Receptor-selective Mutants of Apoptosis-inducing Ligand 2/Tumor Necrosis Factor-related Apoptosis-inducing Ligand Reveal a Greater Contribution of Death Receptor (DR) 5 than DR4 to Apoptosis Signaling* [S]

Received for publication, September 16, 2004, and in revised form, November 1, 2004
Published, JBC Papers in Press, November 1, 2004, DOI 10.1074/jbc.M410660200

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Apoptosis-inducing ligand 2 (Apo2L), also called tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), triggers programmed cell death in various types of cancer cells but not in most normal cells. Apo2L/TRAIL is a homotrimeric protein that interacts with five receptors: death receptor 4 (DR4) and DR5 mediate apoptosis activation, whereas decoy receptor 1 (DcR1), DcR2, and osteoprotegerin counteract this function. Many cancer cell lines express both DR4 and DR5, and each of these receptors can initiate apoptosis independently of the other. However, the relative contribution of DR4 and DR5 to ligand-induced apoptosis is unknown. To investigate this question, we generated death receptor-selective Apo2L/TRAIL variants using a novel approach that enables phage display of mutated trimeric proteins. Selective binding to DR4 or DR5 was achieved with three to six-ligand amino acid substitutions. The DR4-selective Apo2L/TRAIL variants examined in this study showed a markedly reduced ability to trigger apoptosis, whereas the DR5-selective variants had minimally decreased or slightly increased apoptosis-inducing activity. These results suggest that DR5 may contribute more than DR4 to Apo2L/TRAIL-induced apoptosis in cancer cells that express both death receptors.

Apo2L1 (or TRAIL), a member of the tumor necrosis factor (TNF) superfamily, induces apoptosis in a broad spectrum of human cancer cell lines while sparing most normal cells (1). Apo2L/TRAIL triggers apoptosis through binding to the death receptors DR4 (2) and/or DR5 (3, 4). These receptors contain a cytoplasmic death domain that recruits adaptor molecules involved in caspase activation (5). In addition to these two signaling receptors, Apo2L/TRAIL binds to three decoy receptors that inhibit apoptosis induction: DcR1, DcR2, and OPG. DcR1 and OPG lack a cytoplasmic domain, whereas DcR2 has a truncated death domain that is non-functional for apoptosis initiation (1, 6). Like several other members of the TNF superfamily, Apo2L/TRAIL is synthesized as a type II transmembrane protein that can be proteolytically cleaved to release a soluble, homotrimeric molecule. A recombinant version of soluble homotrimeric Apo2L/TRAIL (residues 114–281) induces apoptosis in various cancer cell lines but not in normal cells (7, 8). Administration of soluble Apo2L/TRAIL in mouse xenograft models of human cancer results in marked anti-tumor activity without systemic toxicity (7–9). These results have prompted further evaluation of Apo2L/TRAIL as a potential therapeutic agent for human cancer.

Structural studies show that homotrimeric TNF superfamily ligands bind three receptor molecules (10–12), suggesting that the basic functional signaling unit is trimeric. This notion is further supported by the trimeric structure of certain signaling adaptor molecules that act downstream of the receptors, such as the TNF receptor-associated factors (13, 14). Further cross-linking of receptors beyond the trimeric unit in some cases can lead to stronger signal initiation; this can be modeled by antibody-mediated cross-linking of N-terminally tagged ligand, perhaps mimicking its transmembrane form (1). Binding of Apo2L/TRAIL to DR4 and/or DR5 leads to recruitment of the adaptor FADD (Fas-associated death domain) by the cytoplasmic death domain followed by recruitment and activation of the apoptosis initiators caspase-8 and caspase-10 (5, 15). Studies based on receptor-blocking antibodies indicate that Apo2L/TRAIL can induce apoptosis through either DR4 or DR5 or both, but the relative contribution of each death receptor to apoptosis induction in cells expressing both receptors is unknown (1, 6). Moreover, whereas binding of Apo2L/TRAIL to DR4 and DR5 can result in the formation of homomer as well as heteromeric complexes (5), the importance of heteromeric ligand-receptor complexes for apoptosis stimulation is unclear. Indeed, agonistic monoclonal antibodies specific to either DR4 (16) or DR5 (17) are capable of inducing apoptosis; however, the precise molecular mechanism of death receptor activation by agonistic antibodies is not fully understood.

To investigate the relative importance of DR4 and DR5 for apoptosis induction by Apo2L/TRAIL, we selected ligand variants with relative binding selectivity for DR4 or DR5 through a phage display approach. Although phage display has been used previously for optimizing the target binding affinity of monomeric, homodimeric, or heterodimeric proteins (18, 19), this technique has not been successfully applied as yet to trimeric proteins. Our modifications enabled the display of trimeric
Apo2L/TRAIL on phage and the selection of receptor-selective variants. Investigation of the ability of these variants to induce apoptosis in several cancer cell lines and in normal hepatocytes suggests that DR5 may play a more prominent role than DR4 in mediating apoptosis signals emanating from Apo2L/TRAIL in cells that express both death receptors.

EXPERIMENTAL PROCEDURES

Construction of Apo2L/TRAIL Phage Display Vector—A phagemid vector designed for the expression of Apo2L/TRAIL (residues 96–281) as a fusion to the geneIII protein of M13 bacteriophage was constructed as follows. The DNA encoding the 96–281 portion of Apo2L/TRAIL was amplified by PCR from the plasmid pAPOK5 (20) and ligated into NsiI/BamHI-cleaved pTFFA-g3 (21). The resulting plasmid (pAPOK4) encodes a fragment having the stil bacterial signal sequence fused to the N terminus of the 96–281-residue fragment of Apo2L/TRAIL. A tripeptide linker with the sequence GSA is appended to the C terminus of Apo2L/TRAIL followed by an in-frame amber stop codon and the geneIII product of M13 bacteriophage. The alkaline phosphatase promotor is used to direct expression. pAPOK4.2 was constructed by replacing the alkaline phosphatase promotor in pAPOK4 with the tac promotor.

Construction—The DR4 library was constructed by oligonucleotide-directed mutagenesis (22) using a primer having NNS codons at positions 189, 191, 193, 199, 201, and 209. The template was pAPOK4.2 containing Y123W/S215D mutations and TAA stop codons at 189, 191, 193, 199, 201, and 209. Use of the stop template ensured that any template DNA that did not become mutated and survived the Kunkel selection would not produce functional Apo2L. For the DR5 library, TAA stop codons were introduced into pAPOK4.2 at the library positions 189, 191, 193, 264, 266, 267, and 269. Two oligonucleotides, one specifying NNS codons at the library positions 189, 191, and 193 and the second containing NNS codons at sites 264, 266, 267, and 269, were used in the library mutagenesis reaction. Library mutagenesis, electroporation, and propagation of phage were performed as described (23).

Upon electroporation into SS-320 Escherichia coli the DR5 library gave a single-round infection titering of 500–1000 plaques, containing 2.5–10^6 colonies. Because SS-320 do not have an amber suppressor, the phage pellet was used to infect a 500-ml culture of early log phase XL-1-blue. The culture was grown overnight at 30 °C, and phage were harvested by precipitation with 1/5 volume of 20% polyethylene glycol, 2.5 M NaCl. Phage were amplified by PCR from the plasmid pAPOK5 (20) and ligated into pTFFA-g3. A fixed concentration of phage was then mixed with an increasing concentration of DR4-Fc or DR5-Fc and added to wells coated with DR4-Fc. After incubation to allow binding and washing to remove unbound phage, the bound phage was detected with the horse-radish peroxidase-coupled anti-M13 antibody. Analysis of the ELISA signal as a function of receptor-Fc concentration in solution by using a four-parameter fit yields the IC_50 value.

Expression and Purification of Apo2L/TRAIL Variante—Mutants of Apo2L (114–281) were constructed, expressed in E. coli, and purified as previously described (7, 20). Plasmids designed for E. coli expression of FLAG-tagged Apo2L variants were constructed by oligonucleotide-directed mutagenesis (22) of a plasmid (pFLAG-Apo2L; Scot Marsters, Genentech, South San Francisco, CA) containing 114–281 inserted in pFLAG-MAC (Sigma). pFLAG-Apo2L directs the cytoplasmic expression of N-terminal FLAG-tagged Apo2L/TRAIL (114–281) under control of the tac promotor. For expression of FLAG-tagged Apo2L/TRAIL variants, the mutant plasmids were transformed into E. coli strain strain 43E7. The transformed E. coli were grown to early log phase at 37 °C in 500 ml of 2x yeast/tryptone media containing 50 μg/ml carbenicillin, and expression was induced by the addition of isopropyl 1-thio-β-D-galactopyranoside to a final concentration of 0.4 mM. The FLAG-tagged Apo2L/TRAIL variants were purified as previously described for untagged proteins (20). For both the untagged and tag-tagged proteins, an additional purification step consisting of gel filtration on a 2.6 × 10^6 column of Sephacryl S-200 HR equilibrated and eluted with 0.5 μM sodium sulfamate, GdnCl, and EDTA was added before gel filtration. The pH, 7.5, was used to remove aggregated protein from the preparations.

Assay of Apoptosis Induction—Apoptosis induction in tumor cell lines upon the addition of Apo2L/TRAIL or variants was measured using a fluorescein assay of metabolic activity as previously described (20). Caspase activation in normal cells upon Apo2L/TRAIL addition was determined by using a fluorescein caspase substrate as previously described (24).

Receptor Binding by AlphaQuest® Assay—The binding of the variants to the five known Apo2L/TRAIL receptors (DrR1, DrR2, OPG, DR4, DR5) was examined by using an AlphaQuest® assay. This is a proximity-based assay technology in which emission of singlet oxygen from "donor" beads yields a fluorescent signal from "acceptor" beads brought into proximity by binding interactions. The donor bead was coated with streptavidin and was used to capture biotinylated Apo2L/TRAIL. The acceptor bead was coated with Staphylococcal protein A and was used to capture the receptor-Fc protein. IC_50 values were calculated from binding curves obtained by displacing the biotinylated ligand with ubiquitinylated ligand. Biotinylated Apo2L/TRAIL was prepared by reaction of a surface cysteine mutant of Apo2L/TRAIL (R170C) with biotin-N-hydroxysulfosuccinimidyl ester (Pierce) followed by removal of excess biotin on a PD-10 (Amersham Biosciences) desalting column. The various Apo2L/TRAIL receptors were diluted to 250 ng/ml in assay buffer (phosphate-buffered saline, pH 7.4, containing 0.5% bovine serum albumin and 0.05% Tween 20). Apo2L/TRAIL samples were serially diluted starting at a concentration of 4–20 μg/ml. Receptor (10 μl) and Apo2L/TRAIL (5 μl) were combined in 384-well white Opti-Plates (PerkinElmer Life Sciences)
and incubated for 30 min at room temperature. Biotinylated Apo2L/TRAIL competitor was then added (100 ng/ml, 5 μl per well). After a further 2-h incubation of the plates at room temperature, a mixture of Alphalone streptavidin donor beads and protein A acceptor beads (PerkinElmer Life Sciences) was added (1/10 dilution of each bead in assay buffer, 10 μl/well). The plates were incubated for 1 h at room temperature protected from light and subsequently read using an AlphaQuest® plate reader.

**Surface Plasmon Resonance Measurements of Receptor Binding**—Dissociation constants for the direct binding of Apo2L/TRAIL variants to immobilized receptors were determined by SPR measurements on a BIAcore 3000 instrument as previously described (20). Affinities were also assessed by using a competition assay in which binding of the ligand to immobilized DR5-Fc was competed with receptor in solution. These experiments employed a flow cell having a high density (15,000 resonance units) of immobilized DR5-Fc such that the initial rate of binding was linearly dependent on the concentration of free ligand. Competition experiments were conducted by preparing a series of solutions having a fixed concentration of ligand but a varied concentration of receptor. After incubating for 2 h to allow equilibration to occur, these solutions were injected over the DR5-Fc surface. A linear fit of the observed sensograms was used to extract the binding rate. Analysis of the binding rate as a function of competing receptor concentration enabled calculation of IC50 values by using a four-parameter curve fit.

**RESULTS**

**Phage Display of Apo2L/TRAIL**—The successful application of the phage display approach to the probing of receptor binding determinants requires proper assembly of trimeric Apo2L/TRAIL on the phage surface. To this end we used a construct having the Apo2L/TRAIL gene fused to the geneIII protein of M13 bacteriophage with an in-frame amber stop codon. In strains of E. coli capable of suppressing amber stop codons (supE genotype), a Gln is inserted at the amber stop resulting in secretion of an Apo2L/TRAIL-geneIII fusion protein into the periplasm. Because suppression of amber stop codons is only 10–30% efficient, free Apo2L/TRAIL is secreted as well. When supplied with assembly proteins by co-infection with helper phage, phage particles that incorporate the Apo2L/TRAIL-geneIII fusion protein into their surface can be produced. Trimeric Apo2L/TRAIL can be produced by assembly with either another fusion protein or with free Apo2L/TRAIL. However, because each phage particle will display only a few copies of the geneIII fusion protein (18), it seems likely that trimerization is driven by the free Apo2L/TRAIL produced when the amber stop is not suppressed.

Initial tests of the pAPOK4 vector suggested poor display on the phage of correctly assembled Apo2L/TRAIL. Specific binding increased if the phage were produced by growth at 30 °C rather than 37 °C and by replacement of the phoA promoter with the tac promoter (phagemid pAPOK4.2). Optimum display (~100-fold enrichment) was obtained for phage production at 30 °C with induction of the tac promoter by addition of 1 μM isopropyl 1-thio-β-D-galactopyranoside.

Phage displaying Apo2L/TRAIL showed increased nonspecific binding to a panel of control proteins relative to the nonspecific binding observed for KO7 helper phage (data not shown). In addition, KO7 helper phage displayed high nonspecific binding to purified Apo2L/TRAIL. Sorting of a preliminary Apo2L/TRAIL library having residues 213, 215, 216, 218, 220, and 222 randomized yielded a mutant of Apo2L/TRAIL (Y213W/S215D) that showed decreased helper phage binding. The Y213W/S215D mutant also gave increased display on phage, as measured by binding to an anti-Apo2L/TRAIL antibody, with comparable affinity for DR4-Fc but a 10-fold reduction in apparent affinity for DR5-Fc. The Y213W/S215D substitutions were incorporated into libraries designed for improved affinity against DR4.

**Design and Production of Apo2L/TRAIL Phage Display Libraries**—Residues in Apo2L/TRAIL were chosen for inclusion in the phage display libraries on the basis of an examination of the x-ray structure determined for the Apo2L-DR5-ECD complex (11). “Patch A” on DR5 involves the receptor 60 (residues 67–69) and 90 (residues 91–104) loops, which interact with a cluster of Apo2L/TRAIL residues centered on Gln-205. “Patch B” on DR5 involves the receptor 50 loop (residues 51–65), which makes contact with residues 155–162 and 215–218 of the ligand. Only residues within or near the patch A contact with the receptor (Fig. 1) were considered for mutagenesis. Because patch B is smaller than patch A, it is more hydrophilic, and is composed of ligand residues that are more conserved across TNF superfamily members, it has been proposed that patch A is more important for specificity (11). However, alanine substitution showed that several Apo2L/TRAIL residues in patch A (i.e. Gln-205, Tyr-237, Leu-239) make a large contribution to the free energy of binding for all receptors tested (20). These sites were chosen for mutagenesis to determine whether substitution of these sites increases receptor selectivity when mutated. An example of this type of site is Gln-193, where alanine substitution causes a 1.7-fold decrease in affinity for DR4 but has no effect on binding to DR5 and DcR2 (20). Further changes in receptor specificity might be obtained by substitution of Gln-193 with a residue other than alanine. This rationale was used to design two tailored Apo2L/TRAIL libraries: one including sites 189, 191, 193, 199, 201, and 209 randomized (“DR4 library”), and the other with sites 189, 191, 193, 264, 266, 267, and 269 randomized (“DR5 library”). As shown in Fig. 1, these residues are on the periphery of the patch A contact observed in the x-ray crystal structure (11).

**Sorting for Receptor-selective Variants**—The DR4 library was sorted for two rounds against DR4-Fc coated on microtiter wells followed by three rounds of sorting for DR4 binding in the presence of competing DR5-Fc. For sorting rounds 3, 4, and 5, the phage were incubated with 50, 250, and 750 nM DR5-Fc, respectively, for 30 min before the addition of these solutions to DR4-Fc coated plates. With the DR5 library, phage were sorted for four rounds against DR5-Fc coated wells followed by four rounds with DR4-Fc as competitor. In rounds 5, 6, 7, and 8, phage were incubated with 1, 10, 100, and 500 nM DR4-Fc.
respectively, before selection for DR5-Fc binding. Both the DR4 and DR5 libraries gave strong, specific enrichment even in the presence of the competing receptor.

Individual clones were isolated from the fifth round (DR4 library) or eighth round (DR5 library) of sorting and tested for receptor specificity by phage ELISA (see “Experimental Procedures”), and then the DNA sequence of receptor-selective clones was determined. The amino acid identities deduced from the DNA sequence for the library positions are shown in Supplemental Tables I (DR4-selective) and II (DR5-selective). No spurious sequence changes outside of the library positions were detected. Analysis of four of the clones from the DR4 library by competition phage ELISA (Fig. 2) confirmed that the sorting strategy yielded receptor-selective variants. These four clones bound DR4-Fc with apparent high affinity, whereas binding to DR5-Fc was undetectable. These DR4-selective variants all differed from the wild-type sequence at four of six library positions. Substitution of Tyr-189 with Ala appeared to be important for DR4 selectivity, since all four clones had this mutation.

Variants with selectivity for DR5 (Supplemental Table I) all had at least three amino acid changes, and clones with six substitutions occurred most frequently. Although this library was subjected to eight rounds of sorting, a consensus sequence was not obtained, and some positions, most notably 264, retained high diversity. Substitution of Gln-193 with positively charged Lys or Arg appeared to be important for DR5 selectivity. A negatively charged residue at either position 267 or 269, but not both, was favored. Replacement of Ile-266 with Leu was a frequent change in these variants. Interestingly, the Y189A mutation was also observed in the DR5-selective variants.

Apoptosis Induction by Receptor-selective Variants—To characterize the receptor-selective variants further, we produced FLAG-tagged versions of two of the DR4-selective and three of the DR5-selective mutants. These variants were profiled for receptor binding by using an AlphaQuest® assay and assayed for apoptosis induction on SK-MES lung carcinoma cells. SK-MES cells express DR4 and DR5 at similar levels (data not shown) and are sensitive to apoptosis induction by wild-type Apo2L/TRAIL. Inclusion of the FLAG tag enables testing of oligomerized forms of Apo2L/TRAIL or its variants formed by cross-linking the tagged protein with anti-FLAG antibody. Anti-FLAG cross-linking of tagged wild-type Apo2L/TRAIL resulted in an ~70-fold increased potency for apoptosis induction in SK-MES (Table I).

The AlphaQuest® assay indicated that the two DR4-selective variants retained high affinity binding to DR4-Fc (Table I). Binding to both DR5 and OPG was significantly reduced, whereas the reduction in affinity for DcR1 and DcR2 was more modest. Both of the DR4-selective proteins showed a large decrease in potency for apoptosis induction relative to wild-type Apo2L/TRAIL (ED_{50} = 12.9 ± 5.2 ng/ml) or FLAG-Apo2L/TRAIL (ED_{50} = 48 ng/ml). FLAG-Apo2L.DR4–8 showed no increase in potency upon cross-linking, whereas FLAG-Apo2L.DR4–9 showed a relatively modest 6-fold gain in potency upon cross-linking. The apoptosis-inducing activity of FLAG-Apo2L.DR4–8 on SK-MES could be inhibited by a neutralizing anti-DR4 monoclonal antibody but not by a neutralizing antibody specific for DR5 (data not shown). This finding suggests that the residual activity of FLAG-Apo2L.DR4–8 results from binding to DR4 and does not reflect a weak interaction with DR5.

All of the tested DR5-selective variants showed significant reduction in affinity for DcR1, OPG, and DR4 while maintaining high affinity for DR5 (Table I). The variation in affinity for DR5 ranged from a slight improvement (FLAG-Apo2L.DR5–8) to about a 3-fold decrease (FLAG-Apo2L.DR5–2). The change in affinity for DcR2 was variable and, with the exception of FLAG-Apo2L.DR5–8, was smaller than observed for the other receptors. FLAG-Apo2L.DR5–8 was the most selective variant showing good binding to DR5 but significantly reduced affinity for the other four receptors. Surprisingly, all of the DR5-selective variants retained a high level of apoptosis-inducing activity (Table I). Changes in activity varied from a 2.6-fold increase in ED_{50} for FLAG-Apo2L.DR5–1 to a 6.4-fold decrease for FLAG-Apo2L.DR5–8. The potency of all of the DR5-selective variants increased significantly upon anti-FLAG cross-linking to the same level as measured for the cross-linked wild-type protein. There was not a strict 1:1 correspondence between the affinity measured for DR5 and the potency measured for apoptosis induction. This likely reflects the use of Fc fusion proteins to determine affinities, since the bivalency may suppress affinity differences.

The specificity of a few of the variants was further confirmed by using SPR to measure the dissociation constants for binding to DR4 and DR5. As shown in Table II, FLAG-Apo2L.DR4–8 and FLAG-Apo2L.DR4–9 bind to DR4 with affinity nearly equivalent to that measured for the wild-type protein, whereas binding to DR5 was significantly reduced. The apparent affinity of FLAG-Apo2L.DR4–8 for DR5 was too weak to measure.
by SPR. In contrast, the DR5-selective variant (FLAG-Apo2L.DR5–8) did not appear capable of binding to DR4 but, consistent with the AlphaQuest® data, displayed high affinity binding to DR5. A very slow on-rate was measured for interaction of FLAG-Apo2L.DR4–8 with DR5 and also for binding of FLAG-Apo2L.DR5–8 to DR4. The slow binding kinetics precluded accurate determination of the binding constants by SPR for these weak interactions.

Because FLAG-Apo2L.DR4–8 and FLAG-Apo2L.DR5–8 gave the greatest receptor selectivity, these variants were used for testing the sensitivity of additional cancer cell lines to receptor-specific ligands. Colo205, a colon carcinoma cell line, expresses slightly more DR5 than DR4 and was quite sensitive to trimeric Apo2L/TRAIL (ED50 = 4.2 ± 1.8 ng/ml). FLAG-Apo2L.DR4–8 induced apoptosis only weakly (ED50 = 1150 ng/ml) in Colo205 (Fig. 3A). The addition of anti-FLAG antibody increased the potency of this variant about 8-fold (ED50 = 150 ng/ml). FLAG-Apo2L.DR5–8 showed increased potency against Colo205 cells (ED50 = 0.9 ng/ml; Fig. 3B), and its activity was increased 6-fold by anti-FLAG cross-linking. A similar pattern of results was obtained against Colo320, a colon carcinoma cell line that expresses similar amounts of DR4 and DR5 (data not shown). FLAG-Apo2L.DR4–8 was inactive against Colo320 cells even if cross-linked with anti-FLAG antibody. FLAG-Apo2L.DR5–8 weakly induced apoptosis in Colo320 cells, but the activity of the cross-linked form was equivalent to that observed for anti-FLAG cross-linked wild-type Apo2L/TRAIL.

The breast carcinoma cell line MDA-MB-231 expresses high levels of both DR4 and DR5 yet displays a weaker apoptotic response to Apo2L/TRAIL treatment than observed with Colo205 cells (5). FLAG-Apo2L.DR4–8 was completely inactive against MDA-MB-231 (Fig. 4A). FLAG-Apo2L.DR5–8 showed modest activity against Jurkat cells, and the activity was dramatically increased (ED50 = 0.05 ng/ml) by cross-linking (Fig. 4B). The Jurkat T leukemia cell line expresses DR5 but not DR4. As expected, FLAG-Apo2L.DR4–8 did not induce apoptosis in this cell line even upon cross-linking (Fig. 5A). FLAG-Apo2L.DR5–8 showed modest activity against Jurkat cells, and the activity was dramatically increased (ED50 = 0.05 ng/ml) by cross-linking (Fig. 5B).

**Table I**

| Protein         | IC50 ratio (mutant/wt) | ED50 (ng/ml) |
|-----------------|------------------------|--------------|
| FLAG-Apo2L/TRAIl| 1                      | 48.4/0.7     |
| FLAG-Apo2L.DR4–8| 5.5/15                 | 4000/4100    |
| FLAG-Apo2L.DR4–9| 4.0/18                 | 1000/170     |
| FLAG-Apo2L.DR5–1| >300/32                | 126/1.3      |
| FLAG-Apo2L.DR5–2| >300/5.0               | 67.8/1.0     |
| FLAG-Apo2L.DR5–8| >300/1900              | 7.6/0.3      |

**Table II**

| Variant       | Amino acid changes | Kd ratio (mutant/wt) |
|---------------|--------------------|----------------------|
|               |                    | DR4                  | DR5                  |
| FLAG-Apo2L.DR4–8| Trp-213; Asp-215; Ala-189; Ser-193; Val-199; Arg-201 | 2.3                | NB$^b$               |
| FLAG-Apo2L.DR4–9| Trp-213; Asp-215; Ala-189; Ser-193; Arg-199; Arg-201 | 1.5                | 61                   |
| FLAG-Apo2L.DR5–8| Gln-189; Lys-191; Arg-193; Arg-264; Leu-266; Glu-267 | NB$^b$             | 0.8                  |

$^a$Amino acid changes are relative to the wild-type protein.

$^b$No binding (NB) was detected at the highest concentration (500 nM) used in the SPR measurements.

**Fig. 3.** Assay of apoptosis-induction on Colo205 colon carcinoma cells by FLAG-tagged Apo2L/TRAIl mutants. A fluorescence assay (20) was used to test DR4-selective (panel A) or DR5-selective (panel B) mutants. antiFLAG indicates that 2 μg/ml M2 antibody (Sigma) was added along with the specified concentration of Apo2L/TRAIl mutant. Curves represent fitting using the four-parameter equation. RFU, relative fluorescence units.
signal on Jurkat cells upon cross-linking.

Normal hepatocytes are resistant to Apo2L/TRAIL unless the ligand is aggregated (8). The receptor-selective variants were evaluated for their ability to induce apoptosis in normal hepatocytes using an assay that measures activation of caspase-3 and -7. FLAG-Apo2L.DR4–8 did not induce caspase activation even upon cross-linking with anti-FLAG antibody (Fig. 6). Consistent with previous results (8), Apo2L/TRAIL did not increase caspase activity except after cross-linking. Similar to the wild-type ligand, FLAG-Apo2L.DR5–8 was inactive as a trimer and induced caspase activation upon anti-FLAG cross-linking.

Selection of Substitutions Necessary to Give Selectivity for DR5 Binding—To determine the minimum number of amino acid changes required for DR5 selectivity while maintaining high bioactivity, we did additional phage display experiments followed by testing of selected variants. Two phage libraries were constructed in which the residues at 189, 191, 193, 264, 266, and 267 were allowed to vary as either the wild-type residue, the amino acid found in Apo2L.DR5–8, or a limited set of similar amino acids. These libraries were pooled and sorted for one round against DR5-Fc coated on microtiter wells followed by two rounds of sorting for DR5 binding in the presence of competing DR4-Fc. By the third round of sorting, >1000-fold enrichment was obtained, and individual clones were selected for further analysis. Spot ELISA indicated that 28 of 28 clones tested were positive for DR5 binding, whereas only one was functional for DR4 binding. The amino acid identities deduced
from the DNA sequence at the library positions for the clones positive for DR5 binding, but not DR4 binding, are shown in Supplemental Table III. These results suggest that the residue identity at positions 264 and 267 may not be important for DR5 selectivity since there was no preference at these positions for the amino acid found in variant DR5–8. In contrast, the amino acids found in variant DR5–8 predominated at positions 189, 191, 193, and 266, indicating that one or more of these sites may be important for DR5 selectivity. At position 189 there was a strong preference for Gln, which can be expressed through a Gln codon or through suppression of an amber stop codon.

A few variants (Apo2L.DR5–8E) were produced as an untagged 114–281 fragment (7) of Apo2L/TRAIL and tested for receptor binding and bioactivity. Apo2L.DR5–8E differs from wild-type Apo2L/TRAIL by Y189Q and R191K. Apo2L.DR5–8B has the additional substitution of Q193R, and Apo2L.DR5–8C also has I266L besides these substitutions. Apo2L.DR5–8D has Y189Q, R191K, and I266L. Dissociation constants for receptor binding were determined from SPR data for direct binding of each variant to immobilized receptor. Because all of these variants had decreased binding to DR4-Fc, it was necessary to use a sensor chip with a high density of immobilized DR4-Fc to detect sufficient signal in direct binding measurements. For measurements with wild-type Apo2L/TRAIL, a 4-fold greater binding affinity was calculated from high density as opposed to low density binding surfaces (data not shown). Thus, a competitive binding assay in which Apo2L/TRAIL binding to immobilized DR5–Fc was competed with DR4-Fc or DR5-Fc in solution was also used to assess receptor affinities. As shown in Table III, all of the variants showed decreased binding to DR4. The competition assay suggested a larger magnitude decrease in affinity for DR4 than the direct binding assay. With the exception of Apo2L.DR5–8D, these proteins displayed high affinity binding to DR5. Binding of Apo2L.DR5–8D to either DR4 or DR5 was not detectable, suggesting that this variant was misfolded. Indeed, this sequence was found only in 1 of 51 clones from phage sorting. The two amino acid changes in Apo2L.DR5–8E resulted in a significant decrease in affinity for DR4 and a slight decrease in DR5 binding, but a 16-fold reduction in apoptosis-inducing activity. The addition of Q193R (Apo2L.DR5–8B) had a slight effect on DR5 affinity and did not restore bioactivity. Incorporation of I266L (Apo2L.DR5–8C) resulted in a modest improvement in DR5 affinity and restored full bioactivity. The results for Apo2L.DR5–8 indicate that the H264R and/or D267Q substitutions give further selectivity against DR4 binding while providing modest improvements in DR5 affinity and bioactivity.

**DISCUSSION**

We have shown here that Apo2L/TRAIL can be expressed on M13 bacteriophage in functional form, allowing selection of clones from mutant libraries for receptor-specific binding. To our knowledge this is the first successful application of phage display technology to a TNF-superfamily member. Because most TNF superfamily members bind to their receptors at the interfaces between subunits of the ligand homotrimer, a functional receptor binding site can be formed only when a ligand trimer is properly assembled. Trimer assembly was promoted by using a construct having an amber stop codon since both monomer Apo2L/TRAIL, and a geneIII fusion protein could be produced in the same cell. An excess of free monomer most likely drives oligomerization with the fusion protein. Indeed, in sorting of the second DR5 library (Supplemental Table III) there was selection for an additional amber stop codon at residue 189 where a Gln residue was compatible with DR5 binding. A second amber stop would increase the ratio of monomer to fusion protein because two stops must be suppressed to express the geneIII fusion protein. Functional display of Apo2L/TRAIL was also aided by lowering the growth temperature and by using a strong, inducible promoter. Selection of the DR4 library was enhanced through incorporation of the amino acid substitutions Y213WS215D, which reduced nonspecific binding of Apo2L/TRAIL. For both the DR4 and DR5 libraries, nonspecific binding was further reduced by using competitive selection. As with other protein-protein interactions (25), competitive selection proved to be a powerful approach for increasing the specificity of interaction between Apo2L/TRAIL and its death receptors.

Alanine-scanning mutagenesis has shown that the binding sites on Apo2L/TRAIL for the various receptors are highly similar, with certain key residues (e.g. Gln-205, Tyr-216) making a large free energy contribution to the binding of each of the five receptors (20). Although only the structure of the Apo2L.DR5 complex is known, many of the residues on DR5 that are in contact with Apo2L/TRAIL are conserved in DR4. Thus, it is perhaps not surprising that multiple amino acid substitutions were required to achieve receptor selectivity. By comparison, TNF mutants functional for binding to the p55 receptor, but not the p75 receptor, could be obtained by combining two or three amino acid changes (26). Examination of the Apo2L.DR5 structure (Fig. 1) suggests that the amino acid changes in Apo2L.DR5–8 would disfavor interactions with DR4 residues Lys-67, Asp-69, and Asn-95. The equivalent residues in DR5 are Asp-67, Gly-69, and Asp-95. The R191K substitution may oppose interaction with Lys-67 on DR4. Replacement of Gln-193 with a positively charged residue may interfere sterically with engagement of DR4 residue Asp-69, whereas the Gly in DR5 may accommodate a larger side chain. Ile-266 is a buried residue in Apo2L/TRAIL, and thus, the effect of the I266L substitution must be indirect, perhaps occurring through a change in the conformation of residue 267. D267Q may remove a favorable electrostatic interaction with the positive charge on DR4 residue Lys-67. H264R would be positioned to provide a positive charge for interaction with DR5 Asp-95 while discriminating against neutral residue Asn-95 in DR4. The effect of the Y189Q mutation is difficult to explain, since Tyr-189 interacts with the backbone of DR5 and with residues

| Variant         | Amino acid changes | DR4 binding | DR5 binding | Apoptosis, ED50 |
|-----------------|--------------------|-------------|-------------|-----------------|
| Apo2L.DR5–8     | Gln-189; Lys-191;  | 333         | 3            | 0.5             |
| Apo2L.DR5–8E    | Gln-189; Lys-191;  | 333         | 3            | 0.5             |
| Apo2L.DR5–8B    | Gln-189; Lys-191;  | 333         | 3            | 0.5             |
| Apo2L.DR5–8D    | Gln-189; Lys-191;  | 333         | 3            | 0.5             |
| Apo2L.DR5–8C    | Gln-189; Lys-191;  | 333         | 3            | 0.5             |
that are identical between DR4 and DR5. This substitution may facilitate a conformational change in the binding site that promotes receptor selectivity. A more complete understanding of these results will require the determination of free and receptor-bound structures of Apo2L.DR5–8.

The DR5-selective variants retained a high level of apoptosis-inducing activity against cancer cells, and the activity was significantly increased when the ligand was cross-linked through an N-terminal FLAG tag. In contrast, the DR4-selective mutant showed increased apoptosis-inducing activity, and their potency was only modestly increased by anti-FLAG cross-linking. These results are inconsistent with the conclusions of Muhlenbeck et al. (27), who proposed that DR5 (TRAIL-R2) signals apoptosis only in response to cross-linked Apo2L/TRAIL, whereas the DR4–8 receptor-bound structures of Apo2L.DR5–8.

The treatment was not required to render the cancer cell lines we studied sensitive to the DR5-selective variants. Although the SK-MES and Colo205 cancer cell lines express both DR4 and DR5 at comparable levels, the Apo2L.DR5–8 variant showed increased apoptosis-inducing activity compared with wild-type Apo2L/TRAIL against these cell lines, whereas the Apo2L.DR4–8 variant was much less active. In MDA-MB-231 cells, which also express both DR4 and DR5 and are sensitive to wild-type Apo2L/TRAIL, the Apo2L.DR4–8 mutant was inactive even upon cross-linking, whereas Apo2L.DR5–8 showed weak activity without cross-linking and full activity upon cross-linking. In hepatocytes, which are resistant to non-cross-linked Apo2L/TRAIL but sensitive to the cross-linked ligand, the DR5-selective variant induced the same responses as the wild-type ligand, whereas the DR4-selective mutant was inactive. Taken together, these results suggest that DR5 may contribute more than DR4 to Apo2L/TRAIL-induced apoptosis in cancer or normal cells that express both death receptors. Thus, it may be possible to enhance the anti-tumor activity of soluble trimeric Apo2L/TRAIL further by engineering mutants of this protein that have increased affinity for DR5 but retain a stable trimeric structure.

Acknowledgments—We thank the oligonucleotide chemistry group at Genentech for synthesis of oligonucleotides, the DNA sequencing group for sequencing of phage clones, and Scot Marsters for supplying receptor-Fc fusion proteins.

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Receptor-selective Mutants of Apoptosis-inducing Ligand 2/Tumor Necrosis Factor-related Apoptosis-inducing Ligand Reveal a Greater Contribution of Death Receptor (DR) 5 than DR4 to Apoptosis Signaling

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J. Biol. Chem. 2005, 280:2205-2212.
doi: 10.1074/jbc.M410660200 originally published online November 1, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M410660200

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