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A Splice Variant of CD99 Increases Motility and MMP-9 Expression of Human Breast Cancer Cells through the AKT-, ERK-, and JNK-dependent AP-1 Activation Signaling Pathways*

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The *CD99* gene encodes two distinct transmembrane proteins by alternative splicing of its transcript. To examine the effects of two CD99 isoforms on the invasive phenotypes of breast cancer cells, MDA-MB-231 and MCF-7 human breast cancer cell lines were stably transfected with CD99 cDNAs encoding the major wild-type form (type I) or a minor splice variant (type II). As a result, expression of CD99 type II, but not type I, markedly elevated the motility, binding to fibronectin, MMP-9 expression, and invasiveness of MDA-MB-231 and MCF-7 breast cancer cells. In MDA-MB-435 breast cancer cells expressing both CD99 type I and type II, invasion-related cellular activities were inhibited by the transfection of small interfering RNA (siRNA) targeted to CD99 type II. Meanwhile, CD99 type II-induced MMP-9 expression in MDA-MB-231 cells was shown to be mediated by the binding of AP-1 factors to the *MMP-9* gene promoter. Gel shift assay revealed that ligation of CD99 type II with antibody resulted in the binding of JunD to the AP-1 site of the *MMP-9* promoter region. Initiation of CD99 type II signaling by antibody ligation increased expression of JunD and FosB AP-1 factors, along with phosphorylation of Src, Akt, p38 MAPK, ERK, and JNK. Knockdown of JunD and FosB by siRNA transfection abolished the positive effects of CD99 type II on the motility and MMP-9 expression of MDA-MB-231 cells. Increased expression of JunD and FosB as well as elevated cell motility and MMP-9 expression by CD99 type II ligation were also abrogated by inhibitors, dominant-negative forms, and siRNAs for Akt1, ERK1/2, and JNK1 but not for p38 MAPK. These results suggest that expression of a splice variant of CD99 contributes to the invasive ability of human breast cancer cells by up-regulating AP-1-mediated gene expression through the Akt-dependent ERK and JNK signaling pathways.

CD99, a cell surface glycoprotein with a molecular mass of 32 kDa, was originally described as a human thymus leukemia antigen (1), a Ewing sarcoma-specific membrane marker molecule (2, 3), and a putative adhesion molecule (termed E2) involved in spontaneous rosette formation of T cells with erythrocytes (4–7). CD99 is broadly distributed on many cell types, with particularly strong expression on human cortical thymocytes, Ewing sarcoma cells, and peripheral primitive neuroectodermal tumors (3, 8). The functional role of CD99 is not fully understood, and most information about its functions is derived from triggering CD99-mediated signaling events with agonistic CD99 monoclonal antibodies (mAbs)[2] in hematopoietic cells. In normal cells, CD99 has been functionally implicated in cell adhesion, migration, apoptosis, differentiation, activation, and proliferation of lymphocytes and monocyte extravasation and transport of several transmembrane proteins (9–19). In particular, the role of CD99 in the modulation of cell adhesion has been demonstrated by the ability of anti-CD99 mAbs to induce homotypic aggregation of $CD4^+CD8^+$ thymocytes, whereas other anti-CD99 antibodies block spontaneous T cell-erythrocyte rosette formation (4, 6, 11, 20). It was also shown that antibody ligation of CD99 molecules up-regulated the expression of LFA-1 ($\alpha_L\beta_2$ integrin), and CD99-induced cell aggregation was blocked by the addition of mAbs to LFA-1 or intracellular adhesion molecule 1 (ICAM-1) in a B cell line (20). These results suggest that signal transduction via CD99 modulates cell adhesion of lymphocytes by regulating the expression level of the cell adhesion molecule, integrin LFA-1. Several signal transducing molecules, including MAPKs and protein kinase C, have been found to mediate CD99-dependent cell adhesion of T cells (21, 22). In addition, it was found that CD99 on one cell binds to another neighboring CD99 on the next, indicating that CD99 is a homophilic cell surface interacting protein (18). However, the molecular mechanisms of CD99-mediated signal transduction remain largely unknown.

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[2] The abbreviations used are: mAbs, monoclonal antibodies; MMP, matrix metalloproteinase; AP-1, activator protein-1; FAK, focal adhesion kinase; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK kinase; PI3K, phosphatidylinositol 3-kinase; siRNA, small interfering RNA; RT, reverse transcription; ECM, extracellular matrix; FBS, fetal bovine serum; MTT, 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2*H*-tetrazolium bromide; PBS, phosphate-buffered saline.

The *CD99* gene encodes two distinct proteins produced by alternative splicing of the *CD99* gene transcript (20). Compared with the major wild-type full-length form, the minor splice variant form of CD99 has a relatively short intracytoplasmic fragment (Fig. 1*A*) (23). Interestingly, the minor form of CD99 inhibited homotypic adhesion of B cells, whereas activation of the major form promoted the adhesion process. The opposing effects of the major and minor forms of CD99 on homotypic B cell adhesion were shown to be because of their opposing functions in controlling the expression of the cell adhesion molecule, integrin LFA-1 (20). Because differential expression of alternative splicing products has been shown to be a characteristic feature of many developmentally regulated cell adhesion molecules (24–27), these results suggest that the modulation of adhesion and de-adhesion of lymphocytes occurring during the lymphoid cell differentiation process may be influenced by a balance in the expression level between the major form and splice variant of CD99. In addition, it was reported that expression of the major CD99 form in CD99-deficient Jurkat T cells promotes cell adhesion, whereas co-expression of the two CD99 isoforms induces apoptosis (28). Thus, it appears that differential expression of these two CD99 isoforms can lead to distinct functional outcomes.

Besides developmental processes, various biological phenomena regulated by cell adhesion and de-adhesion could be affected by the differential expression of CD99 molecules. Because alterations in homotypic cell adhesion have been frequently observed in the invasion processes of many types of cancer, the functional role of CD99 molecules in the adhesion process of lymphocytes led us to the assumption that cellular activities related to the invasive potential of cancer cells could in part be regulated by the differential expression of CD99 molecules. To test this possibility, we here investigated the functional effects of CD99 isoforms on several cellular processes involved in cancer invasion and metastasis. As a result, we found that expression of the minor splice variant (type II) of CD99 led to increases in the motility, binding to fibronectin, MMP-9 activity, and invasiveness of human breast cancer cells. Increased cell motility and MMP-9 expression by CD99 type II were found to be mediated by AP-1 factors such as JunD and FosB. CD99 type II-specific signaling events to increase JunD and FosB were regulated by Akt, ERK, and JNK in breast cancer cells. On the basis of our findings, we suggest that expression and activation of the CD99 splice variant enhance the invasive ability of human breast cancer cells by up-regulating AP-1-mediated gene expression through the Akt-dependent ERK and JNK signaling pathways.

EXPERIMENTAL PROCEDURES

Cell Culture, Antibodies, and Reagents—MCF-7, MDA-MB-231, and MDA-MB-435 human breast adenocarcinoma cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin (all obtained from Invitrogen) in 5% CO_2 at 37 °C. Anti-CD99 monoclonal antibody DN16 was purchased from Dinona Bioscience (Seoul, Korea). Antibodies to FAK, phospho-FAK$^{(Tyr925)}$, Src, phospho-Src$^{(Tyr416)}$, Akt, phospho-Akt$^{(Ser473)}$, ERK1/2, phospho-ERK1/2$^{(Thr202/Tyr204)}$, p38 MAPK, phospho-p38 MAPK$^{(Thr180/Tyr182)}$, JNK, phospho-JNK$^{(Thr183/Tyr185)}$, c-Jun, phospho-c-Jun$^{(Ser63/Ser73)}$, JunB, JunD, c-Fos, FosB, Fra1, Fra2, and MMP-9 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PP2, PD98059, SB203580, SP600125, and wortmannin were purchased from Biomol (Plymouth Meeting, PA). All other reagents were from Sigma, unless indicated otherwise.

CD99 Transfection and Selection of Stable Clones—The cDNA expression construct encoding the wild-type form (type I) or splice variant (type II) of CD99 was transfected into MDA-MB-231 and MCF-7 human breast cancer cell lines by using Lipofectamine (Invitrogen) according to the manufacturer's instructions. pcDNA3 vector only was also transfected as a control. Neomycin-resistant clones were isolated by growth in Dulbecco's modified Eagle's medium containing 10% FBS and 0.8 mg/ml G418 (Invitrogen). Stable transfectant clones with high CD99 expression were identified by RT-PCR, immunoblotting, and flow cytometric analysis.

RT-PCR Analysis—Total cellular RNA was purified from the cultured cells using Trizol reagent (Invitrogen) according to the manufacturer's protocol. First strand cDNA synthesis was performed with 1 μg of total RNA using a cDNA synthesis kit (Promega, Madison, WI). For PCR amplification, 5′-GTGCGGCTAGCACCATGGCCCGCGGGGCTG-3′ was used as the sense primer for both cDNAs of CD99 type I and type II. The antisense primers, 5′-TAGTCTCGAGCTATTTCTCTAAAAGAGTACG-3′ and 5′-GCTCTAGACCCTAGGTCTTCAGCCAT-3′, were used for the CD99 type I and type II, respectively. These primer pairs amplify a 583-bp fragment for CD99 type I and a 515-bp fragment for CD99 type II (20). Additionally, cDNAs for MMP-9 and the standard form of CD44 were also subjected to PCR amplification using the following primer pairs: 5′-TGGGCTACGTGACCTATGACAT-3′ (sense) and 5′-GCCCAGCCCACCTCCACTCCTC-3′ (antisense) for *MMP-9* (29); 5′-TTTGCCTCTTACAGTTGAGCCTG-3′ (sense) and 5′-GGTGCCATCACGGTTGACAATAG-3′ (antisense) for *CD44* (30). The reaction mixture was subjected to 25 PCR amplification cycles of 60 s at 95 °C, 90 s at 58 °C, and 90 s at 72 °C. β-Actin amplification was used as an internal PCR control with 5′-GATATCGCCGCGCTCGTCGTCGAC-3′ as the sense primer and 5′-CAGGAAGGAAGGCTGGAAGAGTGC-3′ as the antisense primer. The PCR products were visualized using ethidium bromide in 1% agarose gel.

Western Blotting Analysis—Cells were washed, harvested, and lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 20 μg/ml leupeptin, and 2 mM benzamidine) on ice for 10 min. For phosphoprotein analysis, cell lysis buffer was supplemented with phosphatase inhibitors (1 mM sodium orthovanadate, 1 mM NaF, and 10 mM β-glycerophosphate). After centrifugation at 15,000 × *g* for 10 min, the supernatants were collected and quantified for protein concentration by the Bradford assay. Equal amounts of protein per lane were separated onto 10% SDS-polyacrylamide gel and transferred to an Immobilon-P membrane (Millipore, Bedford, MA). The membrane was blocked in 5% skim milk for 2 h and then incubated with a specific antibody for 2 h. After washing, the membrane was incubated with a secondary antibody conju-

gated with horseradish peroxidase. After final washes, the membrane was developed using enhanced chemiluminescence reagents (Amersham Biosciences).

Flow Cytometric Analysis—Cells were incubated with 20 μg/ml anti-CD99 mAb DN16 for 30 min, washed with cold PBS, and then incubated with saturating concentrations of fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Pharmingen) for 30 min at 4 °C. After washing with PBS, the cells were fixed with 2% formaldehyde in PBS. Cell surface immunofluorescence was analyzed by flow cytometry performed on a FACScan (BD Biosciences).

Transfection of siRNA—Small interfering RNAs (siRNAs) for CD99 type II, JunD, and FosB were designed and synthesized using the software and Silencer™ siRNA construction kit from Ambion (Austin, TX) according to the manufacturer's instructions. CD99 type II siRNA-targeting sequence was 5′-AATGATGGCTGAAGACCTAGG-3′ (sense), which is located between exon 8 and exon 9 in the human *CD99* gene and is found only in the splice CD99 variant (type II) cDNA but not in the major type (type I) cDNA (20). The siRNA-targeting sequences (sense) for JunD and FosB were 5′-GAGAAGGCUCAGCAAGAAG-3′ and 5′-UGUCUGGUAUGAUCCUUCU-3′, respectively. The siRNA control was 5′-UUCUCCGAACGUGUCACGUdTdT-3′ (sense) and 5′-ACGUGACACGUUCGGAGAAdTdT-3′ (antisense), which bears no homology with relevant human genes (31). For siRNA transfection, cells (5×10^5) were seeded in 6-well plates and grown for 24 h to reach 60–70% confluency. The different amounts of siRNA and the Lipofectamine reagent (5 μl) were diluted in 200 μl of Dulbecco's modified Eagle's medium. The diluted siRNA-liposome complex was added to cells in DMEM (800 μl). Following 6 h of incubation, cells were rinsed with fresh medium and grown for 24 h in normal growth medium containing FBS before analysis.

Cell Growth Assay—*In vitro* cell growth was measured using a modified 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) method (32). Cells were seeded in 96-well plates at 2×10^3 cells in 0.1 ml of culture medium per well. After incubation in 5% CO_2 at 37 °C, 10 μl of the MTT solution (5 mg/ml) was added every 24 h to wells of each plate, and the cells were cultured for another 2 h, and 100 μl of SDS (20%) and *N*,*N*-dimethylformamide (50%) solution was added to each well and mixed vigorously to solubilize colored crystals produced within the cells. The ratio of absorbance at 590 nm to absorbance at 630 nm was measured by a microplate ELISA reader (Bio-Tek Instruments, Winooski, VT).

Cell Aggregation Assay—For the measurement of homotypic cell-to-cell adhesion (33), adherent cells were rendered as a single cell suspension by trypsinization followed by incubation with 5 mM EDTA in PBS lacking Ca^{2+} and Mg^{2+} at 37 °C for 5 min and by 7 gentle passes through a 22-gauge needle. After cells were washed with Puck's saline (5 mM KCl, 140 mM NaCl, and 8 mM $NaHCO_3$, pH 7.4), single cell suspensions (1×10^5 cells/ml of Puck's saline) were plated into individual wells of a 24-well culture plate, and incubated in 5% CO_2 at 37 °C with agitation at 70–80 rpm using a orbital shaker. Photographs were taken every 30 min after incubation under a microscope on three predetermined fields, and both the total cell number (*A*) and the number of cells remaining as single cells (*B*) were counted. The results were expressed as the percentage of cells that formed aggregates as follows: $(A - B)/A \times 100(\%)$. For antibody ligation of CD99, the CD99 transfectant cells (5×10^5) suspended in 0.1 ml of serum-free DMEM were pretreated with 2 μg of anti-CD99 mAb DN16 for 2 h and then with 10 μg of goat anti-mouse IgG (γ-chain specific; Sigma) for 3 h before single cell suspensions were made for aggregation assays.

Invasion Assay into Matrigel—Cells were tested for invasive ability through the basement membrane Matrigel (BD Biosciences) *in vitro* in Transwell chambers (Corning Costar, Cambridge, MA), as described previously (34, 35). In brief, 24-well Transwell chamber inserts (Corning Costar, Cambridge, MA) with 8-μm porosity polycarbonate filters were precoated with 80 μg of basement membrane Matrigel (BD Biosciences) onto the upper surface and with 20 μg of gelatin onto the lower surface. Culture supernatant of NIH3T3 fibroblasts in DMEM supplemented with 10% FBS was placed in the lower well. Cells suspended in DMEM/F-12 medium containing 0.1% FBS were added to the upper chambers (2×10^4 cells/well) and incubated for 24 h at 37 °C in 5% CO_2. Cells were fixed and stained with hematoxylin and eosin. Noninvading cells on the upper surface of the filter were removed by wiping out with a cotton swab, and the filter was excised and mounted on a microscope slide. Invasiveness was quantified by counting cells on the lower surface of the filter.

Wound Healing Migration Assay—For the measurement of cell migration during wound healing, cells (5×10^5) were seeded in individual wells of a 24-well culture plate. When the cells reached a confluent state, cell layers were wounded with a plastic micropipette tip having a large orifice. The medium and debris were aspirated away and replaced by 2 ml of fresh serum-free medium. Cells were photographed every 12 h after wounding by phase contrast microscopy. For evaluation of "wound closure," five randomly selected points along each wound were marked, and the horizontal distance of migrating cells from the initial wound was measured.

Attachment Assay—24-Well culture plates were coated with fibronectin, type I collagen, type IV collagen, or laminin (10 μg/cm^2 each; all from Sigma) for 6 h at room temperature and washed with PBS. To block nonoccupied binding sites, the plates were incubated in 1% heat-inactivated bovine serum albumin for 1 h at 37 °C. Cells (5×10^4) suspended in DMEM containing 0.5% bovine serum albumin were dispensed into each extracellular matrix protein-coated well, incubated in 5% CO_2 at 37 °C for 1 h, and gently washed five times with PBS. Cells attached to the bottom of plate were stained with hematoxylin and eosin reagent and counted under a microscope.

Gelatin Zymography—Type IV collagenase activities present in conditioned medium were visualized by electrophoresis on gelatin-containing polyacrylamide gel as described previously (36). Briefly, conditioned medium from cells cultured in serum-free medium was mixed 3:1 with substrate gel sample buffer (40% (v/v) glycerol, 0.25 M Tris-HCl, pH 6.8, and 0.1% bromphenol blue) and loaded without boiling onto 10% SDS-polyacrylamide gel containing type 1 gelatin (1.5 mg/ml). After electrophoresis at 4 °C, the gel was soaked in 2.5% Triton X-100 with gentle shaking for 30 min with one change of detergent solution. The gel was rinsed and incubated for 24 h at 37 °C in

substrate buffer (50 mM Tris-HCl, pH 7.5, 5 mM $CaCl_2$, and 0.02% NaN_3). Following incubation, the gel was stained with 0.05% Coomassie Brilliant Blue G-250 and destained in 10% acetic acid and 20% methanol.

Promoter Assay—A 1305-bp DNA fragment (−1285 to +20), corresponding to the promoter of the human *MMP-9* gene (37), was generously gifted by Dr. Seung-Taek Lee (Yonsei University, Korea) (38). For mt-AP-1 of the *MMP-9* gene promoter, in which distal and proximal AP-1-binding sites (−533 to −527 and −79 to −73, respectively) were destroyed, 5′-TGAGTCA-3′ was changed to 5′-TGAGTtg-3′ (underlined lowercase letters indicate the mutated bases) by the QuickChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA). For mt-NF-κB of the *MMP-9* promoter, in which an NF-κB-binding site (−600 to −590) was destroyed, 5′-GGAATTCCCC-3′ was mutated into 5′-Gatcgat-CCC-3′. After subcloning the mutant *MMP-9* promoters into a promoterless luciferase expression vector, pGL3 (Promega), the corresponding mutations in the constructs were verified by DNA sequencing. The pGL3 vector containing wild-type or mutant *MMP-9* promoter was transfected into MDA-MD-231 cells by using Lipofectamine. Luciferase activity in cell lysate was measured using Promega luciferase assay system according to the instructions of the manufacturer. To normalize luciferase activity, each of the pGL3 vectors was co-transfected with a pRL-SV40ΔEnh, which expresses *Renilla* luciferase by an enhancerless SV40 promoter (38).

Electrophoretic Mobility Shift Assay—Cells were incubated with serum-free medium for 4 h and nuclear extracts were prepared as described previously (39). A double-stranded oligonucleotide probe corresponding to the putative AP-1-binding site (−86 to −66; 5′-TGACCCCTGAGTCAGCACTTG-3′; the AP-1 recognition sequence is underlined) in the proximal *MMP-9* promoter sequences were labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase and purified by a G-50 Sephadex column. The ^{32}P-labeled probes (~40,000 cpm) were then incubated with nuclear extracts (10 μg of protein) for 20 min at room temperature. Samples were resolved on a native 5% polyacrylamide gel, and the gel was dried and subjected to autoradiography. Specificity for binding of AP-1 factors to the corresponding sequence of the *MMP-9* promoter was confirmed by using a cold competitor having a typical AP-1 binding sequence (Promega).

RESULTS

Endogenous Expression of CD99 Isoforms in Human Breast Carcinoma Cell Lines and Generation of Transfectant Clones for Each CD99 Isoform—We first performed RT-PCR analysis using total RNA from three human breast cancer cell lines to examine the production of the two CD99 mRNA isoforms. Nucleotide primers specific for CD99 type I and type II cDNAs generated a 583- and a 515-bp PCR product, respectively, from RNAs obtained from a highly metastatic breast cancer cell line, MDA-MB-435 (Fig. 1*B*). Immunoblotting analysis also showed that both CD99 type I and type II proteins were present in MDA-MB-435 cells. MCF-7, a noninvasive breast cancer cell line, was shown to express CD99 type I at a similar level to MDA-MB-435, whereas CD99 type II transcript and protein were not detected in MCF-7. Meanwhile, an invasive but weakly metastatic cell line, MDA-MB-231, revealed loss of expression of both CD99 types. These results suggest that both *CD99* gene transcription and alternative splicing events generating CD99 type I and type II mRNAs are regulated in a cell lineage-specific manner in human breast cancer. We next transfected the CD99-deficient MDA-MB-231 cells with the two CD99-type cDNAs. Cells transfected with an empty pcDNA3 vector served as the mock transfectant. MCF-7 cells exclusively expressing CD99 type I only underwent CD99 type II transfection. Expression of CD99 type I and type II in the resultant stable transfectant clones was analyzed by RT-PCR and immunoblotting analyses. Among the transfectant clones of MDA-MB-231 cells, a single CD99 expressing clone was identified for each CD99-type construct, although the levels of CD99 mRNA and protein in the CD99 type II transfectant clone were much lower than those in the CD99 type I transfectant clone (Fig. 1*C*). For MCF-7 cells, three CD99 type II transfectant clones displayed a 515-bp PCR product from RT-PCR analysis and 28-kDa protein from immunoblotting analysis, indicating successful expression of the exogenous CD99 type II gene in those transfectant clones. To confirm CD99 protein expression at the cell surface in the CD99 transfectant clones, we examined the protein levels of CD99 proteins by flow cytometry using an anti-CD99 mAb. Cell surface expression of the CD99 protein in both the CD99 type I- and type II-transfected MDA-MB-231 clones was prominent compared with the mock transfectant cells (Fig. 1*D*). A significant increase in the cell surface expression of CD99 proteins was also observed in the three CD99 type II transfectant clones of MCF-7 cells.

Cell Growth, Homotypic Cell Adhesion, and Invasiveness of CD99 Type I and Type II Transfectants—The *in vitro* cell growth rates of the CD99 transfectant clones were measured by MTT assay. Minimal difference in the cell growth rate between both CD99-type transfectants and the mock transfectant was observed in MDA-MB-231 cells (Fig. 2*A*). Also, in MCF-7 cells having endogenous CD99 type I, CD99 type II expression had no significant effect on cell growth rate. These data revealed that neither CD99 type I nor CD99 type II affected the cell growth of these two breast cancer cell lines.

Because CD99 isoforms have been shown to be involved in intercellular adhesion of lymphocytes (11, 20), we examined if the CD99 proteins also regulate the homotypic adhesion of epithelial cells such as breast cancer cells. Single cell suspensions of the CD99 transfectant clones were prepared and allowed to aggregate in saline without Ca^{2+} and Mg^{2+} for 1 h. For MDA-MB-231 cells, homotypic cell adhesion of the CD99 type I transfectant appeared to be ~2-fold higher than that of the mock transfectant (Fig. 2*B*). When cell surface CD99 proteins were ligated with anti-CD99 mAb DN16, the CD99 type I-transfected MDA-MB-231 clone exhibited a 4-fold higher cell aggregation than the mock transfectant. In contrast, CD99 type II transfection did not affect homotypic cell aggregation of MDA-MB-231 and MCF-7 cells. In addition, little effect of CD99 ligation on cell aggregation was observed in the CD99 type II-transfected MDA-MB-231 clone. Furthermore, CD99 type II expression abolished the positive effect of CD99 ligation on the intercellular adhesion of MCF-7 cells having endogenous CD99 type I, indicating that CD99 type II counteracts the cell aggregation-inducing role of CD99 type I. These results suggest that

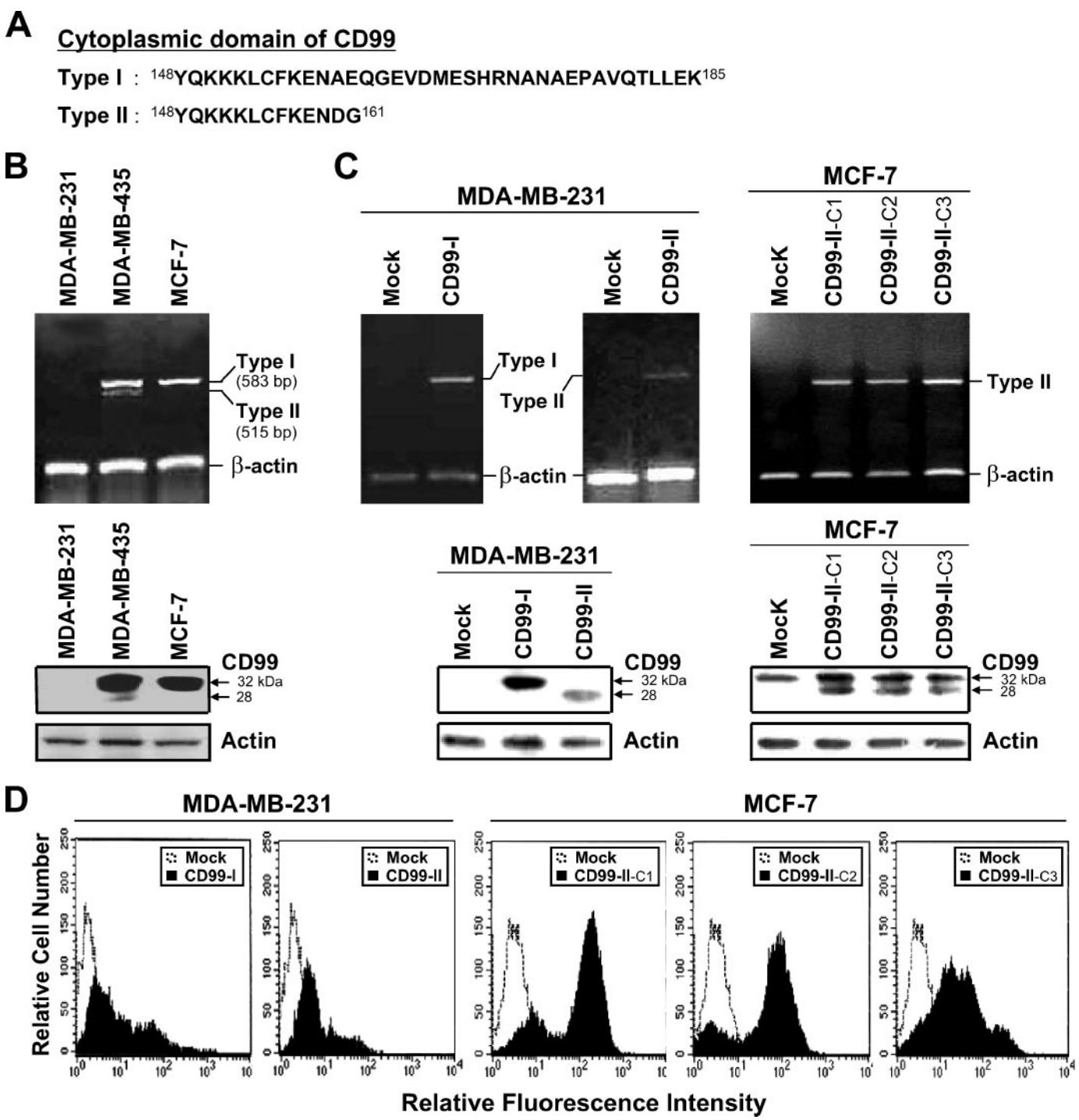


FIGURE 1. **Expression of endogenous and exogenous CD99 type I and type II in human breast carcinoma cell lines.** *A*, amino acid sequences of the cytoplasmic domains of CD99 type I and type II are aligned for comparison. *B* and *C*, RT-PCR and immunoblotting analyses for mRNA and protein levels of CD99 type I and type II in the parental lines and transfectant clones of human breast cancer cells. *Upper panels*, CD99 mRNAs in total RNA (1 μg) were obtained from three human breast carcinoma cell lines, MDA-MB-231, MDA-MB-435, and MCF-7 (*B*), and CD99 type I- or type II-transfected MDA-MB-231 and MCF-7 (*C*). Stable clones were amplified by RT-PCR with specific primers as described under "Experimental Procedures." Each amplified product was electrophoresed on a 1.5% agarose gel. β-Actin mRNA from each cell line and transfectant clone was also analyzed to control for equal RNA amounts. *Lower panels*, total cell lysates (100 μg of protein) from the human breast carcinoma cell lines (*B*) and the CD99 type I or type II transfectant clones (*C*) were electrophoresed on a 15% SDS-PAGE under reducing conditions, transferred to a PVDF membrane, and probed with anti-CD99 mAb DN16. *D*, flow cytometric analysis of CD99 proteins on the cell surface in stable CD99 transfectant clones of MDA-MB-231 and MCF-7 cells. Cells were first stained with DN16 mAb, washed, and stained with fluorescein isothiocyanate-conjugated anti-mouse IgG.

CD99 type I and type II function as a positive and negative regulator, respectively, in homotypic cell adhesion of human breast cancer cells, similar to their roles in lymphocytes (20).

To explore if either of the CD99 isoforms can modulate the invasive ability of breast cancer cells, the invasive efficacy of each transfectant was determined in *in vitro* cell invasion sys-

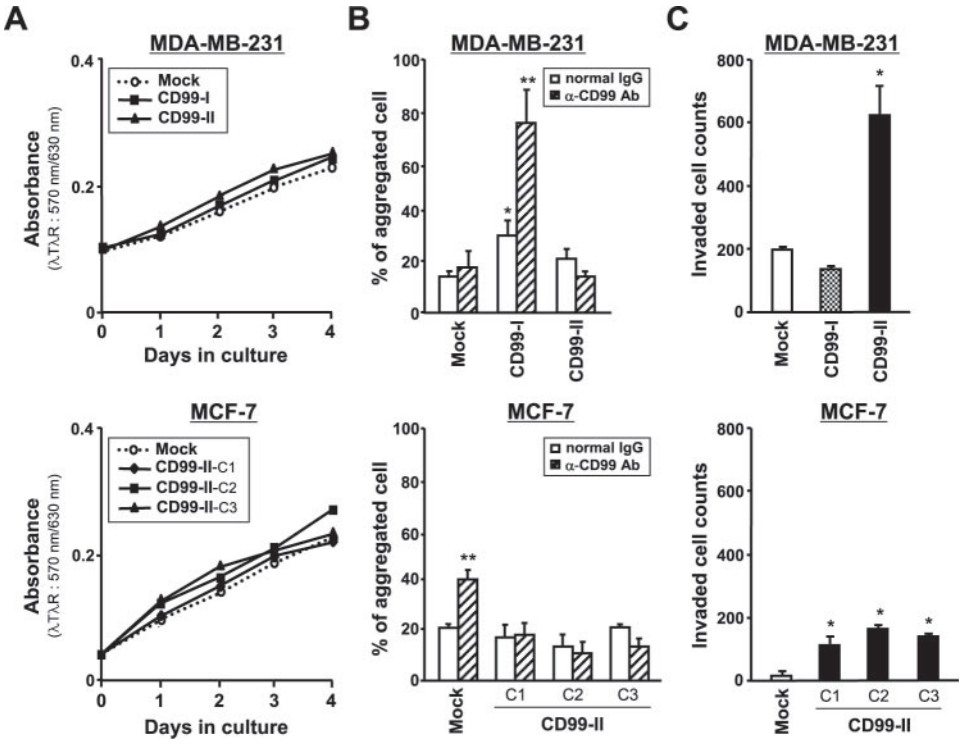


FIGURE 2. **Effect of CD99 type I and type II expression on the proliferation, homotypic aggregation, and invasiveness of MDA-MB-231 and MCF-7 breast cancer cells.** *A*, *in vitro* cell growth analyses of the CD99 type I and type II transfectant clones. Approximately 2×10^3 viable cells were seeded in each well of 96-well culture plates and incubated at 37 °C for the periods indicated, and cell growth was quantified by MTT assay. Data represent the mean of triplicate cultures. *B*, homotypic cell aggregation of the CD99 type I and type II transfectant clones. Each transfectant clone (1×10^5 cells/ml) was pretreated with either anti-CD99 mAb DN16 or mouse IgG for 2 h and then with goat anti-mouse IgG for 3 h. After disrupting cell aggregates by pipetting, the cells were incubated in Puck's saline with orbital shaking. The degree of aggregation was examined under a microscope, and the result was expressed as a percent of total cell numbers forming aggregates at 1 h after incubation. Data represent the mean ± S.E. of triplicate determinations. *Asterisks* indicate that the differences are statistically significant (*, $p < 0.01$ *versus* mock transfectant; **, $p < 0.01$ *versus* normal IgG-treated cells, Student's *t* test). *C*, *in vitro* invasiveness of the CD99 type I and type II transfectant clones. Each transfectant clone (5×10^4 cells) was seeded in a Transwell chamber insert equipped with a Matrigel-coated filter. After 12 h of incubation, cells on the lower surface of the filter were stained with Gill's hematoxylin and counted. Results are mean ± S.E. of triplicate cultures. *Asterisks* indicate that the differences are statistically significant (*, $p < 0.01$ *versus* mock transfectant; Student's *t* test).

tem using a thick Matrigel layer and fibroblast culture supernatant as a cell-migrating barrier and chemoattractant, respectively. As shown in Fig. 2*C*, CD99 type I expression did not affect the invasiveness of the MDA-MB-231 cells. However, the CD99 type II transfectant clone of MDA-MB-231 cells showed significantly increased invasiveness as compared with the mock transfectant. Also, CD99 type II expression appeared to enhance the invasive ability of the poorly invasive MCF-7 cells. These results revealed that CD99 type II, but not the type I, elevates the invasive ability of human breast cancer cells.

Cell Adhesion to Fibronectin, Cell Motility, and Collagen Degrading Activity of CD99 Type I and Type II Transfectants—In addition to homotypic cell adhesion, adhesion of cancer cells to extracellular matrix (ECM) components and basement membrane are frequently altered during the process of invasion and metastasis. Therefore, we examined the effect of CD99 isoform expression on the binding of MDA-MB-231 and MCF-7 cells to ECM proteins by using the cell attachment assay. As a result, the CD99 type II transfectant clones of both MDA-MB-231 and MCF-7 cells exhibited increased binding ability to fibronectin compared with the mock transfectant cells (Fig. 3*A*), indicating that CD99 type II expression facilitates adhesion of breast cancer cells to fibronectin. Because the formation of strong adhesions between the cell and ECM in the substratum has been known to be one of the important biophysical parameters for animal cell migration (40), we next performed a wound migration assay to compare cell motility of the CD99 transfectants. Little difference in the motility of MDA-MB-231 cells was seen between the CD99 type I and the mock transfectant. In contrast, the CD99 type II transfectant clones of MDA-MD-231 and MCF-7 cells exhibited a 5- and 3-fold higher migrating ability than the mock transfectants, respectively (Fig. 3*B*). Thus, CD99 type II appears to stimulate the migrating ability of breast cancer cells.

Because degradation of the basement membrane by cancer cells is a critical event in the cancer invasion and metastasis process, we compared the activity of matrix-degrading enzymes in the culture supernatant between the CD99 isoforms and mock transfectants by using the zymogram assay. As shown in Fig. 3*C*, two types of MMPs exhibiting collagen-digesting activity, MMP-2 (72-kDa type IV collagenase) and MMP-9 (92-kDa type IV collagenase), were detected in the culture supernatant of MDA-MB-231 cells. The CD99 type I transfectant showed significantly reduced activity of MMP-2 when compared with the mock transfectant despite a slight increase in MMP-9 activity. However, a large increase in MMP-9 activity was observed in the CD99 type II transfectant, along with a high MMP-2 activity level similar to that of the mock transfectant. The positive effect of CD99 type II expression on MMP-9 activity was also observed in MCF-7 cells, where MMP-9 activity reached a detectable level only after CD99 type II transfection (Fig. 3*C*). In addition to the enzyme activity of MMP-9, the protein level of MMP-9 in MDA-MB-231 cells was shown to be increased by CD99 type II transfection (Fig. 3*D*), indicating that the stimulating effect of CD99 type II on MMP-9 activity is attributed to the increased expression of MMP-9 by CD99 type II. It thus appears that CD99 type II expression increases the matrix degrading activity of breast cancer cells by up-regulating MMP-9 expression.

Alterations in the Invasive Phenotype of MDA-MB-435 Cells after Knockdown of CD99 Type II by siRNA Transfection—To confirm the positive effect of CD99 type II on the invasive properties of breast cancer cells, an siRNA targeted to the CD99 type

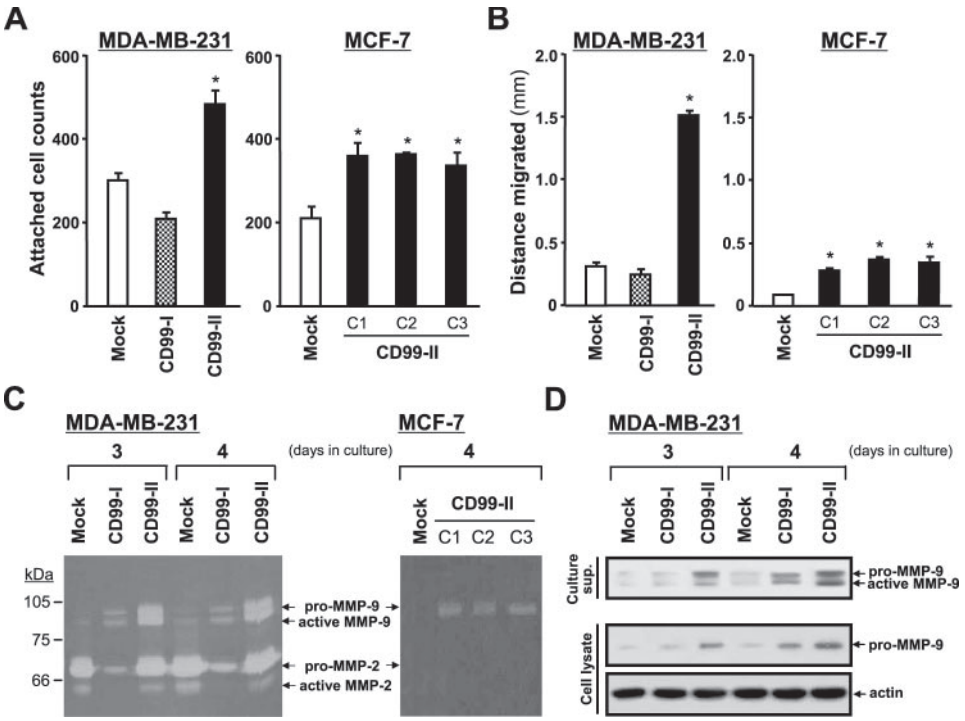


FIGURE 3. **CD99 type II expression increases adhesion to fibronectin, motility, and MMP-9 production of MDA-MB-231 and MCF-7 breast cancer cells.** *A*, adhesion of the CD99 type I and type II transfectant clones to fibronectin. Cells (5×10^4) were dispensed into individual wells of a 24-well plate that had been coated with fibronectin at a concentration of 10 $\mu g/cm^2$. After 1 h of incubation, unbound cells were aspirated, and attached cells were stained with hematoxylin and counted under a light microscope. Data represent the mean ± S.E. of triplicate determinations in two independent experiments. *Asterisks* indicate that the differences are statistically significant (*, $p < 0.03$ *versus* mock transfectant; Student's *t* test). *B*, *in vitro* cell motility of the CD99 type I and type II transfectant clones. Confluent cell cultures were wounded with plastic micropipette tips. Cells were photographed at 48 h after wounding by phase contrast microscopy, and the measurement of cell migration during wound healing was performed as described under "Experimental Procedures." Results are the mean ± S.D. of triplicate cultures. *Asterisks* indicate that the differences are statistically significant (*, $p < 0.01$ *versus* mock transfectant; Student's *t* test). *C*, zymogram depicting matrix metalloproteinase activities in cultures of the CD99 type I and type II transfectant clones. Conditioned medium obtained from each transfectant clone cultured in serum-free medium for the indicated time periods was electrophoresed on a 10% SDS-polyacrylamide gel containing type A gelatin. After removal of SDS, the gels were incubated in gelatinase substrate buffer and then visualized by Coomassie staining. *D*, immunoblotting analysis of MMP-9 protein in culture supernatant and cell lysate of the CD99 type I and type II transfectant clones. Conditioned medium and cell lysate from each transfectant clone was electrophoresed on a 10% SDS-polyacrylamide gel, transferred to a PVDF membrane, and probed with anti-MMP-9 mAb.

II mRNA-specific sequence was transfected into MDA-MB-435 breast cancer cells possessing both endogenous CD99 isoforms. As a result, the protein level of CD99 type II in MDA-MB-435 cells was decreased by CD99 type II siRNA in a dose-dependent manner, whereas CD99 type I level was not affected by the type II siRNA (Fig. 4*A*). In *in vitro* Matrigel invasion assay, knockdown of CD99 type II by siRNA transfection resulted in a significant decrease in invasiveness, demonstrating a positive role of CD99 type II in the invasive ability of MDA-MB-435 cells (Fig. 4*B*). Furthermore, the CD99 type II knockdown cells exhibited much lower migrating ability than the control siRNA-transfected cells retaining the endogenous CD99 type II level (Fig. 4*C*). MMP-9 activity in MDA-MB-435 cell culture was also decreased when the CD99 type II level was reduced by siRNA transfection (Fig. 4*D*). These results strongly support the positive role of CD99 type II in the regulation of invasive potential of human breast cancer cells.

AP-1-mediated Induction of MMP-9 Expression by CD99 Type II—Because MMP-9 appeared to be a target gene up-regulated by the CD99 type II signaling pathway(s) in breast cancer cells, we investigated the transcriptional regulation mode of the *MMP-9* gene by using several mutants of its 5′-proximal promoter region. When a reporter vector containing a wild-type promoter of the *MMP-9* gene was transiently transfected into MDA-MB-231 cells, the CD99 type II transfectant cells showed about a 20-fold higher luciferase activity than the mock transfectant cells (Fig. 5*A*). In contrast to the wild-type promoter, the promoters having mutations at the AP-1-binding sites (mt-5′-AP-1 and mt-3′-AP-1) did not respond to CD99 type II for their activities for the reporter gene expression. However, mutation of the NF-κB-binding site did not abolish the stimulating effect of CD99 type II on *MMP-9* promoter activity. Therefore, these data suggest that CD99 type II-stimulated *MMP-9* gene transcription is mediated by AP-1 transcription factors. To determine whether CD99 type II activation results in increased binding of AP-1 factors to the AP-1 recognition sequence in the *MMP-9* promoter region, we compared the binding of nuclear proteins to the putative AP-1-binding site (−79 to −73) of the *MMP-9* promoter between CD99 type I- and type II-transfected MDA-MB-231 cells. As shown in Fig. 5*B*, the DNA binding activity of AP-1 factors in CD99 type II transfectant cells was significantly increased when CD99 was ligated by the anti-CD99 mAb followed by γ-chain-specific secondary antibody. However, CD99 type I ligation with antibody did not enhance AP-1 binding to the DNA. Interestingly, incubation of the nuclear extract obtained from CD99 type II-activated cells with anti-JunD antibody resulted in a partial supershift of the AP-1-DNA complex in the gel shift assay, whereas incubation with anti-c-Jun and anti-JunB did not. These data indicate that, among the Jun family members, JunD is the AP-1 factor that participates in the complex formation between CD99 type II-induced AP-1 proteins and *MMP-9* promoter DNA. Thus, CD99 type II appears to up-regulate *MMP-9* gene transcription by increasing the binding of the JunD AP-1 transcription factor to the *MMP-9* promoter DNA. In addition to MMP-9 expression, expression of the standard form of CD44, a cell surface molecule that has been shown to be associated with invasion and metastasis of various human cancers (41–45) was also increased by CD99 type II ligation with antibody (Fig. 5*C*). Because the *CD44* promoter was also shown to be AP-1-responsive (46–48), there is a possibility that CD99 type II acti-

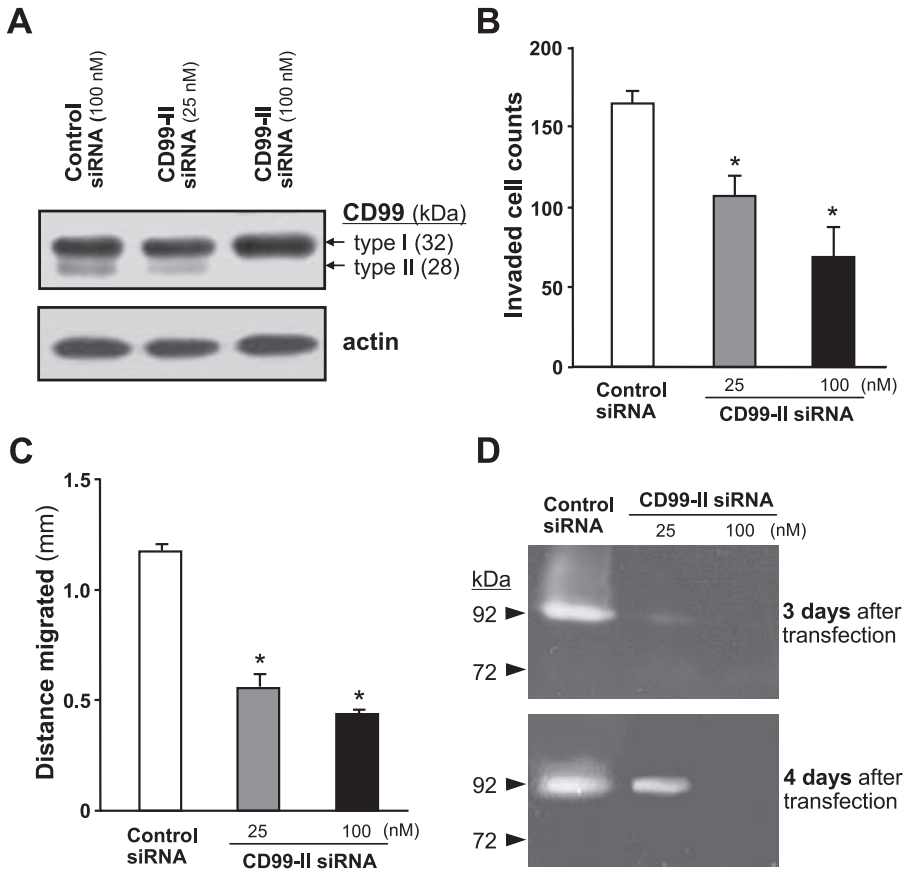


FIGURE 4. **Invasive potential of MDA-MD-435 cells is suppressed by CD99 type II-specific siRNA transfection.** MDA-MB-435 breast cancer cells having endogenous CD99 type I and type II were transfected with control siRNA or CD99 type II siRNA. *A*, protein levels of CD99 isoforms were analyzed by immunoblotting with DN16 mAb after 48 h of siRNA transfection. Invasiveness (*B*), motility (*C*), and gelatinase activity of the siRNA-transfected MDA-MB-435 cells (*D*) were determined in a similar fashion as in Fig. 2*C* and Fig. 3, *B* and *C*, respectively. *Asterisks* in *B* and *C* indicate that the differences are statistically significant (*, $p < 0.01$ *versus* control siRNA-transfected cells, Student's *t* test).

vation may activate a multigenic invasion program through an AP-1-directed increase in expression of invasion-related genes such as *MMP-9* and *CD44*.

Increased Expression of JunD and FosB by CD99 Type II Activation and Its Functional Effect on Cell Motility and MMP-9 Expression—We examined which kinds of AP-1 proteins are induced by CD99 type II-mediated signaling events in MDA-MB-231 breast cancer cells. When CD99 proteins were ligated with anti-CD99 mAb, the CD99 type II transfectant cells exhibited significantly increased protein levels of JunD and FosB, among the seven AP-1 factor members (Fig. 6*A*). In contrast, antibody ligation of CD99 type I did not affect the levels of any of the AP-1 proteins. Although CD99 type I activation also increased the JunD level to some degree, the level of JunD induced by CD99 type I was much lower than that of type II. In addition, incubation of CD99 type II transfectant cells with normal IgG did not increase the expression of JunD and FosB (Fig. 6*B*), demonstrating the specific effect of CD99 type II activation on the expression of JunD and FosB. Meanwhile, both Fra1 and Fra2 protein levels in CD99 type II transfectant cells were found to be higher that those in the type I transfectant cells (Fig. 6*A*). However, no effect of CD99 antibody ligation on the expression of these Fra proteins was observed in CD99 type II transfectant cells or type I transfectant cells. These results indicate that CD99 type II activation, but not type I activation, up-regulates protein levels of JunD and FosB in MDA-MB-231 breast cancer cells.

To investigate whether CD99 type II-stimulated cell motility and MMP-9 expression are mediated by JunD and FosB, we transfected siRNAs targeted to JunD and FosB into CD99 type II transfectant cells. As a result, both JunD and FosB siRNA-transfected cells did not respond to CD99 antibody ligation for the induction of JunD and FosB (Fig. 6*C*). Furthermore, knockdown of either JunD or FosB abolished the stimulating effect of CD99 type II activation on MMP-9 expression. In addition, motility of MBA-MD-231 cells was not increased by CD99 type II activation when JunD or FosB was knock downed (Fig. 6*D*). These data strongly suggest that both JunD and FosB AP-1 proteins are the major transcription factors that control CD99 type II-induced gene expression in MDA-MB-231 breast cancer cells.

Signal Transducing Molecules Involved in CD99 Type II Signaling Pathway(s)—To identify the intracellular signal transducing molecules that participate in CD99 type II signaling pathway(s), the activation status of several signaling mediators was examined after CD99 type II was activated by antibody ligation. As shown in Fig. 7*A*, CD99 type II activation significantly elevated phosphorylation-dependent activation of signaling components such as Src, Akt, p38 MAPK, ERK1/2, JNK, and c-Jun. Interestingly, the phosphorylation levels of Akt and ERK1/2 were increased only by CD99 type II activation, whereas FAK phosphorylation was increased only by the type I activation, indicating a distinct difference between CD99 type I and type II signaling pathways. We next examined the involvement of these signal transducing molecules in CD99 type II signaling in JunD and FosB expression by using inhibitors that block the activation of Src, p38 MAPK, ERK, JNK, and Akt. As a result, induction of JunD and FosB by CD99 type II activation was blocked by a MEK-ERK inhibitor (PD98059), a JNK inhibitor (SP699125), and a PI3K-Akt inhibitor (wortmannin), as well as by an Src kinase inhibitor (PP2) but not by a p38 MAPK inhibitor (SB203580) (Fig. 7*B*). In addition to PP2, wortmannin inhibited CD99 type II-mediated phosphorylation of ERK. Phosphorylation of JNK by CD99 type II signaling was also inhibited by the PI3K-Akt and the MEK-ERK pathway blocker. Therefore, it is likely that pathway cross-talk exists between the Akt and MAPKs signaling pathways in MDA-MB-231 breast cancer cells. These results suggest that activation of ERK, JNK, and Akt may play an important role in

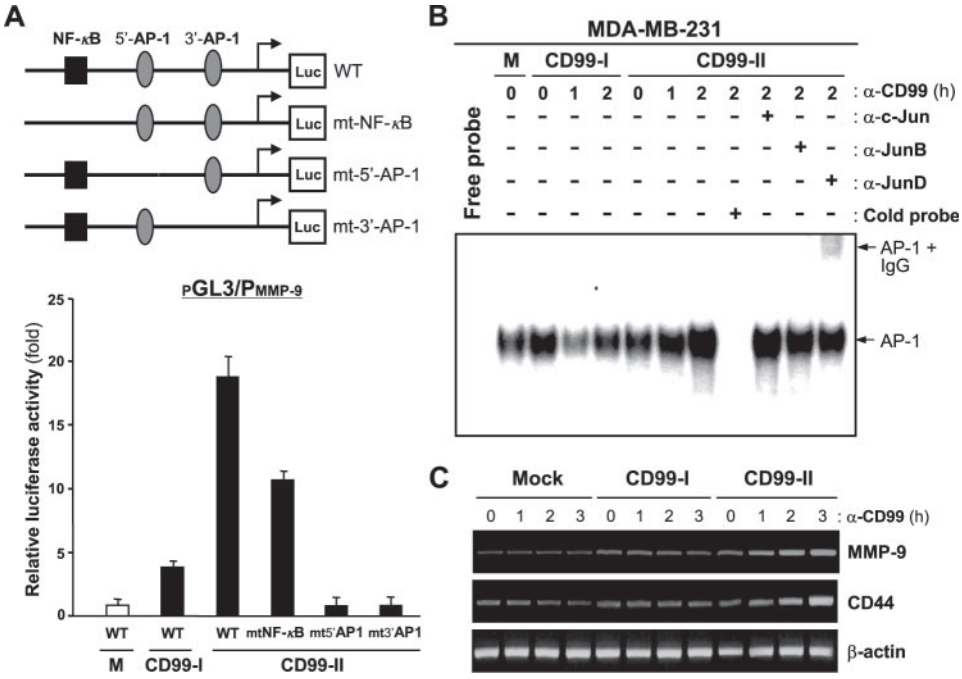


FIGURE 5. **CD99 type II-induced MMP-9 expression is mediated by JunD binding to the AP-1 sites in the *MMP-9* gene promoter.** *A*, human wild-type (*WT*) promoter of *MMP-9* gene and the mutant promoters for an NF-κB-binding site (*mt-NF-κB*) or AP-1-binding sites (*mt-5′-AP-1* and *mt-3′-AP-1*) were fused to a luciferase (*Luc*) reporter gene of a pGL3 vector. Mock (*M*) and CD99 type I and type II transfectants of MDA-MB-231 cells were transiently transfected with the *MMP-9* promoter/luciferase construct (0.2 μg) by using Lipofectamine. At 48 h after transfection, a dual luciferase assay was performed. Normalized luciferase expression + S.D. of triplicate experiments are plotted. *B*, the mock (*M*) and CD99 type I and type II transfectant cells were first serum-starved for 3 h. The cells were treated with anti-CD99 mAb DN16 for 2 h and then with goat anti-mouse IgG for the periods indicated. Nuclear extracts (10 μg of protein) of antibody-treated cells were incubated with a ^{32}P-labeled oligonucleotide representing nucleotides −86 to −66 of the *MMP-9* gene promoter, which contains a putative AP-1-binding site. Specific DNA binding activity of AP-1 factors was determined by electrophoretic mobility shift assay in the absence or presence of a 10-fold excess of cold competitors. Anti-c-Jun, anti-JunB, and anti-JunD antibodies were used for supershift analysis. *C*, serum-starved mock and CD99 type I and type II transfectants of MDA-MB-231 cells were treated with anti-CD99 mAb DN16 for 2 h and then with goat anti-mouse IgG for the indicated time periods. RT-PCR analysis was performed using specific primers for *MMP-9* and the standard *CD44* form as described under "Experimental Procedures."

transducing CD99 type II signals for the induction of JunD and FosB expression.

Functional Involvement of Akt, ERK, and JNK in CD99 Type II Signaling Pathway(s)—To verify the participation of Akt and ERK in the CD99 type II signaling pathways for the induction of cell motility and MMP-9 expression, the expression constructs encoding either an active form or a dominant-negative form of Akt1 and dominant-negative forms of ERK1 and ERK2 were transiently transfected into CD99 type II transfectant cells. The dominant-negative mutant of Akt1 suppressed the CD99 type II-induced expression of JunD and FosB in MDA-MB-231 cells (Fig. 8*A*). CD99 type II-stimulated MMP-9 expression and cell motility were also abrogated by the mutant Akt1. In contrast, the constitutively active form of Akt1 enhanced MMP-9 expression and cell motility as well as JunD and FosB protein levels even without CD99 type II activation. These data indicate an essential role of Akt in CD99 type II signaling events. Moreover, transfection of dominant-negative forms of ERK1 and ERK2 also attenuated the stimulating effect of CD99 type II signaling on the cell motility and expression of JunD, FosB, and MMP-9 (Fig. 8*B*). In addition, knockdown of JNK by siRNA transfection blocked CD99 type II-mediated signaling outcomes (Fig. 8*C*). However, p38 MAPK siRNA-transfected cells did not respond to CD99 type II activation for JunD-FosB-mediated MMP-9 expression and cell motility (Fig. 8*C*), indicating that p38 MAPK does not participate in the CD99 type II signaling pathways leading to increased JunD and FosB expression. Taken together, these results demonstrate that Akt1, ERK1/2, and JNK1 play an essential role in transducing the CD99 type II signal to the nucleus for the induction of JunD and FosB expression in MDA-MB-231 cells.

DISCUSSION

Differential expression of alternative splicing products has been known as physiological and pathological features of many cell adhesion molecules, such as $\alpha_L\beta_1$ integrin, neuronal cell adhesion molecule, platelet endothelial cell adhesion molecule-1, and vascular cell adhesion molecule-1 (25–27, 49). As one of the cell adhesion molecules, CD99 isoforms were also found to be differentially expressed in a cell type-specific manner among various human tumor cells lines and normal tissues (20). A splice variant form (type II) of CD99 was originally reported as an inhibitor for homotypic cell adhesion of lymphocytes, whereas the major CD99 form (type I) acts as an inducer for cell adhesion (20). In this study, homotypic aggregation of human breast cancer cells of epithelial origin was also found to be induced by CD99 type I activation, whereas co-expression of CD99 type II with type I inhibited homotypic cell adhesion induced by type I activation (Fig. 2*B*).

Because many types of cancer cells tend to undergo alterations in homotypic cell adhesion during invasion processes (50), we postulated that differential expression of the CD99 isoforms might affect the invasive potential of cancer cells. In this study, we demonstrated that CD99 type II, but not type I, stimulates the invasiveness of human breast cancer cells (Figs. 2*C* and 4*B*). The positive effect of CD99 type II expression on the invasiveness of breast cancer cells could be a mere result of reduced cell-to-cell adhesion in CD99 type II expressing cells. However, there is a possibility that invasion-related cellular activities other than homotypic cell adhesion, such as cell motility, cell adhesion to matrix, and matrix degradation, could also be altered by CD99 type II expression. We found that the migrating ability of breast cancer cells was significantly increased by CD99 type II (Figs. 3*B* and 4*C*). Also, CD99 type II expression was found to increase the ability of breast cancer cells to adhere to fibronectin (Fig. 3*A*). Although many factors have been known to be involved in the regulation of cell loco-

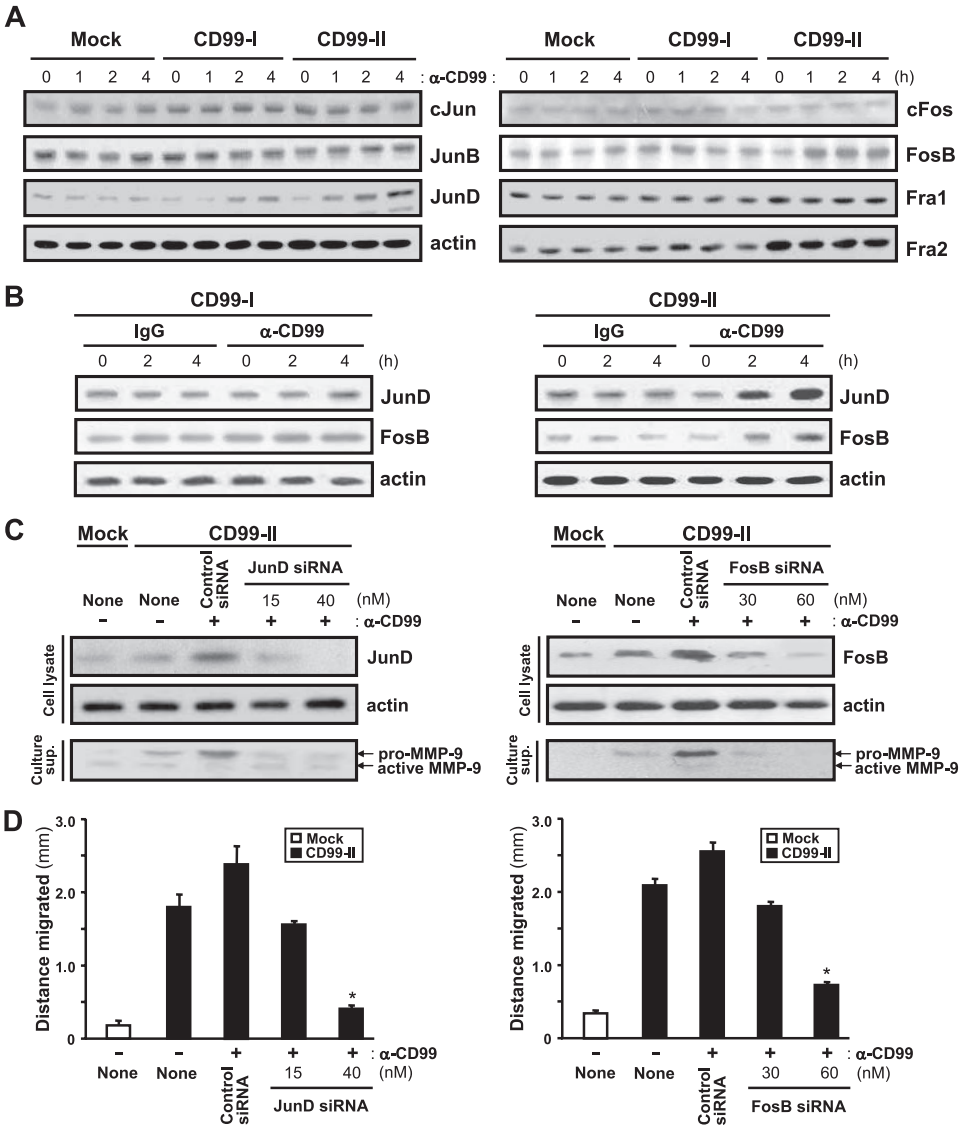


FIGURE 6. **CD99 type II activation by antibody ligation stimulates motility and MMP-9 expression of MDA-MB-231 cells by increasing protein levels of JunD and FosB.** *A*, serum-starved mock and CD99 type I and type II transfectants of MDA-MB-231 cells were treated with DN16 mAb for 2 h and then with goat anti-mouse IgG for the indicated time periods. Cell lysates were analyzed for protein levels of the AP-1 components by immunoblotting using specific antibodies for Jun and Fos subfamily AP-1 members. *B*, the CD99 type I and type II transfectant cells cultured in serum-free medium were treated with either anti-CD99 mAb DN16 or mouse IgG for 2 h and then with goat anti-mouse IgG for the indicated time periods. Protein levels of JunD and FosB in cell lysates were analyzed by immunoblotting using anti-JunD and anti-FosB mAbs. *C*, CD99 type II transfectant cells were transfected with control, JunD, or FosB siRNAs. After 48 h of transfection, the cells were serum-starved for 3 h and treated with either DN16 mAb or normal IgG for 2 h. Following CD99 ligation with secondary antibody, the siRNA-transfected cells were cultured in serum-free medium for 3 days and MMP-9 protein level in the conditioned medium was assessed by immunoblotting analysis with anti-MMP-9 mAb. Protein levels of JunD and FosB in the siRNA-transfected cells were analyzed at 4 h after antibody CD99 ligation. *D*, migration distance of siRNA-transfected cells for 72 h after antibody CD99 ligation was measured in a similar fashion as in Fig. 3*B*. *Asterisks* indicate that the differences are statistically significant (*, $p < 0.01$ *versus* control siRNA-transfected cells, Student's *t* test).

motion, increased cell binding to fibronectin by CD99 type II could contribute to the motility of breast cancer cells. Thus, the data in this study strongly suggest a positive role of CD99 type II in the regulation of breast cancer cell motility and invasiveness.

Because metastatic cells have to penetrate the basement membrane for intravasation as well as extravasation, local destruction of the basement membrane is also essential for cancer cells to metastasize. Among various proteolytic enzymes that are involved in digesting the basement membrane, MMPs have the ability to degrade most ECM proteins (51). In addition, MMPs have been shown to play a role in the initiation of cell movement on ECM (52). Type IV collagen, the main component of basement membranes, is thought to be degraded predominantly by two members of the MMP family, MMP-2 and MMP-9. In a previous report, all of 25 invasive breast cancers, but not any *in situ* malignancy, were found to be immunoreactive to type IV collagenases, suggesting the critical roles of MMP-2 and MMP-9 in the conversion of *in situ* breast cancers to invasive lesions (53). We found here that CD99 type II expression significantly increased the activity and expression of MMP-9 in MDA-MB-231 cells (Fig. 3, *C* and *D*). The positive effect of CD99 type II expression on MMP-9 activity was also seen in both noninvasive MCF-7 cells and metastatic MDA-MB-435 cells, where alterations in the CD99 type II level resulted in changes in MMP-9 activity (Figs. 3*C* and 4*D*). MMP-9 has been specifically associated with the metastatic phenotype (54, 55) and with undifferentiated and aggressive breast cancers (56). Invasiveness and metastases of MDA-MB-435 breast cancer cells were also shown to be effectively inhibited by an agent that suppresses MMP-9 expression (57, 58). Therefore, these results strongly suggest that an increase in MMP-9 expression by CD99 type II may be one of the major factors that cause breast cancer cells to acquire highly invasive and metastatic abilities.

Although alterations in CD99 expression have been demonstrated in a broader range of neoplastic human tissues, the actual relationship of its expression with the development of human cancers has been somewhat controversial. High CD99 expression has been shown in Ewing sarcoma, acute lymphoblastic lymphoma, synovial sarcoma, mesenchymal chondrosarcoma, and rhabdomyosarcoma (2, 3, 59–62). However, the weak or null expression of CD99 has been associated with the development of pancreatic and gastric cancers (63, 64). There is also conflicting evidence for a link between CD99 and the malignant progression of human cancers, such as cancer invasion and metastasis. A statistically significant relationship

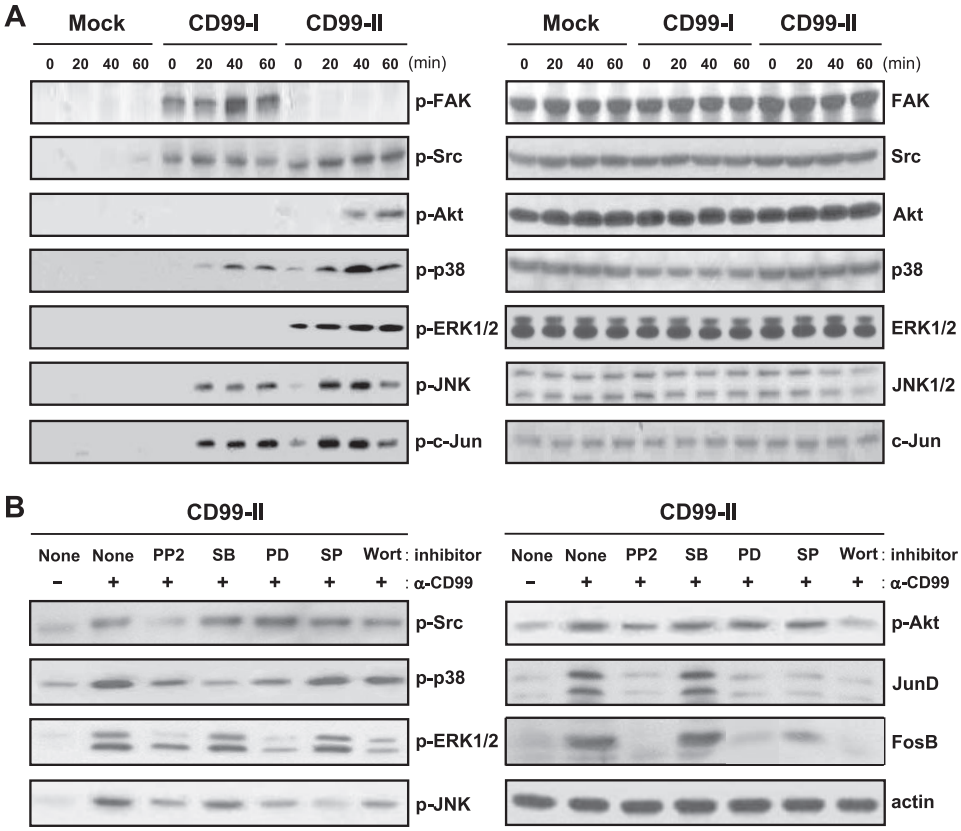
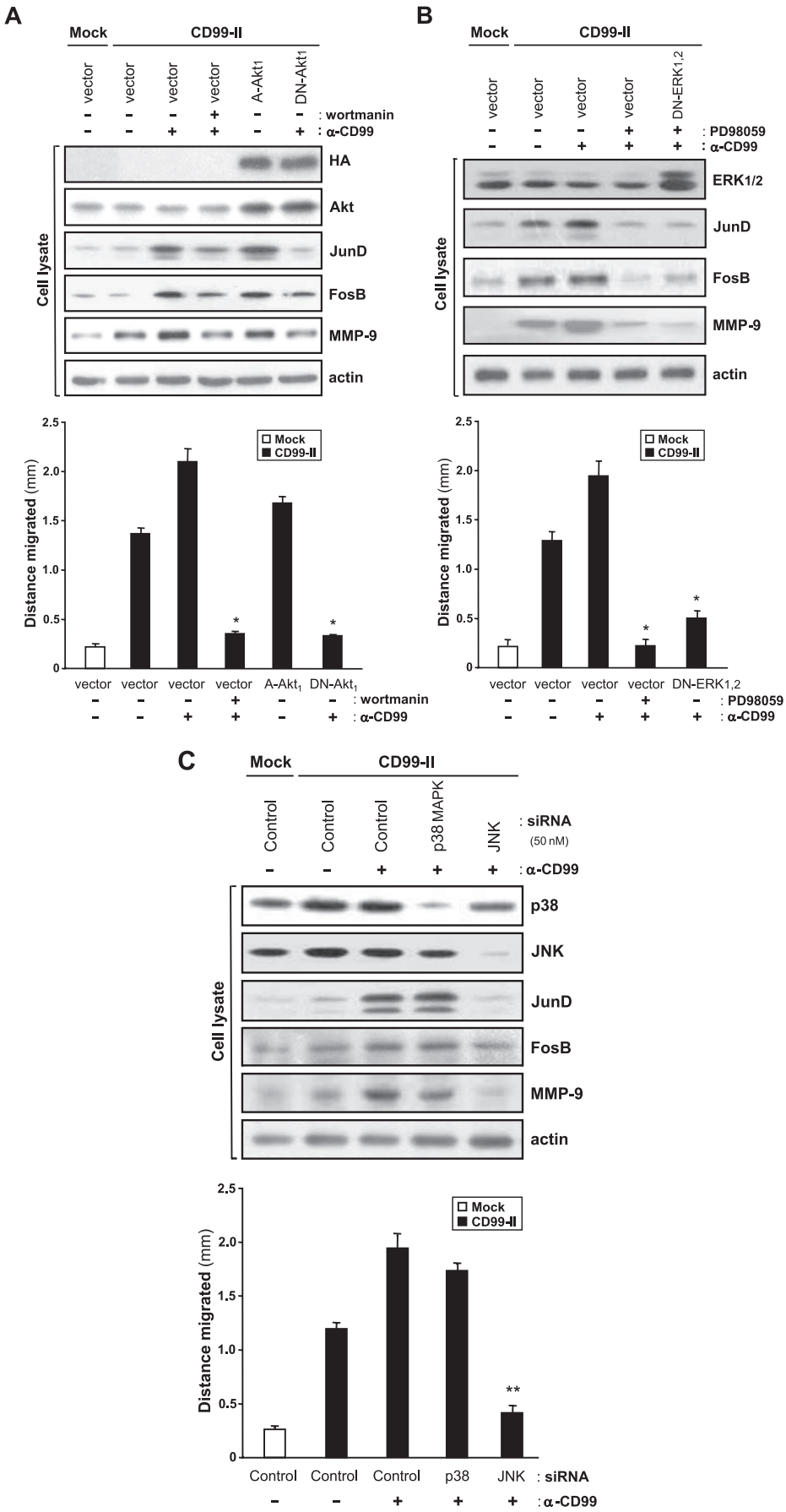


FIGURE 7. **Signal transducing molecules involved in CD99 type II-mediated signaling pathways for the induction of JunD and FosB expression in MDA-MB-231 breast cancer cells.** *A*, alterations in the phosphorylation levels of various intracellular signal transducers during CD99-mediated signaling events. Serum-starved mock and CD99 type I and type II transfectants of MDA-MB-231 cells were treated with anti-CD99 mAb DN16 for 2 h and then with goat anti-mouse IgG for the indicated time periods. Phosphorylation levels of the indicated signaling molecules in cell lysates were compared by immunoblotting analyses using specific antibodies for phospho-FAK$^{(Tyr925)}$, phospho-Src$^{(Tyr416)}$, phospho-Akt$^{(Ser473)}$, phospho-p38 MAPK$^{(Thr180/Tyr182)}$, phospho-ERK1/2$^{(Thr202/Tyr204)}$, phospho-JNK$^{(Thr183/Tyr185)}$, and phospho-c-Jun$^{(Ser63/Ser73)}$. The protein levels of FAK, Src, Akt, p38 MAPK, ERK1/2, JNK, and c-Jun were determined in the same blots by Western blotting analyses using specific antibodies for each protein. *B*, inhibitory effect of various signaling blockers on CD99 type II-induced JunD and FosB expression. The CD99 type II transfectant cells were cultured in serum-free medium containing PP2 (20 μM), PD98059 (*PD*, 50 μM), SB203580 (*SB*, 50 μM), SP600125 (*SP*, 50 μM), wortmannin (*Wort*, 2 μM) or Me_2SO (for vehicle control) for 15 h. The cells were treated with either anti-CD99 mAb DN16 or mouse IgG for 2 h and then with goat anti-mouse IgG for 40 min. Phosphorylation levels of the indicated signaling molecules in cell lysates were examined by immunoblotting analyses using specific phospho-antibodies used in *A*. For measurement of protein levels of JunD and FosB, cell lysates obtained at 4 h after CD99 ligation with secondary antibody were analyzed by immunoblotting with anti-JunD and anti-FosB antibodies.

between CD99-positive cells and the occurrence of local invasion and/or distant metastasis has been found in gastrointestinal and pulmonary neuroendocrine tumors (65). In contrast, a recent report showed that down-regulation of CD99 is associated with the metastatic phenotype of osteosarcoma. Meanwhile, in breast invasive carcinomas, no significant association was found between CD99 immunoreactivity and the metastatic stages of the tumors (66). We now speculate that this discrepancy may not be due only to the difference in cancer types but also because of the difference in the relative expression levels between two CD99 isoforms. The individual expression level of each CD99 isoform in tumors cannot be assessed by CD99 immunoreactivity using anti-CD99 antibodies, which do not distinguish between CD99 type I and type II. Because our current data implicate the functional involvement of CD99 type II, but not type I, in the development of invasive and metastatic phenotypes of human breast cancer cells, the actual relationship between CD99 expression level and malignant cancer progression needs to be examined by evaluating the expression level of each CD99 isoform separately.

Many genes that participate in tumor cell invasion and migration have been identified, including adhesion molecules, small GTPases, cytoskeletal components, and matrix metalloproteinases (67). However, there is little consensus on what controls the expression of these genes and how a program of gene expression is coordinated to manifest an invasive phenotype. In this study, we demonstrate that CD99 type II functions as a positive regulator in the expression of JunD and FosB, components of the AP-1 transcription complex. Increases in JunD and FosB protein levels by antibody ligation of CD99 were observed in CD99 type II transfectant cells but not in CD99 type I transfectant cells (Fig. 6, *A* and *B*), indicating the specific effect of CD99 type II signaling on the expression of JunD and FosB. Increased expression and activity of AP-1 component proteins have been shown to enhance invasion and motility in various model systems (68). Transformation with oncogenic forms of Fos or Jun proteins makes normal rat fibroblast cells invasive (48, 69), but dominant-negative mutants and antisense oligonucleotides for AP-1 component genes inhibit the invasion and migration of oncogenic AP-1-transformed and growth factor-stimulated fibroblasts (48, 70, 71). Overexpression of c-*JUN* induces the invasiveness of chick embryo fibroblasts and MCF-7 breast cancer cells (72, 73). FosB and Fra-1 were also shown to increase the invasiveness and motility of MCF-7 and MDA-MB-231 breast cancer cells (74, 75). Additionally, c-Fos and FosB were found to be significantly up-regulated in inflammatory breast cancer, a rare but particularly aggressive form of primary breast cancer, compared with non-inflammatory breast cancer (76). Interestingly, significant differences in JunD and FosB protein amounts were observed among various breast cancer specimens, along with a relatively uniform expression pattern for c-Jun, JunB, c-Fos, and Fra2 (77), suggesting that JunD and FosB may be the AP-1 proteins regulated during breast cancer development and progression. We show here that the stimulating effect of CD99 type II on motility of MDA-MB-231 cells was abolished when JunD and FosB were knock downed by siRNA transfection (Fig. 6*D*). Thus, the expression and activity of AP-1 factors, including JunD and FosB, are func-



tionally linked to breast cancer cell motility and invasiveness. The functional involvement of AP-1 activity in cancer invasion is more evident in the regulation of *MMP* gene expression. Inducible *MMP* genes share a consensus AP-1-binding site in their promoters, which is responsible for basal expression and responsiveness to various growth factors, cytokines, and tumor promoters (78). The 5′-proximal promoter region of the *MMP-9* gene contains putative binding sites for AP-1 (−79 and −533), NF-κB (−600), Sp1 (−558), and PEA3 (−540) (37). By using *MMP-9* gene promoters with mutations for the putative AP-1 sites, we here demonstrate that both distal and proximal AP-1-binding sites in the *MMP-9* gene promoter are essential for CD99 type II-induced *MMP-9* gene transcription (Fig. 5*A*). Results from gel mobility shift assay indicated that antibody ligation of CD99 type II, but not the type I ligation, increased the binding of nuclear proteins to the *MMP-9* promoter oligonucleotide containing the proximal AP-1-binding site (Fig. 5*B*). In particular, incubation with anti-JunD antibody resulted in a partial supershift of the nuclear proteins-DNA complex in the gel shift assay, indicating that CD99 type II-induced AP-1 proteins includes JunD, among the Jun family members of the AP-1 factors. Also, MMP-9 expression induced by CD99 type II ligation was abrogated by siRNAs targeted to JunD and FosB (Fig. 6*C*). Taken together, the CD99 type II-mediated signaling pathway(s) appears to increase the expression of the AP-1 transcription factors such as JunD and FosB, which, in turn, up-regulate the expression of genes involved in the invasion process of breast cancer cells, including the *MMP-9* gene.

Signaling pathways taken downstream of CD99 have been studied mostly in T lymphocytes, because CD99 has been found to be a co-stimulatory molecule for the T cell receptor (15, 16). CD99 ligation was shown to increase intracellular Ca^{2+} level, protein tyrosine phosphorylation, and phosphorylation levels of MAPKs in T cells, along with homotypic cell aggregation (15, 16, 21). CD99-induced aggregation of Jurkat T cells was inhibited by inhibitors for protein kinase C and protein-tyrosine kinases (22). It was also recently reported that CD99 co-stimulation with T cell receptor/CD3 enhances phosphorylation of JNK and AP-1-responding promoter activity in Jurkat T cells (79). However, it is completely unknown whether CD99-mediated signaling pathways are modulated by the differential expression of CD99 isoforms or whether each CD99 isoform provokes different sets of signaling pathways, although we previously reported the functional involvement of Src in CD99 splice variant-induced cell motility (80). We found here that a different set of signaling molecules is activated between the two CD99 isoforms-mediated signaling events in MDA-MB-231 breast cancer cells (Fig. 7*A*). The phosphorylation level of FAK was increased only when CD99 type I was activated by antibody ligation. In contrast, increased phosphorylation of Akt and ERK1/2 was observed in signaling events triggered by CD99 type II but not by type I. Although both CD99 isoform-mediated signaling pathways increase the phosphorylation levels of Src, p38 MAPK, JNK, and c-Jun, these data indicate that the CD99 splice variant provokes its own signaling pathways different from the major CD99 form. Because CD99 type I and type II differ from each other only in the intracellular C-terminal domain (Fig. 1*A*), it is very likely that the cytoplasmic tail of each CD99 isoform determines the direction of various intracellular signaling pathways.

AP-1 transcription factors are subject to regulation by MAPK signaling pathways with respect to biochemical activity, gene expression, and protein stability (81–83). MAPK pathways also play a critical role in transducing signals from the extracellular environment in normal and malignant breast tissue (84). In this study, among three major MAPK signaling pathways CD99 type II-mediated signaling events to increase the JunD and FosB AP-1 transcription factors were found to be regulated by the ERK and JNK pathways but not by the p38 MAPK pathway. Inhibitors, dominant-negative forms, and siRNAs for ERK1/2 and JNK1, but not for p38 MAPK, suppressed the stimulating effect of CD99 type II activation on JunD and FosB expression as well as cell motility and MMP-9 expression (Figs. 7*B* and Fig. 8, *B* and *C*). It thus appears that ERK1/2 and JNK are the MAPKs that participate in the CD99 type II-mediated JunD and FosB induction pathways. In addition to these two types of MAPKs, Akt/protein kinase B was also found to be involved in the CD99 type II signaling pathway. Pretreatment with wortmannin, a PI3K-Akt pathway blocker, and transfection of a dominant-negative form of Akt1 not only blocked CD99 type II-induced expression of JunD and FosB but also abolished CD99 type II-stimulated cell motility and MMP-9 expression (Figs. 7*B* and 8*A*). Interestingly, wortmannin blocked CD99 type II-mediated activation of ERK1/2 and JNK (Fig. 7*B*), suggesting that functional interplay through pathway cross-talk exists between the Akt and MAPKs signaling pathways in MDA-MB-231 breast cancer cells. The cross-talk between the MEK-ERK and the PI3K-Akt pathways has been demonstrated in MCF-7 breast cancer cells, where ligand type and concentration determine the direction and effect of cross-talk (85, 86). In addition, Akt was shown to directly interact with and phosphorylate MKK4, an upstream activator of JNK, indicating the regulatory function of the PI3K-Akt pathway on JNK signaling (87). It was also recently reported that positive cross-talk between the epidermal growth factor receptor and Akt pathways results in increased expression of c-Jun, JunB, JunD, and FosB, along with increased AP-1 activity, in HT29 human colon cancer cells (88). Taken together, it is very likely that Akt, ERK, and JNK contribute to the induction of AP-1 factors, including JunD and FosB, through pathway cross-talk in CD99 type II-specific signaling cascades in human breast cancer cells.

FIGURE 8. **CD99 type II-stimulated cell motility and MMP-9 expression in MDA-MB-231 breast cancer cells are mediated by Akt-, ERK-, and JNK-dependent AP-1 activation pathways.** The CD99 type II transfectant cells was transiently transfected with either a constitutively active form (*A-Akt1*) or dominant-negative form (*DN-Akt1*) of Akt1 with HA tag (*A*), a mixture of dominant-negative forms (*DN-ERK1,2*) of ERK1 and ERK2 (*B*), or siRNAs targeted to p38 MAPK and JNK (*C*). After 48 h of transfection, the cells were serum-starved for 6 h in the presence of wortmannin, PD98059, or Me_2SO and treated with either DN16 mAb or normal IgG for 2 h. Following CD99 ligation with secondary antibody for 4 h, cell lysates were analyzed for protein levels of the indicated molecules by immunoblotting. The data in the *lower panels* represent the migration distance of each DNA construct- or siRNA-transfected cells for 72 h after antibody CD99 ligation. *Asterisks* indicate that the differences are statistically significant (*, $p < 0.01$ *versus* pcDNA3 vector-transfected cells; **, $p < 0.01$ *versus* control siRNA-transfected cells, Student's *t* test).

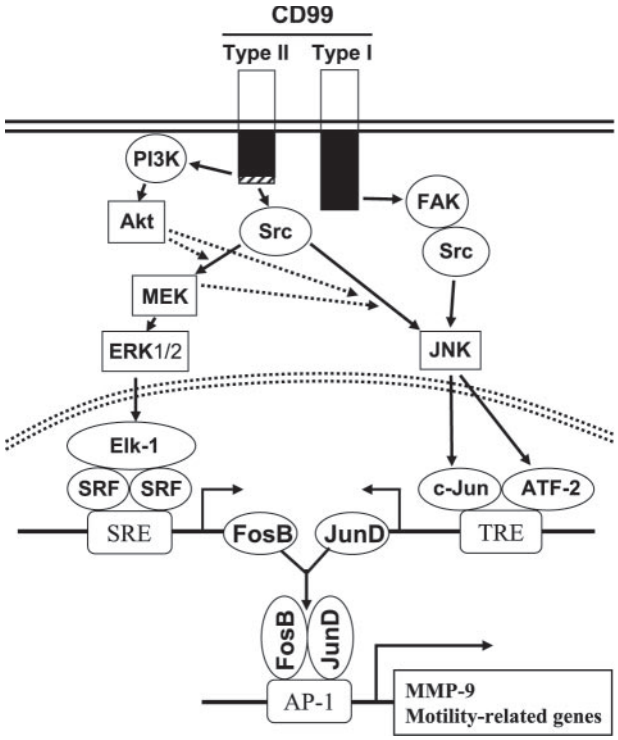


FIGURE 9. **Molecular mechanism of CD99 type II-induced AP-1 activation, AP-1-mediated MMP-9 expression, and cell motility through activation of Akt, ERK, and JNK.** Activation of CD99 type II protein induces the Src-mediated phosphorylation and activation of ERK1/2 and JNK. CD99 type II activation also induces the activation of Akt via PI3K, which leads to full activation of ERK1/2 and JNK through the pathway cross-talk, as illustrated by *broken arrows*. The highly activated ERK1/2 and JNK stimulate the Elk1-mediated *FOSB* gene expression and c-Jun-mediated *JUND* gene expression, respectively. The resultant JunD-FosB complex enhances the transcription of the AP-1-responsive genes, such as *MMP-9* and cell motility-promoting genes.

Based on the data in this study and the general information about the AP-1 activation signaling pathways, we propose the molecular mechanism of CD99 type II-induced JunD and FosB expression in breast cancer cells. As illustrated in Fig. 9, activation of CD99 type II proteins, presumably by their homophilic interaction between neighboring cells (18), induces activation of ERK and JNK taken downstream of Src. This activation was blocked by a PI3K-Akt inhibitor (wortmannin) (Fig. 7*B*), indicating that CD99 type II-mediated ERK and JNK activation is Akt-dependent. Also, inhibition of JNK phosphorylation by a MEK inhibitor (PD98059) indicates positive cross-talk between the MEK-ERK and JNK pathways. Activated ERK and JNK induce the Elk1-mediated FosB and c-Jun-mediated JunD transcription, respectively. The resultant FosB-JunD dimer up-regulates expression of AP-1-responsive genes involved in tumor cell migration and invasion, including *MMP-9* (Fig. 9). Meanwhile, CD99 type I activation may induce JNK activation through the FAK-Src pathway. However, the PI3K-Akt and MEK-ERK pathways were not activated by signals provoked by CD99 type I (Fig. 7*A*). Without activation of the MEK-ERK pathway, there is little or no induction of Elk1-mediated FosB transcription. In addition, the absence of Akt and MEK activation attenuates the JNK pathway that induces the c-Jun-mediated transcription of JunD (Figs. 6*A* and 7*A*). This difference between CD99 type I- and type II-mediated signaling pathways may be because of the different structure of intracytoplasmic fragments (Fig. 1*A*), which is thought to interact with intracellular signaling molecules.

In summary, we have demonstrated for the first time that expression and activation of a CD99 splice variant enhance the invasive ability of human breast cancer cells. The signaling pathways triggered by the CD99 splice variant induce the expression of AP-1 transcription factors such as JunD and FosB, through the activation of Src, Akt, ERK, and JNK, leading to increases in AP-1-mediated MMP-9 expression and cell motility in breast cancer cells. Positive cross-talk between the Akt, ERK, and JNK pathways also contributes to CD99 splice variant-mediated AP-1 activation. These findings may be useful in designing therapeutic interventions that block AP-1 induction via Akt-dependent activation of ERK and JNK by the CD99 splice variant, resulting in the reduction of MMP-9 expression and cell motility and consequently blocking the invasion and metastatic spread of malignant breast cancer.

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