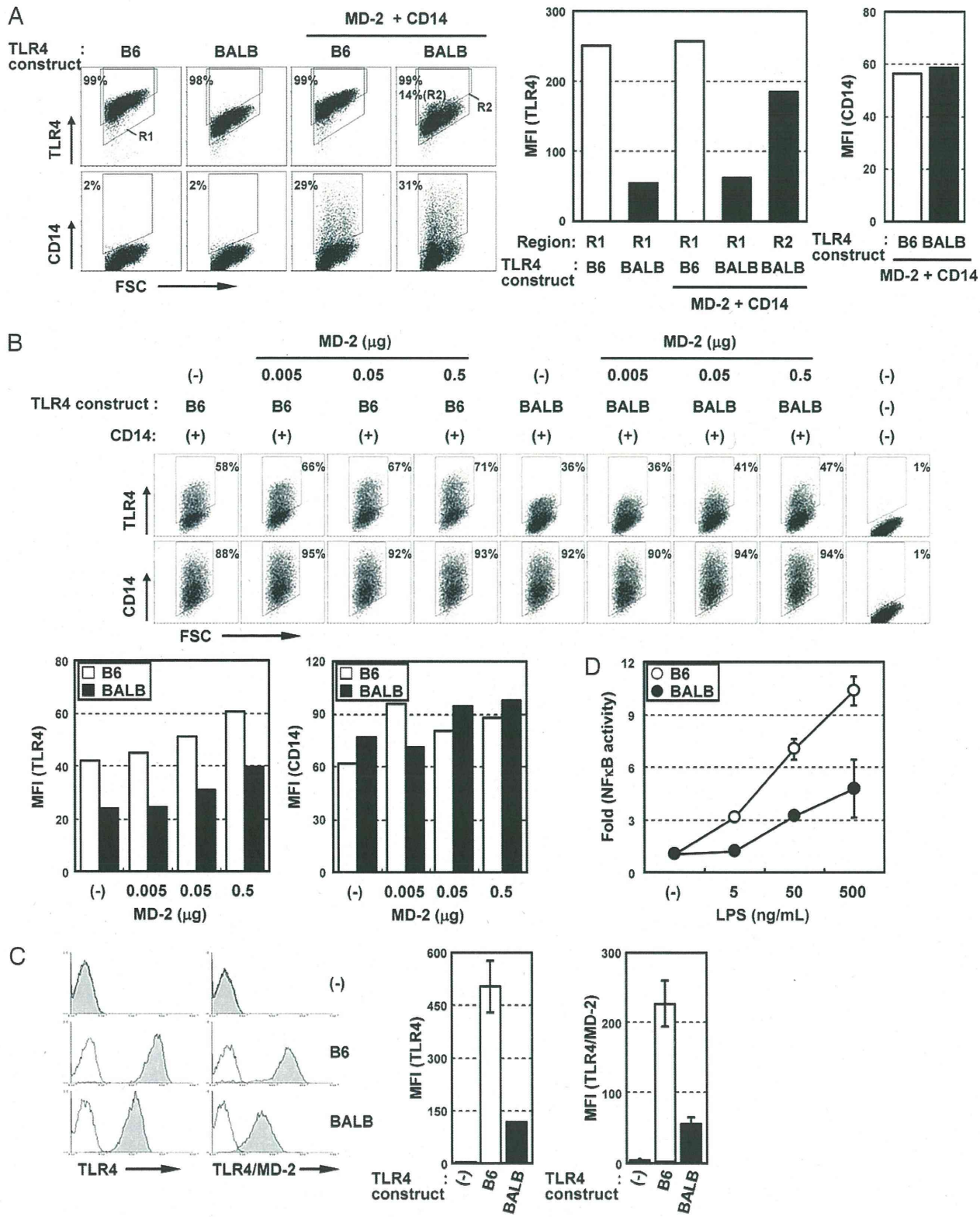

FIGURE 5. BALB TLR4 surface expression and signaling are lower than B6 TLR4 in the presence of MD-2. (**A**) B6 (white columns) or BALB (black columns) TLR4 stably transfected cells established in Fig. 4C were transiently transfected with human CD14 (1.5 μg) and mouse MD-2 (1.5 μg) expression constructs. After 24–48 h cultivation, surface expression of TLR4 and CD14 was determined by flow cytometry using Bio-UT49 and Bio-1B12 followed by PE-stv. Data are represented as MFI in the gated regions (R1 and R2). Percentage of gated cells is depicted in dot plot. Results are representative of three independent experiments (Supplemental Fig. 2). (**B**) B6 (white columns) or BALB (black columns) TLR4 expression constructs (2 μg) were cotransfected into HEK293 cells with human CD14 (0.5 μg) and mouse MD-2 expression constructs at the indicated amounts. After 24–48 h cultivation, surface expression of TLR4 and CD14 was determined and represented as MFI in the gated regions, as in (A). Results are representative of two independent experiments (Supplemental Fig. 3). (**C**) Stable Ba/F3-transfected clones expressing B6 or BALB TLR4 with MD-2 and NF-κB reporter gene were analyzed by flow cytometry using UT49 for TLR4 and UT12 for TLR4/MD-2 followed by PE-conjugated anti-mouse IgG Ab. Open (*Figure legend continues*)

(9, 32, 33), we suggest that differential surface expression levels of TLR4 accounted for at least one mechanism underlying the different sensitivity to LPS between B6 and BALB B cells.

Differences in surface TLR4 expression are not likely due to the amount of TLR4 produced in B6 versus BALB B cells, because the amount of *TLR4* mRNA, and probably of TLR4 protein, produced was similar in these strains of mice. Susceptibility to LPS in B cells was demonstrated to be most closely linked with the locus containing *TLR4* (22). Therefore, we examined the impact of polymorphic variations of the *TLR4* gene on surface expression of this molecule in HEK293 cells. This cell line expresses transfected TLR4 on its cell surface without the help of MD-2 (11). Consistent with B cells, lower surface expression of BALB TLR4, compared with B6 TLR4, was observed when HEK293 cells were transiently and stably transfected with the TLR4 expression vector. NF-κB activation was less inducible to LPS stimulation in BALB TLR4-transfected Ba/F3 cells than B6 TLR4-transfected cells. These findings suggest that genetic control of surface expression of TLR4 and, therefore, LPS sensitivity is directly attributable to SNP(s) within the *TLR4*-coding sequence.

Using B6/BALB chimeric TLR4 constructs as well as site-directed mutagenesis, we identified a V254I mutation as responsible for lower expression of BALB TLR4. Aa254, which is located on the ninth LRR in the central domain of TLR4, is included in the B patch, which is critical for TLR4–MD-2 association (34). Proteins associated with TLR4 (PRAT4A) and gp96 have been identified as chaperones controlling TLR4 translocation in mice. PRAT4A interacts with the E24-F54 region of TLR4, which is close to the A patch, and other regions in TLR4 are not important for their interaction (35). Therefore, it is unlikely that the V254I mutation impacts PRAT4A-mediated TLR4 transportation. Rather, the function of gp96 could be influenced by this mutation. Interestingly, Shibata et al. (36) found that *PRAT4A*$^{-/-}$ macrophages on the BALB background completely lacked surface TLR4, whereas B6 *PRAT4A*$^{-/-}$ macrophages expressed decreased but detectable levels of TLR4 on their cell surface. These findings allow us to speculate that TLR4 is transported by both PRAT4A-dependent and -independent mechanisms on the B6 genetic background, but that the contribution of the PRAT4A-independent mechanism is limited in the BALB background. The PRAT4A-independent mechanism may not support transport of V254I TLR4. Gp96 may be involved in this PRAT4A-independent mechanism. The TLR4 region required for gp96 interaction has not yet been elucidated. It is possible that other processes, after transcription but before cell surface localization of TLR4, are impaired by the V254I SNP (e.g., protein stability, glycosylation), in addition to TLR4 transport.

In human TLR4, aa 299, located on the 10th LRR, has a critical impact on LPS responsiveness, as the naturally occurring D299E mutation interrupts TLR4-mediated LPS signaling (25). Because this mutated amino acid is relatively close to aa 254, located on the ninth LRR, it is possible that human TLR4 with the D299E mutation has an impaired ability to localize to the cell surface.

MD-2 is another candidate controller of TLR4 expression on the cell surface (6). However, we observed that *MD-2* mRNA levels were comparable between B6 and BALB B cells. *MD-2* deficiency in B6 B cells showed an only slightly decreased surface TLR4 level; however, this was still higher than that of BALB B cells. Furthermore, BALB TLR4 expression was low compared with B6 TLR4 with the same amount of cotransfected MD-2 in HEK293 cells. No SNPs between the B6 and BALB *MD-2* coding sequences have been reported in the Mouse Genome Informatics database (http://www.informatics.jax.org/). Stable transfection of MD-2 in Ba/F3 cells failed to express BALB TLR4 at comparable levels of B6 TLR4. These findings suggest that MD-2 is not a critical molecular determinant for genetic control of surface TLR4 expression in B cells.

Previously, genetic control of LPS sensitivity has been reported in other immune cells (23, 37–39) in addition to B cells (21, 22, 40). We revealed that surface TLR4 expression was impaired in BALB BMDCs, albeit to a lesser extent, with decreased TNF-α production by LPS and UT12. In contrast, macrophages did not have strain difference in TLR4 expression and signaling. Therefore, the genetic mechanism underlying impaired TLR4 expression in B cells does not always apply to all immune cells. Presumably, the mechanism for TLR4 surface transportation varies on cell types, and thereby the impact of SNPs in *TLR4* gene on the mechanism could be different among cell types.

Because systemic responses to LPS are influenced by genetic background (41), we challenged mice with agonistic TLR4 mAb. Serum TNF-α, a causative major mediator of endotoxic shock (42), was equally increased in B6 and BALB mice, a finding suggesting similar sensitivity to endotoxic shock in these strains. Considering that macrophage is a major player for the induction of endotoxic shock (10), this finding is compatible with those of surface TLR4 expression and signaling in macrophages as described earlier. We revealed that UT12 induces enlarged spleen at least because of expansion of B cells, which results in the differentiation to CD138^{+} plasma cells with significant increase in B6 mice. LPS unresponsive strains with TLR4 P712H mutation have significantly lower natural IgG3 against LPS than wt strains (43). Therefore, we suggest, as in vivo significance of our findings, that natural Ab and/or innate immunity to invaded bacteria of B cells could be impaired by polymorphic variations of *TLR4* gene in BALB mice.

Rodo et al. (22) reported that the *MHC* locus is strongly linked to LPS sensitivity in B cells, possibly through regulation of RP105 expression. However, it may be difficult to explain low-responder phenotypes of BALB B cells only by the MHC haplotype because of its minor contribution as revealed by their congenic strains (22). B cell responsiveness could be controlled by other primary mechanism(s). It is worth noting that A/J mice, another strain with low LPS responder phenotypes of B cells, have different MHC haplotype from but share identical SNPs in *TLR4* gene with BALB mice (31). This suggests that polymorphic variation, in particular, V254I, is a primary cause of low LPS sensitivity in these strains. Minor contribution of MHC haplotype may explain moderate difference in B cell responses to LPS as previously demonstrated between BALB and A/J mice (40).

As a possible mechanism for genetic control of LPS sensitivity through MHC haplotype, distinctly lower surface expression of RP105 was shown in BALB B cells (22). However, we were unable to observe such low expression. Surface RP105 levels were similar or slightly lower in BALB B cells compared with B6 B cells. Our findings were consistent with the observation that responses to agonistic anti-RP105 mAb are not impaired in BALB B cells, as

histograms represent staining without primary mAbs. Data are summarized as mean ± SD MFI from three independent clones, as in Fig. 4C. Similar results were obtained in two independent experiments. (**D**) Stable clones in (C) were stimulated with LPS (5, 50, 500 ng/ml) or UT12 (10 μg/ml) for 5 h. Luciferase activity was shown as the mean ± SD fold increase against that of nonstimulated cells in triplicate cultures. Similar results were obtained in two independent experiments.

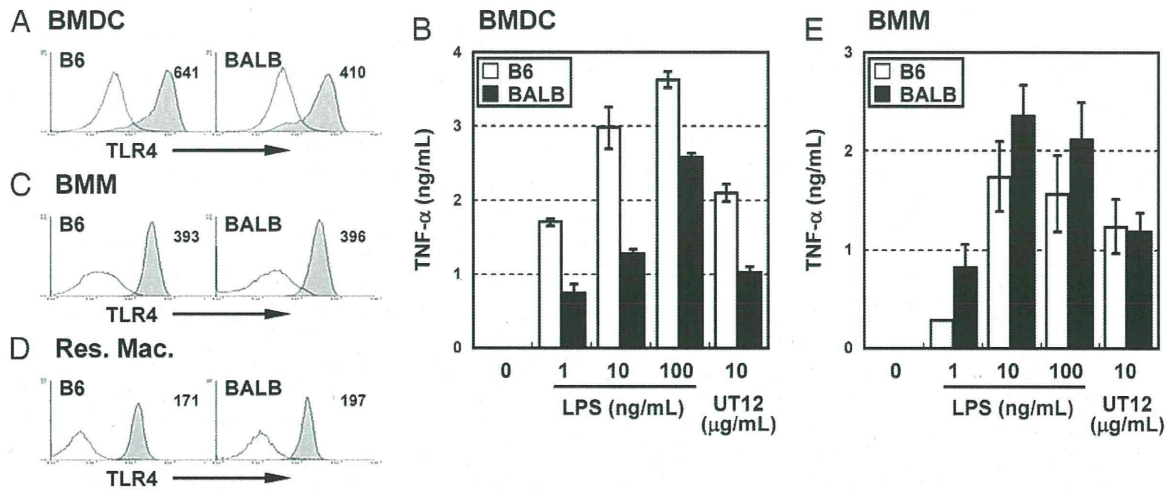

FIGURE 6. Slightly impaired TLR4 expression and signaling in BALB dendritic cells, but not in macrophages. (**A**, **C**, and **D**) B6 or BALB BMDCs (A), BMMs (C), and resident macrophages (D) were stained with Bio-UT49 and FITC-conjugated CD11c (A) or CD11b (C, D) mAbs followed by PE-stv. The expression of TLR4 on $CD11c^+$ cells (A) and on $CD11b^+$ cells (C, D) was analyzed by flow cytometry. Open histogram represents staining with a Bio-isotype control. Numbers in histogram indicate the MFI of staining. Results are representative of three independent experiments. (**B** and **E**) B6 (white columns) or BALB (black columns) BMDCs (B) and BMMs (E) were stimulated with LPS (1, 10, 100 ng/ml) or UT12 (10 μg/ml) for 21 h. TNF-α in cell culture supernatant was shown as the mean ± SD of triplicate cultures. Similar results were obtained in three independent experiments.

assessed by proliferation and cell-surface activation marker expression. A recent article by Vale et al. (21) also showed slightly lower surface expression of RP105 in BALB B cells, which is consistent with our findings. The reason why Vale et al. (21) and our findings regarding RP105 expression were not in agreement with a previous report (22) is unclear. Further investigation is required to reach the conclusion about direct contribution of RP105 to strain-dependent LPS sensitivity via the linkage with MHC locus.

In conclusion, the decreased surface TLR4 expression caused by a V254I point mutation accounts for the low LPS responder phenotype of BALB B cells. The results shown in this study reveal

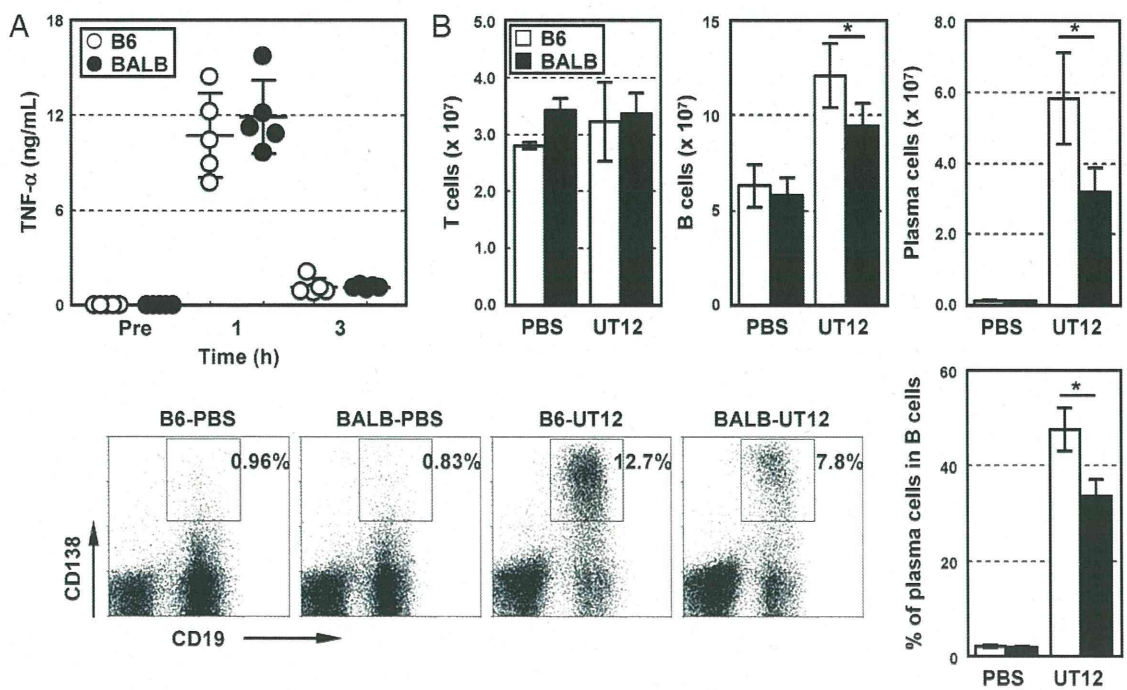

FIGURE 7. BALB mice have similar sensitivity to endotoxic shock but impaired plasma cell differentiation compared with B6 mice. (**A**) Five B6 (open circles) or BALB (black circles) mice were i.p. injected with UT12 (5 μg), and serum was collected 3 d before (Pre) or 1 and 3 h after sublethal challenge. Serum TNF-α was shown as the mean ± SD. Similar results were obtained in two independent experiments. (**B**) Seven days after challenge of B6 (white columns) or BALB (black columns) mice with UT12 mAb (5 μg, $n = 5$) or PBS ($n = 3$), the numbers of $CD3^+$ T cells, $CD19^+$ B cells, and $CD138^+CD19^+$ plasma cells, as well as the ratio of plasma cells to total B cells in spleen, were determined by flow cytometry using the combinations of FITC-CD19/PE-CD3 and of FITC-CD19/PE-CD138 mAbs. Results were shown as the mean ± SD of cell number in spleen or percentage of plasma cells in B cells. Representative dot plots of plasma cell staining were shown with the percentage of gated cells in total spleen cells. $^*p < 0.05$, Student t test. Similar results were obtained in two independent experiments.

the genetic control of LPS sensitivity and also help elucidate the mechanism of directing TLR4 to the proper subcellular compartment and innate immunity by B cells. These data are also useful information for immunologists, because LPS is a widely used mitogen, and B6 and BALB mice are the most frequently used model strains, because they are Th1 and Th2 biased, respectively.

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Disclosures

The authors have no financial conflicts of interest.

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

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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 heregulin β 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 homo- 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 ErbB 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 oligomeriza-

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[2] The abbreviations used are: EGFR, epidermal growth factor receptor; sErbB, soluble ErbB; ETD, Electron-transfer dissociation.

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. It was found that sErbB3 has the strongest effects, and we propose that sErbB3 acts on cell surface receptors but not on heregulin to suppress 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 EQKLISEEDLNMHTGH 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 Ullrich (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 HisTrap HP5 column equilibrated with 100 mM HEPES, pH 7.4, 0.5 M 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 EQKLISEEDLNMHTGH 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 second 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).

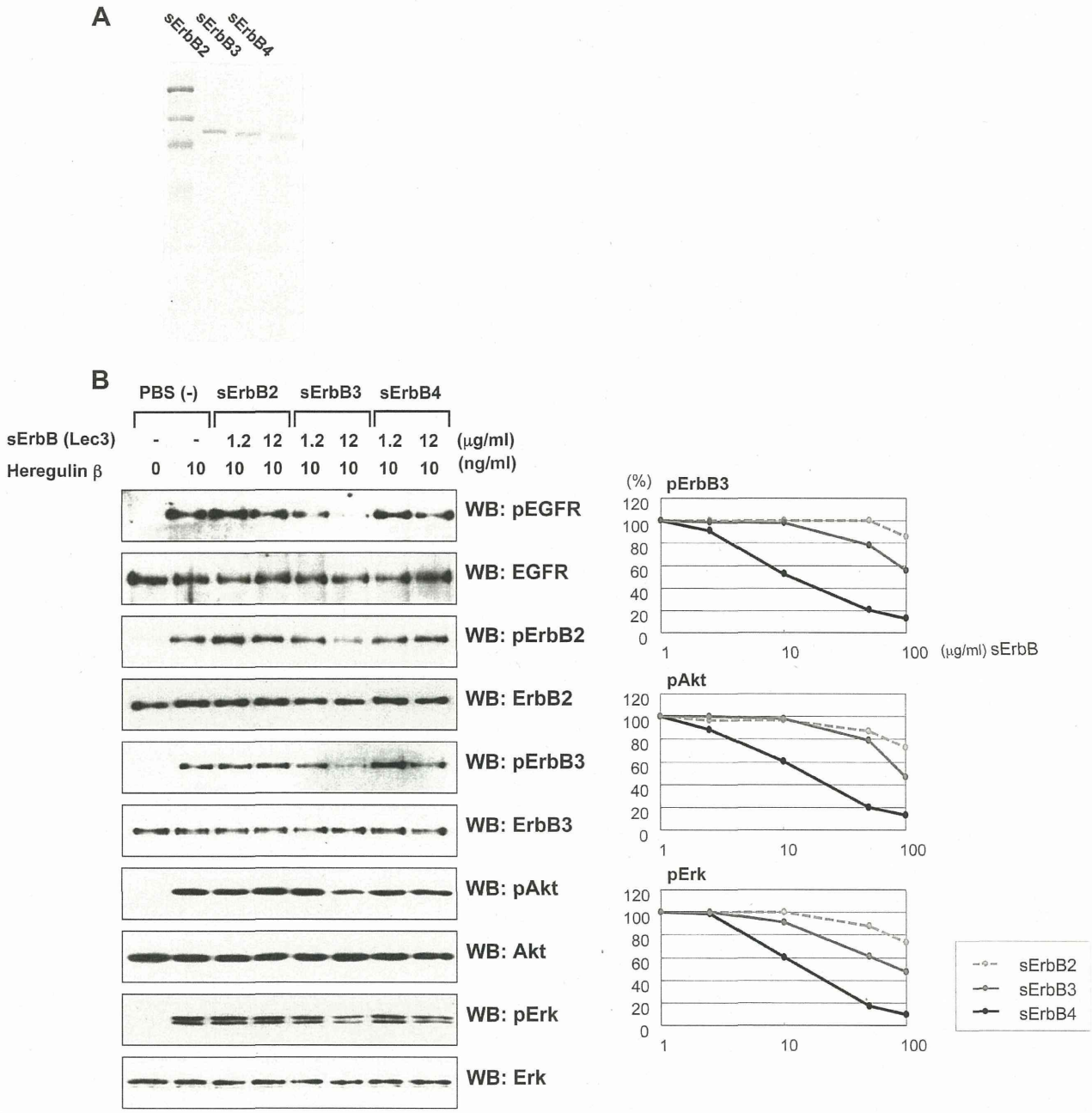


FIGURE 1. **Comparison of suppression of heregulin β signaling by sErbB2, sErbB3, and sErbB4.** *A*, sErbB2, sErbB3, and sErbB4 were produced in Lec3.2.8.1 cells and purified as described under "Experimental Procedures." One μg of each protein was subjected to SDS-PAGE followed by Coomassie Brilliant Blue R-250 staining. *B*, MCF7 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 β for 10 min at 37 °C. For the *left panel*, the cell lysate was prepared, and 15.0 μg protein/lane were subjected to Western blotting (*WB*) using the indicated antibodies. The *right panel* displays the densitometric evaluation. The data are representative of three independent experiments.

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. 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/H_2O (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/H_2O (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 a 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-XL 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 10^6 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 sErbBs on Heregulin β Signaling—First, we examined the heregulin signaling suppressive effects of sErbBs. Myc-His tagged sErbB2, sErbB3, and sErbB4 were expressed in Lec3.2.8.1 cells and purified by column chromatography (Fig. 1*A*). MCF7 cells were treated by each sErbB for 2 h and then stimulated with 10 ng/ml heregulin β for 10 min at 37 °C. As shown in Fig. 1*B*, sErbB3 and sErbB4 suppressed the phosphorylation of EGFR, ErbB2, ErbB3, Akt, and Erk, whereas sErbB2 had little effect. ErbB4 phosphorylation was not detected at this heregulin concentration. When dose dependence was examined, it was revealed that sErbB3 has the strongest suppressive effects; sErbB2 has little effects, and sErbB3 has ~10-fold effects compared with sErbB4.

sErbB3 Acts on Cell Surface Molecules—To determine the mechanisms by which sErbB3 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. sErbB3 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 sErbB3. The results suggested that sErbB3 acts on cell surface molecules but not on ligands.

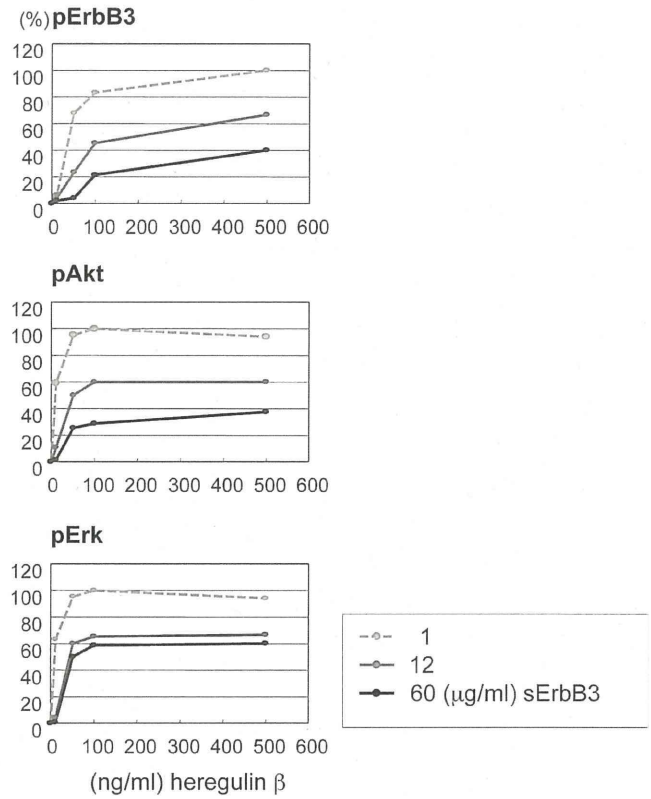
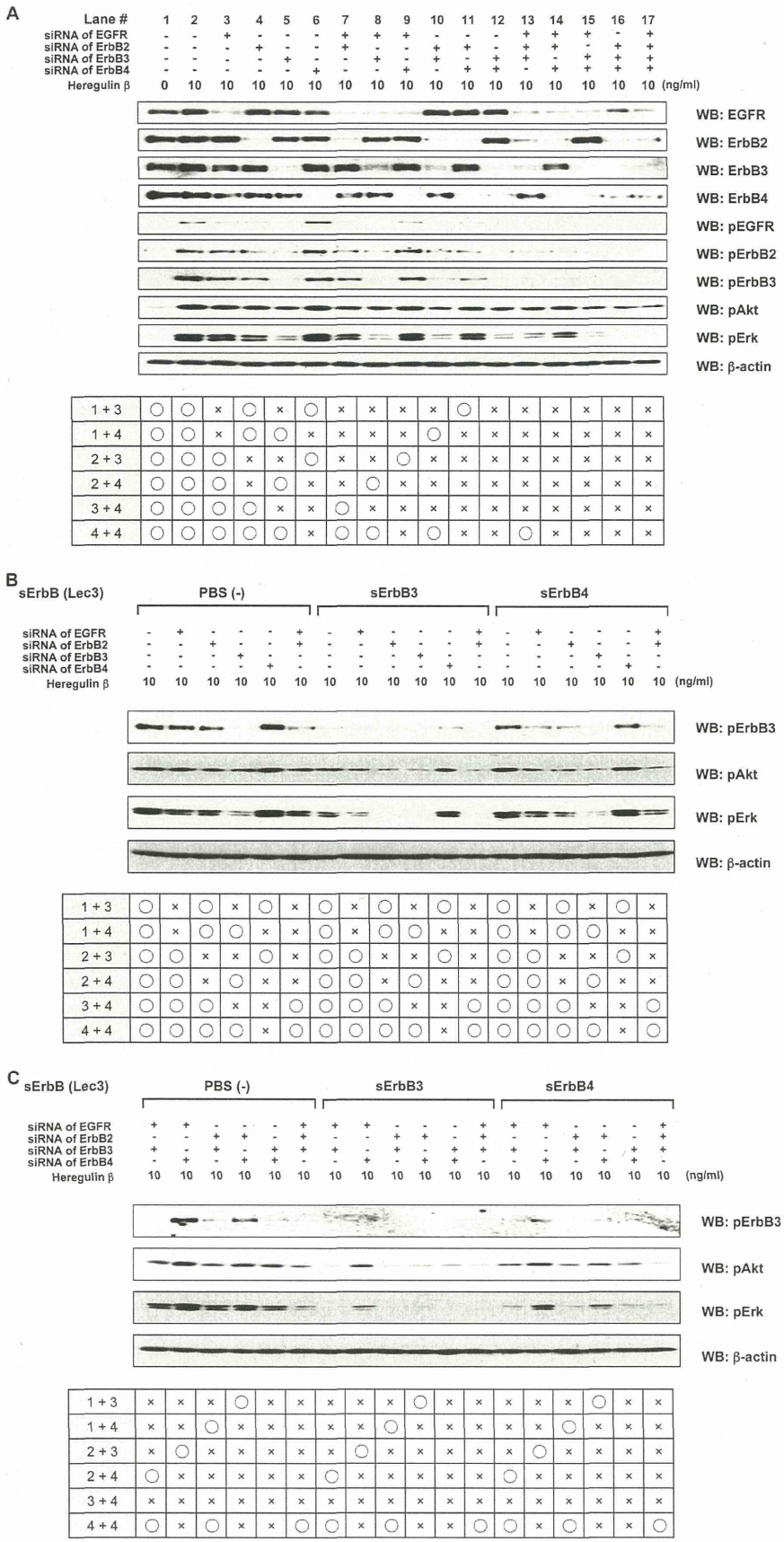


FIGURE 2. **Suppression patterns of heregulin β signaling by sErbB3.** MCF7 cells were serum-starved for 16 h, incubated with 1, 12, or 60 μg/ml sErbB3 for 2 h, and stimulated with indicated concentration of heregulin β for 10 min at 37 °C. The cell lysate was prepared and subjected to Western blotting using the indicated antibodies. Densitometric analysis was performed using a Luminous analyzer. The data are representative of three independent experiments.

sErbB3 Suppresses Heregulin β Signaling through ErbB3-containing Heterodimer in MCF7 Cells—We examined the molecules on which sErbB3 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. 3*A*). 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



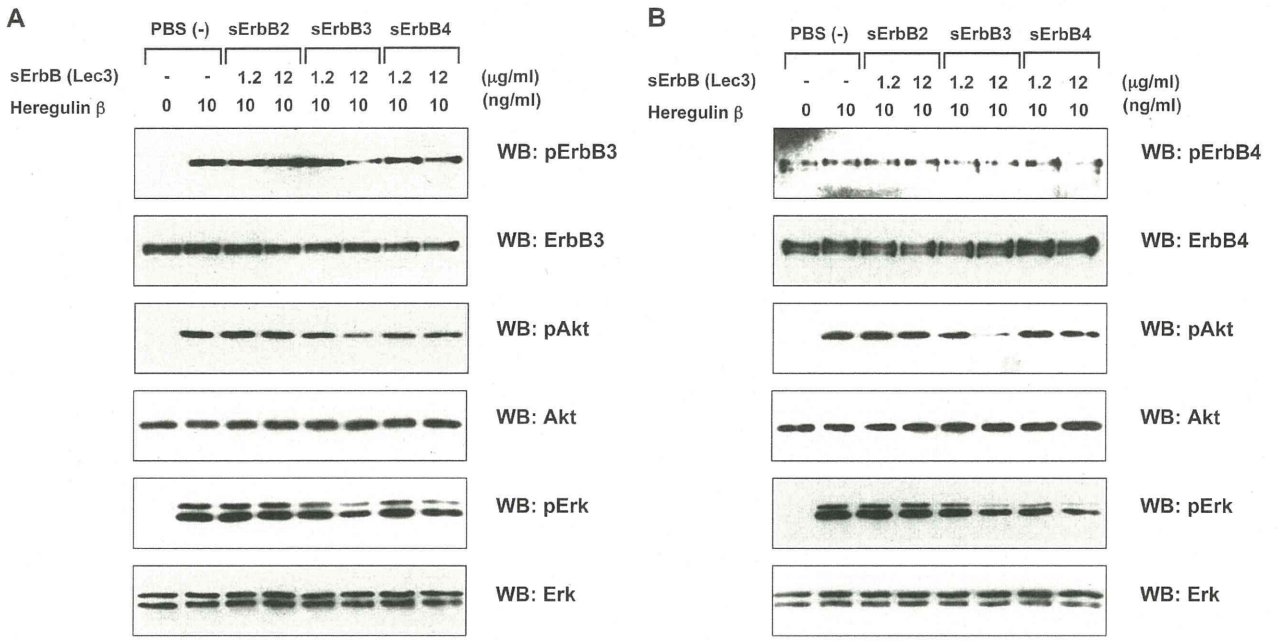


FIGURE 4. **sErbB3 suppresses heregulin β signaling through ErbB2/ErbB3 and ErbB2/ErbB4 heterodimers in CHOK1 cells.** *A*, ErbB3 stable expressing CHOK1 cells were prepared as described under "Experimental Procedures." The cells were serum-starved for 16 h, incubated with indicated concentrations of sErbB2, sErbB3, or sErbB4 for 2 h, and stimulated with 10 ng/ml heregulin β 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.

(*lane 9*), and ErbB2 and ErbB4 (*lane 11*). *Lane 7* represents the heregulin β 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* > *lane 7*.

Next, we examined the heregulin β 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 β 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 β 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 β 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 β 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-

FIGURE 3. **sErbB3 suppresses heregulin β 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 μg/ml sErbB3 or sErbB4 for 2 h, and stimulated with the indicated concentrations of heregulin β for 10 min at 37 °C. The cell lysate was prepared, and 15.0 μg 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.