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Death Domain Mutagenesis of KILLER/DR5 Reveals Residues Critical for Apoptotic Signaling*

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The Fas/tumor necrosis factor (TNF)/TRAIL receptors signal death through a cytoplasmic death domain (DD) containing six α-helices with positively charged helix 2 interacting with negatively charged helix 3 of another DD. DD mutation occurs in head/neck and lung cancer (TRAIL receptor KILLER/DR5) and in *lpr* mice (Fas). We examined the apoptotic potential of known KILLER/DR5 lung tumor-derived mutants (n = 6) and DD mutants (n = 18) generated based on conservation with DR4, Fas, Fas-associated death domain (FADD), and tumor necrosis factor receptor 1 (TNFR1). With the exception of Arg-330 required in Fas or FADD for aggregation or for TNFR1 cytotoxicity, surprisingly major loss-of-function KILLER/DR5 alleles (W325A, L334A (*lpr*-like), I339A, and W360A) contained hydrophobic residues. Loss-of-function of I339A (highly conserved) has not been reported in DDs. Charged residue mutagenesis revealed the following points. 1) E326A, conserved in DR4, is dispensable for death; the homologous residue is positively charged in Fas, TNFR1, and FADD and is critical for DD interactions. 2) K331A, D336A, E338A, K340A, K343A, and D351A have partial loss-of-function suggesting multiple charges stabilize receptor-adapter interactions. Analysis of the tumor-derived KILLER/DR5 mutants revealed the following. 1) L334F has partial loss-of-function *versus* L334A, whereas E338K has major loss-of-function *versus* E338A, examples where alanine and tumor-specific substitutions have divergent phenotypes. 2) Unexpectedly, S324F, E326K, K386N, and D407Y have no loss-of-function with tumor-specific or alanine substitutions. Loss-of-function KILLER/DR5 mutants were deficient in recruitment of FADD and caspase 8 to TRAIL death-inducing signaling complexes. The results reveal determinants within KILLER/DR5 for death signaling and drug design.

The balance between cell division and programmed cell death, or apoptosis, within mammals is crucial for normal development (1–5). An increase in cell death can lead to neurodegenerative diseases, whereas decreased apoptosis or unfettered cellular proliferation can lead to cancer (6). Apoptosis is controlled by multiple pathways that integrate both intra- and extracellular signals, which eventually converge upon cellular proteases, or caspases (7–9). The tumor necrosis factor (TNF)[1] superfamily (TNFR1, Fas, etc.) of trans-membrane receptors respond to soluble or membrane-bound ligands (TNF-α, Fas ligand, TRAIL) to instigate formation of death-inducing signaling complexes (DISCs), which ultimately culminates in caspase activation and death (10, 11).

The TRAIL cytokine controls one such apoptotic pathway by binding a group of extra-cellular receptors belonging to this TNF receptor superfamily (11). Two members of this group, DR4 (TRAIL-R1) and KILLER/DR5 (TRAIL-R2, TRICK2), actively promote apoptosis upon overexpression or TRAIL binding (12–20). TRAIL binding causes the receptors to trimerize, bringing together a 70-amino acid intracellular protein/protein interaction motif, termed the death domain (DD) (10, 21, 22). This domain serves as a platform to recruit an adapter protein which then binds to and activates the initiator caspases (11). In the case of TRAIL ligand, it has recently been demonstrated that FADD (Fas-associated death domain) and caspase 8 are required for cell death signaling (23–26). Both FADD-null and caspase 8-null cells were shown to be deficient in TRAIL-induced apoptosis (23, 24, 26). Two anti-apoptotic members or decoy receptors, TRID (DcR1, TRAIL-R3) and TRUNDD (DcR2, TRAIL-R4), are capable of binding TRAIL but do not transmit an apoptotic signal due to the lack of a functional DD (27–30). The balance between these receptors on the cell and the expression of caspase 8, as well as the presence of intracellular inhibitors, termed cFLIPs, determine the fate of the cell in response to TRAIL treatment (31–33).

The importance of the DD in causing an apoptotic response from DR4 or KILLER/DR5 is underscored by two observations. Experiments in which the DD is deleted renders them nonfunctional (12–20), and the TRAIL receptors, TRID and TRUNDD, are deemed decoys due to their absence of a functional DD (27–30). Studies of the C terminus of TNFR1 including deletion and alanine mutagenesis of DD revealed that this domain is critical for apoptotic signaling (34). Homology studies recognized the presence of a DD within Fas as well (34). Crystal structure analysis of the Fas DD revealed six anti-parallel α-helices (35). Mutagenesis of the Fas DD, as well as similar studies with the pro-apoptotic adapter molecule FADD, revealed the importance of helices 2 and 3 for self-association and protein/protein interactions (35, 36). The interactions appear to act in an antiparallel fashion with FADD helix 2/Fas

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[1] The abbreviations used are: TNF, tumor necrosis factor; DD, death domain; IP, immunoprecipitation; DISC, death-inducing signaling complex; PARP, poly(ADP)-ribose polymerase; FADD, Fas-associated death domain; TNFR, tumor necrosis factor receptor; GFP, green fluorescent protein; EGFP, enhanced green fluorescent protein.

helix 3 and Fas helix 2/FADD helix 3 comprising the bulk of the electrostatic interactions (36).

Naturally occurring mutants of DD-containing receptors have been described. The *lpr*^{cg} mouse, which develops an autoimmune lymphoproliferative disorder, contains a I238N alteration changing an isoleucine to an asparagine within the DD of the mouse Fas receptor (37). Mutations of DD-containing receptors have also been found in human cancers. Rare loss-of-function alterations were initially described in the TRAIL receptor KILLER/DR5 in head and neck cancer specimens. A single-base insertional mutation led to a frameshift in the DD of KILLER/DR5, leading to premature termination of translation and a resulting DD-truncated functionally deficient receptor (38.). More recently, a number of mutations have been reported in the DD of KILLER/DR5 in non-small cell lung cancers in 11 of 104 (10.6%) specimens tested (39). These alterations included eight missense, one nonsense, one splice site, and one silent change within exons 9 and 10 of the human KILLER/DR5 gene (39). None of the substitution changes were functionally characterized, although three of the eight missense alterations involved a C-to-T transition at base pair 1087 leading to an L334F (leucine to phenylalanine change). Leu-334 is the homologous position to the *lpr* mutation in the mouse Fas receptor gene. Alteration in cancer of other receptors has been reported and includes homozygous deletion of DR4 in nasopharyngeal cancer (40), overexpression of DcR3 (a Fas decoy receptor) in colon and lung cancers (41), and overexpression of DcR1 (TRID decoy) in gastrointestinal tumors (42).

Because of their involvement in cancers and the potential therapeutic applications of TRAIL and TRAIL receptor signaling, we undertook a site-directed mutagenesis strategy to identify important aspects of DD transduction of a cell death signal. No detailed mutational analysis or structure-function study has been reported for either of the two pro-apoptotic TRAIL receptors DR4 and KILLER/DR5. Moreover, no detailed comparisons with other TNF receptor family members have been performed in terms of conservation of structural features *versus* functional outcome in death signaling. Thus, the present studies involved the generation of a series of point mutants within human KILLER/DR5 leading to 18 different amino acid substitutions by alanine at positions chosen based on alignments with family members, and information derived from structural evidence with other family members. Finally, we investigated the functional significance of six known DD substitutions isolated from non-small cell lung cancer specimens. Our results provide interesting comparisons into the relative importance of charged *versus* hydrophobic DD amino acid residues and death signaling. Moreover, we show that one tumor-derived KILLER/DR5 mutant displayed a more severe loss-of-function phenotype, whereas a different tumor-derived mutant displayed a much more modest defect in death signaling as compared with their respective alanine substitutions. Finally, a number of tumor-derived mutants were shown to retain apoptotic capability when overexpressed. The present studies provide novel insights into specific structural determinants within the DD that signal downstream caspase activation by the KILLER/DR5 death receptor protein.

MATERIALS AND METHODS

Alanine Scanning Mutagenesis of KILLER/DR5—The KILLER/DR5 cDNA was cloned in frame into pcDNA3.1-Myc-HisA− (Invitrogen) as an *Eco*RI/*Hin*dIII fragment. This C-terminally tagged Myc-His plasmid was subsequently mutagenized using the QuikChange site-directed mutagenesis kit (Stratagene). Due to the size of the template (6.7 kilobases), certain changes were made to the manufacturer's protocol to yield polymerase chain reaction product: inclusion of 10% glycerol, 5% Me_2SO, and 100 ng of template in polymerase chain reaction, decrease of annealing temperature to 50 °C, and 18 cycles of amplification. Mutations were verified by sequencing, and in each case the entire cDNA was checked for the absence of second site mutations.

Transfections, in Vitro Translation, and Western Blotting—The colon cancer cell line SW480 was maintained and transfected as described previously (13). Protein extracts were harvested in 1× Laemmli sample buffer 16 h after transfection. PARP (Roche Molecular Biochemicals; 1:2000) and Myc (Santa Cruz; 1:500) immunoblots were performed following SDS-polyacrylamide gel electrophoresis. Horseradish peroxidase-conjugated secondary antibody (Pierce; 1:5000) treatments were followed by enhanced chemiluminescence (Amersham Pharmacia Biotech). *In vitro* translation reactions were carried out using 1 μg of wild-type or mutant KILLER/DR5 plasmid DNA and the TNT T7-coupled reticulocyte lysate system (Promega).

GFP/PI Fluorescence-activated Cell Sorting Analysis for Sub-G_1 Peak—SW480 cells were transfected with a 1:10 ratio of EGFP-spectrin (43) and KILLER/DR5 expression plasmid. Transfections were harvested at 18 h after transfection, processed, and analyzed for the presence of a sub-G_1 peak as described previously (40). 10,000 GFP-positive cells were analyzed per experiment, and three independent experiments were performed for each mutant. Percentage of apoptosis was calculated by subtracting transfection-induced sub-G_1 peak (vector transfection) from each wild-type or mutant KILLER/DR5 sub-G_1 peak. Figures for Tables II and III were generated by setting wild-type death to 100% (~40% sub-G_1 peak at 18 h) in order to compare point mutants to the wild-type protein.

Blue Cell Method—The blue cell method was performed as previously described (44). Calculations for Table I were carried out by setting the number of vector-transfected blue cells to 100% (at least 300 cells for each independent experiment), and the wild-type and point mutants were determined as a percentage of vector transfected.

TRAIL DISC Immunoprecipitation—293 HEK cells were plated to achieve 80% confluence at the time of transfection in a T75. 30 μg of the indicated KILLER/DR5 plasmid was transfected by calcium phosphate for 16 h to minimize loss of transfected cells. The cells were then trypsinized, spun down, and resuspended in 2 ml of complete medium supplemented with 50 ng/ml His tagged-TRAIL and 1 μg/ml anti-6-histidine antibody (R&D Systems) for 15 min at 37 °C. For untreated samples, only the TRAIL was excluded. The cells were washed twice with ice-cold phosphate-buffered saline and lysed for 30 min on ice in TRAIL DISC IP lysis buffer (30 mM Tris, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100). The lysates were cleared twice by centrifugation at 4 °C. The supernatants were immunoprecipitated overnight with 30 μl of Protein A/G Plus-agarose (Santa Cruz) at 4 °C to isolate the TRAIL DISC. The complexes were subsequently washed four times with TRAIL DISC IP lysis buffer and eluted with Immunopure Gentle Ag/Ab elution buffer (Pierce) with 0.1 M dithiothreitol at room temperature for 2 h. The protein complexes were methanol/chloroform-precipitated and resolved on 15% SDS-polyacrylamide gels. Caspase 8 (Cell Signaling; 1:1000) and FADD (Upstate Biotechnology; 1:2000) Western blots were performed to measure recruitment of these endogenous proteins to the TRAIL DISC along with the exogenously expressed KILLER/DR5 (Myc; Santa Cruz) protein.

RESULTS

Design of KILLER/DR5 DD Alanine Scanning Mutants Based on Homology to DDs of the TNFR Superfamily—Recent mutational and crystal structure studies with Fas and FADD (35, 36) have revealed the importance of helices 2 and 3 of both proteins. Electrostatic interactions between surface residues are thought to mediate DD/DD interactions. DD mutants were designed, based on a number of selection criteria including residue charge, hydrophobicity, conservation, and demonstrated functional significance in other receptor systems. Due to the noted importance of helices 2 and 3 within Fas and FADD, we focused our mutagenesis on charged residues in order to identify residues crucial for receptor/adapter interactions. Fig. 1 depicts an amino acid alignment of the death domains of selected members of the TNF superfamily. Eighteen residues within KILLER/DR5, which were targeted for alanine replacement mutagenesis, are noted with an *asterisk* along with the amino acid position of the short form of the KILLER/DR5 protein.

Fig. 2*A* shows protein expression of each construct following transfection into SW480 colon cancer cells for 16 h. Most of the

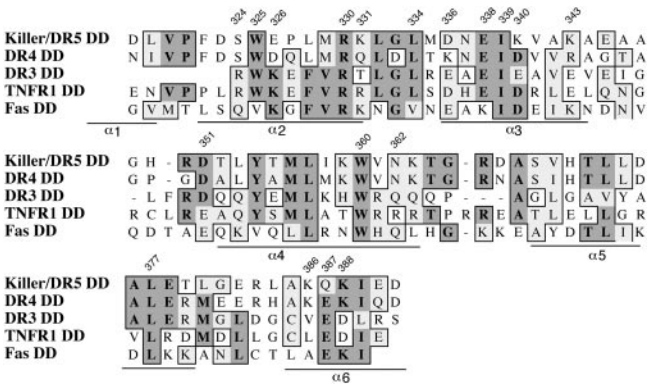

FIG. 1. **Alignment of DD from TNF receptor superfamily members.** *Numbers* denote residues in KILLER/DR5 that were targeted in alanine scanning mutagenesis. *Bold line* below Fas DD denotes α-helices determined from Fas crystal structure studies (35).

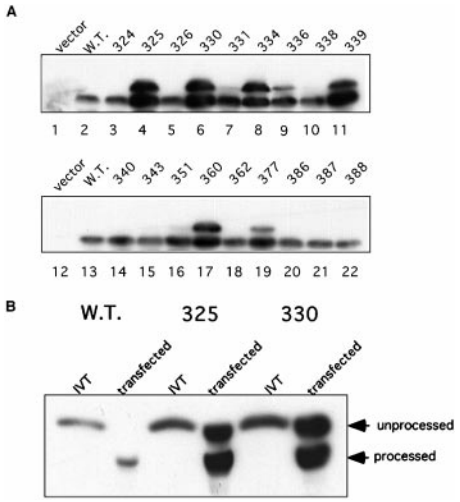

FIG. 2. **Protein expression of wild-type (*W.T.*) and alanine mutant KILLER/DR5 constructs.** *A*, 0.5×10^6 SW480 cells were transiently transfected for 16 h, harvested, and equivalent amounts of protein were run on a 15% SDS-polyacrylamide gel. The gel was then transferred and probed with an anti-Myc antibody, followed by a horseradish peroxidase-conjugated secondary and ECL. *B*, comigration of *in vivo* upper KILLER/DR5 band with unprocessed *in vitro* translation (*IVT*) product. The unlabeled *in vitro* translation reaction was carried out on 1 μg of plasmid as per manufacturer's instructions (Promega). The *in vitro* translation reactions were run out along with transfected lysates and processed as in *A*.

alanine mutants expressed protein at levels equivalent to wild type KILLER/DR5. Selected mutants showed an increase in protein expression levels in addition to the appearance of a lower mobility form of the protein. Interestingly, these mutants with higher levels of expression and the larger form appeared to be either partially or completely defective in inducing apoptosis (see data below). This would explain the higher levels of the transfected protein as the cell was able to withstand the presence of these non-toxic proteins. The lower mobility form could also be explained due to the higher levels of transfected protein being made and withstood by the cell. Fig. 2*B* compares transfected cell lysates to *in vitro* translated proteins, which do not undergo processing of the signal peptide. The TNT reactions yield a lower mobility form of KILLER/DR5, which comigrates with the upper band observed in the transfected cell lysates of mutants, W325A and R330A. In addition, cotransfection of the anti-apoptotic gene, $FLIP_S$, along with wild type KILLER/DR5 protects the cells from death and both the processed and unprocessed forms of the wild-type protein can be detected by Western blot analysis (data not shown).

TABLE I
Blue cell assay for alanine scanning mutants as a percentage of vector

KILLER/DR5 Alanine point mutant	Blue cells
	% of vector
3.1 Myc-His vector	100
Wild-type KILLER/DR5	8 ± 0.2
Loss-of-function mutations	
W325A	72 ± 5.0
R330A	86 ± 2.9
L334A (position equivalent to Fas *lpr*)	86 ± 14.4
I339A	82 ± 31.3
W360A	79 ± 3.1
Partial loss-of-function mutations	
K331A	37 ± 15.7
D336A	50 ± 1.8
E338A	34 ± 3.2
K340A	53 ± 4.1
K343A	47 ± 2.8
D351A	17 ± 4.4
L377A	38 ± 2.2
No loss-of-function mutations	
S324A	37 ± 6.4
E326A	3 ± 0.1
N362A	10 ± 2.0
K386A	14 ± 3.6
Q387A	11 ± 4.3
K388A	9 ± 1.5

Conserved Hydrophobic Residues Are More Critical for Apoptotic Signaling as Compared with Conserved Charged Positions in the KILLER/DR5 DD—In order to functionally characterize the mutant receptors, we employed three methods to assess cell death following transfection. The first method involved cotransfection of the mutant receptor along with pCMV-βgal. Blue cells were counted after 40 h in order to determine relative cell survival as compared with vector-transfected cells (number of blue cells for vector was set as equivalent to 100% survival; Table I). To specifically measure apoptosis, we used a cotransfection based flow cytometric assay to functionally assess apoptotic signaling of each substitution mutant. Each mutant was cotransfected with an EGFP-spectrin construct at a 10:1 ratio (mutant plasmid:EGFP-spectrin plasmid) into SW480 cells for 18 h and the presence of a sub-G_1 peak was used to quantitate apoptotic signaling. The use of the spectrin-bound GFP allowed for the identification of specifically transfected cells, and the spectrin fusion permits cell membrane retention of GFP even if cells lose membrane permeability due to cell death (43). Fig. 3 shows an example of a typical experiment with GFP-positive cells in the *left column* and GFP-negative cells in the *right column*. Vector-transfected (GFP(+)) cells displayed minimal toxicity (as compared with the GFP(−) counterparts) associated with the transfection of DNA into the cells. In contrast, the transfection of wild-type KILLER/DR5 dramatically increased the sub-G_1 peak in addition to decreasing the G_2 peak as compared with the untransfected cell population (Fig. 3). One example of a signaling-competent and -incompetent mutant is also shown for comparison. Table II lists the results of three independent experiments for each mutant compared with the wild-type protein after subtracting out transfection-induced death. Finally, as a further demonstration of each mutant's ability to induce apoptosis, PARP cleavage was measured following 16 h of transfection. Fig. 4 illustrates these results with the *arrow* indicating the cleaved form of the PARP protein, indicative of apoptosis. Vector-transfected cells showed no sign of PARP cleavage at 16 h, whereas wild-type KILLER/DR5 efficiently caused PARP cleavage (Fig. 4, compare *lanes 1* and *2*).

The data from the three methods correlated well with one another (Tables I and II and Fig. 4) and yielded three groups into which the alanine mutants could be divided based on

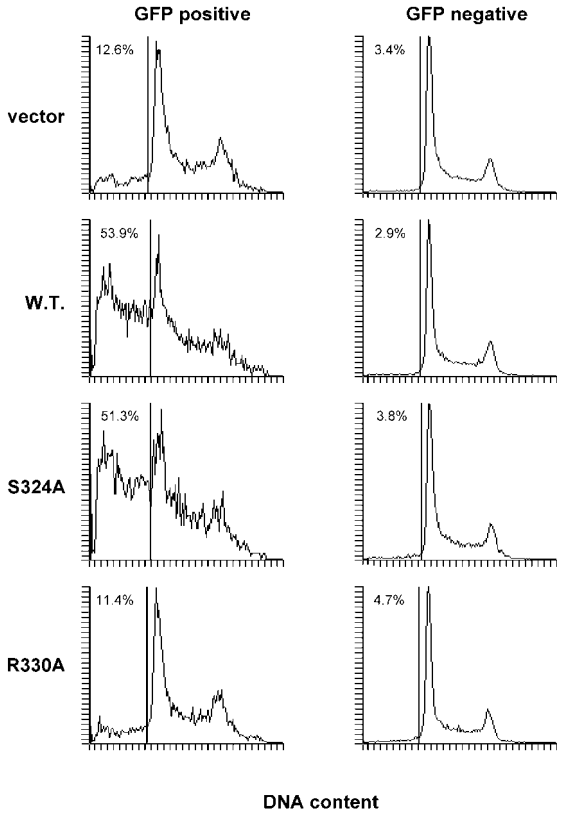

FIG. 3. **Example of flow cytometry/GFP-spectrin based assay to assess sub-G_1 peak following transient transfection of wild-type (*W.T.*) and alanine mutant KILLER/DR5 constructs.** SW480 cells were transfected with a 1:10 ratio of GFP-spectrin:mutant plasmid and harvested at 18 h for flow cytometric analysis. Specifically transfected GFP-positive cells are shown in the *left column*, and untransfected cells are shown in the *right column*. Sub-G_1 peak, indicative of apoptosis, is denoted by the percentage shown to the *left* of the *vertical line* in each graph. One example is given for vector, wild-type KILLER/DR5, signaling competent and incompetent mutant transfected to demonstrate the assay.

TABLE II
Apoptosis (sub-G_1) induced by point mutants expressed as percentage of wild type

KILLER/DR5 point mutant	Sub-G_1 peak	Apoptotic ability[a]
	%	
WT	100	WT
Loss-of-function mutations		
W325A	44 ± 8.4	−
R330A	−1 ± 1.6	−
L334A (position equivalent to Fas *lpr*)	28 ± 14.2	−
I339A	5 ± 3.4	−
W360A	−2 ± 2.5	−
Partial loss-of-function mutations		
K331A	85 ± 5.2	±
D336A	74 ± 11.1	±
E338A	83 ± 7.6	±
K340A	85 ± 12.4	±
K343A	83 ± 8.6	±
D351A	90 ± 4.1	±
L377A	73 ± 5.0	±
No loss-of-function mutations		
S324A	90 ± 12.7	WT
E326A	96 ± 10.6	WT
N362A	98 ± 10.9	WT
K386A	110 ± 7.5	WT
Q387A	110 ± 9.0	WT
K388A	101 ± 11.3	WT

[a] WT, able to induce apoptosis as well as wild-type protein; ±, 20–25% reduction in ability to induce apoptosis; −, completely deficient in ability to induce apoptosis.

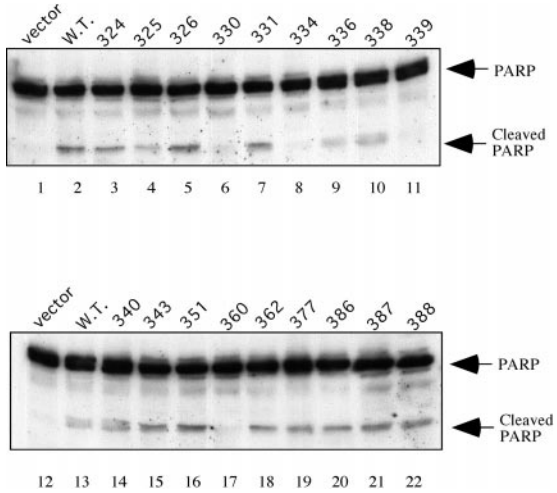

FIG. 4. **PARP cleavage following transient transfection of wild-type (*W.T.*) and alanine mutant KILLER/DR5 constructs.** Lysates from Fig. 2*A* were reprobed with an anti-PARP antibody to measure amount of PARP cleavage induced by each mutant. The *arrow* denotes the cleaved PARP product.

phenotype. The first group contained five mutants, which have a dramatically reduced ability to induce apoptosis: W325A, R330A, L334A (*lpr*-like), I339A, and W360A. The cells expressing these mutants retained ~75% blue cell staining (as compared with 8% for wild type; Table I), demonstrated at least a 75% reduction in sub-G_1 peak at 18 h in four out of the five mutants (Table II), and displayed little to no PARP-cleaving activity at 16 h (Fig. 4). This also correlated with the observation on the protein level that these five mutants had high levels of processed and unprocessed receptor that the cells were able to withstand due to its lack of toxicity (Fig. 2*A*, *lanes 4*, *6*, *8*, *11*, and *17*). Interestingly, four of these (including the *lpr*-like mutation) represent hydrophobic residues scattered throughout helices 2, 3, and 4, which when mutated probably affect overall protein structure due to disruption of the hydrophobic core. Disruption of the *lpr* position in either Fas or FADD results in an inability of the proteins to interact and in TNFR1 a loss of cytotoxicity. Of these five mutants, only R330A might play a role in potentially mediating an electrostatic death domain interaction. This charged residue, which is completely conserved in every proapoptotic TNFR family member, also proved to be critical in TNFR1, Fas, and FADD (34–36), thereby highlighting its importance in transducing an apoptotic signal.

The second class of mutants that was identified by this study demonstrated only a partial loss in cell death signaling: K331A, D336A, E338A, K340A, K343A, D351A, and L377A. These mutants, when overexpressed, displayed only a 10–25% reduction in cell death as measured by sub-G_1 (Table II) and slightly reduced amount of PARP cleavage as compared with wild-type (Fig. 4). With the exception of L377A, these partially defective mutants represent charged residues within helices 2 and 3 and the boundary of helix 4. Interestingly *in vitro* binding studies with Fas (35) and FADD (36) revealed a total loss of interaction if a mutation occurred at the positions corresponding to Lys-331, Asp-336, and Lys-340 and a partial loss at Lys-343. Therefore, these partial loss-of-function mutations may indicate residues important in mediating interactions with an adapter molecule.

The final class of mutants are those that are unaffected by alanine substitution: Ser-324, Glu-326, Asn-362, Lys-386, Gln-387, and Lys-388. They function as the wild-type protein does in all three of the aforementioned assays. In the case of position 326, the homologous residue in FADD when mutated and

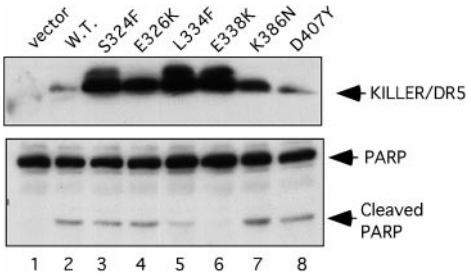

FIG. 5. **Protein expression and PARP cleavage induction by tumor-derived KILLER/DR5 mutants.** The *upper panel* displays protein expression of the mutants as assessed by anti-Myc Western blot, and the *lower panel* measures PARP cleavage induced by each mutant. *W.T.*, wild type.

tested shows an inability to interact with Fas *in vitro*. Examination of charge distribution reveals that, in the case of the TRAIL receptors, DR4 and KILLER/DR5, this residue is negatively charged whereas Fas, FADD, and TNFR1 all retain a positive charge. This residue represents a difference between the TRAIL receptors and the rest of TNFR family DDs not only in charge but also in function. The other residues, which are not affected by mutation, although illustrating examples of subtle differences between family members, are not conserved in identity or charge and to this point have not been demonstrated to be important for function in any of the receptor/adapter systems studied thus far. Meanwhile, mutation of highly conserved hydrophobic residues throughout the DD renders KILLER/DR5 nonfunctional and elimination of charged residues presumed to be the sites of putative protein interactions partially suppresses the apoptotic signal in an overexpression environment.

Some but Not All Tumor-derived KILLER/DR5 Mutants Display Loss of Apoptotic Function When Overexpressed—Due to the incidence of chromosome 8p21–22 loss in human cancers and the lack of an identified tumor suppressor gene in the area, groups have turned their attention toward identifying mutations in DR4 and KILLER/DR5, which have both been mapped to 8p21. The first report of a tumor associated mutation of KILLER/DR5 was in a head neck cancer, which resulted in a truncation of the cytoplasmic domain of the protein (38). In another study, also looking to assign significance to the KILLER/DR5 gene in chromosome 8p21–22 loss of cancers, Lee *et al.* reported alterations in the cytoplasmic domain of the protein in non-small cell lung cancers. Out of 104 samples, 11 mutations were reported including eight missense mutations. This provided us with the opportunity to compare our functional data using alanine mutagenesis to naturally occurring tumor-derived mutants. Three of the tumor mutations occurred at amino acid position 334 (L334F), which corresponds to the same residue altered in the Fas *lpr* case. Coincidentally, four of the remaining five mutations were targeted in our original alanine mutagenesis of the protein: S324F, E326K, E338K, K386N. The remaining point mutation occurred just four amino acids from the end of the protein, D407Y.

The tumor-derived mutants were generated in the same manner as the alanine mutants in order to compare alanine *versus* tumor-specific mutant with the wild-type KILLER/DR5 protein. Mutations were verified by DNA sequencing, and protein expression along with PARP cleavage was evaluated (Fig. 5 and data not shown). The most common naturally occurring mutation (L334F; 3 out of 11), which corresponds to the *lpr* position, actually demonstrated a more severe phenotype as an alanine substitution (Table III). Interestingly, the naturally occurring mutation (L334F) retains the hydrophobicity at the position while decreasing its apoptotic potential by 50%. Conversely, a mutation at a putative protein-protein interaction site (E338K) displayed a complete loss-of-function whereas the alanine mutation displayed only a partial loss-of-function phenotype (Table III). The tumor-specific mutation at position 338 changes the charge from negative to positive, explaining the dramatic loss of apoptotic capacity; however, both Fas and FADD normally have a positively charged lysine at this position. Charge differences such as this between family members may provide a clue to receptor/adapter specificity. Nevertheless, the tumor-specific mutant data provides convincing *in vivo* evidence that positions Leu-334 and Glu-338 are important for proper downstream signaling of cell death.

TABLE III
Apoptosis (sub-G_1) induced by tumor point mutant versus alanine mutant

KILLER/DR5 point mutant	Sub-G_1 peak	Apoptotic ability[a]
	%	
WT	100	WT
Loss-of-function mutations		
L334A	13 ± 9.6	−
L334F	49 ± 12.8	−*
E338A	80 ± 12.4	±
E338K	8 ± 6.2	−
No loss-of-function mutations		
S324A	96 ± 8.4	WT
S324F	80 ± 10.7	WT
E326A	107 ± 4.3	WT
E326K	106 ± 5.2	WT
K386A	105 ± 13.9	WT
K386N	112 ± 11.0	WT
D407Y	123 ± 3.2	WT

[a] WT, able to induce apoptosis as well as wild-type protein; ±, 20–25% reduction in ability to induce apoptosis; −*, 50% reduction in ability to induce apoptosis; −, completely deficient in ability to induce apoptosis.

Unexpectedly, the remaining four missense mutations (S324F, E326K, K386N, D407Y) displayed no loss of apoptotic signaling as assessed by sub-G_1 (Table III) or by PARP cleavage (Fig. 5) in the overexpression studies. Mutation of these residues in the original alanine-mutagenesis yielded no phenotype as well, strengthening the idea of a lesser role played by these sites in the overall structure and signaling of the molecule. This does not rule out the possibility that in these tumors, many of which may have 8p LOH and hence reduced gene dosage, that this mutation may have subtle effects not detectable in these types of overexpression studies.

Expression of Partial or Complete Loss-of-function Mutants Diminishes Endogenous FADD and Caspase 8 Recruitment to TRAIL DISCs—In addition to the receptor/ligand trimers, the TRAIL DISC has recently been demonstrated to contain the adapter molecule FADD and caspase 8 (23–25) in order to propagate the apoptotic signal. Due to an inability to demonstrate a direct interaction between FADD and KILLER/DR5 *in vitro*, we attempted to recapitulate this interaction *in vivo* in the context of the DISC and to determine the effect of exogenous partial and loss-of-function KILLER/DR5 mutants on FADD and caspase 8 recruitment. 293 HEK cells were chosen due to their ability to be transfected and relative resistance to TRAIL-induced apoptosis. Following transfection of the wild-type or mutant KILLER/DR5 plasmid, the cells were harvested 16 h later and treated with His-tagged TRAIL and a cross-linking anti-6-histidine antibody for 15 min at 37 °C. The DISC was then immunoprecipitated and analyzed for the presence of the exogenous KILLER/DR5 receptor along with FADD and caspase 8. As demonstrated in Fig. 6*A*, TRAIL treatment led to the recruitment of the exogenous KILLER/DR5 receptor into DISCs as visualized with an anti-myc antibody. Total caspase 8 was provided as a loading control to ensure equivalent

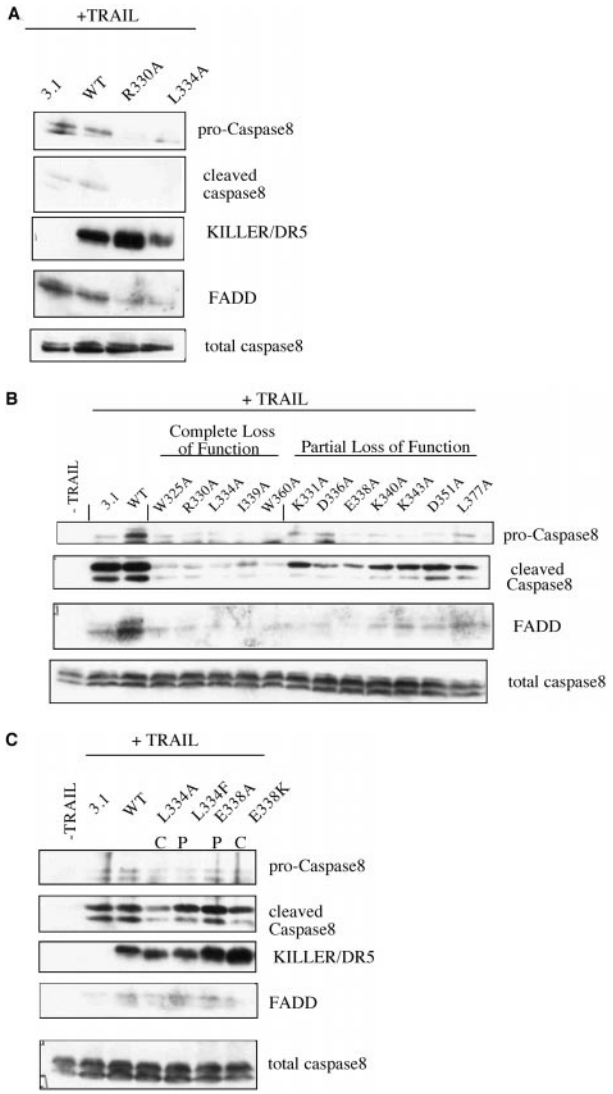

FIG. 6. *A*, TRAIL DISC IP of transfected 293 cells. A T75 (80% confluent) of 293 HEK cells was transfected for 16 h with the indicated wild-type (*WT*) or mutant KILLER/DR5 plasmid followed by a 15-min TRAIL treatment (50 ng/ml) at 37 °C. The DISC immunoprecipitation was carried out overnight at 4 °C followed by SDS-polyacrylamide gel electrophoresis and Western blot analysis for DISC-associated caspase 8, FADD, and exogenous KILLER/DR5. Total caspase 8 is provided as a loading control to ensure equal amounts of protein were used for TRAIL treatment and DISC analysis. *B*, TRAIL DISC IP of panel of total and partial loss-of-function alanine mutants. The DISC IPs were carried out as specified in *A*. The (−) TRAIL lane includes the anti-His_6 antibody and protein A/G-agarose beads as a control for the specificity of the DISC IP. *C*, TRAIL DISC IP of the alanine *versus* tumor loss-of-function mutants. IPs were carried out as detailed above. *P* indicates partial loss-of-function, and *C* indicates complete loss-of-function mutants.

amounts of proteins were treated and processed for DISC analysis. FADD and caspase 8 (both pro- and the cleaved p46 form) were detected in the DISC of vector- and wild-type receptor-transfected 293 cells; however, introduction of either complete loss-of-function receptor, R330A or L334A, led to a dramatic decrease in both FADD and caspase 8 recruitment. This observation helps to explain the lack of apoptosis induction by these mutants in earlier cell death assays (Tables I and II and Fig. 4).

These observations were extended to the panel of complete and partial loss-of-function alanine mutations (Fig. 6*B*) as well as the tumor mutants (Fig. 6*C*). Absence of TRAIL treatment did not result in DISC formation, but TRAIL treatment of vector or wild-type transfected cells showed a robust recruitment of both FADD and caspase 8.

Examination of the amount of cleaved caspase 8 in Fig. 6*B* clearly correlated with the predicted apoptotic potential of each receptor class. The partial loss-of-function mutants showed a decrease in both cleaved caspase 8 and FADD as compared with wild-type. The complete loss-of-function alanine mutants have an even more severe impairment in FADD and caspase 8 recruitment. Finally, in Fig. 6*C*, a similar correlation between DISC components and receptor function was observed. The complete loss-of-function mutation, L334A, showed a decrease in FADD and caspase 8 (pro- and cleaved) as compared with wild-type; however, the corresponding tumor mutation, L334F, exhibited increased binding of both FADD and caspase 8 in accordance with its classification as a partial loss-of-function mutant. Likewise, the partial loss-of-function mutant, E338A, recruited more FADD and caspase 8 than its complete loss-of-function tumor counterpart, E338K. The correlation between FADD/caspase 8 recruitment to TRAIL DISCs and the cell death data for the complete and partial loss-of-function mutants supports the importance of these residues uncovered in the earlier overexpression studies.

DISCUSSION

Mutagenesis of the death domain of KILLER/DR5 revealed hydrophobic residues possibly important for overall protein structure as well as charged residues which may potentially interact with downstream effector molecules. The hydrophobic residues tested (Trp-325, Leu-334, Ile-339, Trp-360, and Leu-377) cause a partial (Leu-377) or complete loss-of-function when mutated to alanine. The complementary residues of Leu-334 and Trp-360 within Fas were shown via crystal structure analysis to comprise part of the hydrophobic core of the death domain (35). Additional studies suggested that disruption of the Leu-334 Fas complementary residue (*lpr)* caused a disruption of the protein structure (35). Future crystal structure studies may determine whether these five hydrophobic residues are truly buried within the protein. Charged residues implicated in mediating electrostatic interactions include Arg-330 (complete loss), Lys-331, Asp-336, Glu-338, Lys-340, Lys-343, and Asp-351. These residues (excluding Glu-338 and Asp-351) were also demonstrated to be important for Fas or FADD self-aggregation as well as protein partner binding (35, 36), signifying their importance in death domain signaling in general. It is possible that mutagenesis of charged residues led only to partial loss-of-function because multiple residual charges were still able to stabilize protein-protein interactions.

Careful attention to charge distribution within helices 2 and 3 of the receptors may begin to explain differences in receptor/adapter specificity. Residue Glu-326 showed a complete loss in Fas/FADD interaction (36), yet no apparent defect was observed when mutated to alanine in KILLER/DR5. It is interesting to note the difference in this case because the charge of this particular position varies within the superfamily of receptors. Only the TRAIL receptors, DR4 and KILLER/DR5, have a negatively charged amino acid at this position which may begin to illustrate differences between TRAIL and other TNFR family receptors. Position 336, also shown to be important in this study, retains a negative charge in all family members except DR4. Future mutational analysis of DR4 will determine whether this residue plays a role in adapter binding by DR4. Lastly, position 338, which has a partial defect in KILLER/DR5-mediated apoptosis, remains untested in the Fas or TNFR1 system; however, inspection of the charge at this position among family members reveals that Fas is the only receptor with a positive charge. Fas is also the only member of the family demonstrated to be able to *directly* bind to the proapoptotic adapter molecule FADD. Although FADD is present in the DISC of other receptors such as TNFR1, DR3, and KILLER/

DR5 (10, 23–25), a direct interaction between these molecules and FADD has not been demonstrated and, in the case of TNFR1, the adapter molecule TRADD is required.

In order to more specifically examine adapter binding to loss-of-function KILLER/DR5 mutants, we utilized a TRAIL DISC immunoprecipitation strategy in which various mutants were introduced and the relative levels of FADD and caspase 8 were assessed. The DD mutants were actively recruited into TRAIL DISCs after TRAIL treatment, signifying that cytoplasmic DD loss-of-function mutations had no effect on ligand binding. The defects, however, could be explained by a decrease in both FADD and caspase 8 recruitment into TRAIL DISCs. As expected, the amount of caspase 8 and FADD recruitment directly correlated with the amount of apoptosis induction, as assessed by the cell death assays. These experiments illustrate the concept that one mutant TRAIL receptor (*i.e.* DR4 or KILLER/DR5) can potentially disrupt cell death signaling through the formation of defective TRAIL DISCs.

The identification of DD tumor point mutants (39) allowed us to functionally characterize their apoptotic capability and FADD/caspase 8 recruitment in addition to comparing their phenotype to the corresponding alanine substitution. The most prevalent mutation occurred in 3 out of 11 samples (L334F) corresponding to the naturally occurring Fas *lpr* mutation, demonstrating the conserved importance of this residue in two receptor systems. The alanine substitution yielded a mutant receptor with a greater reduction in apoptotic potential as well as FADD and caspase 8 recruitment as compared with the tumor-derived mutant (L334F). This divergence in phenotype may be explained by the maintenance of a hydrophobic residue in the tumor-specific mutation. Nevertheless, its prevalence in non-small cell lung cancer points to its importance in maintaining proper protein folding and death signaling. In contrast, the drastic charge change of the tumor mutant E338K resulted in a complete loss-of-death induction by the receptor, whereas the alanine mutant only partially disrupted cell death signaling. The severity of the tumor-specific defect E338K points to an overall disruption of the electrostatic interactions maintained by the residues in helices 2 and 3.

The loss-of-function of these two tumor-specific alleles (L334F, E338K) is not surprising based on the alanine studies; however, the other tumor-associated mutations (S324F, E326K, K386N, D407Y) would not be predicted to affect receptor function. When these mutants were generated and tested, no phenotype was observed, supporting the observations made from the alanine mutagenesis. Physiological levels of these mutant receptors may affect their stability or in the context of ligand binding may demonstrate defective signaling, but these preliminary overexpression studies coupled with the lack of conservation of these residues among family members argue against their importance in KILLER/DR5 structure/function.

In conclusion, our mutational study of the death domain of KILLER/DR5 identified conserved hydrophobic residues and charged amino acids, which are crucial for normal cell death signaling downstream of the receptor. The clustering of specific charged residues critical for apoptosis that are proposed to be in helices 2 and 3 correlates with data from other death domain-containing proteins (Fas, FADD) that these helices are critical for protein/protein interactions. These mutants may be useful in order to better understand the molecular mechanisms behind receptor/adapter specificity.

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