFR was detected by anti-EGFR antibody (upper panel), and high mannose-type N-glycans were detected by ConA (lower panel). Control, A431 membrane fraction from normal A431 cells; ManIB KD, membrane fraction from ManIB-knocked down A431 cells.

rylated EGFR was also observed in situ. The inhibitory effect of GM3 (125 μM) on EGF (20 ng)-induced EGFR phosphorylation was restored to almost 100% of the control level by preincubation of GM3 (125 μM) and penta-antennary GlcNAc terminal N-glycan Os Fr. B (280 μM) for 16 h (Fig. 3, lanes 6 and 13).

Accumulation of High Mannose-type N-Glycans on EGFR—To cause preferential accumulation of high mannose-type N-glycan on EGFR, ManIB encoding α-mannosidase IB (21) was knocked down by antisense treatment. The membrane fraction prepared from ManIB-knockdown (KD) cells was subjected to a lectin blot using mannos-reactive HRP-concanavalin A (ConA). In this blot, dense EGFR bands were observed for EGFR from ManIB KD cells relative to control cells (Fig. 4, lower panel) based on loading equal amounts of EGFR (Fig. 4, upper panel). This indicates that EGFR from ManIB KD cells is enriched for high mannose-type N-glycan.

GM3-mediated Inhibition of EGFR Phosphorylation in the Membrane Fraction from ManIB KD Cells—EGF-induced EGFR phosphorylation and its regulation by GM3 were analyzed using the membrane fraction prepared from control A431 cells or ManIB KD A431 cells by immunoblotting using anti-phospho-EGFR (Tyr1068) antibody and anti-EGFR antibody. EGFR phosphorylation in the presence of GM3 (28 nmol) in the membrane fraction from control A431 cells was about 60% of the control (100 ng of EGF but without GM3) (Fig. 5, lane 3). However, EGF-induced EGFR phosphorylation in the membrane fraction from ManIB KD A431 cells was not inhibited by GM3 (28 nmol) (Fig. 5, lane 6). High mannose-type N-glycan does not bind to GM3 (16), and thus, inhibition of EGFR phosphorylation by GM3 was poor in the membrane fraction from ManIB KD A431 cells likely due to the increased high mannose-type N-glycan content.

N-Glycan Structure Analysis of EGFR—To interpret the relationship between the glycan structures of EGFR and GM3, N-glycan structural analysis was performed. EGFR from control A431 cells, glycosidase-treated A431 cells, and ManIB KD A431 cells were purified using MonoQ and Superdex 200 column chromatography as described under “Experimental Procedures.” Purified EGFR confirmed by silver staining (Fig. 6) was hydrazinolyzed, and the released N-glycans were labeled with 2-aminopyridine and analyzed by HPLC. Only monosialo complex-type N-glycans were detected as charged sugar chains in EGFR. The amount of monosialo complex-type N-glycans was reduced to 11.3% in glycosidase-treated A431 cells and to 52.4% in ManIB KD A431 cells, relative to 78.6% in control A431 cells (Table 1). Neutral oligosaccharides on EGFR from all
samples were analyzed further. The GlcNAc terminal N-glycan content was found to have increased in glycosidase-treated cells from 4.27 to 62.7%, indicating that monosialo complex-type N-glycans were digested by neuraminidase F and β-galactosidase. The high mannose-type N-glycan content increased in ManIB KD cells from 16.7 to 40.2% (Table 1, Fig. 7). These results are consistent with those of the ConA blot (Fig. 4).

**Interaction of EGFR with GM3-coated Polystyrene Beads—**
To evaluate the interaction of GM3 with N-glycan on EGFR, polystyrene beads were coated with GM3, incubated with EGFRs, and analyzed by immunoblotting with anti-EGFR (18). Comparisons were made between the membrane fraction from control, glycosidase-treated, and ManIB KD A431 cells. EGFR from the membrane fraction of glycosidase-treated A431 cells bound the GM3-coated beads strongly (Fig. 8A, treated with glycosidases). However, EGFR from the membrane fraction of control A431 and ManIB KD A431 cells exhibited weak (Fig. 8A, Control) or no binding (Fig. 8A, ManIB KD) to GM3, respectively. The lack of binding between GM3 and EGFR from the membrane fraction of ManIB KD was most likely caused by increased levels of high mannose-type N-glycans. However, we attribute the strong binding between GM3 and EGFR in glycosidase-treated cells to the increased content of GlcNAc terminated N-glycans. These results are consistent with GM3 inhibiting EGFR phosphorylation.

**DISCUSSION**
In this paper, we show that regulation of EGFR kinase phosphorylation by GM3 is dependent on the binding of the GM3 carbohydrate to EGFR N-glycans. This finding complements the study of Yoon et al. (18) in which GM3 regulation of EGFR was reported to be inhibited by addition of purified GlcNAc-terminated N-glycans. Our approach differed in that we altered the type of the N-glycans on EGFR by treatment with two glycosidases and by knocking down ManIB. Thus, we remodeled the N-glycan to enrich for EGFR with either a high affinity or no
affinity for GM3. Phosphorylation of EGFR from the glycosidase-treated cell membrane was strongly regulated by the addition of GM3 in vitro and in situ (Figs. 2 and 3). In contrast, EGFR from the ManIB-knocked down cell membrane that enriched for high mannose-type N-glycans was found to have no affinity for GM3 (16), and consequently, regulation of EGFR phosphorylation was nearly absent (Fig. 5). These results strongly suggest that GM3 binds to N-glycans on EGFR.

The results of the competition assay using Os Fr. B with GlcNAc termini also supported GM3 directly binding N-glycans on EGFR. Carbohydrate to carbohydrate interactions are weak compared with protein to carbohydrate interactions. If GM3 bound to an EGFR peptide, we speculate that a large amount of penta-antennary GlcNAc terminal N-glycan Os Fr. B would be necessary to inhibit binding of GM3 to EGFR because Os Fr. B and GM3 binding involves carbohydrate to carbohydrate interactions. However, equal or similar amounts of Os Fr. B were sufficient to compete with binding of GM3 to EGFR (Fig. 2, lanes 4, 5, 10, and 11 and Fig. 3, lanes 5, 6, 12, and 13). These results also strongly suggest that GM3 binds to N-glycans on EGFR.

N-Glycan structure analysis indicated that A431 EGFR normally contains a variety of sugar chains (Table 1, Fig. 7). The monosialylated complex type was the major structure, however, high mannose and GlcNAc terminal complex types were also present (Table 1). Thus, GM3 can bind to a subset of EGFR glycoforms from A431 under normal conditions to regulate phosphorylation.

In this study, we remodeled the N-glycan structure on EGFR by treating with two glycosidases or with an antisense oligonucleotide specific for ManIB and then examined GM3-mediated inhibition of EGFR phosphorylation in each case. When N-glycan structures with terminal GlcNAc increased (to about 15.7 times that of control cells) in glycosidase-treated cell membrane (Table 1), GM3-mediated inhibition of EGFR phosphorylation was significantly enhanced (Figs. 2 and 3). This suggests that complex-type N-glycans were mostly digested by treatment with neuraminidase F and β-galactosidase, and the increased amount of GlcNAc termini enhanced GM3-mediated inhibition of EGFR phosphorylation. Because N-glycans with terminal GlcNAc were found to strongly bind to GM3, glycosidase-treated EGFR may also strongly bind GM3. This could explain why phosphorylation of glycosidase-treated EGFR was strongly inhibited by GM3. In contrast, ManIB KD EGFR had an increased amount of high mannose-type N-glycan, ranging from 16.7 to 40.2% (Table 1). Because the high mannose-type sugar chain has no binding affinity for GM3, we presume that EGFR from the ManIB KD cells was not regulated by GM3.

Direct evidence for GM3 and EGFR binding was observed in the experiment using GM3-coated beads (Fig. 8). EGFRs from control, glycosidase-treated, and ManIB KD membrane frac-
EGFR was incubated with GM3-coated polystyrene beads, and the binding of EGFR to GM3-coated beads was subsequently described under “Experimental Procedures.” GM3-coated polystyrene beads and each membrane fraction solubilized in 1% SDS were incubated for 1 h at room temperature. After removal of the supernatant by centrifugation, EGFR bound to GM3-coated polystyrene beads was subjected to SDS-PAGE and Western blot analysis using anti-EGFR. A negative control reaction of A431 membrane fraction and polystyrene beads not coated with ganglioside; control, reaction of the A431 membrane fraction and GM3-coated polystyrene beads; glycosidase-treated, reaction of ganglioside (neuraminidase F and β-galactosidase)-treated membrane fraction and GM3-coated polystyrene beads or GM1-coated polystyrene beads; ManIB KD, reaction of membrane fraction from ManIB-knocked down A431 cells and GM3-coated polystyrene beads or GM1-coated polystyrene beads. Western blotting results of 30 μg of protein from membrane fractions of A431 and ManIB-knocked down A431 cells, and glycosidase-treated A431 membrane fraction detected by anti-EGFR antibody. The molecular weight of EGFR from the glycosidase-treated membrane fraction was slightly less than normal EGFR from A431 due to removal of the sialic acid and galactose residues.

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