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Cellular Distribution of a GPI-Anchored Complement Regulatory Protein CD59: Homodimerization on the Surface of HeLa and CD59-Transfected CHO Cells[1]

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Human glycosyl phosphatidylinositol-anchored protein CD59 was solubilized in detergent-insoluble complexes (DICs) and in post-nuclear pellets by a two-step solubilization procedure using Triton X-100 and octylglucoside. CD59 molecules are recovered in both fractions, the amount being greater in the latter fraction in all cell types tested. Specific labeling of surface CD59 molecules revealed that the CD59 detected in DICs originated from intracellular compartments, whereas that in post-nuclear pellets was in part derived from the cell surface. Cross-linking of surface proteins with chemical cross-linker followed by Western blotting with anti-CD59 antibody revealed cross-linked products with molecular masses of 28-36 kDa on HeLa and human CD59 cDNA-transfected CHO cells; the CD59-associating molecules were estimated to be 13-18 kDa in size. The cross-linked products were extracted in the post nuclear pellets, and CD59 existed mainly as a cross-linked form on the cell surface. Two-dimensional electrophoresis of the cross-linked products revealed no trace of molecules other than CD59. The cross-linked products showed the same N-terminal sequences as CD59 and a strikingly similar amino acid composition to that of CD59. Thus, most likely, the cross-linked products are CD59 dimers. The finding that CD59 localized on outer membranes is all in the form of dimers suggests the importance of dimerization for CD59 functioning.

Key words: CD59, detergent-insoluble complex, GPI-anchored protein, nuclear pellet, subcellular distribution.

Various cell surface proteins are anchored to cell membranes *via* a glycosyl-phosphatidylinositol (GPI) anchor. These proteins are found on a wide range of eukaryotic cells and are associated with diverse functions, including enzymatic activity, transport, signal transduction, adhesion, and protection against complement (reviews: Refs. *1* and *2*). The functional importance of the GPI moiety is unknown, although it has been suggested that the anchoring endows proteins with high lateral mobility on the membranes and permits specific release of the proteins by the action of phosphatidylinositol-specific phospholipase C or phospholipase D upon stimulation (*1, 2*).

GPI-anchored proteins are characterized by their insolubility in non-ionic detergents such as Triton X-100 and NP-40 at a low temperature (*3-6*). In detergent extracts, they remain primarily as detergent-insoluble complexes (DICs) with a diameter of 50-100 nm (*5*). These complexes contain tyrosine kinases (*4, 5, 7*) and α-subunits of heterotrimeric G-proteins (*8*), together with GPI-anchored proteins.

In epithelial cells and endothelial cells, GPI-anchored proteins were observed by electron microscopy as clustered in invaginated structures of 50-60 nm diameter called caveolae (*9, 10*). Therefore, it was suggested that DICs corresponded to caveolae. However, it has also been proposed that GPI-anchored proteins are enriched in a membranous fraction distinct from caveolae when isolated without detergents (*11*), and they accumulate in DICs even from cells that lack caveolae (*12, 13*). An association between GPI-anchored proteins and membrane lipids such as glycosphingolipids and cholesterol, has been demonstrated (*12, 14*), suggesting that DICs are membrane subdomains enriched in these lipids together with proteins, including GPI-anchored proteins.

In the previous study on lymphoid cells, GPI-anchored proteins were shown to resist detergent extraction and to remain in post-nuclear pellets after detergent solubilization (*3, 15-17*). In a rat mast cell line, Thy-1 was primarily

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Abbreviations: Ab, antibody; CHO/CD59, CHO cells transfected with human CD59 cDNA; DICs, detergent-insoluble complexes; D-MEM, Dulbecco's modified Eagle's medium; D-PBS, Dulbecco's phosphate-buffered saline; DTSSP, 3,3-dithiobis(sulfosuccinimidyl-propionate); GPI, glycosyl phosphatidylinositol; mAb, monoclonal antibody; PMSF, phenylmethylsulfonyl fluoride; TBS, Tris-buffered saline.

detected in the pellet associated with the Src family kinase Lyn, although small portions were detected in DICs (*18*). Thus, GPI-anchored proteins appear to reside in both DICs and post-nuclear pellets. However, the physiological functions of and interrelations between the proteins in the two fractions remained unclear. Therefore, we established a method to obtain a GPI-anchored protein CD59 from various cell lines by a two-step solubilization procedure using Triton X-100 and octylglucoside that separated the DICs and post-nuclear pellets (see Fig. 1), and the properties of CD59 in these sediments were compared. We found that the CD59 in DICs was originally localized in intracellular compartments, whereas the CD59 in pellets was partially present on the cell surface. Moreover, the latter CD59 molecules form a homodimer on the cell surface.

MATERIALS AND METHODS

Cell Lines, Antibodies, and Reagents—The CHO cells transfected with human CD59 cDNA (CHO/CD59 cells) were obtained by a similar method to that previously described (*19*). The human leukemia cell line, K562, was a gift from Dr. John P. Atkinson (Washington University, St. Louis, MO). The other cell lines were from the Japanese Cancer Research Resources Bank (JCRB). K562 cells and Jurkat cells were maintained in RPMI 1640. CHO/CD59 cells and CHO cells were maintained in Ham F12. HeLa cells were maintained in Dulbecco's modified Eagle's medium (D-MEM). All media were supplemented with 10% fetal calf serum and antibiotics. Cultures were maintained in a 5% CO_2/95% air atmosphere at 37°C.

Monoclonal antibody (mAb) to CD59 (5H8) was a generous gift from Drs. Y. Sugita (Yamanouchi Pharmaceutical, Tokyo) and M. Tomita (Showa University, Tokyo). On flow cytometric analysis, 5H8 was highly reactive and efficiently stained CD59 molecules on various cell types (*20, 21*). Polyclonal antibody (Ab) to CD59 was obtained by immunizing rabbits with CD59 purified from human erythrocytes. mAb to DAF was a generous gift from Dr. T. Kinoshita (Osaka University, Osaka). Polyclonal Ab to caveolin was purchased from Signal Transduction Laboratories (Lexington, KY). Octylglucoside was purchased from Sigma (St. Louis, MO) and a bifunctional chemical cross linker, 3,3-dithiobis(sulfosuccinimidylpropionate) (DTSSP) from Pierce (Rockford, IL). CD59 purified from human erythrocyte membranes was a generous gift from Drs. Y. Sugita (Yamanouchi Pharmaceutical) and M. Tomita (Showa University).

Two-Step Solubilization of Proteins—Cells grown in suspension culture (4×10^7 cells) were washed with Dulbecco's phosphate-buffered saline (D-PBS) and were solubilized with 0.4 ml of lysis buffer 1 [D-PBS containing 10 mM EDTA, 1 mM PMSF (phenylmethylsulfonyl fluoride), and 1% Triton X-100]. After standing at 4°C for 30 min, supernatants and pellets were separated by centrifugation at $10,000 \times g$ for 3 min. The supernatants were further centrifuged at $240,000 \times g$ for 1 h at 4°C to obtain sup 1. The resulting pellet was solubilized with 0.4 ml of lysis buffer 2 (D-PBS containing 10 mM EDTA, 1 mM PMSF, and 60 mM octylglucoside) at 4°C for 30 min with constant agitation, and sup 1′ was obtained by ultracentrifugation at $240,000 \times g$ for 1 h at 4°C. The $10,000 \times g$ pellets of the Triton X-100 lysates were further solubilized with 0.4 ml of lysis buffer 2 at 4°C for 30 min with constant agitation, and sup 2 was obtained by ultracentrifugation at $240,000 \times g$ for 1 h at 4°C. Cells grown on dishes were solubilized similarly to those grown in suspension culture except that cells were solubilized on dishes (5 cm diameter) with 0.5 ml of each lysis buffer. The procedure is summarized in Fig. 1. Supernatants were mixed with Laemmli sample buffer (non-reduced) and stored at −30°C until subjected to electrophoresis.

Western Blotting—Protein samples (50 μl) were resolved on SDS-PAGE (11 or 12% gels) and electroblotted onto nitrocellulose membranes. Membranes were blocked with 10% non-fat dry milk in Tris-buffered saline (TBS) and probed with the first Abs (1 μg/ml) at room temperature for 1 h. After 5 washes in washing buffer (0.1% Tween 20 in TBS), membranes were incubated with 1 : 10,000 diluted horseradish peroxidase-conjugated goat anti-mouse (or -rabbit) IgG (Bio-Rad, Richmond, CA) at room temperature for 1 h and then washed 7 times before detection with an ECL system (Amersham Life Science, Arlington Heights, IL). CD59 incorporated into CHO cells (Fig. 4) was detected by a highly sensitive detection system, ECL Plus (Amersham Life Science).

Cellular Distribution of CD59—Cells grown to confluent on a culture dish (5 cm diameter) were washed with medium, and cell surface CD59 was labeled with anti-CD59 Ab (rabbit IgG, 10 μg/dish) for 45 min on ice. After washing with D-PBS, proteins were solubilized with lysis buffers 1 and 2 as shown in Fig. 1. The sup 1 and sup 2 were incubated with 30 μl of Protein A-Sepharose CL6B (Pharmacia Biotech, Uppsala, Sweden) (50% slurry in lysis buffer 1 or 2) at 4°C overnight. After binding of CD59 molecules labeled with anti-CD59 Ab, unbound proteins were removed [sup (a)] from the beads. The beads were washed twice with washing buffer A (D-PBS containing 1% NP40, 1% BSA, and 1 mM EDTA) and once with washing buffer B (D-PBS containing 0.1% NP40) and were then boiled in 150 μl of Laemmli sample buffer to release bound CD59 (extracellular CD59). The sup (a) was pre-cleared with 30 μl of Protein A-Sepharose CL6B (50% slurry in lysis buffer 1 or 2) at 4°C for 1 h, and the CD59 molecules in the sup were immunoprecipitated with anti-CD59 Ab (rabbit IgG, 10 μg) and Protein A-Sepharose CL6B at 4°C for 1 h. The CD59 molecules on the beads were released

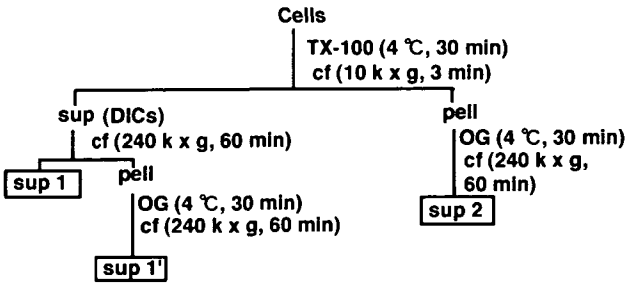

Fig. 1. **Two-step solubilization procedure for detecting GPI-anchored proteins.** Cells were solubilized with 1% Triton X-100 at 4°C. The supernatant containing DICs was ultracentrifuged and sup 1 was obtained. The $240,000 \times g$ pellet was solubilized with 60 mM octylglucoside and ultracentrifuged to obtain sup 1′. On the other hand, the $10,000 \times g$ pellet of Triton X-100 was solubilized with 60 mM octylglucoside, and sup 2 was obtained after ultracentrifugation.

similarly to extracellular CD59 after washing in buffers A and B (intracellular CD59). Fifty microliters of each sample was analyzed by Western blotting using mAb to CD59 (5H8).

Incorporation of Purified CD59 into CHO Cells—CHO cells grown on culture dishes (10 cm diameter) were thoroughly washed with D-PBS and incubated with CD59 purified from human erythrocytes (1 μg/ml D-PBS) for 15 min in a 5% CO_2/95% air atmosphere at 37°C. Cells bearing CD59 were washed with medium and the cellular distribution of incorporated CD59 was determined as described above.

Cross-Linking and Analysis of Cell Surface CD59—HeLa cells and CHO/CD59 cells grown on culture dishes (5 cm diameter) were washed with D-PBS and cross-linked with an impermeable bifunctional chemical cross-linker, DTSSP (1 mg/ml D-PBS), at 4°C for 30 min. The cells were washed 3 times with 5 ml of cold D-PBS, then proteins were solubilized with lysis buffers 1 and 2 as shown in Fig. 1. The sup 1 and sup 2 were subjected to Western blotting with anti-CD59 mAb (5H8).

For two-dimensional SDS-PAGE analysis, CHO/CD59 cells (harvested from 8 dishes, 5 cm in diameter) were surface-labeled with iodine-125 (Amersham) as described previously (*22*). After the cross-linking treatment with DTSSP, cells were solubilized as in Fig. 1. The sup 2 was pre-cleared with 10 μg of mouse IgG and Protein G-Sepharose 4 Fast Flow (Pharmacia Biotech), and then CD59 and the cross-linked products were immunoprecipitated with 5H8 and Protein G-Sepharose. The samples were resolved on two-dimensional SDS-PAGE (first dimension non-reducing and second dimension reducing), and visualized by autoradiography.

Purification of the Cross-Linked Products—Cross-linked products from CHO/CD59 cells grown on culture dishes (10 cm diameter, 207 dishes) were solubilized as described above. Sup 2 was applied to an anti-CD59 Ab (5H8) affinity column pre-equilibrated with D-PBS containing 60 mM octylglucoside, 1 mM EDTA, and 0.1 mM PMSF. The column was extensively washed with the above buffer, and bound proteins were eluted with 0.1 M glycine HCl buffer containing 60 mM octylglucoside, 1 mM EDTA, and 0.1 mM PMSF (pH 3.0). The eluted fractions were neutralized with 1 M Tris-HCl (pH 7.5), and thoroughly dialyzed against H_2O.

Amino Acid Composition and N-Terminal Amino Acid Sequence of the Cross-Linked Products—Desalted and lyophilized purified products were added to 0.2 ml of 5.7 N HCl, in a container, which was then sealed under reduced pressure, and hydrolysis was carried out at 110°C for 24 h. Amino acid analysis was performed on a Hitachi L-8500 amino acid analyzer.

For N-terminal amino acid sequence, the purified proteins were separated by non-reducing SDS-PAGE and transferred to a PVDF membrane. Proteins were stained with Coomassie Brilliant Blue R-250, and the stained bands were excised for analysis by an automated G1005A protein sequencing system (Hewlett Packard, Corvallis, OR).

RESULTS

1) Two-Step Solubilization Method for Detecting CD59 and Its Distribution in Solubilized Supernatants—The turbid 10,000 × *g* supernatants solubilized with non-ionic detergents were employed for Western blotting. By these methods, however, we failed to establish a reproducible means to detect the GPI-anchored proteins CD55 and CD59 in the detergent extracts from leukemia cell lines, particularly after storage. This was not caused by inefficiency of the extraction conditions used, since they were detected at neither higher TX-100 concentration (up to 2%) nor lower cell density (2.5×10^7 cells/ml). In addition, their detection was often achieved after immunoprecipitation or gel filtration.

Recovery of CD55 and CD59 was more easily accomplished by a combination of sedimentation and ultracentrifugation (Fig. 1). Briefly, DICs were subjected to ultracentrifugation, resulting in a clear supernatant (sup 1) which reproducibly contained Western blot-detectable GPI-anchored proteins (Fig. 2). The pellets after ultracentrifugation of DICs were further solubilized with octylglucoside (sup 1′). No GPI-anchored proteins were detected in this sediment. On the other hand, the debris remaining after the extraction of Triton X-100 was further solubilized with octylglucoside and ultracentrifuged (sup 2). This fraction also contained Western blot-detectable GPI-anchored proteins (Fig. 2). Ultracentrifugation of each extract was essential for the consistent detection of CD59. We interpreted this to mean that some components co-solubilized in the DICs and pellets of leukemia cell lines may disrupt the Western blot detection by interacting with GPI-anchored proteins.

In all cell types tested, CD59 molecules were detected in both sup 1 and sup 2 by Western blotting. The amount of CD59 in sup 2 was greater than that in sup 1. In the Jurkat cells, most of the CD59 was detected in sup 2. Another GPI-anchored protein, CD55, showed a similar distribution to CD59 (Fig. 2B), whereas transmembrane protein MCP (CD46) was primarily detected in sup 1 (Fig. 2A). Caveolin was distributed mainly in sup 2 in HeLa, CHO/CD59 (Fig. 2A), and other caveolin-positive cells (MDCK and H1080, not shown). The results were reproducible and could be obtained with samples stored for more than 2 months at −20°C.

2) Cellular Distribution of CD59—To test if the CD59 molecules recovered in sup 1 and sup 2 differ in cellular localization, we labeled cell surface CD59 with anti-CD59 polyclonal Ab on intact cells. After solubilization of the cells by the procedure in Fig. 1, labeled CD59 molecules in the sups were adsorbed on Protein-A Sepharose. Non-labeled CD59 molecules which were not sedimented were recovered by immunoprecipitation with anti-CD59 polyclonal Ab (intracellular CD59) (Fig. 3). This method discriminated properly between extracellular and intracellular proteins in a control experiment shown in Fig. 4. As the extracellular control we evaluated the distribution of purified CD59 added exogenously to CHO cells, otherwise bearing no CD59. In this model CD59 molecules were recovered exclusively as an extracellular protein in sup 1. In sup 2, more than 90% of CD59 was recovered as an extracellular protein, although a trace of CD59 was recovered as an intracellular protein. We consider that the CD59 molecules incorporated into membranes might be internalized during the incubation and thus be detected as an intracellular protein. Alternatively, CD59 molecules in caveolae structure were detected as an intracellular protein. Caveolin was

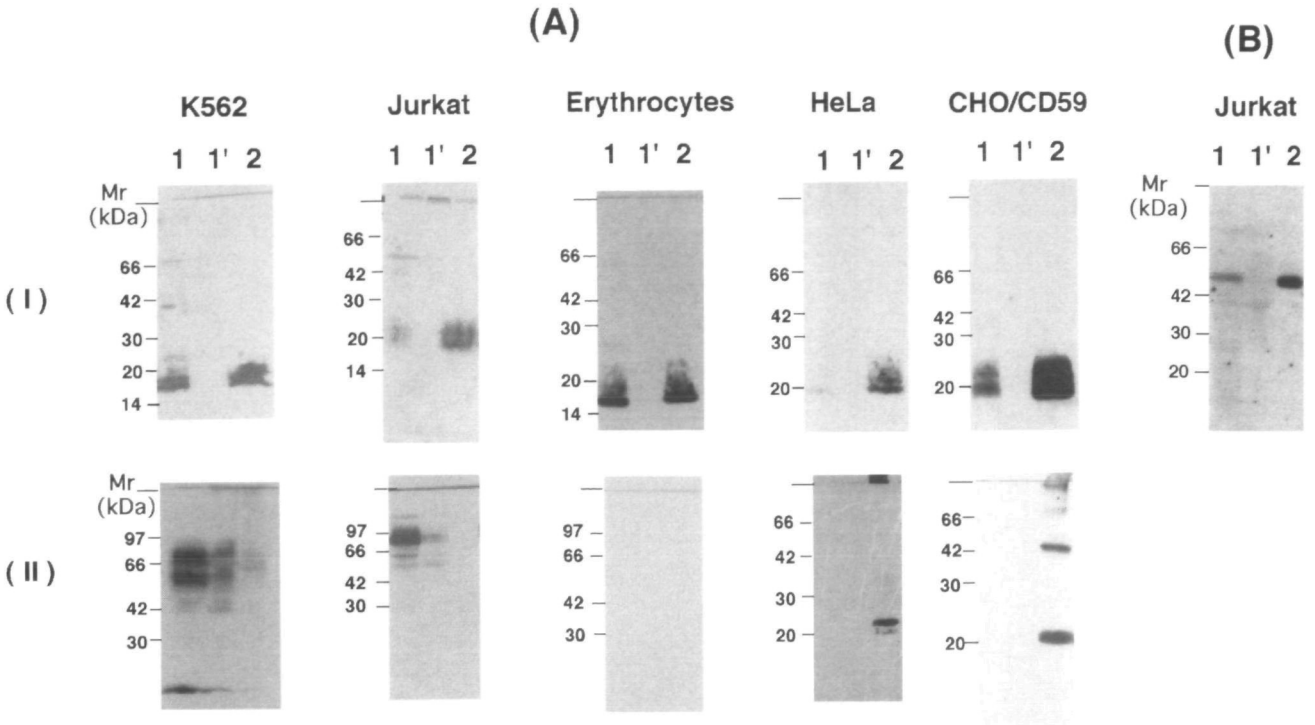

Fig. 2. **Distribution of GPI-anchored proteins in the solubilized supernatants.** Supernatants from various cells obtained by the two-step solubilization procedure were run on 10% or 11% SDS gel under non-reducing conditions. Panel A-(I), CD59 in the supernatants was detected by Western blotting with anti-CD59 mAb. Panel A-(II), MCP (K562, Jurkat, and Erythrocytes), and caveolin (HeLa and CHO/CD59) in the supernatants were detected by Western blotting with anti-MCP mAb and anti-caveolin polyclonal Ab, respectively. Panel B, DAF in the supernatants of Jurkat cells was detected by Western blotting with anti-DAF mAb.

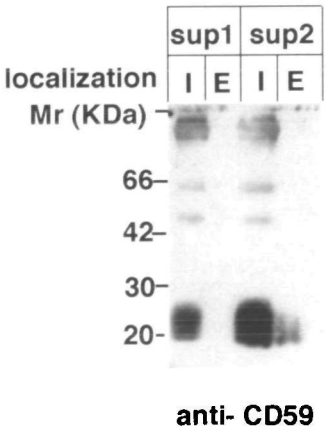

Fig. 3. **Cellular distribution of CD59.** Cell surface CD59 on CHO/CD59 cells was labeled with polyclonal Ab to CD59 for 45 min on ice. The cells were washed and proteins were solubilized by the two-step solubilization procedure described in Fig. 1. The labeled CD59 (extracellular CD59) was sedimented with Protein A Sepharose CL6B. Pre-clearing was carried out with Protein A Sepharose CL6B, then non-labeled CD59 (intracellular CD59) was immunoprecipitated with polyclonal Ab to CD59 and Protein A Sepharose CL6B. Extra- and intracellular CD59 in sup 1 and sup 2 were detected by Western blotting using anti-CD59 mAb. I, intracellular; E, extracellular.

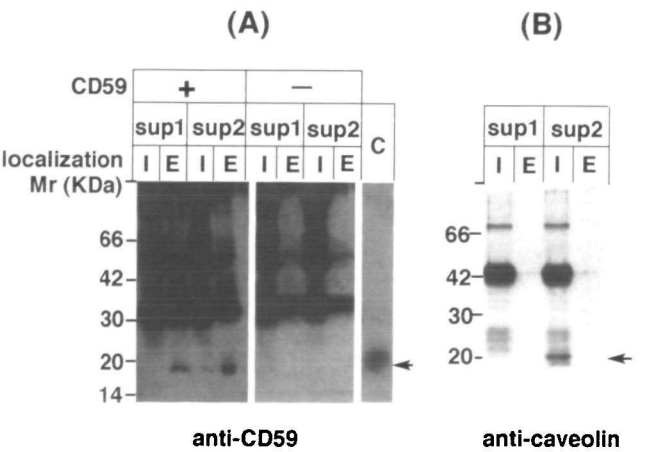

Fig. 4. **Cellular distribution of incorporated CD59 and endogenous caveolin.** Panel A, CHO cells were incubated with (+) or without (−) purified CD59 at 37°C for 15 min. They were washed with the medium, and the distribution of incorporated CD59 molecules in membranes was determined as described in Fig. 3. Panel B, extra- and intracellular caveolin in sup 1 and sup 2 were detected as described in Fig. 3, except that polyclonal Ab to caveolin was used in sedimentation and Western blotting analysis. I, intracellular; E, extracellular; C, purified CD59 for the control.

recovered exclusively as an intracellular protein in sup 2 (Fig. 4B), which is in accordance with the previous reports that showed caveolin facing only the cytosol in caveolae structure (*23, 24*).

In CHO/CD59 cells, CD59 molecules in sup 1 arose exclusively from intracellular compartments and no cell surface CD59 was detected in sup 1. On the other hand, in sup 2, although CD59 molecules were primarily from the intracellular compartment, considerable amounts were detected in cell surface fractions (Fig. 3). Similar results

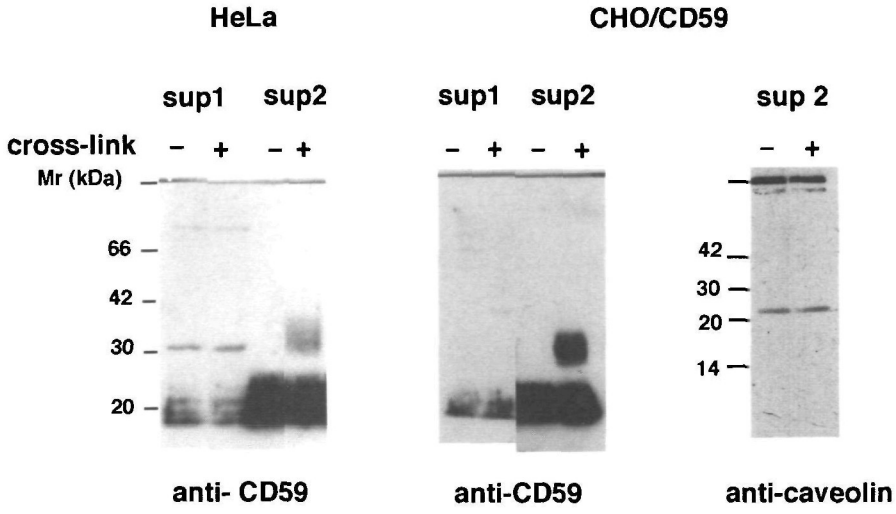

Fig. 5. **Cross-linking of cell surface CD59 by chemical cross-linker, DTSSP.** *Cell surface proteins of HeLa and CHO/CD59 cells were cross-linked with DTSSP, and proteins were solubilized as described in Fig. 1. The cross-linked products were detected by Western blotting with anti-CD59 mAb. Sup 2 from CHO/CD59 cells was Western-blotted with polyclonal Ab to caveolin (right panel).*

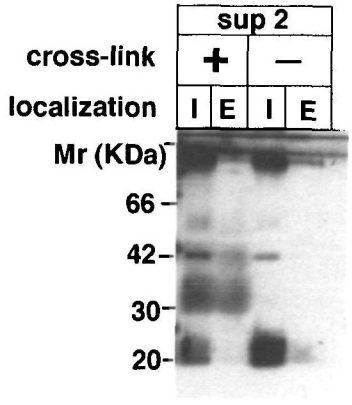

Fig. 6. **Cellular distribution of cross-linked products.** *Cell surface CD59 on CHO/CD59 cells were labeled with polyclonal Ab to CD59 for 45 min on ice. The cell were washed and cell surface proteins were cross-linked with DTSSP and solubilized by means of the two-step solubilization procedure as in Fig. 1. The distribution of cross-linked products was determined as described in Fig. 3. I, intracellular; E, extracellular.*

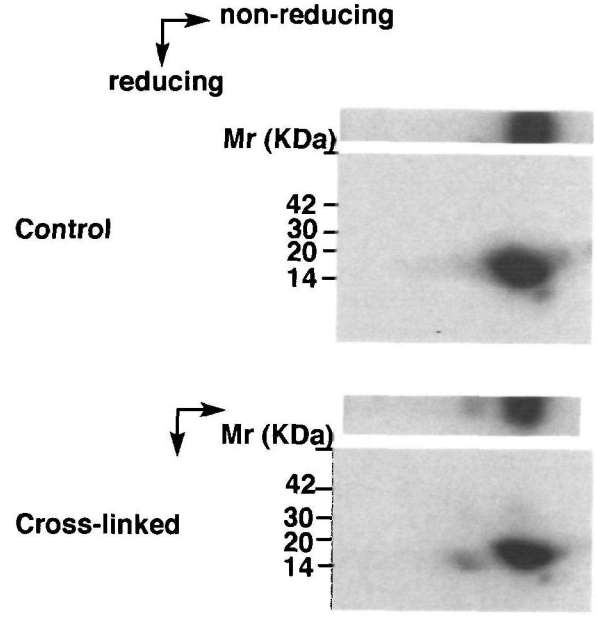

Fig. 7. **Two-dimensional SDS-PAGE of the cross-linked products.** *Surface proteins of CHO/CD59 cells were labeled with ^{125}NaI and cross-linked with DTSSP. The cellular proteins were solubilized as described in Fig. 1. CD59 and cross-linked products in sup 2 were immunoprecipitated with mAb to CD59 and separated on SDS-PAGE (non-reducing in the first dimension and reducing in the second dimension). The band of the cross-linked products was faint compared to that in the Ab-detection experiment (Fig. 5), suggesting that Ab-accessible but ^{125}NaI-inaccessible states of CD59 may present on membranes.*

were obtained with HeLa cells (data not shown).

3) Cross-Linking of CD59 Molecules on Cell Surface—To test if the CD59 molecules are linked to some other molecules on cell membranes, we cross-linked the surface proteins of HeLa cells and CHO/CD59 cells with a membrane-impermeable chemical cross-linker, DTSSP. First, we cross-linked proteins on the intact cells, solubilized membrane proteins and then detected cross-linked products in sup 1 and sup 2 by means of Western blotting using anti-CD59 mAb (5H8). The cross-linked products in the molecular weight range of 28-36 kDa were clearly detected in both HeLa and CHO/CD59 cells. The cross-linked products were found in sup 2, but not in sup 1 in both cell lines (Fig. 5). Next, we labeled cell surface CD59 with anti-CD59 polyclonal Ab after cross-linking with DTSSP and detected its cellular distribution by the method similar to that in the case of Fig. 3. As shown in Fig. 6, the cross-linked products were detected in both the intracellular and cell-surface fractions of sup 2. It should be noted that CD59 exclusively existed as a cross-linked form in the cell surface fraction, while it existed as both the cross-linked and non-cross-linked forms in the intracellular compartment. By subtracting the molecular weight of CD59, that of the CD59-associated protein was estimated to be 13-18 kDa. The relevant protein was not caveolin since its molecular mass differed from that of caveolin (22 kDa) and no trace of cross-linked products was observed by blotting with anti-caveolin Ab (Fig. 5).

4) Characterization of CD59-Associated Protein—We

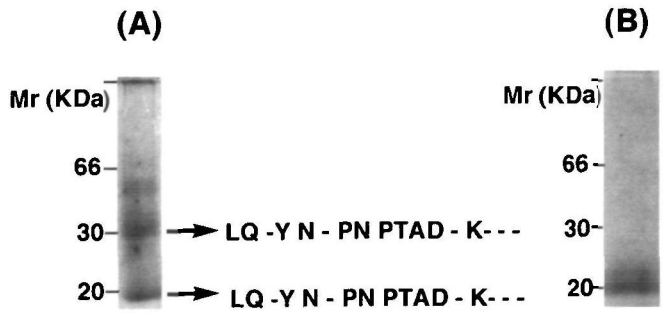

Fig. 8. **N-terminal amino acid sequences of cross-linked products.** (A) Purified cross-linked products from CHO/CD59 cells were separated on non-reducing SDS-PAGE, transferred to a PVDF membrane, and stained with Coomassie Brilliant Blue R-250. The bands were excised, and N-terminal amino acid sequences were determined. (B) On reducing SDS-PAGE, the cross-linked products showed a single band as assessed by silver staining.

tried to characterize the CD59-associated protein by two-dimensional SDS-PAGE (first dimension non-reducing and second dimension reducing). Thus, cell-surface proteins were labeled with ^{125}NaI, cross-linked and solubilized, and ^{125}I-labeled cross-linked products in sup 2 were used as the sample. As shown in Fig. 7, when the cross-linked products (28-36 kDa) were separated on the 2nd SDS-PAGE, they migrated to a position corresponding to CD59. This suggested that the CD59-associated protein is another CD59 molecule and that the cross-linked products are CD59 dimers. To clarify this point, we obtained the cross-linked products from CHO/CD59 cells and purified them to homogeneity on reducing SDS-PAGE. The CD59 monomer and cross-linked products separated on non-reducing SDS-PAGE were transferred to a PVDF membrane, and the N-terminal sequences were determined. As shown in Fig. 8, the cross-linked products showed the same N-terminal sequences as those of CD59 molecules, supporting the idea that the cross-linked products are CD59 dimers. The possibility could be excluded that another molecule with a blocked N-terminal is linked to CD59, since the amino acid composition of the cross-linked products was highly similar to that of CD59 (Table I).

TABLE I. **Amino acid composition of cross-linked products.**

	Theoretical value (residues/molecules)	Cross-linked products (residues/molecules)
Asp+Asn	14	13.8
Thr	6	5.4
Ser	2	2.1
Glu+Gln	8	6.9
Pro	2	0
Gly	1	—[a]
Ala	4	2.7
Val	3	4.1
Met	0	0
Ile	1	0.03
Leu	7	7.3
Tyr	4	3.1
Phe	4	3.6
Lys	6	7.0
His	1	0.5
Arg	2	0.8

Purified cross-linked products from CHO/CD59 cells were hydrolyzed with 5.7 N HCl as described under "MATERIALS AND METHODS". [a]Glycine content was not correctly evaluated since it was accompanied with contamination through the purification step and this was not removed even after extensive dialysis.

DISCUSSION

In this study, we solubilized CD59 in DICs (sup 1) and in post nuclear pellets (sup 2) by a two-step solubilization procedure using Triton X-100 and octylglucoside. Using this method, we demonstrated that CD59 molecules are recovered in both fractions and that the amount was greater in the latter fraction in all cell types tested. Another GPI-anchored protein, CD55, was similarly extracted (Fig. 2B), suggesting that the distribution pattern may be general for GPI-anchored proteins. Additionally, the latter fraction contained less than 10% of the total cellular proteins; thus, we were able to purify CD59 and the cross-linked products by one step purification through affinity chromatography. Our method may provide an efficient purification of GPI-anchored proteins in general.

Cross-linking of GPI-anchored proteins with antibodies causes activation of T cells and neutrophils (*25-27*). The activation is likely to be mediated through Src family tyrosine kinases. By specific labeling of surface CD59 molecules, we demonstrated that the CD59 detected in DICs was localized intracellularly, whereas that in post-nuclear pellets was partly present on the cell surface. This finding is surprising because DICs were suggested to be a specialized membrane region rich in signaling molecules, such as Src family kinases and G-proteins (*28*), and may act as a signaling center for GPI-anchored proteins, and thus, should be accessible from the extracellular side. GPI-anchored proteins become detergent-insoluble only after having been sorted in the early *trans*-Golgi network (*14*), which may explain the properties of DICs representing solubilized complexes of Golgi network components. In contrast, CD59 recovered in post-nuclear pellets was originally at least partially localized on the cell surface and accessible to Ab, indicating a signaling capacity.

CD59 molecules in post-nuclear pellets formed non-covalent dimers on HeLa and CHO/CD59 cells. On the cell surface, CD59 existed mainly as homodimers. Although the cross-linker used in this study was impermeable, dimers were also found in the intracellular fraction. It is not known to which fractions proteins in caveolae structures distribute, although caveolin that faces only the cytosol was detected in the intracellular fraction. It is likely that the CD59 molecules residing in caveolae structures were detected in the intracellular fraction: they can be cross-linked, since the cross-linker was small enough to enter caveolae, but can not be monitored by surface-labeling Ab since Ab was too large to enter. Alternatively, dimers may be translocated inside the cells, but the mechanisms involved remain unclear.

First, we expected to detect signaling molecules bridging both CD59 and Src kinases by the cross-linking experiment. However, under the conditions tested, we only detected cross-linked products of CD59 dimers on these cells. Although the possibility remains that signaling molecules would be detected under some more efficient cross-linking conditions, we favor the mechanism suggested by Dráberová *et al.*; activation of GPI-anchored proteins may induce aggregation of co-localizing Src kinases, thus

inducing their transphosphorylation, which leads to cellular stimulation (*18*).

A number of signal-transducing molecules have been reported to form dimers to facilitate each other's activation (*29-32*). Most of them are transmembrane or intracellular proteins. Regarding short transmembrane and GPI-anchored proteins (LIF, CNTF, *etc.*), gp130 and LIFβ can transmit signals to intracellular second messengers. In addition, CD55 forms homodimers on human and monkey cells (*33*), and CD59 may be dimerized on human platelets (*34*). Dimer formation may affect the physiological functions of CD59, such as inhibition of MAC formation and signaling. These findings led us to analyze whether the dimers are implicated in the effective ligand capturing or signaling mechanisms. Since CD59-mediated signaling is not observed in epithelial cells, we are now investigating the importance of CD59 dimer formation in lymphocytes or neutrophils, in which signaling actually passes through GPI-anchored proteins.

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