Tumor Necrosis Factor-related Apoptosis-inducing Ligand (TRAIL) Induces Death Receptor 5 Networks That Are Highly Organized*‡

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Background: Whether ligand-induced clusters of DR5 have a specific structural organization is unknown.

Results: Ligand binding results in the formation of death receptor dimers that exist within high molecular weight networks.

Conclusion: Ligand-induced DR5 clusters are highly organized networks formed through dimerization of receptor trimers.

Significance: The biophysical character of DR5 networks may have implications for future rational design of DR5-targeted therapeutics.

Recent evidence suggests that TNF-related apoptosis-inducing ligand (TRAIL), a death-inducing cytokine with anti-tumor potential, initiates apoptosis by re-organizing TRAIL receptors into large clusters, although the structure of these clusters and the mechanism by which they assemble are unknown. Here, we demonstrate that TRAIL receptor 2 (DR5) forms receptor dimers in a ligand-dependent manner at endogenous receptor levels, and these receptor dimers exist within high molecular weight networks. Using mutational analysis, FRET, fluorescence microscopy, synthetic biochemistry, and molecular modeling, we find that receptor dimerization relies upon covalent and noncovalent interactions between membrane-proximal residues. Additionally, by using FRET, we show that the oligomeric structure of two functional isoforms of DR5 is indistinguishable. The resulting model of DR5 activation should revise the accepted architecture of the functioning units of DR5 and the structurally homologous TNF receptor superfamily members.

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily of ligands (1) that triggers the extrinsic apoptotic pathway via death receptors 4 and 5 (DR4 or TRAIL-R1 and DR5 or TRAIL-R2, respectively) (2–4). These type I membrane receptors serve as viable targets for cancer therapeutics as pre-clinical models have demonstrated that recombinant TRAIL (5–7) and anti-body agonists (8–13) targeting this pathway have potent anti-tumor activity without exhibiting systemic cytotoxicity (7, 11). Therefore, given the potential as a promising target in cancer, it is critical to characterize the precise mechanism by which death receptors initiate apoptosis to optimize existing TRAIL receptor-targeted therapeutic strategies (9, 14–18). However, despite the well established sequence of signaling events downstream of receptor activation (19, 20), how the initiating events in TRAIL-induced cell death (specifically events in the membrane) propagate a death signal remains unknown.

The crystal structure of the extracellular domain (ECD) of the TRAIL-DR5 complex (21–23) suggests that three receptor monomers tightly associate with the trimerized TRAIL (24) to form a symmetric ligand-receptor complex (Fig. 1A, Protein Data Bank code 1d0g, shown is a top view of the complex). Additionally, based on the last resolved amino acid of the ECD, the trimeric receptor arrangement places the transmembrane (TM) domains of DR5 ~50 Å apart (Fig. 1A, red dashed line), and therefore interactions between the TM domains are presumed to have no role in TRAIL-DR5 signaling. The TRAIL-DR5 trimeric structure is consistent with the crystal structure of LTα-TNFRI (25), a related TNF ligand-receptor pair, which is also a complex of three noninteracting receptor monomers and a tightly bound homo-trimeric ligand. Thus, based upon these crystal structures, it has reasonably been presumed that the relevant signaling event in the TNF receptor superfamily is ligand-induced extracellular trimerization and subsequent trimerization of these receptors’ intracellular regulatory domains (death domains in the case of DR5). However, crystal structures and functional studies of downstream domains and proteins (including the death domain, FADD, and caspase-8) suggest that the process may instead be dictated by receptor dimerization, not trimerization (26–29). An additionally intriguing piece of evidence is a dimeric crystal structure of the ECD of TNFR1 in the absence of ligand (30), which is stabilized by the well studied pre-ligand assembly domain (PLAD) (31–33) as well as by membrane-proximal residues, for which no
known role exists. In both the TNF receptors and DR5, the PLAD has been identified as crucial for ligand binding, but there has been no definitive study to clarify the role of the dimeric TNFR1 complex in signaling. To date, no ligand-independent oligomeric structure of DR5 exists nor is it known whether there is a relevant dimeric form of DR5.

Recently, our understanding of TNF receptors has been further complicated by a number of studies that show activation is associated with supramolecular receptor clustering within the membrane (34–38), with the most recent example being DR5 (39, 40). Visualized in fluorescence microscopy studies, these ligand-induced aggregates of receptors, previously referred to as signaling protein oligomeric transduction structures, or SPOTS, are on the order of 300–500 nm in diameter. Despite considerable speculation about the physical character of SPOTS (41, 42), the mechanism through which clusters form and whether they have a specific structural organization are unknown, leaving open the following significant and fundamental question. Are SPOTS simply co-localized aggregates formed via random nonspecific interactions between receptors? Or, more interestingly, are they highly structured networks that possess stabilizing and targetable motifs? Because of the difficulty of studying endogenous membrane receptors in their native states, no specific structural information has been available to begin to address this question.

Although no experimental data exist to support a molecular model of SPOTS, several hypothetical models of TNF ligand-receptor network structure have been presented (41, 42), two of which are schematized in Fig. 1. In the absence of ligand, pre-formed receptor oligomers, shown as trimeric complexes (described below as our preferred model for DR5) but also suggested to be dimeric (e.g. in the case of TNFR1), assemble in the plasma membrane via the membrane-distal residues of the PLAD (Fig. 1B). Based on available crystal structures (21–23), ligand binding first results in the formation of a symmetric trimer complex, which consists of three noninteracting receptor monomers held together by the trimeric ligand-bound crystal structure, as shown in A, D. ligand-induced structural changes allow for interactions of multiple ligand-receptor trimers resulting in early stage receptor clustering via dimerization or trimerization of ligand-receptor trimers, shown above and below, respectively. E, combination of trimeric ligand-receptor interactions (i.e. crystal structure trimer) and receptor-receptor interactions (either dimeric or trimeric) drives receptor clustering and the formation of large organized receptor networks.

FIGURE 1. A current model for organized ligand receptor networks. A, TRAIL-DR5 crystal structure (center, Protein Data Bank code 1d0g, top view), a schematic representation (left) and an overlay (right). Dashed red line indicates the previously predicted transmembrane domain separation. B, in the absence of ligand, DR5 forms pre-ligand trimers via interactions within the pre-ligand assembly domain, although pre-ligand dimers have also been proposed. C, addition of TRAIL causes a structural reorganization from a preligand state to the ligand-induced trimer state, as inferred by the trimeric ligand-bound crystal structure, as shown in A. D, ligand-induced structural changes allow for interactions of multiple ligand-receptor trimers resulting in early stage receptor clustering via dimerization or trimerization of ligand-receptor trimers, shown above and below, respectively. E, combination of trimeric ligand-receptor interactions (i.e. crystal structure trimer) and receptor-receptor interactions (either dimeric or trimeric) drives receptor clustering and the formation of large organized receptor networks.
tor interaction embedded within the network of trimeric ligand-receptor complexes) as the intracellular activating unit. An alternative model suggests that the network is held together by trimeric, not dimeric, receptor interactions (Fig. 1, D and E, bottom) (41).

Here, we have evaluated whether TRAIL-induced rearrangement of DR5 involves the formation of a stable receptor dimer species and whether that dimeric species exists within large structured networks. In the process, we offer the first evidence that TM α-helices within TNF receptors form tightly associated bundles and the first structural and functional interrogation of two distinct, alternatively spliced DR5 isoforms.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents**—Jurkat cells were cultured in RPMI 1640 media (HyClone). HEK293 cells were cultured in DMEM (HyClone). BJAB-derived cells (43, 44) were cultured in RPMI 1640 with HEPES, sodium pyruvate, and L-glutamine (ATCC). All media were supplemented with 10% FBS, L-glutamine, penicillin, and streptomycin. All cultures were maintained at subconfluence at 37 °C and 5% CO₂ in a water-jacketed incubator. FLAG-sTRAIL (residues 114–281) was expressed in *Escherichia coli* and purified as described previously (45) using the pT7-FLAG-1 inducible expression vector and anti-FLAG resin (Sigma). DR5 antibody agonist (mAb631), DR5 surface-staining antibody (mAb6311), and fluorescent secondary antibody (NL637) were purchased from R&D Systems. Antibodies for Western blots, DR5 (3696) and β-actin (5125), were purchased from Cell Signaling Technologies. BJAB DR5-deficient and BJAB DR5-deficient + DR5-S cells were a kind gift from Andrew Thorburn (43, 44).

**Cloning and DNA Constructs**—Complementary DNA (cDNA) for full-length DR5-S (residues 1–411) and DR5-L (residues 1–440) was cloned into pcDNA3.1(+) for transient expression in HEK293 cells. For transient expression in BJAB DR5-deficient cells, DR5-S(1–411) and DR5-L(1–440) were inserted into pIRE2-EGFP vector. For FRET analysis, extracellular and TM residues for DR5-S(1–211) and DR5-L(1–240) were inserted in-frame into pECFP-N1 and pEYFP-N1 vectors using EcoRI and BamHI sites. Both pECFP-N1 and pEYFP-N1 vectors contain the monomeric mutation A206K to the CFP or YFP preventing constitutive fluorophore clustering (46), and CFP/YFP shows no affinity for each other (Fig. 3C). Mutation of DR5 constructs was carried out by two-step PCR mutagenesis. All constructs were verified by sequencing, and expression was verified by Western blot and cell surface staining. CFP and YFP constructs were additionally verified individually by fluorescence microscopy using both CFP and YFP filters. pECFP-N1 and pEYFP-N1 constructs were a gift from David D. Thomas and the CFP-YFP tandem constructs was a gift from Stanley G. Nathenson (47).

**FRET**—Briefly, subconfluent HEK293 cells were transfected by the calcium phosphate method of transfection with CFP- and/or YFP-tagged constructs shown in Fig. 3A with a 2-fold excess of acceptor. Twenty four to 48 h post-transfection, cells were imaged using a Nikon Eclipse TE2000 inverted microscope and a ×40 objective lens. Fluorescent proteins were illuminated using a mercury lamp (XCite 120-watt Fluorescence Illumination System). Filters for excitation and emission of CFP (430/24 and 470/24 nm, respectively) and YFP (500/20 and 535/30 nm, respectively) were controlled using an automated filter wheel (Ludl MAC6000). Steady-state images were taken in 5–20-s intervals throughout the course of photobleaching, which took an average of 5 min. Images were acquired using MetaMorph and analyzed using ImageJ software. Instrument-independent FRET efficiency was calculated using Equation 1, where $F_{DA}$ is the donor fluorescence in the presence and $F_D$ is the donor fluorescence after acceptor photobleaching, as described previously (48). Results for each experiment are based on ~50 cells taken from a single transfection over ~5 regions of interest, with each cell treated as an independent sample, and cells having fluorescence primarily within the plasma membrane were studied. Reported averages and standard errors were calculated over the full sample of cells. All FRET experiments were repeated at least three times to confirm results.

$$E = 1 - \frac{F_{DA}}{F_D} \quad (\text{Eq. 1})$$

In a separate experiment, multiple populations of cells were transfected at a CFP/YFP ratio ranging from 1:0.5 to 1:4. FRET was measured using photobleaching as described, and FRET efficiency was plotted as a function of YFP intensity. FRET efficiency versus YFP intensity data were fit using a two-parameter saturable binding model, where the measured FRET efficiency (FRET%) is a function of the local YFP intensity, described by Equation 2 and described previously (46, 48–50).

$$\text{FRET} = \frac{\text{FRET}_{\%\text{max}} \cdot \text{YFP}}{\text{YFP} + K_{da}} \quad (\text{Eq. 2})$$

From this model fit of the data were extracted two parameters as follows: the maximum FRET efficiency (FRET%max) and the relative dissociation constant ($K_{da}$), which yield information about the fluorophore separation (i.e. structure) and binding affinity, respectively, for comparison of DR5 isoforms and mutants. Results shown are from three separate transfections at varying CFP/YFP ratios, and the results were pooled, with each point representing data from a single cell. These experiments (i.e. multiple transfections at different CFP/YFP ratios) were repeated to verify reproducibility, although data from transfections done on separate days were not pooled.

**Confocal Microscopy**—Imaging of ligand-receptor clusters in Jurkat cells was done as described previously (37, 39). Briefly, cells were washed with PBS and treated with a DR5-specific mouse antibody (R&D Systems, MA6311) agonist at 5 μg/ml for 1 h. After washing, cells were treated with an anti-mouse secondary antibody labeled with either NorthernLights557 or NorthernLights637 (R&D Systems) used at 5 μg/ml for 30–60 min and washed. All incubations and washes were done in PBS + 1% FBS and at 4 °C to prevent receptor internalization. Labeled cells were transferred to pre-chilled polylysine-coated 35-mm glass-bottom culture dishes (MatTek Corp.) for ~5–10 min at RT before imaging to allow cells to settle and clusters to form. NorthernLights637-labeled clusters were imaged in an Olympus IX81 inverted microscope equipped with a FluoView
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FV1000 laser scanning confocal head (633 nm laser excitation, 645–745 nm emission). Either ×60 (1.42NA) or ×100 (1.3NA) oil immersion objective lenses were used.

Z-stacks of NorthernLights557-labeled clusters were acquired using a Zeiss Cell Observer Z1 microscope equipped with a Yokogawa CSU-X1 confocal head using a ×100 (1.40NA) oil immersion objective (561 nm laser excitation, 617/73 nm emission). The spinning disc confocal allowed image acquisition at multiple depths overcoming the sample photobleaching problem in the laser scanning confocal. Orthogonal sectioning and maximum intensity projections of Z-stacks were performed using the Zeiss Axiosview software.

Cross-linking and Western Blot Analysis—Jurkat or BJAB cells (DR5-deficient and re-expressing either DR5-S or DR5-L) were washed and treated with TRAIL or DR5-specific antibody agonist for 1 h at 4 °C in PBS (pH 8.0) with rotation. Cells were subsequently cross-linked with 0.5–1 mM BS3 (Pierce; a homobifunctional, amine-reactive, noncleavable, and membrane-impermeable cross-linker with an 11.4 Å spacer arm), for 30 min at room temperature. Cross-linked samples were quenched with 20 mM Tris-HCl (pH 7.5) for 15 min at room temperature. Cells were pelleted by centrifugation and lysed in RIPA buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% Nonidet P-40) supplemented with protease inhibitors and 10 mM iodoacetamide for 1 h at 4 °C. Total protein concentration of lysates was determined by BCA assay (Pierce), and equal amounts of total protein (typically ~100 μg) were mixed with 2× Laemmli sample buffer with DTT and β-mercaptoethanol, boiled at 100 °C for 10 min, and loaded on 4–12% BisTris SDS-polyacrylamide gel (Invitrogen). For nonreducing conditions, lysates were mixed with 2× Laemmli sample buffer in the absence of DTT and β-mercaptoethanol, but samples were treated identically otherwise. Proteins were transferred to nitrocellulose membrane and probed using antibodies as described. To account for potential nonspecific cross-linking in the presence of ligand, the same lysates were separated via SDS-PAGE and Coomassie-stained (see supplemental material).

Caspase Activity and Surface Staining—BJAB DR5-deficient cells, five million cells in 400 μl of serum-free media, were transfected by electroporation (200 V, 975 microfarads) with the indicated plasmid and moved back into 10 ml of complete media. Several hours after electroporation, live cells were isolated by Ficoll gradient. Twenty four to 48 h after transfection, cells were treated with a DR5-specific antibody agonist (mAb631) at 1 μg/ml for 4 h at 37 °C. Caspase activity was measured using CaspGLOW Red Active Caspase 8 staining kit (BioVision), gating on live GFP-positive cells by flow cytometry (FACSCalibur). Additionally, DR5 surface expression was measured on unstimulated cells by antibody staining using a DR5 antibody and fluorescent secondary antibody. DR5 surface expression was determined by flow cytometry using an identical gating scheme for live GFP-positive cells. Data were analyzed using FlowJo software (Tree Star, Inc.).

Prediction of Helical Transmembrane Domains—The sequence-based prediction of helical transmembrane domains was based on a hidden Markov model via the TMHMM Server Version 2.0, and protein sequences were obtained via the NCBI gene data base.

Replica Exchange Molecular Dynamics (REMD) Simulations—REMD simulations were run using the CHARMM software package (51) and force-field version parameter set 22 (52) with CMAP correction (53) and the MMTSB toolset (54). All simulations were run using an implicit bilayer model, generalized born with switching function (55–56). For the monomer simulations, the bilayer hydrophobic thickness was set to 25, 30, or 35 Å, with a switching length of 0.3 Å in each case. The starting configuration was a single ideal helix (sequence: GTKHS-GEVPAVEETVTSSPPTASPDSLGSIGIGVTAAVAVLAVFVCKSLWK), placed in the center of the bilayer, and aligned with the bilayer normal axis. For the dimer simulations, TM helices (sequence: GTKHS-GEVPAVEETVTSSPPTASPDSLGSIGIGVTAAVAVLAVFVCKSLWK) were embedded in a bilayer of hydrophobic thickness of 32 Å. REMD simulations were run using 16 replicas, over a temperature range of 300–600 K. A switch was attempted every 2 ps, with an observed switching frequency of ~25%. All analysis was performed on the lowest temperature replica (300 K). Simulations were run using a 2-fs time step for a duration of 10 ns for each state (i.e. a total of 160 ns when considering the number of replicates).

Synthesis, Purification, and Analysis of DR5-TM—Solid-phase peptide synthesis of DR5-TM (TPASPDSLGSIGIVGTVAAVAVVFVCKSLWK) was carried out with a PE Biosystems Pioneer™ using standard double-coupling cycles of Fmoc/tBu-chemistry with HATU/DIEA as coupling reagents, in the presence of NMP. The sequence was assembled on Fmoc-Lys-PEG-PS resin (initial load of 0.18 mmol/g). The synthesis was optimized using pseudoproline dipeptides: Fmoc-Val-Ser (ψMe,Me-Pro)-OH, Fmoc-Val-Thr (ψMe,Me-Pro)-OH, and Fmoc-Leu-Ser (ψMe,Me-Pro)-OH (Novabiochem, San Diego). Final cleavage from the resin and side chain deprotection was carried out by treatment with reagent K: TFA, phenol, thioanisole, 1,2-ethanediol, water (82.5, 5, 2.5, and 5%), for 4 h at 25 °C. The cleavage mixture was filtered, and the resin was washed with a small amount of reagent K. Combined filtrates were concentrated under nitrogen and precipitated in 30 ml of diethyl ether at 0 °C. Precipitated peptide was collected by centrifugation and washed with ice-cold diethyl ether. The crude peptide was dissolved in 50% TFA and purified by HPLC on a C-4 column (Vydac, 214TP1010) using a Gold Beckman Coulter system. Protein elution was achieved with a linear gradient from 0 to 63% B (95% isopropyl alcohol, 4.9% H2O, 0.1% TFA) in 40 min at a flow rate of 2.0 ml/min with detection at 220 nm. The HPLC fractions were collected and analyzed by MALDI-TOF MS. The pooled fractions found to be essentially pure were lyophilized to yield 22% based on starting resin.

Analysis of DR5-TM by MALDI-TOF—Mass spectral data were acquired with a Bruker Biflex III Matrix-assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) system. The sample was co-crystallized with the matrix 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid), and the data were collected in the linear mode, positive polarity, with an accelerating potential of 19 kV. Each spectrum is the accumulation of 100–400 laser shots. Mass spectroscopy for DR5-TM yielded an m/z...
value of 3647 [M + H]^+", which is in agreement with the predicted value of 3646 Da. HATU is N-[(dimethylamino)-1H-1,2,3-triazolo[4, 5-b]pyridino-1-ylmethylene]-N-methylmethaninium hexafluorophosphate N-oxide; DIEA is N,N-diisopropylethylamine; and NMP is N-methyl-2-pyrrolidone.

RESULTS

**DR5 Is Expressed as Two Functional Isoforms That Differ in Their Transmembrane Domains**—DR5 is expressed as two isoforms that differ by the insertion of 87 bp resulting from alternative splicing and the inclusion of an intron (57). Translation of DR5 with and without this insertion results in the expression of long and short isoforms of DR5 at the protein level, respectively, referred to here as DR5-L and DR5-S (Fig. 2A). DR5-L contains an additional 29 amino acids at the junction between the extracellular and predicted transmembrane (TM) domains, including a cysteine residue at position 209. The organization of DR5 networks via ligand-induced dimerization may allow for interaction of DR5 TM α-helices as receptor TM domains are no longer confined to the 50 Å separation observed in the TRAIL-DR5 complex crystal structure. Therefore, we wondered if physical interactions between the TM domains of DR5 receptor monomers play a role in the stabilization of receptor network.

We first sought to confirm the function of DR5-L and DR5-S. Both isoforms are functionally active, as expression of either DR5-S or DR5-L in DR5-deficient (DR5-def) cells results in activation of caspase-8, both in a ligand-independent manner, consistent with previous results (58), and in a ligand-induced manner (Fig. 2B). Surface staining and flow cytometry confirmed approximately equal expression of DR5-S and DR5-L on the cell surface relative to the DR5-deficient control cell line (Fig. 2C). Furthermore, protein lysates from cells expressing exclusively DR5-S or DR5-L illustrate the size difference by SDS-PAGE, running at approximately their predicted molecular masses of 40 kDa (DR5-S) and 43 kDa (DR5-L), respectively (Fig. 2D). Jurkat cells, which have been shown to express both isoforms at the mRNA level (57), also express both isoforms at the protein level (Fig. 2D). Importantly, although we show here the first evidence that both isoforms of DR5 are ligand-sensitive, and DR5-S appears to activate caspase-8 to a greater extent than DR5-L (Fig. 2B, compare DR5-S and DR5-L), we do not suggest that DR5-S is necessarily more active as there are noticeable differences in the expression level. Whether DR5-S and DR5-L differ in their oligomeric structure, a primary focus of this study, has not been investigated to date. However, given the location of Cys-209 of DR5-L (which, as we will describe below, is located within the contiguous TM α-helix), we wondered whether this cysteine residue could be used to identify potential interactions, covalent and noncovalent, within the DR5 TM domain.

**Ligand-induced Dimerization Mediates DR5 Network Formation**—It has been shown previously that TNF ligands (including TRAIL and a DR5 antibody agonist) induce receptor clustering within the plasma membrane (37, 39, 40). Therefore, we first used fluorescence microscopy to identify whether DR5 agonistic antibody binds and induces ligand-receptor clusters. Fluorescent-labeled agonist ligand binds DR5 on the surface of Jurkat cells, which endogenously express both DR5-S and DR5-L, and forms large clusters representing ligand-receptor complexes within the plasma membrane (Fig. 3A). Images were taken from a single confocal xy plane at approximately the midplane of the cell. Shown are the fluorescent receptor-bound agonist ligand (Fig. 3A, panel i), transmitted light image (Fig. 3A, panel ii), and the overlay (Fig. 3A, panel iii) demonstrating that cluster formation occurs within the plasma membrane. Estimates of the cluster sizes based on these fluorescent images are on the order of 200–500 nm in diameter, consistent with the estimated size of FasL-induced Fas clusters (37). To determine whether the ligand-receptor clusters are distributed throughout the plasma membrane of the entire cell, multiple confocal images (in the xy-plane) were acquired at varying

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**FIGURE 2. DR5 is expressed as two functional isoforms.** A, transcription of two alternatively spliced isoforms results in the expression of DR5-S and DR5-L differing by 29 amino acids (bold) within the extracellular domain and predicted transmembrane domain (underlined). B, DR5-S or DR5-L (or empty vector control) were transiently transfected into BJAB DR5-deficient (DR5-def) cells and treated with a DR5-specific agonist antibody (α-DR5) as indicated. Flow cytometry analysis of caspase-8 activity shows a regain of function and ligand sensitivity with expression of DR5-S or -L. Results represent the level of caspase-8 activity (mean ± S.E.) for three independent experiments. C, surface expression in transiently expressing cells was quantified by surface staining and flow cytometry to show equal surface expression of DR5-S (blue) and DR5-L (green) and increased levels over vector transfected (red). D, whole-cell lysates from Jurkat cells, BJAB DR5-def cells, and DR5-def cells re-expressing DR5-S or DR5-L confirm the predicted size of DR5-S (40 kDa) and DR5-L (43 kDa).
depths (in $z$). Axial reconstruction at the cell mid-plane in each image clearly shows the distribution of fluorescent ligand-receptor clusters throughout the plasma membrane (Fig. 3B, $xy$, $yz$, and $xz$-planes). Maximum intensity projection in the $xy$-plane illustrates the size and distribution of these clusters. C. Jurkat cells were treated with TRAIL or DR5-specific agonist ($\alpha$-DR5) and cross-linker (x-link) as indicated, and whole-cell lysates were run under nonreducing SDS-PAGE and probed for DR5. Highlighted are monomeric (M), dimeric (D), trimeric (T), and oligomeric (O) forms of DR5. D and E, a similar experiment was run using BJAB DR5-deficient (DR5-def) cells + DR5-S or + DR5-L using $\alpha$-DR5. Agonist causes the formation of a disulfide dimer in DR5-L cells but not in DR5-S cells, and again this dimer species exists within a high molecular weight complex. F, Jurkat, BJAB DR5-deficient, DR5-deficient cells + DR5-S, and DR5-deficient cells + DR5-L were treated with a DR5 agonist, and lysates were run under nonreducing conditions. Shown is the dimeric form of DR5, present upon treatment with ligand and only when DR5-L is expressed.

FIGURE 3. Ligand-induced DR5 dimers within high molecular weight networks. A, Jurkat cells were treated with an agonistic antibody specific to DR5 and subsequently stained with fluorescent secondary antibody. Confocal microscopy shows the formation of ligand-receptor clusters on the cell surface. Shown is the fluorescent-labeled agonist (panel i), transmitted light (panel ii), and an overlay (panel iii). Scale bar represents 2 $\mu$m. B, confocal microscopy of agonist treated Jurkat cells in $x$, $y$, and $z$-dimensions shows ligand-receptor clustering in the $xy$, $yz$, or $xz$-focal plane at approximately the mid-line of the cell. Maximum intensity projection (MIP) in the $xy$-plane illustrates the size and distribution of these clusters. C. Jurkat cells were treated with TRAIL or DR5-specific agonist ($\alpha$-DR5) and cross-linker (x-link) as indicated, and whole-cell lysates were run under nonreducing SDS-PAGE and probed for DR5. Highlighted are monomeric (M), dimeric (D), trimeric (T), and oligomeric (O) forms of DR5. D and E, a similar experiment was run using BJAB DR5-deficient (DR5-def) cells + DR5-S or + DR5-L using $\alpha$-DR5. Agonist causes the formation of a disulfide dimer in DR5-L cells but not in DR5-S cells, and again this dimer species exists within a high molecular weight complex. F, Jurkat, BJAB DR5-deficient, DR5-deficient cells + DR5-S, and DR5-deficient cells + DR5-L were treated with a DR5 agonist, and lysates were run under nonreducing conditions. Shown is the dimeric form of DR5, present upon treatment with ligand and only when DR5-L is expressed.

To begin to characterize the molecular architecture of ligand-induced DR5 clusters, and thus to illuminate new details regarding the various models of how DR5 oligomers stabilize networks (e.g. those in Fig. 1E), we used cross-linking of surface proteins with a membrane-insoluble amine-reactive cross-linker. This approach allowed us to capture the native oligomeric states of the receptor as they are in the cell membrane before they are lost on dissociating gels. Based on hypothetical network models (Fig. 1E) and the sequence of the DR5 TM domain, we hypothesized that TRAIL- or agonist-induced networks may be stabilized in part via close contacts between DR5 monomers, including interactions between TM domains. To date, it is unknown whether ligand-induced structural re-arrangement of DR5 results in stabilization of TM interactions, including disulfide bond formation via Cys-209 unique to DR5-L. First, Jurkat cells were treated with TRAIL or DR5 antibody agonist as indicated, and cell lysates were analyzed via nonreducing SDS-PAGE (Fig. 3C) (59, 60). The addition of the membrane-impermeant cross-linker results in the formation of a ligand-independent receptor trimer, at the approximate molecular weight of three receptor monomer units (~120 kDa, Fig. 3C, compare 1st and 4th lanes). This result provides the first structural insight into pre-ligand receptor assembly and stoichiometry of DR5, which differs from the presumed pre-ligand dimeric state of TNFR1 (55) and motivates our choice in Fig. 1B. RNAi studies were used to confirm that this band is indeed DR5 (see supplemental material).

The addition of either TRAIL or antibody agonist causes the formation of high molecular weight DR5 clusters (200+ kDa, Fig. 3C, 5th and 6th lanes), reflecting the ligand-induced receptor clusters shown in Fig. 3, A and B. In the absence of cross-linker, the addition of TRAIL or agonist produces a distinct
band at ~85 kDa, the expected molecular mass of two DR5 monomer units (Fig. 3C, compare 1st lane with 2nd and 3rd lanes), indicating the presence of a disulfide-linked receptor dimer species. That is this in fact a DR5 monomer-monomer interaction is further corroborated with mutational analysis and FRET experiments described below. That cross-linking of either TRAIL- or agonist-treated samples causes the disappearance of this dimer band suggests that ligand-induced DR5 dimers are embedded within the high molecular weight clusters and provides the first molecular details of the ligand-induced rearrangement of DR5 and the higher order structure of the TRAIL-DR5 network (Fig. 1E) and agonist-DR5 network. Analysis under reducing conditions abolishes the DR5 dimer in both the TRAIL and antibody agonist-treated samples (see supplemental material), indicating that ligand-dependent dimerization is driven through a cysteine disulfide bond, suggesting this may be DR5-L (further discussed below).

We note that the addition of ligand does not result in a molecular mass shift of the 120 kDa pre-assembled receptor trimer (Fig. 3C, 4th to 6th lanes). Thus, we see no specific band at the predicted molecular weight of the ligand-receptor trimer complex (the crystal structure, which should be at ~180 kDa). Like the dimer, we interpret this to most likely mean that the ligand-bound trimeric structure (Fig. 1C) exists only transiently on its own before becoming embedded within the DR5 clusters. However, this accounting does not explain the persistence of the 120-kDa trimer band upon addition of TRAIL. It is possible that a steady-state level of pre-ligand assembled trimer is maintained in the membrane. Alternatively, this lack of shift in molecular weight may correspond to an inability to cross-link the TRAIL to the trimeric receptor. The distinction between these various possibilities remains unclear. Numerous attempts to identify TRAIL on these blots has proven unsuccessful, possibly due to a lack of sensitivity in detecting ligand bound to endogenous receptor or due to epitope masking (e.g. as a result of residing in a complex or due to cross-linker masking of the antibody epitope).

Because Jurkat cells express both isoforms of DR5, we tested whether the observed disulfide-bonded dimer is indeed DR5-L, likely given its additional cysteine residue. We generated two cell lines derived from a BJAB cell line previously selected for DR5 deficiency (43, 44) expressing either the DR5-S or DR5-L isoform. The addition of a DR5-specific agonist induces clustering in both DR5-S and DR5-L lines (Fig. 3, D and E), although somewhat more so in the short isoform. Importantly, however, the ligand-dependent formation of disulfide-linked receptor dimer occurs exclusively in cells expressing DR5-L (Fig. 3, D and E, compare 2nd lane in each Western blot). This result is recast in a separate noncross-linked Western blot, focusing on the dimer molecular weight from four cell lines (Jurkat, BJAB DR5-deficient, BJAB-DR5-S, and BJAB-DR5-L) (Fig. 3F). That disulfide-linked dimers of DR5-L do not occur in the absence of ligand despite ligand-independent trimerization suggests the following. 1) The free cysteine residue in unliganded DR5-L is inaccessible, either by the receptor conformation or possibly by being buried in the membrane, and is thus unable to form disulfide linkages. 2) Ligand binding causes conformational changes that expose the free cysteine and thus promote disulfide bond formation. We note that in both Jurkat and BJAB cell lines, there is an overall increase in DR5 intensity at all nonmonomeric molecular weights upon addition of the cross-linker. Experiments with a reversible cross-linker suggest that cross-linking does not up-regulate the receptor (data not shown); therefore, this difference in apparent total DR5 levels may more likely be due to epitope masking of certain populations of receptor and/or poor detection of monomeric DR5 under nonreducing SDS-PAGE.

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**DR5 Dimers Are Stabilized via Covalent and Noncovalent Interactions**—Cells expressing DR5-S show no disulfide-linked dimer; however, the similar presence of high molecular weight clusters indicates that DR5-S may also cluster via ligand-dependent dimerization, albeit exclusively through noncovalent interactions not detected by nonreducing SDS-PAGE. Moreover, in both DR5-S- and DR5-L-expressing cells, cross-linking of surface proteins results in the formation of a ligand-independent receptor trimer (Fig. 3, C–E). Therefore, we tested whether DR5-S and DR5-L are able to form stable receptor oligomers, including both receptor dimers and trimers, through either covalent (i.e. disulfide) or noncovalent interactions in transiently transfected HEK293 cells. To assess disulfide-linked dimerization, HEK293 cells were transiently transfected with either DR5-S or DR5-L (Fig. 4A), as well as cysteine mutants (Fig. 4B), and lysates were isolated and run under reducing or nonreducing conditions as indicated. Transient expression of DR5-L results in disulfide-linked dimerization, whereas DR5-S is unable to form disulfide-linked dimers, and reducing either DR5-S or DR5-L expressing cells produces entirely monomeric receptors (Fig. 4A). Mutational analysis of free cysteine residues within the TM domains of DR5-S (Cys-203) and DR5-L (Cys-209 and Cys-232) demonstrates that disulfide dimers of DR5-L involve Cys-209, the cysteine residue unique to DR5-L. Mutation of the TM Cys-203/-232 (a conserved TM cysteine residue in DR5-S and DR5-L, respectively, see Fig. 2A) has no effect on dimerization (Fig. 4B).

To evaluate noncovalent association of receptors, HEK293 cells were transiently transfected with DR5-S or DR5-L, and surface receptors were cross-linked as described and cell lysates analyzed via SDS-PAGE. Interestingly, although noncross-linked receptors are entirely monomeric, cross-linking of surface receptors shows that oligomeric receptor structures are stable within the membrane at high levels of receptor expression in the absence of ligand (Fig. 4C). Noncovalent dimers of DR5-S are present, although the short isoform has a greater tendency to form high molecular weight aggregates in the absence of ligand. That DR5-S exists as a dimeric species when concentrated at the cell surface suggests that, like DR5-L, it may exist as a dimer within ligand-receptor networks (despite lacking the cysteine) (Fig. 1E). Under these overexpressed conditions, DR5-L primarily forms receptor dimers and trimers (Fig. 4C), with a notable reduction in higher order molecular weight clusters as compared with DR5-S, consistent with the difference in the BJAB cells (Fig. 3, D and E). The presence of oligomeric receptor species in transiently expressing cells indicates that overexpression due to the unregulated CMV promoter, and thus receptor crowding in the membrane, enables DR5 to adopt an active oligomerized conformation, consistent with the
observation that overexpression results in ligand-independent DR5 activity (58). We note the lower level of background nonspecific bands in transiently expressing HEK293 cells (compared with Jurkat and BJAB results in Fig. 3), which is a result of receptor overexpression relative to other proteins.

To verify these Western blot results that, at overexpressed receptor levels, DR5 clusters occur in the absence of ligand, DR5-S and DR5-L were tagged with a C-terminal YFP fluorophore, and the resulting plasmid transfected into HEK293 cells for fluorescence imaging. The function of both DR5-S-YFP and DR5-L-YFP was confirmed by their ability to activate caspase-8, for fluorescence imaging. The function of both DR5-S-YFP and DR5-L-YFP was confirmed by their ability to activate caspase-8.

FIGURE 4. DR5 dimerization and network formation via covalent and non-covalent interactions. A, HEK293 cells were transiently transfected with DR5-S or DR5-L, and lysates were run on SDS-PAGE in the absence or presence of reducing agents (red). Transient overexpression of DR5-L, but not DR5-S, in HEK293 cells causes spontaneous disulfide dimer formation. B, cysteine mutagenesis within the transmembrane domain, including Cys-203 in DR5-S and Cys-209 and Cys-232 in DR5-L, demonstrates that disulfide dimerization of DR5-L occurs via Cys-209, a cysteine residue unique to the long isoform. Highlighted are the monomeric (M) and dimeric (D) forms of DR5. C, HEK293 cells transiently expressing DR5-S or -L (or vector control) were surface cross-linked (x-link) and run on SDS-PAGE gel. Surface cross-linking shows the similarities and differences in the organizations of DR5-S and DR5-L. Consistent with stabilization of an active conformation, dimer formation occurs in both DR5-S and DR5-L under transient overexpression. DR5-S forms high molecular weight clusters, whereas DR5-L is primarily dimeric and trimeric. Highlighted are monomeric (M), dimeric (D), trimeric (T), and oligomeric (O) forms of DR5. D, full-length YFP-tagged DR5-S (panel i) shows a high degree of receptor clustering within the membrane, forming large receptor aggregates, consistent with cross-linking experiments. Full-length YFP-tagged DR5-L (panel ii) shows some degree of clustering in the membrane, but it has a more diffuse pattern than DR5-S. Consistent with previous studies, removal of the cystolic domain results in homogeneous localization throughout the plasma membrane, as observed with DR5-S-YFP lacking a cytosolic domain (panel iii).

DR5 Transmembrane α-Helices Form Dimeric Bundles in Both Isoforms—Accordingly, to further measure receptor structure in the membrane of a living cell, we performed fluorescence resonance energy transfer (FRET), using CFP- and YFP-tagged DR5 and tumor necrosis factor receptor 1 (TNFR1) as a control (Fig. 5A). Placement of the fluorophore immediately downstream of the TM domain allows for analysis of TM separation. Similar constructs have been used to study receptor assembly in DR5, Fas, and TNF systems using FRET (61). Although truncation of the cytosolic domain precludes the formation of ligand-independent receptor clusters (Fig. 4D), this placement of the fluorophore immediately downstream of the TM domain still yields measurable energy transfers. This demonstrates that truncated receptors retain the ability to oligomerize via extracellular and transmembrane residues (the focus of this study), and it further allows for detailed analysis of TM domain oligomerization and the relative role of TM residues, including Cys-209. The function of fluorescent protein-tagged DR5 constructs, both full-length and truncated, was evaluated in BJAB DR5-deficient cells (see supplemental material).

FRET was measured by acceptor selective photobleaching (62), where an increase in donor fluorescence after selective photobleaching of the acceptor provides a quantitative measure of energy transfer efficiency between fluorophores (Fig. 5B). Transfection of DR5-CFP and -YFP constructs yields significant energy transfer over donor-only controls, with DR5-L,
0.402, having a significantly higher energy transfer than DR5-S, 0.327 (p < 0.05) (Fig. 5C). Although co-transfection of DR5-S and DR5-L leads to measurable levels of hetero-oligomeric FRET, 0.164, the amount of energy transfer is significantly lower (p < 0.001). Mutation of DR5-L C209A causes a significant reduction in DR5-L FRET to a level indistinguishable from DR5-S. Note: * indicates statistically significant with p < 0.05; ** indicates statistically significant with p < 0.001.

The observation of measurable steady-state FRET efficiencies clearly indicates that oligomeric receptor complexes exist in the cell membrane. FRET efficiency increases with either the fraction of receptors in oligomeric complexes or the proximity of donors and acceptors within the complex. Thus, it was unknown whether the observed differences in FRET efficiency between DR5-S, DR5-L and DR5-L C209A are due to a change in the equilibrium of association or a change in the structure of the dimer (i.e. the separation of the fluorophores). To elucidate
these two independent factors, FRET measurements were made in HEK293 cells at varying YFP concentrations and FRET efficiency was plotted as a function of YFP intensity (Fig. 5, E–G). FRET efficiency (FRET) increases with increasing YFP intensity ([YFP]), and the data were fit to a hyperbolic saturable binding curve of the form FRET = (FRETmax [YFP])/((Kd + [YFP]) as described previously (46, 50). From this fit two parameters were extracted as follows: the maximum FRET efficiency (FRETmax, the theoretical limit of FRET efficiency at large [YFP]) and the relative dissociation constant, Kd, (the YFP concentration at which half-maximum FRET is observed), yielding information about fluorophore separation and relative binding affinity, respectively. DR5-S and DR5-L C209A show a similar increase in FRET efficiency with increasing acceptor concentration, whereas DR5-L shows a sharp increase in energy transfer at low levels of YFP, thus favoring the complexed state. Despite differences in the equilibrium of oligomerization, the maximum FRET in each case is approximately equal, indicating that fluorophore separation in the receptor-complexed state is the same for DR5-S, DR5-L, and DR5-L C209A (Fig. 5H). These results suggest that receptor assembly involves TM dimerization, forming similar oligomeric structures even in the absence of disulfide bond formation.

In general, interactions between TM domain α-helices can provide a driving force for intermolecular assembly and are involved in the physiologic function and pathogenic dysfunction in a number of TM signaling molecules (63–66). However, in the crystal structures of the ECD of the TRAIL-DR5 complex (which do not include any of the additional residues in the DR5L isoform), the last resolved residues place the three TM α-helices ~50 Å apart. This has led to the conclusion that the TM helices of DR5 are independent and noninteracting, a presumption that is propagated in all molecular schematics of TNF receptors. Conversely, the pre-ligand dimeric structure of TNFR1 places the TM helices less than 20 Å apart, suggesting that if this is the dimer structure that is embedded within a supramolecular network, then the TM helices may in fact form a stable dimeric bundle within the membrane. Therefore, we next tested whether synthetic DR5 TM peptides, in the absence of regulation by soluble domains, have the propensity for self-association. Synthetic DR5 TM peptides were reconstituted into dodecylphosphocholine micelles in the absence and presence of reducing agents and were then cross-linked, or not, with glutaraldehyde. We observe the covalent dimerization of TM peptides via Cys-209 disulfide bonding with samples prepared in the absence of reducing agents (Fig. 6A). The addition of reducing agents during sample preparation precludes dimer formation. Moreover, cross-linking of reduced samples shows the DR5 TM domain has only a low propensity to dimerize via noncovalent interactions, suggesting that noncovalent interactions within the TM domain alone may not be sufficient for receptor dimerization. Nonreduced, cross-linked samples show that the disulfide-linked dimer species may be able to recruit an additional monomer or dimer, indicating some level of higher order TM domain clustering through both covalent and noncovalent interactions, consistent with results in HEK293 cells expressing the full-length protein (Fig. 4C). Time-resolved FRET analysis using labeled peptides also confirms that DR5 TM peptides exist as oligomers in detergent, both in the presence and absence of reducing agent (data not shown).

We predicted the structure of the monomeric and disulfide-linked TM domain using REMD simulations (67). As expected from sequence-based α-helical TM prediction tools (see Fig. 2A), several of the residues in the long isoform form a contiguous helix and insert in the membrane (Fig. 6B). Additionally, the structural prediction of the DR5 TM monomer suggests a potential role for the widely conserved GG4 (GXXXG) helix dimerization motif (68), which has approximately the same circumferential location on the
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α-helix as Cys-209. REMD simulation of the disulfide-linked DR5 TM dimer further shows a noncovalent GG4 interaction in the disulfide-linked state (Fig. 6C). In the symmetric dimer, this dimerization motif lies on the same helical interface as the Cys residue forming a CXXXGXXXG sequence in the long isoform. It is unclear how often a cysteine residue occurs in proximity to a GG4 motif, although a brief bioinformatics search reveals that several membrane proteins contain the CXXXGXXXG motif within the TM domain, including LST1, a protein that spontaneously forms disulfide-linked homodimers (69), and death receptor 4, a related TRAIL receptor for which no known TM interactions exist (although we note that the motif is located in a different region of the predicted TM helix than in DR5). Collectively, these results show a strong role for TM α-helix dimers in the overall architecture of activated DR5.

DISCUSSION

The current understanding of the structure-function relationship in the TRAIL-DR5 complex is based not on native full-length protein expressed at endogenous levels but rather on crystals of fragmented soluble domains, the behavior of overexpressed receptors in model cell lines, and fluorescence imaging at the whole-cell level. These studies have provided highly useful information about the fundamental role of the PLAD, ligand-binding, and death domains and suggested a role for supramolecular clustering. However, little has been done to elucidate the structural and molecular mechanisms in early events of DR5 signaling. It is therefore of great interest and difficulty to understand the nuanced role of not just the specific interactions between TRAIL and DR5 but also between DR5 monomers and exactly how the sum of such interactions result in the orchestration of macromolecular aggregates visible by fluorescence microscopy (39).

Supramolecular clustering of receptors, including TNFR1, Fas, and DR5 has emerged as a potentially powerful paradigm shift in the field of apoptotic signaling. The canonical view of a single trimeric ligand-receptor structure is giving way to a revised picture of highly organized networks of ligands and receptors driven through stable receptor-receptor interactions in multiple domains that vary in their stoichiometry to generate large aggregates of ligand-receptor trimeric structures (41, 42). The first network model was put forth by Chan (41) and suggests that ligand causes aggregation of pre-assembled dimeric receptors via extensive receptor-receptor interactions in a tri-fold symmetry. A second network model was put forth by Ozsoy et al. (42) suggesting that trimeric TNF ligands engage a pre-formed receptor dimer complex and cause a 120° rotational conformational change resulting in dimeric activation and formation of a network in the form of a hexagonal mosaic of ligand-receptor trimers. Although both models are intriguing, they lack the necessary diverse experimentation and evidence to support their foundation, which is a ligand-induced dynamic reorganization of pre-ligand assembled dimers that enables specific receptor-receptor interactions that stabilize the network.

Our results from an endogenous system (Jurkat cells) and corroborated in model systems (BJAB and HEK293 cells) provide the first substantive support for these types of models, shown in Fig. 1E, in which TRAIL-induced networks display an organization via DR5 dimerization through membrane-proximal residues. In the case of DR5, our data suggest that preformed receptor trimers, different from the pre-assembled dimers observed in the case of TNFR1, are engaged by trimerized TRAIL (24) to form a TRAIL-DR5 trimer complex during which the receptors undergo a conformational change that exposes the dimeric interaction motifs. We have shown that this motif includes interactions between TM domain α-helices (e.g. the Cys-209 in the case of DR5-L and possibly GXXGX in the case of both DR5-L and DR5-S), but we do not rule out the likely possibility that the interaction is further stabilized by interactions in the ECD and intracellular domains as well (for example those in or near the PLAD and death domain). This ligand-induced structural rearrangement promotes formation of receptor dimers that tether the trimeric ligand-receptor complex (Fig. 1D) thus driving the formation of organized receptor networks (Fig. 1E). Therefore, formation of the network results serves two general purposes. First, network formation stabilizes the receptor dimer species that may in turn stabilize the dimerization of intracellular death domains as well as caspase-8, as suggested by structural and functional data to be the active conformation. Also, network formation may provide a high, local concentration of active dimeric intracellular death domains able to overcome inhibitory pathways.

In the context of the hypothetical network models (Fig. 1E), one interpretation of our data is that ligand-induced dimerization via TM residues (including both Cys-209 disulfide bond formation and noncovalent association) occurs between neighboring trimeric structures of ligand and receptor. An alternative and equally valid interpretation of the data is that ligand induces a dimeric association (via Cys-209 disulfide bond or noncovalent interactions) within a trimeric ligand-receptor structure. i.e. TM association occurs between two receptor monomers within a crystal structure unit. This intra-trimeric TM association would likely tend to oppose network formation, as intra-trimeric association would leave only one free TM domain to associate with an adjacent trimeric unit. Thus, in the extreme case where every ligand-independent, pre-assembled trimer forms an intra-trimeric association upon ligand binding, one would expect a maximum of two trimeric ligand-receptor units per cluster. In the more realistic case, where intra-trimeric association occurs in a percentage of these complexes, network size would tend to be limited due to a lack of free TM helices. In the context of DR5-L, if irreversible intra-trimeric Cys-209 disulfide bond formation occurs in a fraction of all trimeric ligand-receptor complexes, we would expect less network formation and a potentially less functional isoform of DR5. Interestingly, all of our data are consistent with this interpretation that DR5-L tends to form networks to a lesser extent than DR5-S (see Figs. 3, D and E, and 4C) and is less functional (see Fig. 2B). Therefore, we cannot rule out the possibility for intra-trimeric TM association, which may be reversible in the case of DR5-L. Additionally, we cannot rule out the possibility that receptor networks may be stabilized via trimeric receptor bundles (Fig. 5E, compare top (42) and bottom (41) models) or potentially even by tetrameric receptor bundles via combina-
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...tions of covalent (Cys-209 disulfide) and noncovalent (GG4) interactions, as predicted by studies with the purified TM peptide (see Fig. 6A). Ongoing efforts include quantification of cluster size and differences in cluster size between DR5-S and DR5-L isoforms.

A second important facet of this study, in particular for the ongoing efforts to target DR5 in the treatment of cancer, is the validation and further understanding of two DR5 isoforms expressed on the protein level in human cancer cells. To date, no pattern of DR5-S or DR5-L expression in normal versus tumor cells is known, although it is intriguing to postulate that the presence of DR5-S and/or DR5-L isoforms in tumor cells may serve as a viable predictive biomarker for TRAIL-based targeted therapy and/or any agent that may rely on engaging this pathway. Although the expression of two DR5 isoforms (at the mRNA level) was first noticed relatively early after the discovery of DR5 (57, 70), no studies have investigated the difference, structural or functional, between the two, and only a few publications have acknowledged the expression of the two isoforms at the protein level (58, 71–72). The functional role of the 29 amino acid insertion in DR5-L is yet unknown, although the sequence is rich in structurally active residues, such as proline, as well as chemically active residues, such as serine or threonine residues that may be modified by phosphorylation or glycosylation as shown previously (58). It is possible that, with the addition of 29 amino acids, the cysteine residue evolved to hold the TM helices in an active conformation that is otherwise less favorable due to the increased flexibility from the additional residues upstream.

Whether there is functional significance to our finding that DR5-S and DR5-L preferentially form homo-oligomers over hetero-oligomers remains unknown. Mutation of C209A does not increase the ability of DR5-L to interact with DR5-S (data not shown), suggesting that the reduced propensity for hetero-oligomerization is not solely due to the disulfide bonds between DR5-L monomers. That DR5-S and DR5-L have a low propensity to form hetero-oligomers begs the question of whether one has a higher affinity for decoy receptors (which do not have free cysteines), thus influencing its activity. More generally speaking, studying the functional difference of the two isoforms in their native endogenous states is remarkably difficult and reflects a ubiquitous challenge to biophysicists attempting to make headway in natural cellular systems. Any measure of protein function (e.g. caspase activation or cell death) must be normalized to the total active protein content in the plasma membrane. However, even in our stable BJAB lines expressing exclusively one of the two isoforms, we were unable to quantify relative receptor efficiency because of uncontrollable and non-quantifiable differences in total protein expression. Even if one were able to quantify exact receptor densities in the plasma membrane and further determine how many of these proteins are active, it is unclear that the relationship between receptor density and function is strictly linear, further complicating a determination of an isoform-dependent efficiency per receptor. Quantifying these aspects of receptor activation may become possible through ongoing studies that employ higher resolution microscopy and time-resolved FRET measurements or may require a more complicated combination of cell biology and biophysics as has been attempted for DR5, as well as in the study of a separate class of receptor networks (36, 73, 74).

CONCLUSION

We have shown that TRAIL-induced cell death via DR5 involves structural re-organization and receptor dimerization within large protein complexes and that dimerization is mediated by membrane-proximal residues. This is the first evidence to suggest that ligand-induced receptor clusters are highly organized and that they are likely regular arrays or regularly structured networks. Moreover, given the structural and functional evidence that dimerization of intracellular domains as well as downstream signaling proteins is critical for their activation, the ligand-induced receptor dimerization event appears to be a crucial step in DR5 activation. Here, we took advantage of a free cysteine residue in DR5-L to identify a novel protein-protein interaction motif and show that the fluorophore separation in the DR5-L disulfide-linked structure is indistinguishable from that of DR5-S. Thus, at endogenous receptor levels, ligand-induced dimerization occurs, at least in part, via the TM domain, a domain in the TNF receptor superfamily that has remained largely unstudied. More broadly, given their structural homology, both monomeric and ligand-bound, many other TNF receptor superfamilies are likely to undergo a similar dimerization event that is critical for signal propagation through the membrane. Ongoing efforts to understand the functional relevance of TNF receptor networks should clarify whether they exist as a mechanism to concentrate intracellular signals (to overcome biochemical thresholds), whether the network formation itself causes the key conformational change associated with signaling, or both.

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