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Analysis of the Dimerization of Human CD99 Using Bimolecular Fluorescence Complementation Technique

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Two isoforms of human CD99 have been identified, but only heterotypic interaction between the isomers was recently demonstrated. In this study, we performed bimolecular fluorescence complementation analysis to further characterize the interaction *in vivo.* Upon transiently transfecting plasmids expressing either of the two isoforms fused with yellow fluorescent protein (YFP) fragments, all the YFP-tagged CD99 molecules were properly localized on cell surfaces, and formed fluorescent dimers. Interestingly, however, unlike the previous report, the homodimers formed as efficiently as the heterodimer *via* their extracellular domains, implying its distinct regulatory role through modulating the complex profile.

Keywords: CD99, BiFC, isoform, dimer formation, YFP

Human CD99 is an integral transmembrane protein present on the surfaces of most cells from normal tissues, and has been implicated in various cellular events such as cell-cell adhesion during hematopoietic cell differentiation, selection process of thymocytes, apoptosis of neuronal cells, and T-cell activation [3, 5, 11, 19, 22].

CD99 was initially identified as a highly glycosylated 32 kDa protein with sialylation, in which the fully processed sugar residues account for 14 kDa [2, 9]. Interestingly, however, the *CD99* gene was later found to encode another 28 kDa protein produced by alternative splicing of its transcript, which contains a relatively short truncated intracytoplasmic fragment [11]. Recently, the opposing roles of the two isoforms have been reported in several cases. In B cells, the expression of the full-length long form promoted cell adhesion, whereas the truncated short form inhibited the process [1, 4]. Similar outcomes have been observed in some malignancies. The short-form CD99 remarkably favors migration and metastasis of osteosarcoma, whereas the long-form CD99 dramatically inhibits the phenomena, implying the opposite roles of the two isoforms as an oncoprotein and a tumor suppressor, respectively [17, 18]. It was also reported that when the two isoforms are coexpressed, CD99 heterodimers are formed and localized in rafts, but other remaining CD99 molecules are present as a CD99 monomeric form outside the rafts, indicating the absence of the homodimeric interaction [1].

Despite accumulated data on the CD99 functions, the biochemical and molecular analyses on the molecule have not been well established. Considering the opposite roles of the two CD99 molecules, the differential expression and interaction between them may determine the distinct fate of cells. Thus, in this study, to examine *in vivo* interactions between the CD99 molecules, we utilized the Bimolecular Fluorescence Complementation (BiFC) technique, in which each of two putative interacting protein partners is fused respectively to the complementary nonfluorescent half fragments of an autofluorescent protein, and depending on dimer formation between the test proteins, the subsequent positive interaction of the two nonfluorescent complementary fragments may result in restoration of fluorescence [12–16, 20, 21].

For the experiments, we fused the N-terminus (from 1 to 172 amino acid; YN) or the C-terminus (from 173 to 238 amino acid; YC) of YFP to the cytoplasmic ends of the two CD99 isoforms, the full-length long (L) form and the truncated short (S) form, resulting in four recombinant CD99 expression constructs (Figs. 1A and 1B). Thus, pCD99L/YN and pCD99L/YC contain the YN and the YC fragments fused to the cytoplasmic end of the L form, respectively, and similarly, pCD99S/YN and pCD99S/YC were designed to express the YN and the YC fragments fused to the S form of CD99.

To demonstrate that the recombinant proteins were properly expressed, we transfected each construct into 293T cells, in which CD99 is lacking in contrast to Jurkat T cells expressing both the 32 kDa L form and the 28 kDa S form (Fig. 1C). As the YN and the YC fragments generate

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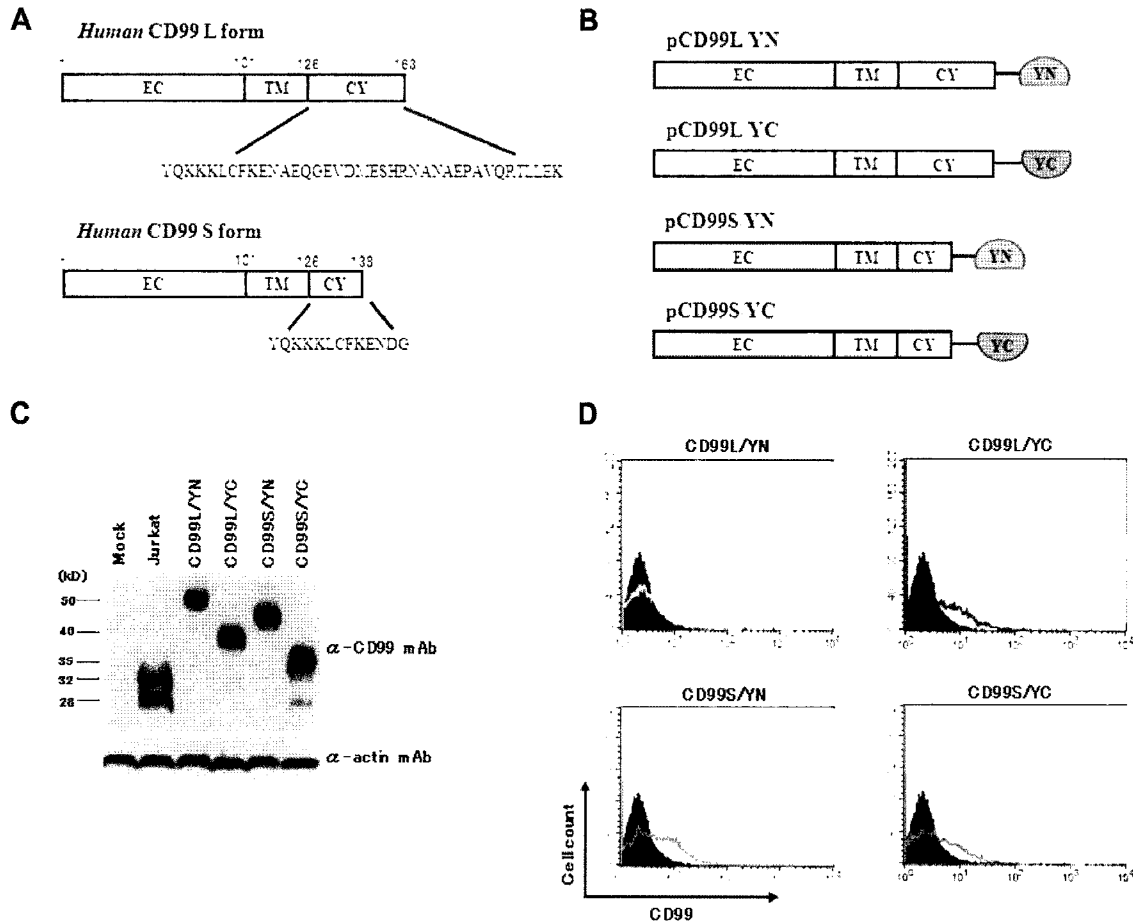

Fig. 1. CD99 isoforms fused to the YFP fragments are successfully expressed on the cell surfaces.
A. Schematic diagrams of human CD99 isoforms, the L form and the S form. Amino acid sequences of cytoplasmic domains of the two isoforms are indicated. The leader sequence is excluded in the diagram. The extracellular, the transmembrane, and the cytoplasmic domains are denoted as EC, TM, and CY, respectively. **B.** Diagrams of the YFP fragment-fused CD99 isoforms constructed for BiFC analysis. The N-terminal and the C-terminal regions of YFP are denoted as YN and YC, respectively. **C.** Western blot analysis of the CD99 isoforms fused with YN and YC fragments. The transiently transfected 293T cells with various CD99 fusion constructs were lysed, and analyzed with a specific mAb for CD99 (DN16; Dinona Inc., Seoul, Korea) or β-actin (C4; Santa Cruz Biotechnology, CA, U.S.A.). The Jurkat cells and the mock transfected 293T cells were used as a positive and a negative control, respectively. **D.** Flow cytometry analysis of surface expression of the four CD99 isoform constructs. Cells were stained with FITC-conjugated anti-CD99 mAb (DN16-FITC). In each panel, open and solid profiles represent cells transfected with the recombinant CD99 isoform constructs and the vector control DNA, respectively.

polypeptides with molecular masses of 19 kDa and 7.3 kDa, respectively, the recombinant proteins from pCD99L/YN, pCD99L/YC, pCD99S/YN, and pCD99S/YC migrated with molecular masses of 51, 39.3, 47, and 35.3 kDa, respectively (Fig. 1B), confirming that the constructs are properly expressing the YFP fragment-tagged recombinant proteins with the expected sizes. To ensure that the recombinant protein from each construct was successfully localized on cell surfaces like CD99, an anti-CD99 mAb that has affinity with an epitope in the extracellular domain of CD99 [10] was treated for flow cytometry. In all the four recombinant CD99 constructs, between 11% and 16% of the transfectants displayed the extracellular CD99 moiety on their surfaces (Figs. 1C and 1D), suggesting that the YFP-tagged CD99 constructs are localized on cell surfaces.

It has been reported that the two isoforms of CD99 molecules covalently link to form heterodimers in Jurkat T cells but not homodimers, the existence of which in lipid rafts appeared to be crucial for the induction of apoptosis [1]. However, it is necessary to examine if the existence of the heterodimers in Jurkat cells can be generalized in other normal and malignant cells in which CD99 was previously reported to have functional roles. To investigate whether CD99 exists only as heterodimers in non-T cells, we transiently transfected the four YN- or YC-fused CD99 isoform constructs. Each of the four constructs was transfected alone, or cotransfected in combinations such as pCD99L/YN and pCD99L/YC, pCD99S/YN and pCD99S/YC, pCD99L/YN and pCD99S/YC, or pCD99L/YC and pCD99S/YN, into 293T cells. On BiFC analysis, each of the four YN- or YC-tagged proteins alone failed to produce any fluorescent signal, whereas the transfectants with both homodimer sets, regardless the L form or the S form, as well as the heterodimer sets surprisingly appeared to produce strong

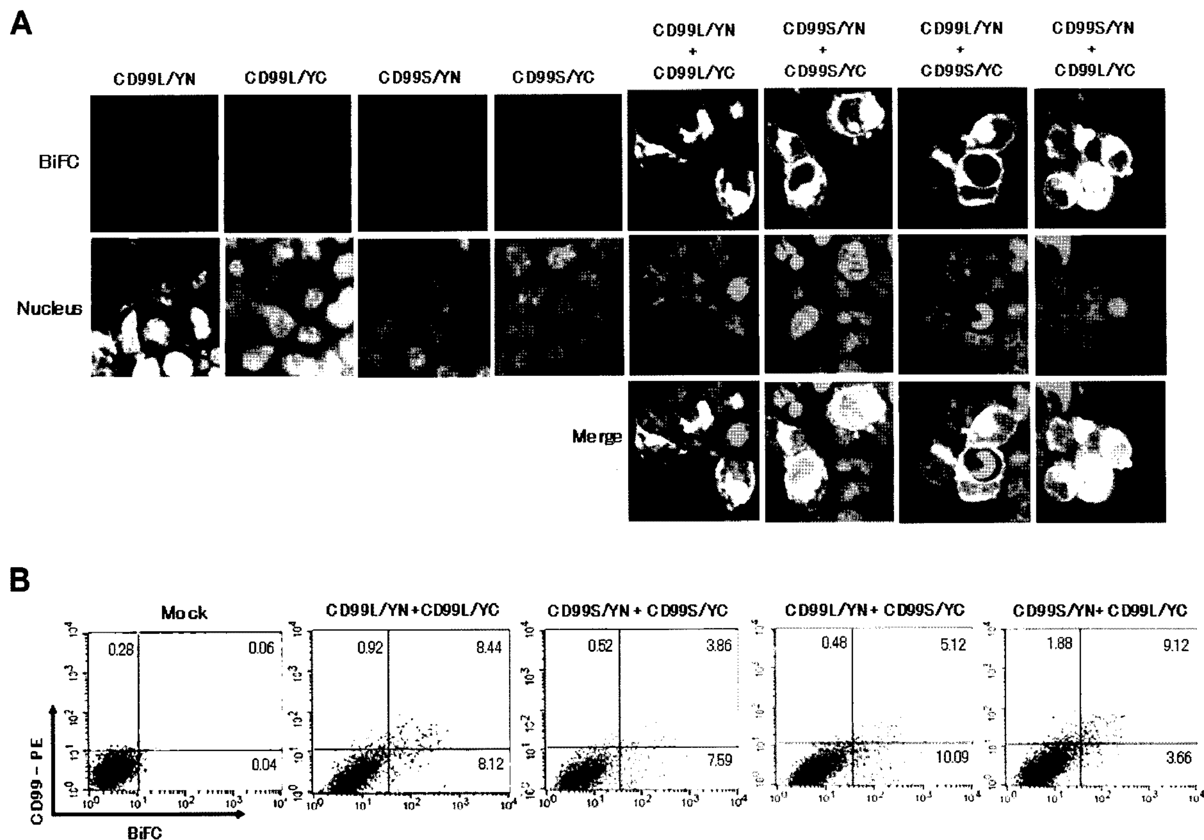

Fig. 2. Human CD99 molecules form homodimers as well as heterodimers.
A. BiFC analysis of the YFP fragment-fused CD99 isoform. To examine the dimerization, 0.8 μg of the YFP fragment-fused CD99 isoform alone or in combination was transfected into 1×10^5 of 293T cells grown on coverslips in each well of 24-multiwell plates. Forty-eight hours after transfection, the cells were incubated at 25°C for 3 h, and fixed, and treated with anti-CD99 mAb. DNA was counterstained with TO-PRO-3 (Molecular Probes, Eugene, OR, U.S.A.), and observed using a confocal laser microscope (Axiovert 200, Carl Zeiss). Green fluorescence indicates the dimer formation. **B.** Flow cytometric analysis of the CD99 fusion proteins expressed on the cell surface. Cells were surface-stained with PE-conjugated anti-CD99 mAb (DN16-PE). The two-color FACS data display BiFC formation at the x-axis and surface CD99 expression at the y-axis.

fluorescent signals (Fig. 2A), suggesting that CD99 molecules can form not only heterodimers but also homodimers. Since CD99 is a transmembrane protein, the tagged CD99 dimers being correctly processed should appear along the endomembrane system. As expected, all the dimers formed by the tagged CD99 isoforms appeared to be localized not only to the plasma membrane but also to the endomembrane system with intense fluorescence. Thus, it is likely that the dimer formation may begin to arise at the Golgi, and then they migrate to the cell surfaces. Since unpaired CD99 molecules may exist as a monomeric form on the surfaces, we performed FACS analysis to examine the ratio between the dimers and the unpaired monomers. In contrast to the mock transfection, 3.86% to 9.12% of the cells in matching pair combinations exhibited not only high YFP fluorescence values but also CD99 positivity, percentages of which comprise 83% to 91% of total cells expressing extracellular CD99 shown as CD99-PE positive (Fig. 2B). Since the proportions of the cells showing CD99-positive but BiFC-negative are very low, it is expected that most of the CD99 molecules on the surfaces exist as a dimeric form.

To narrow down the domain essential for the dimerization, we utilized chimeric expression constructs, in which the extracellular domain and the transmembrane and cytoplasmic domains of CD99 are substituted by the corresponding domains of the human CD4 molecule, and fused to the C-terminus of YFP. One construct, pCD99EC/CD4TM.CY/YC, contains the CD99 extracellular domain followed by the transmembrane-cytoplasmic domain of CD4, and the other construct, pCD4EC/CD99TM.CY/YC, holds the CD4 extracellular domain followed by the CD99 transmembrane-cytoplasmic domain (Fig. 3A). The negative control partner pCD4/YC has the CD4 sequence fused to the YFP C-terminal fragment. Each of these chimeric constructs was transiently cotransfected with pCD99L/YN into 293T cells. The transfectant with the constructs containing the CD4 extracellular domain displayed high CD4 reactivity, shifting up along the CD4-PE axis, whereas the constructs with the CD99 extracellular domain had no effect on reactivity to CD4 (Fig. 3C). All the transfectants except for the mock exhibited extracellular CD99 expression because of the cotransfected CD99L/YN construct in

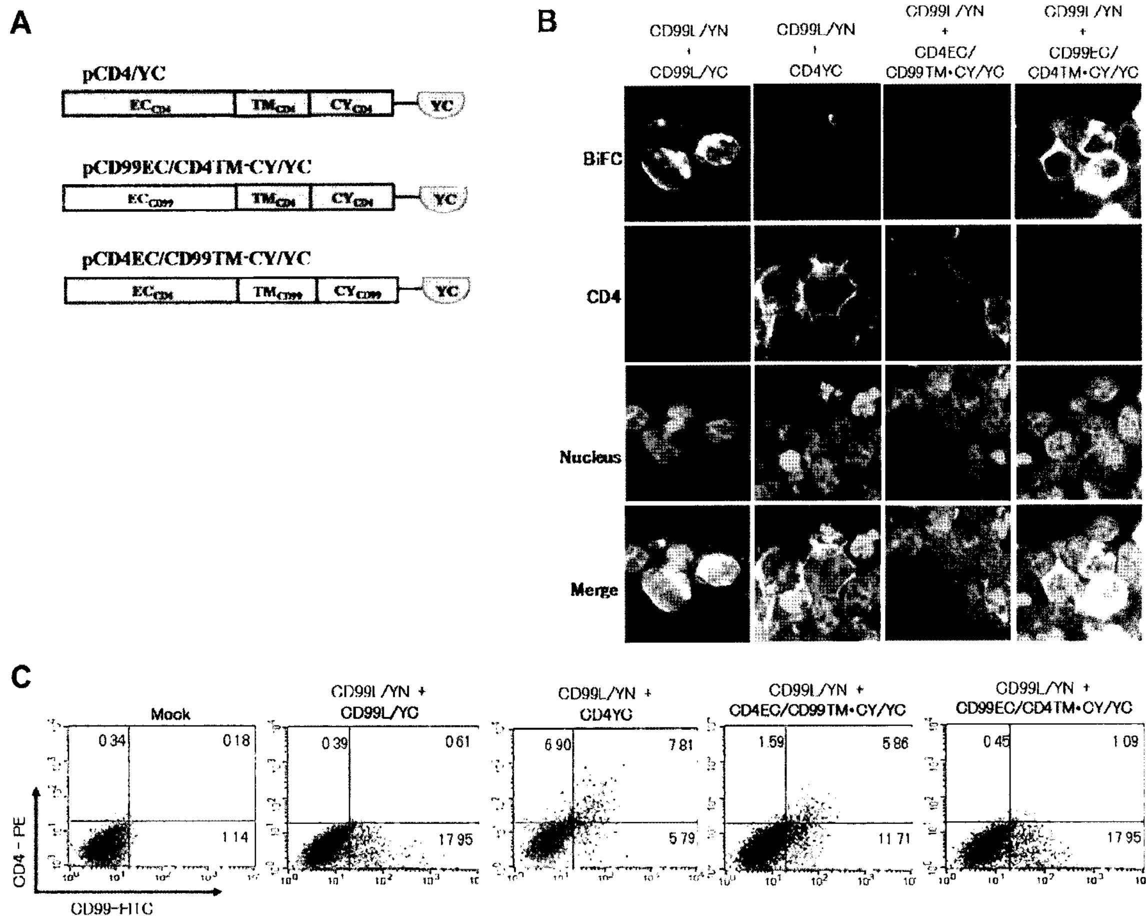

Fig. 3. The CD99 isoforms dimerize through their extracellular domains.
A. Schematic diagrams of CD4-CD99 hybrid constructs for BiFC analysis. B. BiFC analysis of CD4-CD99 hybrid proteins with CD99 L form. The results of BiFC are shown in the upper row. In the second row, the surface CD4 expression was detected with PE-conjugated anti-CD4 mAb (YG22-PE; Dinona Inc.). C. Flow cytometric analysis of CD4-CD99 hybrid proteins. Cells were analyzed for the presence of CD4 and CD99 moieties on the surfaces by flow cytometry.

FACS analysis. When the construct containing the CD99 extracellular domain, pCD99EC/CD4TM.CY/YC, was cotransfected with pCD99L/YN, the cells produced fluorescence, whereas neither of the constructs containing the CD4 extracellular domain, pCD4EC/CD99TM.CY/YC or pCD4/YC, did (Fig. 3B), suggesting dimer formation *via* the extracellular domain of CD99. Recently, using the BiFC technique, murine CD99 was shown to form a homotypic dimer by interacting *via* its extracellular domain [6], which strongly supports our finding that human CD99 molecules dimerize *via* their extracellular domains regardless of the types of the isoforms.

Many transmembrane proteins that mediate signaling pathways frequently form dimers on cell surfaces [7, 8]. Our observation that CD99 can naturally dimerize without any stimulus supports an idea that CD99 might be a receptor that exists as a dimer that becomes active upon stimulation by ligands. Further functional roles and the significance of the existence of various isoform complexes in diverse cellular situations should be elucidated.

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