Engagement of CD99 triggers the exocytic transport of ganglioside GM1 and the reorganization of actin cytoskeleton

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Abstract **We studied the role of lipid rafts and actin cytoskeleton in CD99-mediated signaling to elucidate the mechanism of protein transport upon CD99 engagement. CD99 engagement in Jurkat cells elicited the exocytic transport of GM1 as well as several surface molecules closely related with CD99 functions. In addition, CD99 molecules were rapidly incorporated into lipid rafts and appeared to rearrange the actin cytoskeleton upon CD99 stimulation. Association of CD99 with actin cytoskeleton was inhibited by methyl-β-cyclodextrin, while CD99-mediated GM1 clustering was inhibited by cytochalasin D. Therefore, we suggest that CD99 may play a role in the vesicular transport of transmembrane proteins and lipid rafts from the intracellular location to the cell surface, possibly by effecting actin cytoskeleton reorganization.**

Key words: T lymphocyte; Cellular activation; Vesicular transport; CD99; Costimulatory molecule; Lipid raft; Cytoskeleton

1. Introduction

The process of regulated transport of transmembrane proteins is critical for correct and immediate functions of lymphocytes, and thus protein transport has long been recognized as an important mechanism for T cell activation. Many cell surface proteins are not constantly present on the cell surface, but undergo constitutive or ligand-induced internalization into endosomes, and subsequent recycling back to the cell surface or degradation in the lysosomal compartment. In the case of T cell receptors, they are rapidly internalized and recycled in T cells in the absence of external stimuli [1], whereas some cell surface proteins, such as Fas ligand or cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), are stored in the intracellular compartments, but transported to the cell surface only when required for their functions [2,3]. However, molecular mechanisms for regulation of exocytosis are currently far from being completely understood, and cell surface proteins involved in this process remain to be exploited. Early works demonstrated that the cytoskeletal reorganization is closely linked to protein trafficking in response to cell stimulation [4]. Although some movement of cargo-laden vesicles can be driven by diffusion, most cells require active transport along cytoskeletal tracks powered by molecular motor proteins [5]. For exocytosis and endocytosis, cytoskeleton–membrane interactions are also suggested to be important for the control of vesicular transport, and some signaling processes have been reported to regulate the events [6].

The plasma membrane is not homogeneous, but composed of laterally associated 'lipid rafts' enriched in cholesterol, sphingolipids and a subset of proteins that float in a 'sea' of otherwise glycerophospholipid-rich plasma membrane. The raft hypothesis proposed that attractive forces between sphingolipids and cholesterol mediate the formation of lateral lipid clusters in an unsaturated glycerophospholipid environment [7]. Glycosphingolipid GM1 has commonly been used as a marker of lipid rafts recognized by cholera toxin (CTx), but when detergent-permeabilized cells in the resting state were labeled with fluorescein isothiocyanate (FITC)-conjugated CTx, GM1 was shown to be mainly present in intracellular membranes [8]. Similarly to cell surface proteins such as CTLA-4, GM1 was exported to the plasma membrane upon T cell activation through T cell receptor.

The CD99 molecule is a 32-kDa transmembrane glycoprotein, which is highly expressed on thymocytes, memory lymphocytes, pancreatic islet cells, and primitive neuroectodermal cells [9]. CD99 has been functionally implicated in cell migration, apoptosis, cell adhesion, Th1 cell differentiation, and intracellular transport of transmembrane proteins [10–15], but the basic molecular mechanism of CD99-mediated signal transduction is not well known. Previously, we showed that CD99 engagement led to extracellular signal-regulated kinase (ERK) activation and homotypic aggregation [16]. In this report, we investigated whether CD99 engagement affects the translocation of GM1 to the plasma membrane, and actin cytoskeleton reorganization. We show that GM1 is rapidly exported with transmembrane proteins in response to CD99 stimulation, and provide several lines of evidence for a critical

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Abbreviations: CTLA-4, cytotoxic T lymphocyte-associated antigen 4; ERK, extracellular signal-regulated kinase; LFA-1, leukocyte function-associated antigen 1; ICAM-1, intercellular adhesion molecule 1; DIG, detergent-insoluble glycolipid-enriched domain; MCD, methyl-β-cyclodextrin; CTx, cholera toxin; mAb, monoclonal antibody

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role of CD99 in vesicular transport and actin cytoskeleton redistribution.

2. Materials and methods

2.1. Cells, antibodies, and reagents

Jurkat T cells were cultured at 37°C and 5% CO_2 in RPMI 1640 (Gibco, Grand Island, NY, USA) medium supplemented with 10% fetal calf serum (Hyclone, Logan, UT, USA), 2 mM L-glutamine, 400 U/ml penicillin, and 150 μg/ml streptomycin. Anti-CD99 (DN16), anti-leukocyte function-associated antigen 1 (LFA-1) (P21), anti-intercellular adhesion molecule 1 (ICAM-1) (AP7), and anti-CD5 (P7) antibodies were obtained from DiNonA (Seoul, Korea). Anti-CD3ε antibody (Ab) (M-20) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). FITC-conjugated CTx, cytochalasin D, and methyl-β-cyclodextrin (MCD) were purchased from Sigma (St. Louis, MO, USA). Polystyrene latex microspheres (Polysciences, Warrington, PA, USA) coated with anti-CD99 monoclonal antibody (mAb) were prepared according to the manufacturer's protocol. Briefly, 400 μg of anti-CD99 was incubated with 0.5 ml of a 2.5% suspension of the beads (3 μm diameter) in 1 ml of 0.1 M borate buffer pH 8.5 at room temperature overnight. The beads were then washed and incubated with 10 mg/ml bovine serum albumin in 0.1 M borate buffer pH 8.5 to block any remaining protein-binding sites. The beads were stored in 1 ml of phosphate-buffered saline (PBS), pH 7.4, containing 10 mg/ml bovine serum albumin (BSA).

2.2. Immunofluorescence, laser confocal microscopy, and flow cytometric analysis

For CD99 engagement in suspension, cells were prepared at 1×10^6/100 μl in PBS and incubated with anti-CD99 mAb (10 μg/ml) and then secondary goat or rabbit anti-mouse IgG for cross-linking for indicated durations. To visualize F-actin, cells were incubated with tetramethylrhodamine isothiocyanate (TRITC)-conjugated phalloidin (Sigma). Next, cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. Then cells were washed twice in cold PBS, harvested with cytospin onto Silane-coated glass slides, and air-dried. The samples were mounted and processed for microscopy. In some experiments, cells were adhered to poly-L-lysine-coated coverslips and then stimulated with anti-CD99 mAb and rabbit anti-mouse IgG for the indicated time intervals. Then cells were fixed in 4% (w/v) paraformaldehyde, rinsed, and treated with 10 mM glycine. Cells were washed, permeabilized with 0.1% Triton X-100, and incubated with 3% BSA. Cells were sequentially incubated with appropriate antibodies. To visualize the distribution of filamentous actin and lipid rafts marker, GM1, cells were incubated with phalloidin-TRITC and CTx-FITC, respectively. Coverslips were mounted on slides and confocal images were obtained with a Zeiss Axioplan photomicroscope and Zeiss LSM510 (Zeiss, Hamburg, Germany). Images were processed using a Workstation using Program. For flow cytometric analysis, resting and activated cells were stained with FITC-labeled antibodies. Cells were acquired on a FACSCalibur™ flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and analyzed with CELLQuest™ (Becton Dickinson) software.

2.3. Preparation of lysates from CD99-engaged cells and immunoblotting

Jurkat T cells pretreated with given concentrations of MCD for 10 min at 37°C were washed with cold PBS, resuspended at a concentration of 1×10^7 cells/ml in serum-free RPMI 1640 and then stimulated with anti-CD99 mAb at 10 μg/ml and then with rabbit anti-mouse IgG at 20 μg/ml for 30 min. Cells were washed twice in cold PBS and solubilized in lysis buffer [10 mM Tris–HCl, pH 7.6, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, and 10 μg/ml leupeptin] on ice for 15 min. The lysates were clarified by centrifugation at $16\,000\times g$ for 15 min at 4°C. Proteins in the lysates separated by SDS–polyacrylamide gels were transferred to polyvinylidene difluoride (PVDF) membrane, which was blocked for 60 min in 5% non-fat milk. The membranes were incubated with primary antibody for 60–120 min, washed three times in Tris-buffered saline–Tween, incubated for 60 min in horseradish peroxidase (HRP)-conjugated rabbit anti-mouse or goat anti-rabbit immunoglobulin, and then washed three times. Finally, a chemiluminescence detection system (Amersham, Arlington Heights, IL, USA) was used for visualization of relevant proteins.

2.4. Surface biotinylation and immunoprecipitation

Jurkat T cells (1×10^7) were washed three times with ice-cold PBS and biotinylated for 30 min with 10 μl of 10 mg/ml sulfosuccinimidobiotin (sulfo-NHS-SS-biotin; Pierce, Rockford, IL, USA) in PBS at 4°C according to the published protocol [17]. Cells were then washed with PBS and excess NHS-SS-biotin was quenched with 0.2% BSA and 0.1 M glycine in PBS. Surface-biotinylated cells were stimulated with 10 μg/ml of anti-CD99 and 20 μg/ml of anti-mouse Ig for the indicated durations. Then, cell surface biotin was cleaved by treatment of glutathione cleavage buffer (50 mM glutathione in 75 mM NaCl and 10 mM EDTA containing 1% BSA and 0.075 N NaOH) so that only the internalized biotin was protected. After the lysis of cells, the clarified lysates were incubated with 30 μl of streptavidin-agarose (ImmunoPure immobilized streptavidin; Pierce) at 4°C for 2 h selectively to collect only the internalized cell surface proteins. The amounts of biotinylated CD3 protein were assessed by immunoblotting with anti-CD3ε in recovered precipitates. Densitometric analysis was performed to determine the internalization rate. The background value at time 0 was subtracted, and the data were normalized to the loading level of CD3ε. The internalization rate is expressed as a percentage of the total surface-labeled CD3ε that is internalized at the indicated time. Data shown are representative of three independent experiments which all showed similar results.

2.5. Isolation of detergent-insoluble membrane fractions

Cells were washed with cold MES-buffered saline (MBS; 25 mM MES, 0.15 M NaCl, pH 6.5) and then lysed with 1 ml of ice-cold MBS lysis buffer (25 mM MES pH 6.5, 0.15 M NaCl, 0.05–1% Triton X-100, 1 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin). Following 30 min incubation on ice, the lysates were homogenized with 20 strokes of a loose-fitting Dounce homogenizer, gently diluted with an equal volume of 80% sucrose (w/v) in MBS, and transferred to the bottom of a SW41Ti centrifuge tube. The sample was then overlaid with 6.5 ml of 30% sucrose and 3.5 ml of 5% sucrose in MBS (with 1 mM Na_3VO_4) and centrifuged for 18 h at $200\,000\times g$ at 4°C in a Beckmann SW41Ti (Fullerton, CA, USA). One milliliter fractions were harvested serially from the top of the gradient. The same volume of each fraction was used for the immunoblotting analysis.

2.6. Detergent extraction of CD99

Detergent extraction was done essentially as described [18]. After appropriate treatments, Jurkat T cells were washed with cold PBS and incubated for 10 min with gentle shaking on ice in extraction buffer (15 mM Tris–HCl, pH 7.5, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 150 mM NaCl, 10 μg/ml leupeptin, aprotinin, and PMSF as protease inhibitors, and 0, 0.1, 0.4, or 1.0% Triton X-100 detergent). The extracted material was collected after centrifugation and washed twice with cold extraction buffer. 1 M KI was added to the extraction buffer to disrupt possible interactions with cytoskeletal proteins. Samples of the soluble and insoluble fractions were boiled, separated by SDS–PAGE, and assayed by immunoblotting.

3. Results

3.1. CD99 engagement triggers upregulation of cell surface proteins and clustering of lipid rafts

Previous reports have demonstrated that engagement of CD99 upregulates the surface expression of T cell receptors and MHC molecules in thymocytes [14,19]. To test whether the upregulation of the cell surface proteins was specific to CD99 engagement, we investigated proteins involved in cell–cell adhesion and signaling through T cell receptors since the interaction between LFA-1 and ICAM-1 was implicated in homotypic aggregation and CD99 was also proposed to be a costimulatory molecule for T cell receptor. In the comparison of the expression of several cell surface proteins upon CD99 or CD5 engagement, CD99 engagement upregulated CD3, LFA-1, ICAM-1, and CD5 in Jurkat T cells, while the engagement of CD5, a lipid raft-associated molecule, did not affect the surface expression of these molecules (Fig. 1).

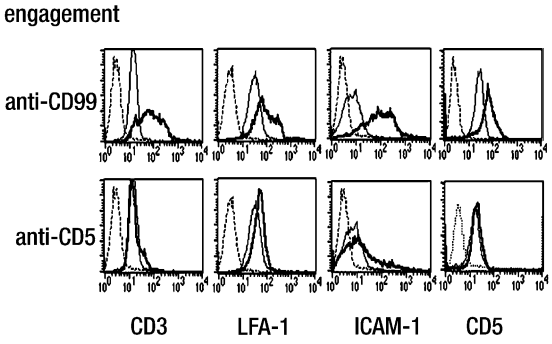

Fig. 1. Upregulation of cell surface proteins by CD99 engagement. Jurkat T cells were incubated in the presence (thick lines) or the absence (thin lines) of the indicated antibodies, anti-CD99 or anti-CD5. After the treatment, the expression levels of surface molecules such as CD3, LFA-1, ICAM-1, and CD5 were analyzed on a flow cytometer. The background fluorescence is shown as dotted lines.

These data suggested that CD99 affects the transport of the tested cell surface proteins.

To test whether CD99 affects the localization of transmembrane proteins by influencing the movement of lipid rafts, we investigated whether CD99 engagement redistributes glycosphingolipid GM1, a marker of lipid rafts, by incubating Jurkat cells with anti-CD99 Ab-coated microbeads, and then visualizing the distribution of lipid rafts using FITC-conjugated CTx that labeled GM1. Homotypic aggregation was induced, and GM1 was clustered into cell–cell contact sites and around the areas between microbeads and cells (Fig. 2A,B). To investigate the effect of CD99 engagement on the redistribution of lipid rafts independently of homotypic aggregation, a single cell with a microbead was focused, in which GM1 was shown to be concentrated around the microbead (Fig. 2B). These findings indicate that CD99 engagement leads to the clustering of GM1.

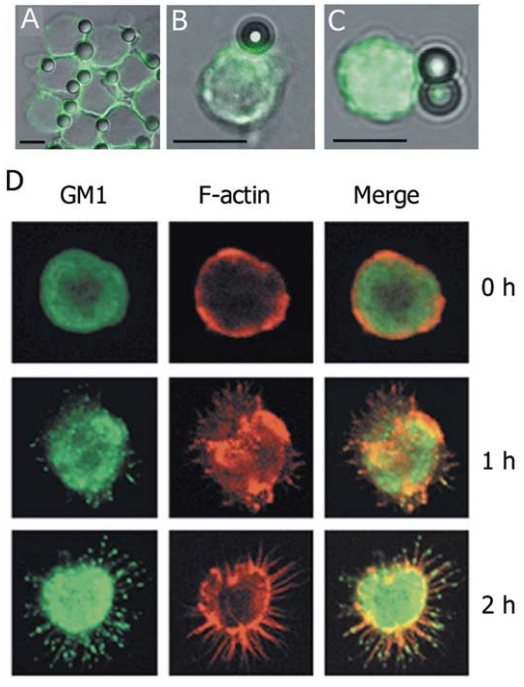

Fig. 2. CD99 engagement triggers the clustering of lipid rafts and the transport of GM1 from the intracellular location to the cell surface with the formation of filopodia and lamellipodia. Jurkat T cells were engaged with anti-CD99-coated microbeads and then stained with FITC-labeled CTx (A). An isolated cell with microbead is shown in B with magnification. In C, a cytochalasin D-treated cell adhering to the anti-CD99-coated microbead is shown. Inset bars indicate 10 μm. Jurkat cells were adhered to poly-L-lysine-coated coverslips and then stimulated with anti-CD99 mAb and rabbit anti-mouse IgG for the indicated time. Cells were fixed, permeabilized, and stained with CTx-FITC and phalloidin-TRITC to visualize GM1 and filamentous actin, respectively (D). Merged views are shown.

3.2. *CD99 engagement triggers the transport of GM1 from the intracellular location to the cell surface and the formation of filopodia and lamellipodia*

Next we asked whether CD99 engagement also affected the transport of GM1 in a similar way as the cell surface transmembrane proteins examined. To stimulate cells without homotypic aggregation, we incubated Jurkat cells to become adherent on the poly-L-lysine-coated coverslides, and then the cells were stimulated with anti-CD99 Abs. When cells were permeabilized and stained with FITC-labeled CTx, GM1 was shown to be mainly present in intracellular membranes in the resting state (Fig. 2D, upper panel), as reported earlier [8]. With CD99 engagement, GM1 was prominently clustered in intracellular compartment within 1 h (Fig. 2D, middle panel) and then exported along the filopodial extensions at least in 2 h (lower panel). With labeling of filamentous actin by staining with phalloidin-TRITC, we could clearly observe prominent filopodia formation. Most cells showed filopodia and thickening of cortical filamentous actin

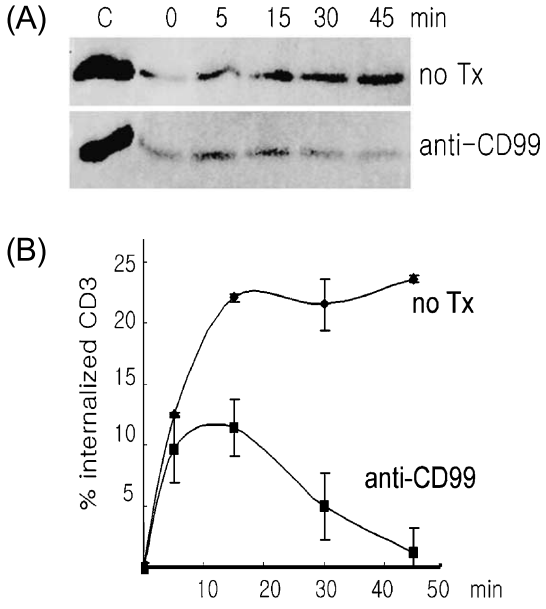

Fig. 3. Export of recycling TCR–CD3 complex by CD99 engagement. Jurkat cells were labeled with NHS-SS-biotin on the cell surface and incubated in the absence (no Tx) or presence (anti-CD99) of anti-CD99 mAb. After the indicated time, biotinylated proteins on the cell surface were stripped with the cleavage buffer. Internalized proteins were protected and precipitated with streptavidin beads. Unstripped CD3 at 0 min after labeling is shown in lane C. With time, more CD3 proteins were internalized and shown. Densitometric analysis was performed as described in Section 2. A representative autoradiogram from three independent experiments is shown in the upper panel (A) and the graph at the bottom (B) shows the percent internalization rate from averages of three experiments.

(lamellipodia). Clustered GM1 was clearly detected at the tip of the filopodial extensions. These data suggest that CD99 affects the transport of GM1 as well as cell surface proteins along with rearrangement of actin cytoskeleton, independently of homotypic aggregation.

3.3. Export of recycling CD3 upon CD99 engagement

To confirm that CD99 engagement leads to the translocation of CD3 to the plasma membrane biochemically, we examined the effect of CD99 ligation on the recycling of CD3–TCR complex by using a biotinylation assay. In Fig. 3, T cells were treated with sulfo-NHS-SS-biotin so that only cell surface molecules were labeled. Cells were placed in culture, and removed at the indicated time points for cleavage in glutathione cleavage buffer. This treatment cleaves the labeled biotin from all the surface CD3 molecules by reduction of the SS bond, while internalized CD3 molecules retain their biotin. Subsequently, remaining biotinylated proteins were precipitated with streptavidin-agarose. Under resting conditions, CD3 underwent time-dependent endocytosis (Fig. 3A, top, no Tx) that reached about 23% of all labeled CD3 in 15 min with a densitometric analysis. The proportion of CD3 internalization did not increase significantly after 15 min. This result is consistent with the previously reported estimation [1]. With CD99 engagement, less CD3 was protected from the cleavage buffer treatment (Fig. 3A, bottom). By 45 min, most CD3 molecules were shown to be on the cell surface upon CD99 engagement (Fig. 3B). These results suggest that CD99 engagement may accelerate the export of recycling CD3 molecules or block CD3 endocytosis. It is more likely that CD99 engagement influences the exocytosis of CD3 since initial endocytosis of CD3 was only slightly affected by CD99 engagement.

3.4. A fraction of activated CD99 molecules are associated with lipid rafts

Next we investigated whether CD99 resides in the lipid raft microdomain. Detergent-insoluble glycolipid-enriched domains (DIGs) equivalent to lipid rafts were isolated by sucrose density gradient centrifugation from Jurkat T cells lysed in a buffer containing a non-ionic detergent, Triton X-100. Aliquots of each fraction were subjected to dot blotting, and the DIG fractions were identified by positive reactivity with CTx that recognizes ganglioside GM1 (Fig. 4C). Although the majority of CD99 were present in intracellular and heavy membrane fractions (10–12 in Fig. 4A), a very small amount of CD99 molecules was detected in the DIG fractions with use of 0.1% Triton X-100. It appeared that the association of a small amount of CD99 with DIG fractions was sensitive to a high amount of detergent as exemplified in the case of CD45

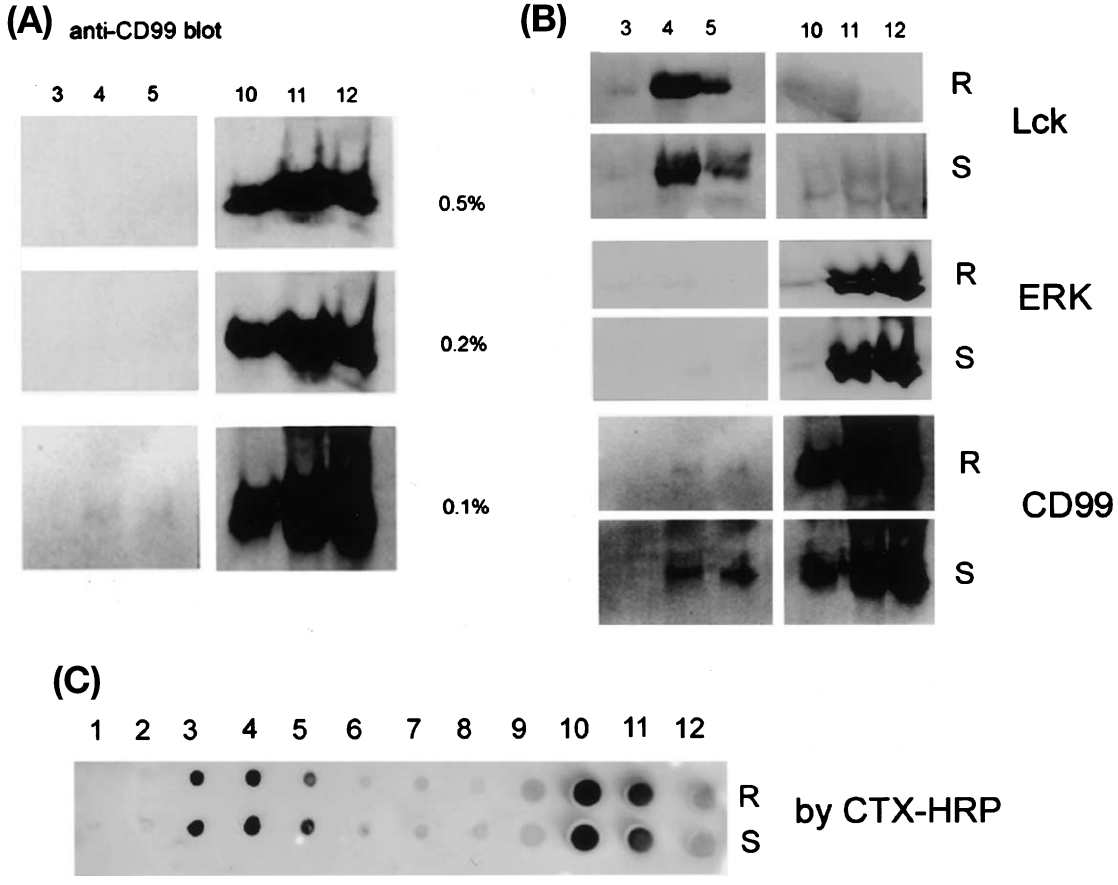

Fig. 4. Localization of CD99 in lipid rafts upon CD99 engagement. To separate lipid raft fractions, cell lysates from 1×10^7 cells were placed at the bottom of a sucrose gradient. After ultracentrifugation, low density fractions (3–5) and high density fractions (10–12) were analyzed by immunoblotting with given antibodies. A: Fractions were separated with use of given concentrations of Triton X-100, and blotted with anti-CD99 mAb. B: Fractions prepared using 0.1% Triton X-100 MBS lysis buffer were subjected to immunoblotting with given antibodies. R and S indicate resting and stimulated with anti-CD99 mAb, respectively. C: The fractions were spotted onto the PVDF filter and blotted with CTx-HRP.

[20]. The amount of CD99 in the DIG fraction was increased with CD99 engagement (Fig. 4B, bottom) when the DIG fractions were separated with 0.1% Triton X-100. It was clearly shown that the DIG fractions were adequately prepared since the presence of Lck, a lipid raft-resident protein, was restricted in fractions 3–5 while ERK, a cytoplasmic kinase, was detected in fractions 10–12 (Fig. 4B). These results suggest that CD99 engagement leads to its partition into the DIG fractions that are equivalent to lipid rafts.

3.5. Lipid raft integrity is necessary for the association of CD99 molecules with the cytoskeletal fraction upon CD99 engagement

Since many receptors associate with the cytoskeletal fraction upon ligand binding and subsequent activation, we then tested whether CD99 partitioned into the cytoskeletal fractions of the resting and the CD99-engaged cells. Differential detergent solubility assays were performed to investigate the interaction between CD99 and the cytoskeleton. In the assay, the detergent-insoluble fraction remains at the pellet, containing interconnected cytoskeletal proteins such as actin and spectrin, and tightly associated membrane and signaling proteins as well as DIG-resident proteins. As shown in Fig. 5A in resting Jurkat T cells, most CD99 molecules are present in the soluble fraction. However, when cells were stimulated with anti-CD99 mAb, the amount of CD99 in the detergent-insoluble fraction increased, implying that CD99 molecules are incorporated into the cytoskeleton/heavy membrane fraction on CD99 engagement (Fig. 5B). To confirm that CD99 becomes actually associated with the cytoskeleton when stimulated, we added KI to the extraction buffer to break protein–cytoskeletal interactions. The detergent-insoluble CD99 proteins almost disappeared with KI treatment (Fig. 5C). These data suggest that CD99-mediated signaling induces a tight interaction between CD99 and cytoskeleton.

Next we tested whether this signaling is also dependent on the presence of intact lipid rafts. When Jurkat T cells were

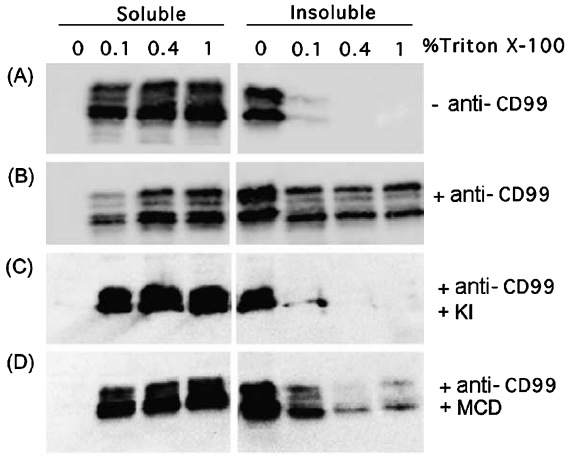

Fig. 5. Association of CD99 with the actin cytoskeleton upon its engagement and its dependence on the intact lipid rafts. Jurkat cells engaged in the absence or presence of anti-CD99 mAb were treated with buffer containing the indicated concentrations of Triton X-100. Soluble and insoluble fractions were collected separately, and analyzed by immunoblotting using anti-CD99 mAb. In C, 1 M KI was added to the extraction buffer at the time of lysis. In D, Jurkat cells pretreated with 10 mM MCD were stimulated with anti-CD99 mAb, and then fractions were collected.

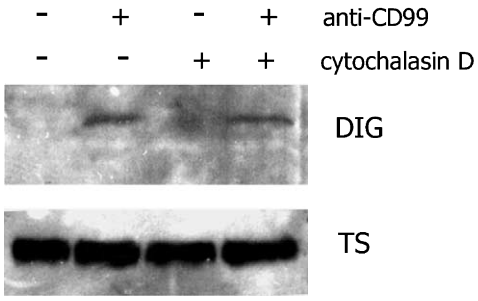

Fig. 6. Association of engaged CD99 with lipid rafts in the presence of cytochalasin D. The Jurkat cell line was treated with or without anti-CD99 mAb in the presence or absence of 10 μM cytochalasin D as indicated. Lipid raft fractions were separated by the same protocol given in Fig. 4. The DIG fraction (4) and the Triton X-soluble fraction (TS) (11) were taken and immunoblotted with anti-CD99 DN16 mAb.

stimulated with anti-CD99 Ab after pretreatment with 10 mM MCD, the association of CD99 with the cytoskeletal fraction was significantly reduced (Fig. 5D). These results indicate the requirement of lipid rafts for the association between CD99 and the actin cytoskeleton.

3.6. CD99-mediated clustering of GM1 is dependent on actin cytoskeletal rearrangement

Next we investigated whether the rearrangement of actin cytoskeleton is required for the two events, the association of engaged CD99 with lipid rafts and CD99-mediated signaling. When we incubated Jurkat cells with anti-CD99 Ab-coated microbeads in the presence of 10 μM cytochalasin D, the clustering of GM1 around anti-CD99 Ab-coated microbeads was abolished even though cells and microbeads adhered to each other (Fig. 2C). This finding suggests that CD99-mediated signaling requires the rearrangement of the actin cytoskeleton.

The association of CD99 with lipid rafts in the presence of 10 μM cytochalasin D was then investigated by the fractionation of the DIG fraction over the sucrose density gradient. Partition of engaged CD99 into lipid raft fractions was shown when we prepared the DIG fractions with or without the treatment of cytochalasin D (Fig. 6). In the presence of cytochalasin D, the amount of CD99 in the DIG fraction still increased upon CD99 engagement. This finding suggests that the incorporation of CD99 into the lipid raft fraction is largely independent of actin cytoskeletal rearrangement.

4. Discussion

Here we provide evidence for a role of CD99 in the vesicular export of transmembrane proteins and GM1 as well as a role of lipid rafts in CD99-mediated signaling. We have demonstrated that engagement of CD99 elicits the export of transmembrane proteins such as LFA-1, ICAM-1, CD5, and CD3, and GM1, and the association of CD99 with the cytoskeletal compartment in a lipid raft-dependent manner. Furthermore, the actin cytoskeletal rearrangement, as revealed by the formation of filopodia and lamellipodia, was accompanied with the export events.

In this study, we were interested in determining whether CD99 was localized in lipid rafts and whether CD99-mediated signaling was linked to lipid rafts. We showed that most

CD99 molecules were not present in lipid rafts during the resting state, but the fraction of CD99 molecules in lipid rafts increased significantly upon CD99 engagement. One of the important properties of lipid rafts is that they can include or exclude proteins to variable extents. Several cell surface proteins, especially glycosylphosphatidylinositol-anchored proteins, have been shown to be constitutively present in lipid rafts. However, many functionally important receptors, such as T and B cell receptors, are located outside the lipid rafts, but partition into lipid rafts upon their engagement or other activation events [7,21,22]. CD99 appeared to be a cell surface protein which partitioned into the lipid raft compartment in a regulated manner. The association of CD99 with lipid rafts was sensitive to a high concentration of Triton X-100. So far the basis for the sensitivity to the high detergent concentration is not clear, but it appeared that the detergent sensitivity reflects a weak interaction between CD99 and lipid rafts similarly to a reported weak interaction between CD45 or FcεRI and lipid rafts [20,23,24]. The identification of proteins or lipids that directly interact with CD99 molecules would help us understand the molecular basis of the interaction between CD99 and lipid rafts.

Here it was shown that lipid rafts and actin cytoskeleton had an essential role in CD99-mediated signaling in spite of the presence of only a small fraction of CD99 molecules in lipid rafts or the actin cytoskeletal compartment in the resting state. With CD99 engagement, CD99 molecules became associated with the actin cytoskeleton as well as lipid rafts. This finding supports the cell biological mechanism of CD99-mediated homotypic aggregation since homotypic aggregation requires not only the interaction between the adhesion molecules but also the appropriate involvement of the cytoskeleton [18,25,26]. It appeared that the initial entrance of CD99 into lipid rafts upon CD99 engagement occurs by their clustering by anti-CD99 mAb, but the downstream effects by CD99 engagement required the actin cytoskeleton. It remains to be investigated how CD99 signaling elicits the association of the cytoskeleton and CD99 or lipid rafts, and whether CD99 influences the cytoskeletal association of other cell surface proteins.

CD99 engagement led to the transport of GM1 along the filopodial extensions. In the resting cells, GM1 is present in the intracellular locations as well as plasma membrane and is known to undergo recycling through plasma membrane, endosome, Golgi complex, and endoplasmic reticulum [27]. Recently, it was shown that the synthesis and transport of GM1 was increased upon T cell activation through T cell receptors [8]. Our data indicate that CD99 may be one of the regulators that determine the distribution of GM1, controlling the amount of GM1 on the cell surface relative to the amount in the intracellular location. The total amount of GM1 was not altered during 3 h stimulation of CD99 when CD99-engaged Jurkat cells were permeabilized and stained by CTx-FITC (data not shown).

Regulated expression of transmembrane proteins is critical for the correct biological functioning of lymphocytes. For example, CTLA-4 as well as adhesion or activation-related molecules are upregulated on the cell surface upon T cell activation by the export from the intracellular locations [2,28]. Thus, the T cell activation processes may trigger the related exocytic events as well as endocytic events. We suggest that CD99 may have a certain role in the activation-related exocytic processes. In this context, it is worth noting that CD99 is highly upregulated in memory T and B cells, which are ready to express effector molecules which are stored in the intracellular reservoir such as endosomes or secretory lysosomes [9,29]. Therefore, CD99 may be not just another costimulatory T cell surface molecule, but a molecule that has an effector function, such as target cell lysis by cytotoxic T cells or NK cells. To explore these functions of CD99, the functions of CD99 in effector T cells, NK cells, mast cells, or macrophages need to be investigated.

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