Suppression of Heregulin β Signaling by the Single N-Glycan Deletion Mutant of Soluble ErbB3 Protein*

Motoko Takahashi‡1, Yoshihiro Hasegawa‡, Yoshitaka Ikeda‡, Yoshinao Wada‡, Michiko Tajiri§, Shigeru Ariki‡, Rina Takamiya†, Chiaki Nishitani‡, Motoko Araki‡, Yoshiaki Yamaguchi‡, Naoyuki Taniguchi‡, and Yoshio Kuroki‡

From the †Department of Biochemistry, Sapporo Medical University School of Medicine, Sapporo 060-8556, Japan; ‡Division of Molecular Cell Biology, Department of Biomolecular Sciences, Faculty of Medicine, Saga University, Saga 849-8501, Japan; §Department of Molecular Medicine, Osaka Medical Center and Research Institute for Maternal and Child Health, Osaka 594-1101, Japan, and †Systems Glycobiology Research Group, RIKEN-Max Planck Joint Research Center, Global Research Cluster, RIKEN, Wako 351-0198, Japan

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Background: Extracellular domain of ErbBs (sErbBs) down-regulates growth factor signaling.

Results: sErbB3 acts on ErbB3-containing heterodimers to suppress heregulin signaling, and the effects are enhanced by single N-glycan deletion.

Conclusion: N-Glycan on Asn-418 controls the ability of sErbB3 to suppress heregulin signaling.

Significance: Provides new insights toward understanding the mechanisms by which N-glycan regulates ErbB receptors.

Heregulin signaling is involved in various tumor proliferations and invasions; thus, receptors of heregulin are targets for the cancer therapy. In this study we examined the suppressing effects of extracellular domains of ErbB2, ErbB3, and ErbB4 (soluble ErbB (sErbB)) on heregulin β signaling in human breast cancer cell line MCF7. It was found that sErbB3 suppresses ligand-induced activation of ErbB receptors, PI3K/Akt and Ras/Erk pathways most effectively; sErbB2 scarcely suppresses ligand-induced signaling, and sErbB4 suppresses receptor activation at ~10% efficiency of sErbB3. It was revealed that sErbB3 does not decrease the effective ligands but decreases the effective receptors. By using small interfering RNA (siRNA) for ErbB receptors, we determined that sErbB3 suppresses the hergulin signaling by interfering ErbB3-containing heterodimers including ErbB2/ErbB3. By introducing the mutation of N418Q to sErbB3, the signaling-inhibitory effects were increased by 2–3-fold. Moreover, the sErbB3 N418Q mutant enhanced anticancer effects of lapatinib more effectively than the wild type. We also determined the structures of N-glycan on Asn-418. Results suggested that the N-glycan-deleted mutant of sErbB3 suppresses heregulin signaling via ErbB3-containing heterodimers more effectively than the wild type. Thus, we demonstrated that the sErbB3 N418Q mutant is a potent inhibitor for heregulin β signaling.

The ErbB family consists of four members, ErbB1 (EGFR),2 ErbB2, ErbB3, and ErbB4. They are type I transmembrane glycoproteins comprising a ligand binding extracellular domain, a transmembrane domain, an intracellular tyrosine kinase domain, and a C-terminal regulatory region. Without ligand stimulation, ErbB receptors exit as a “tethered form” in which molecules are folded in such a way as to prevent dimerization. By binding to a ligand, conformational rearrangement occurs that gives rise to a “extended form” in which the dimerization arm projecting from domain II mediates homodimers and heterodimers and is followed by the activation of downstream signaling such as Ras/Erk pathway or PI3K/Akt pathway (1–4). The signals are involved in a wide variety of cellular events such as proliferation, differentiation, migration, and adhesion. Aberrant expression or dysregulation of these receptors has been implicated in cell transformation and cancer (5).

Several reagents targeting ErbB signaling are developed for the treatment of cancer. For example, monoclonal antibodies against EGFR (e.g. cetuximab) (6), ErbB2 (e.g. trastuzumab) (7, 8), or tyrosine kinase inhibitors (e.g. gefitinib, erlotinib, and lapatinib) (9–11) are approved and clinically used for cancer therapy. A possible alternate to those reagents are soluble ErbB (sErbB), truncated extracellular domains of the ErbB receptor; herstatin, a naturally occurring ectodomain of ErbB2 that consists of the first 340 amino acids of the ErbB2 extracellular domain followed by a novel C terminus derived from exon 8 of the ErbB2 gene (12), inhibits EGFR and ErbB3 activation (13). A splice variant ErbB3 (p85-sErbB3) has also been reported to inhibit heregulin-stimulated activation of ErbB2 receptors and downstream signaling (14). Lindzen et al. (15) designed a fusion protein comprising truncated extracellular domains of EGFR and ErbB4 (TRAP-Fc) and demonstrated its anti-cancer function. Herstatin was reported to bind to the full-length ErbB2 to inhibit the activation of ErbB2-containing heterodimers; however, p85-sErbB3 and TRAP-Fc are suggested to bind to ligands as decoy receptors to suppress downstream signaling.

The functional regulation of ErbB receptors by N-glycan has been reported (16–24). EGFR, ErbB2, ErbB3, and ErbB4 contain 11, 8, 10, and 11 potential glycosylation sites in their extracellular domains, respectively. In a previous study N-glycan on Asn-420 of EGFR was reported to play an important role in the suppression of ligand-independent spontaneous oligomerization-
tion (19). We also demonstrated that N-glycan on Asn-418 of ErbB3 is involved in ligand-induced ErbB2-ErbB3 heterodimer formation and downstream signaling (23). It is possible that N-glycan in domain III of ErbB is involved in the structural maintenance of extracellular domains (24).

In the present study we prepared the extracellular domain of ErbB2, ErbB3, and ErbB4 (sErbB), whose crystal structures have been previously described (25–28), and compared their suppressive effects on heregulin signaling. We developed the N418Q mutant of sErbB3 and found that the suppressive effects are significantly enhanced. Moreover, the mutation augmented synergistic effects on the anticancer drug lapatinib. The results indicate that N-glycan is involved in the regulation of physicochemical properties of ErbB3, and manipulation of N-glycan of sErbB may be a useful strategy to develop a novel therapy of cancer.

EXPERIMENTAL PROCEDURES

Materials—Human recombinant heregulin β EGF domain was purchased from Millipore (Billerica, MA). Antibodies to specific phosphorylated proteins and polyclonal antibodies to EGFR, Akt, and Erk were purchased form Cell Signaling Technology (Beverly, MA). The monoclonal antibody to ErbB2 was purchased from Leica Biosystems (Wetzlar, Germany). The monoclonal antibody to ErbB3 was from Thermoscientific (Waltham, MA). The polyclonal antibody to ErbB4 was from Abcam (Cambridge, UK). An antibody against synthesized peptide EQKLI SEEDLNHTGH was prepared by Transgenic Inc. (Kumamoto, Japan). The polyclonal antibody to the His tag was from MBL (Nagoya, Japan). Alexa Fluor 488 and Alexa Fluor 594-conjugated secondary antibodies were from Molecular Probes. Lapatinib was from Synkinase (Melbourne, Australia). All other chemicals and reagents were purchased from Wako Pure Chemicals (Osaka, Japan) unless otherwise noted.

Cell Culture—The Lec3.2.8.1 cell line was kindly provided by Dr. Pamela Stanley (Department of Cell Biology, Albert Einstein College of Medicine) and maintained in a Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% (v/v) fetal bovine serum (FBS) (29, 30). The MCF7 cells were obtained from RIKEN (Wako, Japan) and maintained in the same medium. The Flp-In CHO cell line was obtained from Invitrogen and maintained in Ham's F-12 medium (Sigma) with 10% (v/v) FBS.

Establishment of sErbBs and ErbB Receptor Stable Expressing Cells—cDNA for human ErbB2 and ErbB4 (JMa/CYT1) were kindly provided by Dr. Tadashi Yamamoto (The University of Tokyo) and Dr. Axel Ulrich (Max Planck Institute of Biochemistry), respectively. sErbB2 (residues 1–622 of the mature protein), sErbB3 (residues 1–620 of the mature protein), sErbB4 (residues 1–625 of the mature protein), or non-tagged ErbB receptors (ErbB2, ErbB3, and ErbB4) were subcloned in a pcDNA5/FRT expression vector. N418Q-mutated sErbB3 was generated by the QuikChange site-directed mutagenesis kit (Stratagene). To establish sErbBs and ErbB receptor stable expressing cells, the Flp-In system (Invitrogen) was used; for host cell lines, Lec3.2.8.1 cells transfected with pFRT/lacZeo2 or Flp-In CHO cells (Invitrogen) were used. cDNA encoding sErbBs or ErbB receptors were transfected into host cells with pOG44 plasmids using Lipofectamine 2000 (Invitrogen). The stable expressing cells were selected with 600 μg/ml hygromycin B (Calbiochem).

Purification of Recombinant sErbBs—sErbBs stable expressing cells were cultured for 10 days, and the medium were collected. After filtration through a 0.45-mm membrane filter (Millipore), the expressed myc-His tagged sErbBs were purified by a series of column chromatographies on HisTrap HP5 (GE Healthcare), Mono Q (GE healthcare), an anti-myc-His peptide antibody column, and HiLoad Superdex 200 pg (GE Healthcare) using the AKTA purifier system (GE Healthcare). In detail, the filtrated medium was applied onto a HiTrap HP5 column equilibrated with 100 mM HEPS, pH 7.4, 0.5 mM NaCl, 20 mM imidazole, and the protein was eluted by a gradient of imidazole up to 500 mM. Fractions containing sErbBs were then applied onto a Mono Q column equilibrated with 20 mM Tris-HCl, pH 8.0, and the protein was eluted by the gradient of NaCl up to 500 mM. The anti-myc-His peptide antibody column was prepared using a rabbit antibody against synthesized peptide EQKLI SEEDLNHTGH and the Protein G column (GE Healthcare) and cross-linker dimethyl pimelimidate, and the protein was eluted by 0.1 M glycine-NaOH, pH 10.0. Finally, fractions containing sErbBs were applied onto a HiLoad Superdex 200 pg column equilibrated in a phosphate-buffered saline (PBS) and purified to homogeneity. Purity of proteins was confirmed by SDS-PAGE.

Protein Sample Preparation and Western Blotting—Cells were rinsed twice with ice-cold 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) glycerol, 5 mM sodium pyrophosphate, 10 mM NaF, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate, 1 mM PMSF, 2 μg/ml aprotinin, 5 μg/ml leupeptin, and 1 mM dithiothreitol), and centrifuged at 15,000 × g for 10 min at 4 °C, and the supernatant was used as a protein sample. The protein concentrations were determined using a Bio-Rad Protein Assay kit (Bio-Rad). The samples were separated by 10% SDS-PAGE and transferred to PVDF membranes (Millipore). After blocking, the blots were probed with an indicated antibody and then incubated with an HRP-conjugated secondary antibody, and immunoreactive bands were visualized using a chemiluminescence reagent (SuperSignal West Pico; Pierce). Densitometric analysis was performed by using a Luminous analyzer.

Small Interfering RNA (siRNA) Transfection—siRNA of EGFR, ErbB2, ErbB3, and ErbB4 were obtained from Cell Signaling Technology, and cells were transfected with the siRNA using the Lipofectamine RNAiMAX reagent (Invitrogen) following the manufacturer's instructions.

Immunofluorescence Staining—MCF7 cells were incubated with or without sErbB3 for 2 h, rinsed with PBS (–), fixed with 4% paraformaldehyde/PBS for 10 min, and rinsed with PBS (–). After blocking with 5% BSA, PBS for 30 min, cells were stained with anti-ErbB2, anti-ErbB3 and anti-His antibodies for 16 h at 4 °C followed by staining with Alexa Fluor 488 and Alexa Fluor 594 conjugated secondary antibodies for 1 h. The images were obtained using a fluorescence microscope (Keyence).
**Cell Proliferation Assay**—MCF7 cells were plated in quadruplicate in a 96-well plate (2000 cells/well). After serum starvation for 16 h, the cells were treated with the indicated concentrations of lapatinib for 4 h and then incubated with sErbB3 for 2 h and finally stimulated with 20 ng/ml heregulin β for 10 min at 37 °C. Cell proliferation was assayed after 72 h using a WST-1 reagent (Dojindo Molecular Technologies).

**Isolation of Glycosylated Peptides**—The purified sErbB3 and sErbB3 N418Q mutant were S-carbamidomethylated and digested with 1% (w/w) each of lysyl endopeptidase (Wako Pure Chemicals) and trypsin (Promega) at 37 °C for 16 h. The glycosylated peptides in the digest were enriched by the hydrophilic affinity method as described previously (31). Briefly, a 100-μg digest was mixed with 15 μl of packed volume of Sepharose CL-4B (GE Healthcare) in 1 ml of an organic solvent of 1-butanol/ethanol/H2O (4:1:1, v/v) and incubated for 45 min. The gel was washed twice with the organic solvent and incubated with an aqueous solvent of ethanol/H2O (1:1, v/v) for 30 min, and the solution phase was recovered and dried using a SpeedVac concentrator. Reversed phase chromatography was carried out on
an Inertsil WP300 C8 column (1.0 × 150 mm, 300 Å, GL Sciences) using an isocratic elution with 3.5% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid for 10 min followed by a gradient elution of acetonitrile (3.5–55%, v/v) in 0.1% (v/v) trifluoroacetic acid for 75 min. The glycosylated peptides were isolated and analyzed by MS.

**Mass Spectrometry**—The glycan profile and amino acid sequence of glycosylated peptides were obtained by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) using a pulsed nitrogen laser (337 nm). For glycan profiling, MALDI time-of-flight (TOF) measurements were performed using a Voyager DE Pro mass spectrometer (AB Sciex, Framingham, MA) in linear mode (32). For amino acid sequencing, collision-induced dissociation spectra were obtained by an AXIMA-QIT mass spectrometer (Shimadzu Corp., Kyoto, Japan). In these MALDI measurements, a 0.5-μl aliquot containing 0.1–1 pmol of glycosylated peptide was mixed with an equal volume of 10 mg/ml 2,5-dihydroxybenzoic acid dissolved in 50% (v/v) acetonitrile solution and then loaded onto a MALDI sample target plate for pulsed nitrogen laser (337 nm) irradiation. Measurements were carried out in positive ion mode. Electron-transfer dissociation (ETD) tandem MS for identification of glycosylation site was performed by an LTQ-XT mass spectrometer (Thermo Fisher Scientific, Waltham, MA) (33). Glycosylated peptide samples were dissolved in a 0.1% acetic acid and 50% (v/v) methanol solution and directly infused into the mass spectrometer using a nanospray tip. ETD was performed using 10⁶ anions of fluoranthene for reaction, and the ion/ion reaction time was set to 100 ms. The ETD tandem mass spectra was acquired by 200 scans.

**RESULTS**

**Comparison of Suppression Effects of sErB3 on Heregulin β Signaling**—First, we examined the heregulin signaling suppressive effects of sErB3s. Myc-His tagged sErB2, sErB3, and sErB4 were expressed in Lec3.2.8.1 cells and purified by column chromatography (Fig. 1A). MCF7 cells were treated by each sErB for 2 h and then stimulated with 10 ng/ml heregulin β for 10 min at 37 °C. As shown in Fig. 1B, sErB3 and sErB4 suppressed the phosphorylation of EGFR, ErbB2, ErbB3, Akt, and Erk, whereas sErB2B had little effect. ErbB4 phosphorylation was not detected at this heregulin concentration. When dose dependence was examined, it was revealed that sErB3 has the strongest suppressive effects; sErB2 has little effects, and sErB3 has ~10-fold effects compared with sErB4.

**sErB3 Acts on Cell Surface Molecules**—To determine the mechanisms by which sErB3 suppresses the heregulin signaling, the suppression patterns of heregulin β signaling were observed under increasing amounts of ligands. As shown in Fig. 2, the phosphorylation levels of ErbB3, Akt, and Erk increased in accordance to heregulin concentrations but almost reached a plateau at around 100 ng/ml. sErB3 suppressed the phosphorylation levels in a dose-dependent manner even at a heregulin concentration of 500 ng/ml, and the heregulin concentration that caused plateau levels of phosphorylation was not altered at any concentration of sErB3. The results suggested that sErB3 acts on cell surface molecules but not on ligands.

**sErB3 Suppresses Heregulin β Signaling through ErbB3-containing Heterodimer in MCF7 Cells**—We examined the molecules on which sErB3 act to suppress heregulin β signaling. First, we determined the ErbB homodimer(s) or heterodimer(s) that transmits heregulin β signals in MCF7 cells by knocking down each ErbB receptor by siRNA (Fig. 3A). The knockdown efficiency of each siRNA was confirmed by Western blotting. When ErbB3 was knocked down (lane 5), all phosphorylation levels of EGFR, ErbB2, ErbB3, Akt, and Erk were significantly suppressed. This result suggested that heterodimers that contain ErbB3, such as EGFR/ErbB3, ErbB2/ErbB3, and ErbB3/ErbB4, are crucial for the downstream signaling of heregulin β. This notion was also reinforced by the fact that phosphorylation levels of ErbB3 and Erk were almost correlated (lanes 1–17). We next tried to determine which heterodimer is the most crucial for heregulin signaling among ErbB3-containing heterodimers. The siRNA for ErbB4 failed to suppress the phosphorylation levels of both Akt and Erk, suggesting that the ErbB3/ErbB4 heterodimer is not very crucial for the downstream signaling (lane 6). Moreover, compared with siRNA for EGFR, siRNA for ErbB2 suppressed the downstream signaling more effectively, suggesting that the ErbB2/ErbB3 heterodimer is more crucial for downstream signaling than the EGFR/ErbB3 heterodimer (lane 3 and lane 4). This conclusion is also supported by the examination of the results regarding the combination of siRNA of EGFR and ErbB2 (lane 7), EGFR and ErbB4.
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### A

| Lane # | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 |
|-------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|
| siRNA of EGFR | - | - | * | - | * | - | * | - | * | - | * | - | * | - | * | - | - |
| siRNA of ErbB2 | - | - | * | - | * | - | * | - | * | - | * | - | * | - | * | - | - |
| siRNA of ErbB3 | - | - | * | - | * | - | * | - | * | - | * | - | * | - | * | - | - |
| siRNA of ErbB4 | - | - | * | - | * | - | * | - | * | - | * | - | * | - | * | - | - |
| Heregulin β | 0 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |

- WB: EGFR
- WB: ErbB2
- WB: ErbB3
- WB: ErbB4
- WB: pEGFR
- WB: pErbB2
- WB: pErbB3
- WB: pAkt
- WB: pErk
- WB: β-actin

### B

#### siRNA of EGFR
- sErbB (Lec3) [PBS (-)]
- sErbB3
- sErbB4
- Heregulin β

| Lane # | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
|-------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|
| siRNA of EGFR | - | - | * | - | * | - | * | - | * | - | * | - | * | - | * | - | - |

- WB: pErbB3
- WB: pAkt
- WB: pErk
- WB: β-actin

### C

#### siRNA of EGFR
- sErbB (Lec3) [PBS (-)]
- sErbB3
- sErbB4
- Heregulin β

| Lane # | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
|-------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|
| siRNA of EGFR | - | - | * | - | * | - | * | - | * | - | * | - | * | - | * | - | - |

- WB: pErbB3
- WB: pAkt
- WB: pErk
- WB: β-actin
(lane 9), and ErbB2 and ErbB4 (lane 11). Lane 7 represents the heregulin \( \beta \) signaling transmitted by the ErbB3/ErbB4 heterodimer, lane 9 represents ErbB2/ErbB3, and lane 11 represents EGFR/ErbB3. Phosphorylation levels of Akt and Erk were lane 9/Lane 11.

Next, we examined the heregulin \( \beta \) signaling-suppressive effects of sErbB3 with siRNA of ErbB receptors. As shown in Fig. 3, B and C, sErbB3 suppressed signaling more effectively than sErbB4 under any of the conditions examined. In particular, the phosphorylation of ErbB3 was suppressed effectively by sErbB3, suggesting that heterodimer formation involving ErbB3 was suppressed.

In conclusion, in MCF7 cells, heregulin \( \beta \) signaling was shown to be transmitted by receptor heterodimers containing ErbB3, especially ErbB2/ErbB3, and sErbB3 effectively suppresses the signaling from those heterodimers.

sErbB3 Suppresses Heregulin \( \beta \) Signaling In ErbB-transfected CHOK1 Cells—By using ErbB3- or ErbB4-transfected CHOK1 cells, in which endogenous ErbB2 is expressed, the effect of sErbB3 on heregulin \( \beta \) signaling transmitted by the ErbB2/ErbB3 heterodimer or ErbB2/ErbB4 heterodimer plus ErbB4/ErbB4 homodimer was examined. Fig. 4, A and B, show the results with ErbB3-transfected CHOK1 cells and ErbB4-transfected CHOK1 cells, respectively. Both results indicate that sErbB3 suppressed downstream signaling of the ErbB2/ErbB3 heterodimer or ErbB2/ErbB4 heterodimer plus ErbB4/ErbB4 homodimer more effectively than sErbB4.

Colocalization of sErbB3 and ErbB2 or ErbB3 on the Cell Surface—We examined the localization of sErbB3 on the cell by immunofluorescence staining. MCF7 cells were incubated with sErbB3 for 2 h, rinsed with PBS (-), and stained with anti-ErbB2 or anti-ErbB3 and anti-His antibodies. As shown in Fig. 5, sErbB3 colocalized with cell surface ErbB2 and ErbB3. This observation suggested that sErbB3 interacts with cell surface ErbB2 and ErbB3.

sErb3 N418Q Mutant Suppresses Heregulin \( \beta \) Signaling More Effectively Than Wild Type—In the previous study it was found that the ErbB3 N418Q mutant forms a heterodimer with ErbB2 and homodimer in the absence of ligands (23). We hypothesized that the ErbB3 N418Q mutant might change the structure from the tethered form to the extended form with less energy. In our next approach we examined the signaling-inhibitory effects of the sErbB3 N418Q mutant. Myc-His tagged wild type and the N418Q mutant of sErbB3 were expressed in CHOK1 cells or Lec3.2.8.1 cells and purified by column chromatography (Fig. 6 A). As shown in Fig. 6 B (MCF7 cells) and Fig. 6 C (ErbB3 transfected CHOK1 cells), the sErbB3 N418Q mutant suppressed heregulin signaling via the ErbB2/ErbB3 heterodimer more effectively than the wild type. Similar results were obtained in T47D and BT474 breast cancer cells (Fig. 6, D and E). Results from the ErbB4-transfected CHOK1 cells suggested that the sErbB3 N418Q mutant also suppressed heregulin signaling via ErbB2/ErbB4 heterodimer more effectively than the wild type (Fig. 6 F). The sErbB3 N418Q mutant pro-

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**FIGURE 3.** sErbB3 suppresses heregulin \( \beta \) signaling through ErbB3-containing heterodimers in MCF7 cells. MCF7 cells were treated with the indicated combinations of siRNA for ErbB receptors, serum-starved for 2 h, incubated with (B and C) or without (A) 10 \( \mu \)g/ml sErbB3 or sErbB4 for 2 h, and stimulated with the indicated concentrations of heregulin \( \beta \) for 10 min at 37 °C. The cell lysate was prepared, and 15.0 \( \mu \)g of protein/lane were subjected to Western blotting (WB) using indicated antibodies. The lower panel indicates the possible active combination of ErbB receptor heterodimers in each sample.

**FIGURE 4.** sErbB3 suppresses heregulin \( \beta \) signaling through ErbB2/ErbB3 and ErbB2/ErbB4 heterodimers in CHOK1 cells. A, ErbB3 stable expressing CHOK1 cells were prepared as described under “Experimental Procedures.” The cells were serum-starved for 16 h, incubated with indicated concentrations of sErbB2, sErbB3, or sErbB4 for 2 h, and stimulated with 10 ng/ml heregulin \( \beta \) for 10 min at 37 °C. The cell lysate was prepared and subjected to Western blotting (WB) using the indicated antibodies. B, the same experiment as panel A was performed using ErbB4 stable expressing CHOK1 cells.
duced in CHOK1 cells was also shown to have suppressing effects (Fig. 6G). When dose dependence was examined, it was revealed that the N418Q mutant of sErbB3 has ~2–3-fold inhibitory effects of heregulin signaling in ErbB3-transfected CHOK1 cells (Fig. 6H).

sErbB3 N418Q Mutant Exhibits Synergy with Anticancer Drug, Lapatinib—We next examined the combined signaling-inhibitory effects of sErbB3 with lapatinib, an ErbB2 tyrosine kinase inhibitor. As shown in Fig. 7A, sErbB3 augmented the inhibitory effects of lapatinib, and the additive effects were more significant in the sErbB3 N418Q mutant. We also examined the combined effects of sErbB and lapatinib on cell proliferation of MCF7 cells. As shown in Fig. 7B, sErbB3 augmented the inhibitory effects of lapatinib, and the sErbB3 N418Q mutant exhibited more significant effects.

Structures of N-Glycan on Asn-418 in sErbB3—Although it was suggested that the N-glycan on Asn-418 of ErbB3 is involved in the functional regulation of ErbB3 and sErbB3, whether Asn-418 of ErbB3 is really glycosylated has not been determined, and if it is, the structure of the N-glycan has also not been determined. To clarify this issue, purified sErbB3 was subjected to enzymatic proteolysis, and the glycosylated peptide was then isolated by reversed-phase chromatography. The glycosylated peptide was identified in wild type sErbB3 but was missing in the N418Q mutant sample (Fig. 8A). Amino acid sequencing of the peptide backbone by collision-induced dissociation MS/MS indicated that the isolated peptide contains Asn-418 (Fig. 8B), and ETD MS/MS confirmed that Asn-418 is glycosylated (Fig. 8C). We determined the N-glycan profiles on Asn-418 of sErbB3 from Lec3.2.8.1 cells (Fig. 8D) and from CHOK1 cells (Fig. 8E) using MALDI linear TOF MS. The results indicate that the dominant population of N-glycan on Asn-418 of sErbB3 produced from Lec3.2.8.1 cells was a high mannose type composed of five mannosos (so called Man5), and that from CHOK1 cells was LacNAc2Man3GlcNAc2 with or without sialic acid. Furthermore, any unglycosylated peptide containing Asn-418 (m/z 1120.6) was not found in a tryptic peptide mixture, indicating that Asn-418 is nearly 100% glycosylated in wild type sErbB3.
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DISCUSSION

In this study we have compared the suppressive effects of sErbB2, sErbB3, and sErbB4 on heregulin signaling and determined that sErbB3 suppresses signaling most effectively. It was suggested that sErbB3 suppresses heregulin signaling through the ErbB3-containing heterodimer in MCF7 cells and that it acts not on ligands, but on cell surface molecules, probably ErbB receptors. Immunohistochemical analysis also suggested the binding of sErbB3 to cell surface ErbB2 and ErbB3. By deleting N-glycan on Asn-418, the suppressive effects were enhanced by 2–3-fold, and synergistic effects for lapatinib were noted (Fig. 7B). The cell lysate was prepared, and 15.0 μg of protein/lane were subjected to Western blotting (WB) using indicated antibodies. C, The same experiment as panel B was performed with sErbB3-transfected CHOK1 cells. G, the same experiment as panel B was performed with sErbB3-transfected CHOK1 cells. The heregulin β concentration used was 1 ng/ml. H, ErbB3-transfected CHOK1 cells were serum-starved for 16 h, incubated with the indicated concentrations of wild type or N418Q-mutated sErbB3 produced in CHOK1 cells. The heregulin β concentration used was 1 ng/ml. H, ErbB3-transfected CHOK1 cells were serum-starved for 16 h, incubated with the indicated concentrations of wild type or N418Q-mutated sErbB3 for 2 h, and stimulated with 10 ng/ml heregulin β for 10 min at 37 °C. The left panel shows Western blotting with indicated antibodies, and the right panel displays the densitometric evaluation. The data are representative of three independent experiments.

Sugar chains play a role in a variety of biological events by affecting the physicochemical properties of glycoproteins. It has been reported that protein structure, stability, hydrophilicity, and protein-protein interactions are affected by glycosylation. Because most of the molecules involved in cell–cell communication are glycosylated, it is important to determine the mechanisms by which glycosylation regulates protein functions for signal transduction study. To date, we have been focused on the functions of N-glycans of cell surface molecules, which are implicated in signaling regulation (40–44). Among them, N-glycans of ErbB receptors are of interest in matters regarding cancer biology (19–24, 45). Based on our observations that deletion of N-glycan on Asn-420 of EGFR leads to ligand-independent oligomerization (19) and deletion of N-glycan on Asn-418 of ErbB3 leads to ligand-independent ErbB2/ErbB3 heterodimer formation and activation of PI3K/Akt and Ras/Erk pathways (23), we hypothesized that N-glycans in domain III of ErbB receptors are involved in prevention of ligand-independent dimer formation. Ligand binding is thought to induce rotation of a ridged body containing domains I and II, which leads to the structure changing from the tethered form to the extended form and allows dimerization of ErbB receptors (1–4). In the structural model of the extended form, the N-glycan on Asn-418 is placed in between domain I and III, potentially causing steric hindrance (Fig. 9B). The N-glycan might be involved in the maintenance of ErbB receptors in the tethered form in the absence of the ligand, and therefore, the ErbB3 N418Q mutant might change the structure from the tethered form to the extended form with less energy. Interestingly, the structural studies have revealed that ErbB2 (26) and Drosophila EGFR (46), which lack the corresponding N-glycan in domain III, are in the extended form without ligand stimulations. It has been reported that the addition of an N-glycan alters the aspar-
agine side chain torsion angle distribution and reduces its flexibility, and it may be involved in stabilizing the protein folding (47). Similar effects might act on oligomerization of ErbB receptors. Because it has been suggested that N-glycans are involved in the oligomerization of other receptors as well (48, 49), it is assumed that some N-glycans might act to prevent unnecessary protein-protein interactions.

In the present study we focused on the anticancer effects of sErbB3 and found that the deletion of N-glycan on Asn-418 of sErbB3 significantly enhanced the suppressive effects on heregulin signaling. Previous studies on p85-sErbB3 and TRAP-Fc suggested that they trap ligands to suppress downstream signaling; however, we have shown that sErbB3 acts on cell surface molecules (Figs. 2 and 5). We assume that sErbB3 binds to ErbB2/ErbB3 heterodimers on the cell surface, and the N418Q mutant of sErbB3 binds at a higher frequency. Because we have confirmed that ErbB2/ErbB3 heterodimers could form in the Lec3.2.8.1 cells (data not shown), sErbB3 produced in

**FIGURE 8.** Determination of the structures of N-glycans on Asn-418 in sErbB3. A, reversed phase chromatograms of proteolytically treated wild type (upper) and N418Q mutated (lower) sErbB3. The purified wild type and N418Q mutated sErbB3 were S-carbamidomethylated and digested with lysyl endopeptidase and trypsin. The glycosylated peptides in the digest were enriched by hydrophilic affinity and then purified by reversed phase chromatography using an isocratic elution with 3.5% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid for 10 min followed by a gradient elution of acetonitrile (3.5–55%, v/v) in 0.1% (v/v) trifluoroacetic acid for 75 min. The arrow indicates the glycosylated peptide that exists in wild type sErbB3 but not in the N418Q mutant. B, MALDI QIT mass spectra of the glycosylated peptide. MALDI ion trap MS caused a loss of N-linked glycan from the glycosylated peptide due to ion activation upon ionization, and the resulting unglycosylated peptide ion was observed at m/z 1120.6 indicated by an arrowhead (upper spectrum). The ion at m/z 2743.2 contained an intact glycan. Collision-induced dissociation MS/MS of the precursor ion at m/z 1120.6 generated the product ions specific to the sequence shown in the inset (lower spectrum). C, ETD MS/MS spectrum of the glycosylated peptide ion at m/z 2743.2, which is a triple-charged precursor ion containing an N-glycan of 1623 Da. ETD generated the product ions at m/z 763.4 and m/z 2501.3 for z7 and z8 ions, respectively (arrows). The mass of the latter ion indicates that Asn-418 is glycosylated. Assignment of product ions is indicated below the mass spectrum. D and E, MALDI linear TOF mass spectrum of the glycosylated peptide containing Asn-418 from sErbB3 produced in Lec3.2.8.1 cells (D) or CHOK1 cells (E). The illustration indicates the structure of N-glycan on Asn-418 of sErbB3. The intensity of the signals allows a rough estimation of the relative abundances of the molecules. Blue square, N-acetylglucosamine; green circle, mannose; yellow circle, galactose; purple diamond, sialic acid; red triangle, fucose.
Lec3.2.8.1 cells could bind to ErbB2 on the cell surface. It is likely that sErbB2, which lacks an intramolecular tether and is in extended form without ligands, binds to cell surface ErbB receptors more easily than sErbB3 or sErbB4. However, as shown in Fig. 1B, the suppressive effects of heregulin signaling of sErbB2 were far less pronounced than other sErbBs. Alvarado et al. (46) has suggested that conformational change of the ErbB receptor from the tethered form to the extended form is not sufficient for dimerization or activation. They consider that the fact sErbB2 does not form homodimers or heterodimers in vitro (50, 51) suggests sErbB2 is stringently autoinhibited, and they presume the existence of unknown ligands for ErbB2. Deletion of N-glycan on Asn-418 of ErbB3 might cause not only conformational changes from tethered extended form to extended form but also other changes that increase the affinity to cell surface ErbB receptors.

Because both of the sErbB3 with high mannose type and complex type N-glycans have shown similar results (Fig. 6, B–G), it seems that the extent of the effect does not vary with the structural difference of the N-glycan. However, for better understanding of the role of the N-glycan, the importance of the terminal structure remains to be determined. Moreover, lectin blotting with peanut agglutinin suggested that ErbB3 has no terminal structure remains to be determined. Moreover, lectin understanding of the role of the structural difference of the sErbB2 were far less pronounced than other sErbBs. Alvarado et al. (46) has suggested that conformational change of the ErbB receptor from the tethered form to the extended form is not sufficient for dimerization or activation. They consider that the fact sErbB2 does not form homodimers or heterodimers in vitro (50, 51) suggests sErbB2 is stringently autoinhibited, and they presume the existence of unknown ligands for ErbB2. Deletion of N-glycan on Asn-418 of ErbB3 might cause not only conformational changes from tethered extended form to extended form but also other changes that increase the affinity to cell surface ErbB receptors.

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In conclusion, the results indicate that sErbB3 exhibits strong suppressive effects on heregulin signaling by acting cell surface ErbB receptor dimers, especially those containing ErbB3. Manipulation of N-glycan of sErbB3 enhances the suppressive effects, and up-regulates the synergistic effects with lapatinib, thereby suggesting that N-glycan is involved in the physicochemical property of ErbB3. It seems likely that sErbB3 plays a role in regulating the functional properties of the cells, such as migration, invasion, or metastasis, via inhibiting heregulin signaling. Because sErbB3 occurs naturally, the sErbB3 N418Q mutant might be a potent anticancer drug with fewer side effects.

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