GDP-mannose-4,6-dehydratase (GMDS) Deficiency Renders Colon Cancer Cells Resistant to Tumor Necrosis Factor-related Apoptosis-inducing Ligand (TRAIL) Receptor- and CD95-mediated Apoptosis by Inhibiting Complex II Formation*

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Background: An impaired TRAIL-induced apoptosis by GMDS deficiency resulted in tumor progression.

Results: Caspase-8 activation at FADD-dependent complex II upon TRAIL or CD95L stimulation was inhibited by GMDS deficiency.

Conclusion: GMDS regulated the transition from a primary DISC to a secondary complex II upon TRAIL or CD95L stimulation.

Significance: Our findings develop a better understanding about death receptor signaling complex.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces apoptosis through binding to TRAIL receptors, death receptor 4 (DR4), and DR5. TRAIL has potential therapeutic value against cancer because of its selective cytotoxic effects on several transformed cell types. Fucosylation of proteins and lipids on the cell surface is a very important post-translational modification that is involved in many cellular events. Recently, we found that a deficiency in GDP-mannose-4,6-dehydratase (GMDS) rendered colon cancer cells resistant to TRAIL-induced apoptosis, resulting in tumor development and metastasis by escape from tumor immune surveillance. GMDS is an indispensable regulator of cellular fucosylation. In this study, we investigated the molecular mechanism of inhibition of TRAIL signaling by GMDS deficiency. DR4, but not DR5, was found to be fucosylated; however, GMDS deficiency inhibited both DR4- and DR5-mediated apoptosis despite the absence of fucosylation on DR5. In addition, GMDS deficiency also inhibited CD95-mediated apoptosis but not the intrinsic apoptosis pathway induced by anti-cancer drugs. Binding of TRAIL and CD95 ligand to their cognate receptors primarily leads to formation of a complex comprising the receptor, FADD, and caspase-8, referred to as the death-inducing signaling complex (DISC). GMDS deficiency did not affect formation of the primary DISC or recruitment to and activation of caspase-8 on the DISC. However, formation of secondary FADD-dependent complex II, comprising caspase-8 and cFLIP, was significantly inhibited by GMDS deficiency. These results indicate that GMDS regulates the formation of secondary complex II from the primary DISC independent of direct fucosylation of death receptors.

Apoptosis is essential to many biological processes in multicellular organisms, including embryonic development, immune responses, tissue homeostasis, and normal cell turnover. Deregulation of apoptosis is an important aspect of cancer pathogenesis and has been widely recognized as a hallmark of most types of cancer (1). Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL2/Apo-2L) has been shown to induce apoptosis in cancer cells by its engagement with cognate receptors, TRAIL receptor 1 (death receptor 4 (DR4)) and TRAIL receptor 2 (DR5) (2). These receptors transmit a caspase-activating death signal through a cytoplasmic death domain (DD). In contrast, neither TRAIL receptor 3 (Decr1) nor TRAIL receptor 4 (Decr2) can mediate apoptosis due to the complete or partial absence of DD, respectively. TRAIL is expressed on cytotoxic T cells and natural killer (NK) cells and is an important immune effector molecule in the surveillance and elimination of developing cancers (3). Thus, inactivation of the TRAIL pathway followed by escape from TRAIL-mediated immunosurveillance has an important role in cancer onset and progression (4). As with the TRAIL receptors, CD95 (Fas/Apo-1) is also a member of the DR family, which belongs to the TNF receptor superfamily (5). Cross-linking of CD95 with its natural ligand CD95L (CD178) or with agonistic antibodies induces apoptosis in a wide variety of cells, including lympho-

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2 The abbreviations used are: TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; DR, death receptor; GMDS, GDP-mannose-4,6-dehydratase; DISC, death-inducing signaling complex; NK, natural killer; DD, death domain; FADD, Fas-associated death domain; cFLIP, cellular FADD-like interleukin-1 β-converting enzyme-inhibitory protein; DD, death effector domain; PARP, poly(ADP-ribose) polymerase; NF-κB, nuclear factor-κB; HPRT, hypoxanthine guanine phosphoribosyltransferase; Z, benzoyloxycarbonyl; fnk, fluoromethyl ketone; PNGase F, peptide/N-glycosidase F, AAL, A. aurantia lectin.
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cytes (6). CD95-mediated apoptosis plays a pivotal role in activation-induced cell death, termination of immune responses, elimination of autoreactive cells, immune privilege, and cancer elimination through effector function of T and NK cells (7).

TRAIL- and CD95L-mediated apoptosis mechanisms are executed through similar signaling pathways (8). Activation of these DRs leads primarily to the formation of the death-inducing signaling complex (DISC) formed by the recruitment of the Fas-associated death domain (FADD), caspase-8 (and, in some cases, caspase-10), and the cellular FADD-like interleukin-1β-converting enzyme (FLICE)-inhibitory protein (cFLIP). In a homotypic interaction, the DD of FADD binds to the DD of these DRs. The death effector domain (DED) of FADD in turn interacts with the DED of procaspase-8 and thereby recruits this proenzyme to the CD95, DR4, and DR5 DISCs. Pro-caspase-8 is proteolytically cleaved and thereby activated at the DISC. Activated caspase-8 then initiates the apoptosis-executing intracellular signaling pathway.

In recent reports, we demonstrated that a deficiency in GDP-mannose-4,6-dehydratase (GMDS) leads to escape from NK cell-mediated tumor surveillance through the acquisition of resistance to TRAIL-induced apoptosis, followed by tumor progression and metastasis (9, 10). This study indicated the significance of GMDS as a regulator of TRAIL-induced apoptosis. GMDS is an enzyme responsible for the synthesis of GDP-fucose, which is an indispensable donor substrate for cellular fucosylation (11, 12). Fucosylation involves the attachment of an 1-fucose residue to an oligosaccharide or protein and is one of the most important oligosaccharide modifications in cancer (13). Most cell surface receptors, including epidermal growth factor receptor (14), transforming growth factor-β receptor (15), E-cadherin (16), and integrins (17), are fucosylated. Fucosylated oligosaccharides regulate many physiological and pathological events, including cell growth, migration, embryogenesis, and tumor invasion, by affecting the folding and structure of these cell surface receptors (18). Recently, Wagner et al. (19) reported that O-glycosylation regulated TRAIL-induced apoptosis by promoting ligand-induced clustering of DR4 and DR5, which in turn mediated the recruitment and activation of caspase-8. This result clearly showed that glycosylation is one of the most important regulators of TRAIL signaling. However, the mechanisms by which GMDS regulates TRAIL-induced apoptosis remain unknown. In the present study, we demonstrated that GMDS deficiency results in impairment of CD95- and TRAIL-induced apoptosis by suppressing the formation of complex II, which comprises FADD, caspase-8, and cFLIP.

EXPERIMENTAL PROCEDURES

Cell Lines and Materials—GMDS- and mock-rescued HCT116 cells were previously established (9). Briefly, pcDNA3.1/Hyg (Invitrogen) vector carrying human wild-type GMDS was transfected into HCT116 cells, which were purchased from the American Type Culture Collection (Manassas, VA), with the FuGENE 6 transfection reagent (Roche Applied Science) according to the manufacturer's protocol. Selection was performed by adding Hygromycin B (Calbiochem) at 150 μg/ml. These cells were cultured in RPMI1640 supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum. For Western blotting, rabbit polyclonal anti-DR5, rabbit polyclonal anti-cleaved PARP (Asp-214), rabbit polyclonal anti-β-actin (Cell Signaling, Beverly, MA), rabbit polyclonal anti-DR4 (BD Biosciences), mouse monoclonal anti-caspase-8 (12F5), mouse monoclonal anti-cFLIP (NF6) (Enzo Life Sciences, Farmingdale, NY), rabbit polyclonal anti-FADD(125–140) (Calbiochem), and rabbit polyclonal anti-CD95 (C-20) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antibodies were used. For immunoprecipitation, mouse monoclonal anti-DR4 (DR-4-02), mouse monoclonal anti-DR5 (B-D37), rabbit polyclonal anti-FADD (H181) (Santa Cruz Biotechnology, Inc.), and mouse monoclonal anti-FLAG (M2) (Sigma) antibodies were used. For flow cytometry, mouse monoclonal anti-DR4 (catalog no. 69036), mouse monoclonal anti-DR5 (catalog no. 71908), mouse monoclonal anti-DR1 ( catalog no. 90906), and mouse monoclonal anti-DcR2 (catalog no. 104918) (R&D Systems, Minneapolis, MN) antibodies were used. A mouse monoclonal anti-CD95 antibody (DX2) purchased from BD Biosciences was used for both flow cytometry and induction of apoptosis. A goat polyclonal anti-DR5 agonistic antibody was purchased from R&D Systems and used for apoptosis induction. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Promega, Madison, WI), anti-mouse IgG1 (Enzo Life Sciences), and anti-rabbit IgG (Cell Signaling) secondary antibodies were used. Alexa488-labeled anti-mouse IgG was purchased from Invitrogen. Recombinant TRAIL was purchased from BIOMOL (Plymouth Meeting, PA). Z-VAD-fmk and Z-IETD-fmk were purchased from R&D Systems. LY294002, PD98059, SP600125, and etoposide were purchased from Merck. SN50 and SN50M were purchased from Enzo Life Sciences. Recombinant Protein G from Streptococcus sp., 5-fluorouracil, rapamycin, and cisplatin were purchased from Sigma. PNGase F was purchased from Roche Applied Science.

Western Blotting and Lecin Blotting—Proteins were subjected to SDS-PAGE under reducing conditions and then transferred to a polyvinylidine difluoride membrane (Millipore, Woburn, MA). After blocking with phosphate-buffered saline (PBS) containing 5% skim milk for 1 h at room temperature, the membranes were incubated with primary antibodies overnight at 4 °C. After washing the membrane with Tris-buffered saline containing 0.05% Tween 20 (TBST) (pH 7.4), the membrane was incubated with HRP-labeled secondary antibodies. For lectin blotting, the protein-transferred membrane was blocked with 3% bovine serum albumin (BSA) overnight at 4 °C. Then the membrane was incubated with biotinylated Aleuria aurantia lectin (AAL; Seikagaku Corp., Tokyo, Japan). AAL is a lectin that specifically binds to fucosylated oligosaccharides (20). After washing, the membrane was incubated with diluted avidin-peroxidase conjugates (ABC kit, Vector Laboratories, Burlingame, CA). Enhanced chemiluminescence (ECL) development was performed using an ECL kit (GE Healthcare) or Immobilon Western (Millipore) according to the respective manufacturer's protocol.

Transfection of siRNA—Cells were transfected with 50 nm siRNA against each TRAIL receptor using a TransIT TKO transfection reagent (Mirus BIO LLC, Madison, WI), according to the manufacturer's procedure. The sequences of each siRNA
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Table 1

| Genes     | Analysis   | Sequences*                  | Annealing | Cycle |
|-----------|------------|-----------------------------|-----------|-------|
| DR4       | RT-PCR     | F:\'\'-CAGAAGCTTTAAGAGCCTTGCAAGCTACG-3\' | 66        | 30    |
| DR5       | RT-PCR     | R:\'\'-AGGATGCGTCACTTCAAGCTACG-3\' | 66        | 30    |
| DcR1      | RT-PCR     | F:\'\'-CAGAAGCTTTAAGAGCCTTGCAAGCTACG-3\' | 61        | 32    |
| DcR2      | RT-PCR     | R:\'\'-AGGATGCGTCACTTCAAGCTACG-3\' | 65        | 32    |
| GAPDH     | RT-PCR     | F:\'\'-AACCCTCTAGGTGCTTCAAGCTACG-3\' | 61        | 32    |
| DR5       | Real time  | F:\'\'-TGTAACATTACACCGAGCTTACGAC-3\' | 66        |       |
| DcR1      | Real time  | R:\'\'-TTCTGAGATCCGGCACTACGAC-3\' | 66        |       |
| DcR2      | Real time  | F:\'\'-AGGAGGTAGCCCGTCAAGCTACG-3\' | 66        |       |
| HPRT      | Real time  | R:\'\'-TGACACTGGGCAAACCAATGCA-3\' | 66        |       |
| DR4       | siRNA      | 5:\'\'-GGACACCUUCCGGAAGACAGCT-3\' |          |       |
| DR5       | siRNA      | 5:\'\'-GAAGACCGUAGAGAAGACAGCT-3\' |          |       |
| DcR1      | siRNA      | 5:\'\'-CAAACAGUAAAGUAGACAGCT-3\' |          |       |
| DcR2      | siRNA      | 5:\'\'-GAACAUUCAAGGAAAGACAGCT-3\' |          |       |
| Negative 1 | siRNA     | 5:\'\'-UCUAAAGGGAACGUAAGCAGCT-3\' |          |       |
| Negative 2 | siRNA     | 5:\'\'-GCGCAUAAAGCAGCAAGAGCT-3\' |          |       |

* F, forward primer; R, reverse primer.

are provided in Table 1. All siRNAs were designed by and purchased from Takara Bio (Shiga, Japan).

Reverse Transcription (RT)-PCR—Total RNA was extracted from cells using the TRIzol reagent (Invitrogen). The RNA concentration was determined spectrophotometrically, and samples were then stored at −80 °C until use. cDNA was synthesized from 1 μg of total RNA using oligo(dT) primers and SuperScript III reverse transcriptase, according to the manufacturer’s procedure (Invitrogen). Then PCR amplification was performed using Ex TaqHS polymerase (Takara Bio). Denature and extension steps were performed at 94 °C for 30 s and at 70 °C for 2 min, respectively. Annealing temperature and amplification cycle were described in Table 1. The PCR products were visualized on a 1.5% agarose gel stained with ethidium bromide. For real-time RT-PCR, 500 ng of RNA was reverse transcribed using the PrimeScript RT reagent kit (Takara Bio), according to the manufacturer’s procedure. Each synthesized cDNA was diluted 10-fold. Two μl of each diluted cDNA was adjusted to a 20-μl solution containing 1× SYBR Premix Ex TaqII (TAKARA BIO) and 0.4 μM forward and reverse primers. Real-time PCR analysis was carried out using a Chromo4 Real-Time PCR Detector (Bio-Rad). After initial polymerase activation and a denaturation step of 10 s at 95 °C, the samples underwent 40 amplification cycles, each comprising 5 s at 95 °C and 20 s at 66 °C. Fluorescence was measured at the end of each cycle to monitor the progress of amplification. A melting curve was recorded by heating slowly at 0.5 °C/s from 66 to 95 °C and holding for 3 s. Fluorescence was measured continuously during the slow temperature increase to monitor the dissociation of synthesized double strand PCR products. The primer sequences for the genes used in this study are provided in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and hypoxanthine guanine phosphoribosyltransferase (HPRT) were used as internal controls. The results of real-time RT-PCR analysis were normalized relative to HPRT.

Immunoprecipitation—Cells were lysed in TNE buffer (10 mM Tris-HCl (pH 7.8), 1% Nonidet P-40, 0.15 mM NaCl, and 1 mM EDTA), including a protease inhibitor mixture (Complete Mini EDTA-free, Roche Applied Science), and then protein was extracted. The concentration of protein was quantified using a bicinchoninic acid kit (Thermo Fisher Scientific, Waltham, MA). For immunoprecipitation, antibodies were coupled to Protein G-Sepharose (GE Healthcare) for 3 h at 4 °C. Cell lysates were preincubated with Protein G-Sepharose for 3 h at 4 °C to exclude nonspecific binding to Protein G-Sepharose. After centrifugation, supernatants containing proteins were added to Protein G-Sepharose-coupled antibodies and then rotated overnight at 4 °C. The Protein G-Sepharose was then washed five times with TNE buffer and then resuspended in sample buffer for SDS-PAGE. After boiling, supernatants were subjected to SDS-PAGE.

Flow Cytometry—Cells were harvested with PBS containing 1 mM EDTA and then incubated with each primary antibody (5 μg/ml) in PBS containing 0.1% BSA for 20 min on ice. After washing once, the cells were incubated with Alexa488-labeled anti-mouse IgG (8 μg/ml) in PBS containing 0.1% BSA for 20 min on ice. Flow cytometric analysis was performed using a FACScan® flow cytometer operated with CellQuestPro software version 5.2, (BD Biosciences). Cell debris that showed low values of forward and side scatters was gated out. Ten thousand events were acquired in each sample.

Sub-G1 Assay—Cells were treated with recombinant TRAIL and anti-CD95 agonistic antibody (DX2). Anti-CD95 antibody was cross-linked with recombinant Protein G by rotation for 30 min just before use. After treatment for 24 h, cells were harvested in PBS containing 1 mM EDTA, and fixation was then performed in ice-cold 50% ethanol for 40 min. Subsequently, the cells were resuspended in PBS containing 1 mg/ml RNase and warmed at 37 °C for 20 min to degrade cellular RNA. Cellular DNA was then stained for 10 min at 4 °C in PBS containing 100 μg/ml propidium iodide (Sigma). The cells were sorted
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according to their DNA content using a FACSCalibur flow cytometer and analyzed by Modifit software (version 3.0). Cell debris and fixation artifacts were gated out. Ten thousand events were acquired in each sample. The cells containing sub-G1 DNA were calculated as a percentage of total cells.

Clonogenic Survival Assay—Cells were plated in a 6-well plate at 1.2 × 10^3 cells/well. After overnight attachment, the cells were treated with the various concentrations of each anti-cancer drug for 2 days. The medium was subsequently changed every 3 days. After 10 days, the surviving colonies were fixed with formalin and stained with crystal violet. The area of survival cells was quantified with ImageJ software.

Measurement of Caspase-3 and -8 Activities—Caspase-3 and -8 activities were determined using the caspase-3 and -8 colorimetric assay kits, respectively (BioVision, Mountain View, CA), according to the manufacturer’s procedure. Briefly, cells were plated in a 6-cm dish at 9.0 × 10^3 cells/dish. After 48 h, the cells were treated with each reagent for 2.5 h. The cells were detached and lysed in chilled cell lysis buffer for 10 min at 4 °C. After centrifugation, supernatants were prepared as cytosolic extracts and quantified. These cytosolic extracts were reacted with DEVD-p-nitroanilide or IETD-p-nitroanilide substrate in reaction buffer containing 10 mM DTT for measurement of caspase-3 or caspase-8 activities, respectively. The p-nitroanilide light emission was quantified using a BenchMark microplate reader (Bio-Rad) at 405 nm.

Analysis of DISC or Complex II Formation by Immunoprecipitation—DISC analysis using FLAG-tagged TRAIL (FLAG-TRAIL) or FLAG-tagged CD95L (FLAG-CD95L) (Enzo Life Sciences) was performed as described previously (21). For ligand affinity precipitation, at 48 h after 7.5 × 10^5 cells were seeded onto 15-cm culture dishes, cells were incubated for the indicated times in the presence of 500 ng/ml FLAG-TRAIL or FLAG-CD95L complexed with 2 μg/ml anti-FLAG M2 antibody by rotation for 30 min. As a control experiment, cells were incubated with only 2 μg/ml anti-FLAG M2 antibody. Then cells were lysed by the addition of 1 ml of lysis buffer (30 mM Tris/HCl (pH 7.4), 150 mM NaCl, 5 mM KCl, 10% glycerol, 2 mM EDTA, and 1% Triton). Aliquots containing 1 mg of proteins were subjected to immunoprecipitation using Protein G-Sepharose and an additional 1 μg/ml anti-FLAG M2 antibody overnight at 4 °C. Ligand affinity precipitates were washed and subjected to SDS-PAGE under reducing conditions. For analysis of complex II, cells were incubated for the indicated times with either 50 ng/ml recombinant TRAIL or 2.5 μg/ml anti-CD95 agonistic antibody (DX2), which was cross-linked with 5 μg/ml recombinant Protein G for 30 min. As a control for the experiment using anti-CD95 agonistic antibody, mouse IgG1 isotype control (BD Bioscience) was used. After cell lysis, the protein complexes were immunoprecipitated using anti-FADD antibody (H181) overnight, and the precipitates were washed and subjected to SDS-PAGE under reducing conditions.

RESULTS

Fucosylation on N-Glycan of DR4, but Not DR5, Is Restored in GMDS-rescued HCT116 Cells—In our previous study (9), we found that a colon cancer cell line, HCT116, had deletion mutation of GMDS followed by a deficiency of fucosylation. We fur-
ilar between mock- and GMDS-rescued cells (Fig. 1B). Mean fluorescence intensity of the data indicates no influence of a deficiency of fucosylation on protein expression and trafficking to the cell surface membrane. DR4, but not DR5, has a putative N-glycosylation site. Treatment with PNGase F, which removes N-glycans from proteins, decreased the molecular weight of DR4, indicating the existence of N-glycan on DR4 (Fig. 1C). Restoration of fucosylation of DR4 in GMDS-rescued cells was confirmed by DR4 immunoprecipitation followed by lectin blotting using AAL, which binds to fucosylated glycans (Fig. 1D). Because the previous study by Wagner et al. (19) demonstrated the existence of O-glycans on DR4 and DR5, we examined whether fucose residues were attached to N-glycans and/or O-glycans. As shown in Fig. 1E, fucosylation of DR4 in GMDS-rescued cells disappeared upon treatment with PNGase F, indicating the attachment of fucose residues to N-glycans, but not O-glycans, on DR4. Subsequently, we examined whether fucosylated glycans were observed on DR5. We could not detect AAL binding at the molecular weight corresponding to DR5 in GMDS-rescued cells when DR5 immunoprecipitation followed by lectin blotting was performed (Fig. 1F). This result indicates that DR5 in GMDS-rescued cells did not have fucosylated glycans.

**DR4- and DR5-mediated Apoptosis Is Increased in GMDS-rescued Cells**—It has been shown that, in cancer cells that express both receptors, the relative contributions of DR4 and DR5 to TRAIL-induced apoptosis are dependent on cell type (23, 24). To examine which receptor is primarily responsible for the increased TRAIL-induced apoptosis in GMDS-rescued cells, siRNAs against each TRAIL receptor were used. In Fig. 2A, the specificity and efficiency of siRNA were determined by real-time RT-PCR (top) and flow cytometry (bottom) analyses. Mock- and GMDS-rescued cells were transfected with siRNAs against DR4 and DR5. At 24 and 48 h after transfection, real-time RT-PCR analysis and flow cytometry analyses were performed, respectively. * and **, p < 0.05 (siCont1 mock versus siDR4 or siDR5 mock (*) and siCont1 WT-GMDS versus siDR4 or siDR5 WT-GMDS (**), Mann-Whitney U test). The inset numbers indicate the difference of mean fluorescence intensity of staining in the presence (solid line) and absence (dashed line) of the first antibody. Error bars, S.D. B, at 48 h after transfection with DR4 and/or DR5 siRNA, transfected cells were treated with TRAIL for 2.5 h. Lysates were then extracted, and the expression of cleaved PARP was determined by Western blotting using 8% polyacrylamide gel. C, the involvement of DR5 was determined using anti-DR5 agonistic antibody. Mock- and GMDS-rescued cells were treated with the indicated concentrations of anti-DR5 agonistic antibody (R&D Systems) for 2.5 h. The expression of cleaved PARP was then determined by Western blotting.

**FIGURE 2.** Both DR4 and DR5 contribute to increased TRAIL-induced apoptosis in GMDS-rescued cells. A, the specificity and efficiency of siRNA were determined by real-time RT-PCR (top) and flow cytometry (bottom) analyses. Mock- and GMDS-rescued cells were transfected with siRNAs against DR4 and DR5. At 24 and 48 h after transfection, real-time RT-PCR analysis and flow cytometry analyses were performed, respectively. * and **, p < 0.05 (siCont1 mock versus siDR4 or siDR5 mock (*) and siCont1 WT-GMDS versus siDR4 or siDR5 WT-GMDS (**), Mann-Whitney U test). The inset numbers indicate the difference of mean fluorescence intensity of staining in the presence (solid line) and absence (dashed line) of the first antibody. Error bars, S.D. B, at 48 h after transfection with DR4 and/or DR5 siRNA, transfected cells were treated with TRAIL for 2.5 h. Lysates were then extracted, and the expression of cleaved PARP was determined by Western blotting using 8% polyacrylamide gel. C, the involvement of DR5 was determined using anti-DR5 agonistic antibody (R&D Systems) for 2.5 h. The expression of cleaved PARP was then determined by Western blotting.

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indicating that both DR4 and DR5 contribute to increased TRAIL-induced PARP cleavage in GMDS-rescued cells. Furthermore, to confirm the involvement of DR5 in TRAIL-induced apoptosis in GMDS-rescued cells, we used an anti-DR5 specific agonistic antibody. Treatment with this antibody induced PARP cleavage more significantly in GMDS-rescued cells than in mock-rescued cells (Fig. 2C), indicating that DR4- and DR5-mediated apoptosis was significantly increased by the restoration of GMDS despite the absence of fucosylation on DR5.

Decoy Receptors Do Not Contribute to Increased TRAIL-induced Apoptosis in GMDS-rescued Cells—We examined whether DcR1 and DcR2 are involved in the observed increase in TRAIL-induced apoptosis in GMDS-rescued cells. No alteration in gene expression of DcR1 and DcR2 was observed (Fig. 3A). Cell surface expression of DcR1 and DcR2 was also quite similar between mock- and GMDS-rescued cells (Fig. 3B). Treatment with DcR1 siRNA resulted in no alterations in TRAIL-induced PARP cleavage. When mock- and GMDS-rescued cells were treated with DcR2 siRNA, TRAIL-induced PARP cleavage was increased in both mock- and GMDS-rescued cells; however, differences of sensitivity in TRAIL-induced PARP cleavage between both types of cells were not abolished (Fig. 3C). The specificity and efficiency of DcR1 and DcR2 siRNA were examined by real-time PCR and flow cytometry analysis (Fig. 3D). These results indicate that decoy receptors are not responsible for acquiring resistance to TRAIL-induced apoptosis in GMDS deficiency.

CD95-mediated, but Not Anti-cancer Drug-mediated, Apoptosis Is Increased in GMDS-rescued Cells—CD95 exhibits relatively high homology to TRAIL receptors in the TNF superfamily and shares an apoptosis-inducing mechanism with them. We therefore examined CD95-mediated apoptosis in mock- and GMDS-rescued cells. The expression of CD95 on the cell surface was quite similar between mock- and GMDS-rescued cells (Fig. 4A). When cells were treated with anti-CD95 agonistic antibody, increases in cleaved PARP expression and the sub-G1 population were observed in GMDS-rescued cells compared with mock-rescued cells (Fig. 4B and C). As with cleaved PARP, the sub-G1 population serves as a marker of cells undergoing apoptosis. These results indicate that GMDS deficiency leads to a decrease in CD95-mediated apoptosis. To examine the sensitivity to other apoptosis-inducing stimuli in mock- and GMDS-rescued cells, we treated cells with anti-cancer drugs. No alterations in cell death were observed upon treatment with etoposide, 5-fluorouracil, rapamycin, or cisplatin (Fig. 4D and E). Altogether, GMDS deficiency results in the acquisition of resistance to extrinsic apoptotic stimuli through TRAIL receptors and CD95 but not to intrinsic signals caused by certain anti-cancer drugs.

FIGURE 3. Neither DcR1 nor DcR2 is involved in the increased TRAIL-induced apoptosis in GMDS-rescued cells. A, gene expression levels of DcR1 and DcR2 was determined by RT-PCR. RT, reverse transcription. B, expression levels of DcR1 and DcR2 on the cell surface were determined by flow cytometry. As first antibodies, anti-DcR1 (catalog no. 90906) and DcR2 (catalog no. 104918) antibodies were used. The inset numbers indicate the difference of mean fluorescence intensity of staining in the presence (solid line) and absence (dashed line) of the first antibody. C, at 48 h after transfection with DcR1 or DcR2 siRNA, the cells were treated with TRAIL for 2.5 h. Cell lysates were extracted, and the expression of cleaved PARP was determined by Western blotting. D, the specificity and efficiency of siRNA were determined by real-time RT-PCR (top) and flow cytometry (bottom) analysis. Mock- and GMDS-rescued cells were transfected with siRNAs against DcR1 and DcR2. At 24 and 48 h after transfection, real-time RT-PCR and flow cytometry analyses were performed, respectively. * and **, p < 0.05 (siCont1 mock versus siDR4 or siDR5 mock (*) and siCont1 WT-GMDS versus siDR4 or siDR5 WT-GMDS (**), Mann-Whitney U test). Error bars, S.D.
The Restoration of GMDS Augments TRAIL- and CD95-mediated Caspase-8 Activation—To determine the step in apoptosis signaling at which TRAIL receptor- and CD95-mediated apoptosis is inhibited by GMDS deficiency, we examined the activation of caspase-3 and -8 because these are late and early events after ligand-receptor binding, respectively. After treatment with TRAIL, the augmented activation of caspase-3 and -8 was observed in GMDS-rescued cells compared with mock-rescued cells (Fig. 5A). In addition, Western blotting using anti-caspase-8 antibody also confirmed the augmentation of caspase-8 activation upon TRAIL treatment in GMDS-rescued cells (Fig. 5C). When mock- and GMDS-rescued cells were treated with anti-CD95 antibody, the same results were obtained (Fig. 5B and D).

The Increase in TRAIL-induced Apoptosis in GMDS-rescued Cells Is Dependent on Augmented Activation of Caspase-8—To confirm whether augmented caspase-8 activation is responsible for the increase of TRAIL-induced apoptosis, a caspase-8 inhibitor, Z-IETD-fmk, was used. As shown in Fig. 6A, treatment with Z-IETD-fmk resulted in almost complete inhibition of TRAIL-induced PARP cleavage. The caspase-3 inhibitor, Z-VAD-fmk, also inhibited TRAIL-induced PARP cleavage almost completely. In addition, the increase of sub-G1 population upon TRAIL stimulation in GMDS-rescued cells was completely blocked in the presence of Z-VAD-fmk or Z-VAD-IETD (Fig. 6B). These results indicate that GMDS deficiency leads to resistance to TRAIL receptor- and CD95-mediated apoptosis by affecting events upstream of caspase-8 activation. Recently, emerging information indicates that DRs can promote numerous non-cytotoxic signaling pathways related to cell proliferation, differentiation, chemokine production, and inflammatory responses by activating nuclear factor-κB (NF-κB), mitogen-activated protein kinase (MAPK), phosphatidylinositol-3-kinase (PI3K), and c-Jun N-terminal kinase (JNK) (25). Thus, we examined the involvement of the non-cytotoxic function of TRAIL in increased apoptosis in GMDS-rescued cells using inhibitors of each signaling pathway. As shown in Fig. 6C, none of the inhibitors were able to abolish the difference in sensitivity to TRAIL-induced PARP cleavage between mock- and GMDS-rescued cells, indicating that GMDS deficiency does not affect the non-cytotoxic function of TRAIL.

Formation of DR4/5 or CD95 DISCs Does Not Change between Mock- and GMDS-rescued Cells—The activation of caspase-8 occurs through its clustering at the DISC followed by self-processing. Therefore, we examined the formation of the primary DISCs upon treatment with FLAG-tagged recombinant TRAIL or CD95L by immunoprecipitation using anti-FLAG antibody and Western blotting (Fig. 7, A and B). From
15 min after treatment, we were able to detect DR4/5 and CD95 in these immunoprecipitates. However, there were no differences between mock- and GMDS-rescued cells. In addition, procaspase-8 (p53/55), cleaved caspase-8 (p41/43), p43 cFLIP, and FADD were recruited to the primary DISCs in a similar fashion in both mock- and GMDS-rescued cells. In contrast, when the supernatants from the primary DISC immunoprecipitation experiments were subjected to Western blotting, significant increases in cleaved caspase-8 (p41/43) were observed in GMDS-rescued cells compared with
mock-rescued cells. One interpretation of this data is that caspase-8 was highly activated upon TRAIL or CD95L stimulation at some other complex dissociated from the primary DISC in GMDS-rescued cells.

**TRAIL Receptor- and CD95-mediated Complex II Formation Is Increased in GMDS-rescued Cells**—Following the primary DISC, TRAIL or CD95L induces the formation of a secondary cytosolic complex, involving FADD, caspase-8, and cFLIP. This FADD-dependent complex, referred to as complex II, further augments caspase-8 activation and cell death (26, 27). We therefore performed an immunoprecipitation using anti-FADD antibody after treatment with TRAIL or CD95 agonistic antibody and then examined the association of caspase-8 and cFLIP with FADD by Western blotting. As shown in Fig. 8, caspase-8 activation was increased in FADD immunoprecipitates from GMDS-rescued cells at 1 h after treatment. In addition, an increased association of p43 cFLIP with FADD was also observed in GMDS-rescued cells at 1 h after treatment. Given that no alterations in caspase-8 activation at the DISCs were observed (Fig. 7), these results indicate that the increased activation of caspase-8 in GMDS-rescued cells occurs at the FADD-dependent complex II, distinct from the primary DISC.

**DISCUSSION**

In this study, we examined the mechanism by which GMDS deficiency leads to resistance to TRAIL-induced apoptosis. Fucosylated glycans have been known to regulate many kinds of receptor-mediated signaling pathways (13). We found that DR4, but not DR5, was modified by fucosylated N-glycans in GMDS-rescued cells (Fig. 1, C–F). A previous report, using liquid chromatography–mass spectrometry, showed detailed oligosaccharide structures of O-glycan on DR5 in CHO cells, which express high levels of fucosylated glycans (19). This study determined that DR5 was modified by sialyl T antigen (NeuAcα2–3Galβ1–3GalNAc-O-Ser/Thr) or disialyl T antigen (NeuAcα2–3Galβ1–3(NeuAcα2–6)GalNAc-O-Ser/Thr) but not fucosylated glycans. Moreover, when we examined oligosaccharide structures of O-glycans derived from mock- and GMDS-rescued cells in detail using HPLC and mass spectrometry, we observed no restoration of fucosylation on O-glycans in GMDS-rescued cells.3 Thus, we expected that DR4, but not DR5, is responsible for increased TRAIL-induced apoptosis in GMDS-rescued cells. Surprisingly, however, we observed significant involvement of DR5 in the increased TRAIL-induced apoptosis in GMDS-rescued cells. These results suggest that GMDS deficiency renders the cells resistant to TRAIL-induced apoptosis independent of direct fucosylation of DR4 and DR5. Jin et al. (28) previously reported that there are no differences in TRAIL sensitivity between wild-type and mutant DR4 (whose N-glycosylation site was disrupted). We found that GMDS deficiency also sensitizes cells to CD95-mediated apoptosis but not to cell death mediated by anti-cancer drugs (Fig. 4). These results suggest that there are fucosylated glycoproteins or glycolipids that regulate extrinsic apoptotic signals via DR4/5 and CD95. Future studies are needed to determine whether GMDS deficiency causes desensitization to apoptosis induced by TNF, a member of the DR family.

3 M. Kinoshita, K. Moriwaki, E. Miyoshi, and K. Kakehi, manuscript in preparation.
GMDS Regulates Formation of FADD-dependent Complex II

**FIGURE 8.** GMDS deficiency inhibits TRAIL receptor- and CD95-mediated complex II formation. Cells were incubated with 50 ng/ml TRAIL (A) or 5 μg/ml anti-CD95 antibody (DX2) precomplexed with recombinant Protein G (B). Cell lysates were then immunoprecipitated (IP) with anti-FADD antibody (H-181) and Protein G. The immunoprecipitates were subjected to Western blotting for caspase-8 (12F5) and cFLIP (NF6). Anti-mouse IgG (Promega) and anti-mouse IgG1 (Enzo Life Sciences) secondary antibodies were used for caspase-8 and cFLIP, respectively. The supernatants (sup) were also subjected to Western blotting of cleaved PARP and β-actin. Arrowheads, IgG background bands.

Wagner et al. (19) reported that O-glycosylation facilitates ligand-induced clustering of DR4 and DR5, which in turn promotes DISC recruitment and caspase-8 activation. However, we observed no alterations in DR4/5 DISC formation between mock- and GMDS-rescued cells (Fig. 7). As shown in Fig. 8, GMDS deficiency significantly affects the formation of FADD-dependent complex II. Thus, our findings in this study using HCT116 cells are independent of O-glycan-mediated regulation of TRAIL-induced apoptosis. This theory is supported by the fact that fucosylation on TRAIL receptors is not involved in the mechanism underlying the formation of complex II remains unclear. It has been reported that receptor internalization upon death ligand stimulation is required to fully induce apoptosis (33, 34). However, it is still controversial whether TRAIL-induced receptor internalization is universally required to elicit apoptosis (35, 36). Indeed, when we examined the involvement of receptor internalization in our findings by treating mock- and GMDS-rescued cells with FLAG-TRAIL coupled with anti-FLAG antibody at 4 °C followed by a chase at 37 °C, we observed no alterations in receptor internalization (data not shown). Thus, inhibition of complex II formation by GMDS deficiency might be independent of ligand-stimulated receptor internalization. CD95 DISC formation is accompanied by the building of stable microaggregates followed by the formation of signaling protein oligomeric transduction structures, leading to receptor clustering into large platforms (37). These clusters contain proteins or lipids other than DRs. TRAIL receptor also forms a large SDS-stable complex upon TRAIL stimulation (19). Fucosylated glycans often bring about qualitative but not quantitative changes in many kinds of proteins by affecting their conformation on the cell surface. Thus, GMDS deficiency might result in a qualitative, conformational change in the cell surface complex, promoting the dissociation of DED-containing adaptor proteins from the DISC. Identification of the molecular mechanism by which GMDS regulates TRAIL- and CD95-induced apoptosis would shed light on the regulation of complex II formation.

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