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Death Receptor 5, a New Member of the TNFR Family, and DR4 Induce FADD-Dependent Apoptosis and Activate the NF-κB Pathway

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Summary

Death receptor 4 (DR4) is a recently described receptor for the cytotoxic ligand TRAIL that reportedly uses a FADD-independent pathway to induce apoptosis and does not activate the NF-κB pathway. We have isolated a new member of the tumor necrosis factor receptor (TNFR) family, designated DR5, which bears a high degree of sequence homology to DR4. However, contrary to the previous reports, both DR4- and DR5-induced apoptosis can be blocked by dominant-negative FADD, and both receptors can activate NF-κB using a TRADD-dependent pathway. Finally, both receptors can interact with FADD, TRADD, and RIP. Thus, both DR5 and DR4 use FADD, TRADD, and RIP in their signal transduction pathways, and FADD is the common mediator of apoptosis by all known death domain–containing receptors.

Introduction

The tumor necrosis factor receptor (TNFR) family of proteins plays an important role in the mediation of apoptosis or programmed cell death in diverse biological systems (Smith et al., 1994; Gruss and Dower, 1995; Baker and Reddy, 1996). These type 1 membrane proteins share significant sequence homology in their extracellular domains, owing to the presence of highly conserved cysteine residues in the cysteine-rich pseudorepeats, a hallmark of this family (Smith et al., 1994; Gruss and Dower, 1995; Baker and Reddy, 1996). In addition, three members of this family, TNFR1, Fas/Apo-1 (CD95), and death receptor 3 (DR3) (also called Wsl-1, Apo-3, and TRAMP), possess a conserved domain of approximately 80 amino acids near their C terminal called the death domain, which is required for induction of apoptosis by these receptors (Itoh and Nagata, 1993; Tartaglia et al., 1993; Cleveland and Ihle, 1995; Chinnaiyan et al., 1996a; Kitson et al., 1996; Marsters et al., 1996; Bodmer et al., 1997).

Death domains are also found in Fas-associating protein with death domain (FADD) (or MORT1), TNFR1-associated death domain (TRADD), and receptor-interacting protein (RIP), three cytoplasmic adapter proteins implicated in the mediation of apoptosis by the death domain–containing receptors (Boldin et al., 1995; Chinnaiyan et al., 1995; Hsu et al., 1995, 1996a; Stanger et al., 1995). FADD is an adapter molecule that possesses a C-terminal death domain through which it binds to the death domain of Fas/Apo-1 (Boldin et al., 1995; Chinnaiyan et al., 1995). Despite its sequence homology to the similar domains present in the death domain–containing receptors, the death domain of FADD cannot induce apoptosis when overexpressed in mammalian cells and, in fact, can block the apoptosis mediated by Fas and TNFR1 in a dominant-negative fashion (Chinnaiyan et al., 1995; Hsu et al., 1996b). FADD possesses another domain called the death effector domain at its N terminus, which can induce apoptosis when overexpressed in mammalian cells (Chinnaiyan et al., 1995; Hsu et al., 1996b). Through its death effector domain, FADD binds to the proapoptotic apical caspase, Caspase 8 (also called FLICE, MACH, or Mch5) (Boldin et al., 1996; Fernandes-Alnemri et al., 1996; Muzio et al., 1996). Caspase 8 is the most proximal caspase in the cascade of caspase, activation of which eventually leads to cell death (Boldin et al., 1996; Fernandes-Alnemri et al., 1996; Muzio et al., 1996). While Fas/Apo-1 can bind directly to FADD, it is generally believed that TNFR1 and DR3 bind to FADD via the intermediate death domain–containing adapter molecule TRADD (Chinnaiyan et al., 1996a, 1996b; Hsu et al., 1996b; Kitson et al., 1996). Thus, FADD is the final common link between the death domain–containing receptors Fas/Apo-1, TNFR1 and DR3, and Caspase 8 (Boldin et al., 1995; Chinnaiyan et al., 1995, 1996a, 1996b; Hsu et al., 1996b; Kitson et al., 1996).

In addition to recruiting FADD, TRADD recruits two additional molecules to the aggregated receptor complex of TNFR1 or DR3: the death domain–containing protein RIP and TRAF2, which lacks a death domain (Hsu et al., 1995, 1996a, 1996b). While recruitment of FADD leads to activation of caspases and eventual cell death, recruitment of RIP and TRAF2 leads to the activation of the NF-κB pathway, which may protect cells from TNF-induced apoptosis (Beg and Baltimore, 1996; Hsu et al., 1996a, 1996b; Liu et al., 1996; Van Antwerp et al., 1996; Wang et al., 1996).

DR4 is the most recently described death domain–containing receptor and was shown to be the receptor for the cytotoxic ligand TRAIL (Apo-2 ligand) (Pan et al., 1997a). It was reported that DR4-induced apoptosis cannot be blocked by dominant-negative FADD, and DR4 cannot coimmunoprecipitate FADD, TRADD, or RIP (Pan et al., 1997a). These results are in agreement with a previous report of the inability of dominant-negative FADD to block TRAIL-induced apoptosis (Marsters et al., 1996) and led to the conclusion that DR4 is unique among the death domain–containing receptors in using a FADD-independent pathway to activate caspases (Pan et al., 1997a). In the same study it was reported that DR4 is incapable of activating the NF-κB pathway (Pan et al., 1997a).

During the course of investigating a new death domain–containing receptor designated DR5, we discovered that while it resembled DR4 in overall structure, it resembled TNFR1 and DR3 in using FADD and NF-κB as mediators of its signal transduction pathway. This

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led to a comparative analysis of the signal transduction pathways utilized by DR5, DR4, and DR3, which is the focus of the present study.

Results

Molecular Cloning and Sequence Analysis of DR5

Two human expressed sequence tag (EST) clones (IMAGE Consortium clones 650744 and 664665) were found to have statistically significant homology to the extracellular domain of human TNFR1. To obtain the full-length clone, 5′ and 3′ rapid amplification of cDNA ends (RACE) was used. The full-length clone was designated DR5 and encoded a protein of 411 amino acids with characteristics of a cell-surface receptor, including a signal peptide of 51 amino acids at the beginning and a transmembrane region of 23 amino acids (residues 183–205) present in the middle (Figure 1A). The initiating methionine was surrounded by the sequence CCGCCATGG, which is highly homologous to the consensus Kozak sequence CCA/GCCATGG (1996). An in-frame stop codon present 10 amino acids upstream of this methionine established it as the true start site (data not shown). The extracellular domain contained cysteine-rich pseudorepeats, a hallmark of the TNFR family, and had the highest degree of homology to DR4 (58% identity and 70% similarity) (Figure 1A). The extracellular domain also shared weaker homology with TNFR1 (27% identity and 46% similarity), Fas (25% identity and 45% similarity), DR3 (21% identity and 40% similarity), and other members of the TNFR family. DR5 has a cytoplasmic tail of 212 amino acids, with a death domain near its C terminus possessing significant sequence homology to the death domains of DR4, TNFR1, DR3, and Fas (Figure 1B). In addition, several amino acids, which have been shown to be essential for death signaling by TNFR1 and Fas/Apo-1 (Itoh and Nagata, 1993; Tartaglia et al., 1993), are also conserved in the death domain of DR5 (Figure 1B). This includes amino acid L334, corresponding to the site of *lpr* mutation in the mouse Fas receptor (Figure 1B) (Watanabe-Fukunaga et al., 1992).

Alignment of the protein sequences of DR5 and DR4 revealed that methionine at position 59 might represent the true start site for DR4 (Figure 1A). Similarly, the true signal peptide for DR4 might lie between amino acid residues 59-109 (Figure 1A). This conclusion was supported by the hydrophobicity plots, which revealed a highly hydrophobic segment between amino acids 32-49 and 86-104 for DR5 and DR4, respectively (Figure 1C).

Expression of DR5

Tissue distribution of DR5 revealed the presence of a major transcript of approximately 4.4 kb, present in all tissues tested, including spleen, thymus, prostate, testis, ovary, small intestine, colon mucosa, and peripheral blood leukocytes (Figure 2).

Overexpression of DR5 Induces Apoptosis in Mammalian Cells

Overexpression of death domain–containing receptors can induce apoptosis in a ligand-independent manner (Boldin et al., 1995; Chinnaiyan et al., 1996a). Therefore, we tested the ability of DR5 to induce apoptosis in MCF7, a human breast carcinoma cell line; 293T, a subclone of human embryonic kidney cells; and BHK, a baby hamster kidney cell line. Transient transfection of a full-length DR5 construct induced rapid apoptosis in all three cell lines tested (Figure 3A and data not shown). Two C-terminal deletion mutants of DR5, lacking either the cytoplasmic tail (DR5ΔCP) or the death domain (DR5ΔDD), failed to induce apoptosis, indicating that the death domain is essential for transmitting the death signal (Figure 3A). A mutant construct, containing a leucine-to-asparagine substitution at amino acid 334 (DR5-L334N), also failed to induce apoptosis (Figure 3A). Thus, like other death domain–containing receptors, amino acid L334, which corresponds to the site of *lpr* mutation, is essential for signaling apoptosis by DR5 (Watanabe-Fukunaga et al., 1992; Itoh and Nagata, 1993; Tartaglia et al., 1993; Kitson et al., 1996).

Dominant-Negative FADD Can Block Apoptosis by DR5 and DR4

Mediation of apoptosis by the death domain–containing receptors TNFR1, Fas, and DR3 involves recruitment of the adapter molecule FADD/MORT1 either directly or via an intermediate adapter molecule TRADD (Boldin et al., 1995; Chinnaiyan et al., 1995, 1996a, 1996b; Hsu et al., 1995, 1996b). Two recent studies, however, suggest that DR4 uses a unique and distinct FADD-independent pathway to transmit its death signal (Marsters et al., 1996; Pan et al., 1997a). This conclusion is based on the inability of a dominant-negative mutant of FADD (DN-FADD) to block apoptosis mediated by DR4 effectively and the inability of DR4 to coimmunoprecipitate FADD or TRADD (Pan et al., 1997a). As DR5 bears a high degree of sequence homology to DR4 in its cytoplasmic tail, including the death domain, we had expected it also to act independently of FADD. Surprisingly, DN-FADD (amino acids 80-208) was able to partially block apoptosis mediated by DR5 in MCF7 cells (Figure 3B). To resolve the discrepancy between our study and the published reports, we tested the ability of increasing amounts of DN-FADD to block apoptosis mediated by DR3, DR4, and DR5 in 293T cells. DR3 possesses a death domain highly homologous to TNFR1, and like TNFR1, its apoptosis can be blocked by DN-FADD (Chinnaiyan et al., 1996a). Increasing amounts of DN-FADD led to progressive inhibition of apoptosis by all three receptors, with almost complete inhibition of apoptosis seen at a receptor to DN-FADD ratio of 1:10 in all the three cases (Figures 3C, 3D, and 3E). These results indicate that contrary to the previous reports, DR4 and DR5 also mediate apoptosis by a FADD-dependent pathway. Hence, all known death domain–containing receptors use FADD as the final common link with the downstream apoptotic caspases.

DR5 and DR4 Induce Apoptosis by a Caspase-Dependent Mechanism

The next step in the mediation of death signal by Fas, TNFR1, and DR3 is the recruitment of the interleukin-1β-converting enzyme- like protease Caspase 8 (FLICE/MACH1) by FADD to the aggregated death receptor

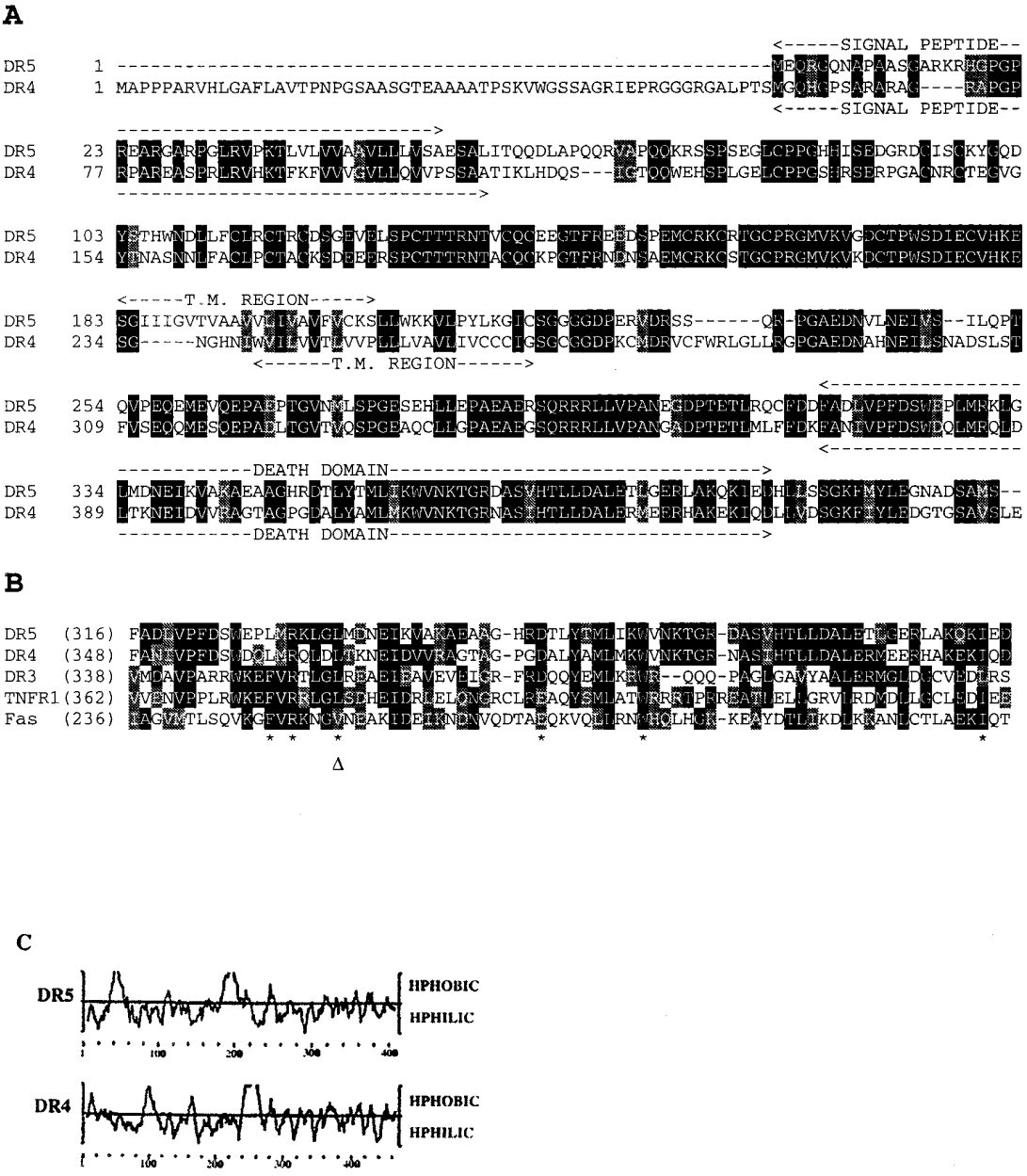

Figure 1. The Nucleotide and Predicted Amino Acid Sequences of DR5 and Sequence Analysis

(A) The predicted amino acid sequence of DR5 is aligned with the sequence of DR4. Black; identical amino acids; gray, homologous residues. The positions of the predicted signal peptides, transmembrane regions, and the death domains are shown above and below the sequences of DR5 and DR4, respectively. Unlike the report by Pan et al. (1997a), the start site is numbered as amino acid 1.

(B) Sequence alignment of the death domains of DR5, DR4, DR3, TNFR1, and Fas/Apo-1. Black, residues identical in more than 30% of sequences; gray, those homologous in more than 30% of sequences. Asterick, residues essential for the mediation of apoptosis by the human TNFR1. Δ, residues corresponding to the site of the *lpr* mutation in the mouse Fas receptor. The residues are numbered with the start site representing the first amino acid.

(C) Hydrophobicity plots of DR5 and DR4. Numbers refer to the position of the amino acid residues. HPhobic, hydorphobic; Hphilic, hydrophilic.

complex (Boldin et al., 1996; Chinnaiyan et al., 1996a; Muzio et al., 1996). To test whether DR5-induced apoptosis also involves recruitment of FLICE (MACH1), we tested the ability of a dominant-negative mutant of FLICE (Boldin et al., 1996) containing a cysteine-to-serine substitution at the catalytic active site (FLICE-C360S mutant) to block apoptosis mediated by DR5. As shown in Figures 3B and 3F, the FLICE-C360S mutant was able to block apoptosis mediated by DR5, DR4, and DR3 effectively. Finally, z-VAD-fmk and CrmA, two inhibitors of proximal caspases, also blocked apoptosis mediated by DR5 in MCF7 cells (Figure 3B) (Tewari and Dixit, 1995; Muzio et al., 1996). Thus, DR5 and DR4 resemble other death domain–containing receptors in sharing the same

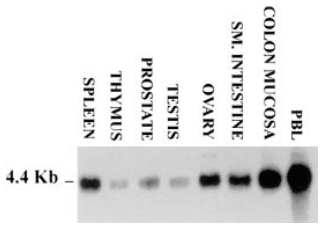

Figure 2. Northern Blot Analysis of DR5 mRNA in Human Tissues

Human multiple tissue Northern blots (Clontech) were probed with a DR5 encoding cDNA probe. PBL, peripheral blood leukocytes; Sm. Intestine, small intestine.

proximal and distal signaling molecules for transmitting the death signal.

Both DR5 and DR4 Activate NF-κB

Activation of TNFR1 and DR3 has been shown to induce NF-κB activation, whereas DR4 has been shown to lack this activity (Tartaglia and Goeddel, 1992; Chinnaiyan et al., 1996a; Kitson et al., 1996; Marsters et al., 1996; Bodmer et al., 1997; Pan et al., 1997a). To test whether DR5 can induce NF-κB, we cotransfected different receptor constructs with a NF-κB-luciferase reporter plasmid and a *lacZ* expression plasmid into 293T cells. Overexpression of DR5 led to significant activation of NF-κB in 293T cells, whereas the two C-terminal deletion mutants, DR5ΔCP and DR5ΔDD, as well as the L334N point mutant, failed to do so (Figure 4A). Therefore, the death domain of DR5 is essential for both induction of apoptosis and NF-κB activation. Based on the sequence homology to DR4, we did not expect DR5 to induce NF-κB activation. Our result led us to reexamine the ability of DR4 to induce NF-κB. Contrary to the previous report (Pan et al., 1997a), DR4 effectively activated NF-κB in 293T cells (Figure 4A). Thus, DR5 and DR4 resemble TNFR1 and DR3 in their ability to activate NF-κB.

We also tested the ability of DR5, DR4, and DR3 to induce NF-κB activation in MCF7 cells (Figure 4B). While DR3 could effectively activate NF-κB in these cells, DR5 and DR4 could do so only slightly. However, in the presence of CrmA, both DR5 and DR4 could effectively activate NF-κB, an effect comparable to that seen with DR3. These results suggest that the relatively poor ability of DR4 and DR5 to induce NF-κB in these cells is secondary to their rapid induction of apoptosis, and they also indicate that the ability of these receptors to activate NF-κB is independent of their ability to induce apoptosis.

Dominant-Negative TRADD Can Block NF-κB Activation by DR5, DR4, and DR3

As demonstrated above, the death domain of DR5 is responsible for the mediation of apoptosis as well as NF-κB activation. Previous studies have shown that the death domains of TNFR1 and DR3 are similarly responsible for both these activities, and these results can be explained mechanistically by the ability of their death domains to recruit TRADD (Tartaglia et al., 1993; Kitson et al., 1996). TRADD has the ability to activate the apoptotic pathway through FADD or the NF-κB pathway by binding to RIP and TRAF2 (Hsu et al., 1996a, 1996b). However, no physical interaction could be demonstrated between DR4 and either TRADD or RIP (Pan et al., 1997a); based on the sequence homology between the death domains of DR4 and DR5, DR5 was expected to behave in a similar fashion. To resolve this discrepancy, we decided to reexamine the possibility that TRADD may be the adapter that binds to the death domains of DR5 and DR4 and mediates apoptosis and NF-κB activation. We started by testing the ability of an N-terminal deletion mutant of TRADD (ND-TRADD) to block NF-κB activation by DR5, DR4, and DR3. This deletion construct lacks the N-terminal 102 amino acids and thus does not possess a complete TRAF2-binding domain, but it does possess a complete RIP/FADD-binding death domain (Hsu et al., 1996a, 1996b). A similar N-terminal deletion construct of TRADD (residues 25–312) was previously shown to block TNFα-induced NF-κB activation in the 293 cells (Park and Baichwal, 1996). As shown in Figure 4C, ND-TRADD could block NF-κB activation effectively by all three death domain receptors. These results indicate that DR5 and DR4 resemble DR3 and TNFR1 in using a TRADD-dependent pathway to activate NF-κB.

DR5 and DR4 Interact with FADD, TRADD, and RIP in Coimmunoprecipitation Assays

To confirm the physical interactions of DR5 and DR4 with the adapter molecules FADD, TRADD, and RIP, coimmunoprecipitation experiments were carried out. Both DR5 and DR4 could coimmunoprecipitate AU1-FADD to the same extent as DR3 and Fas (Figure 5A). Coexpression of ND-TRADD did not lead to any further increase in the binding of FADD to any of the receptors (data not shown). These results suggest that either DR4 and DR5 can bind to FADD independent of TRADD or that the observed effect is due to the presence of endogenous TRADD, which is not limiting in amount. Both DR4 and DR5 could also coimmunoprecipitate HA-tagged ND-TRADD and RIP (Figures 5B, 5C and 5D). These results suggest that both DR4 and DR5 resemble TNFR1 and DR3 in involving FADD, TRADD, and RIP in their signal-transduction pathway and that FADD is the common mediator of apoptosis by all known death domain–containing receptors.

Discussion

DR5 is the fifth death domain–containing member of the TNFR family and is highly homologous to the TRAIL receptor, DR4. Like other members of this family, the extracellular domain of DR5 contains cysteine-rich pseudorepeats that play a crucial role in binding to cognate ligand (Smith et al., 1994). The extracellular domain of DR5 is most homologous to DR4 (58% identity; 70% similarity) (Pan et al., 1997a). This high degree of homology extends to the cytoplasmic tail and the death domain as well, suggesting that DR5 and DR4 are more closely related evolutionarily to each other than to the other members of this family and may represent a new subfamily among the TNFR family members.

DR5 possesses a highly conserved death domain in

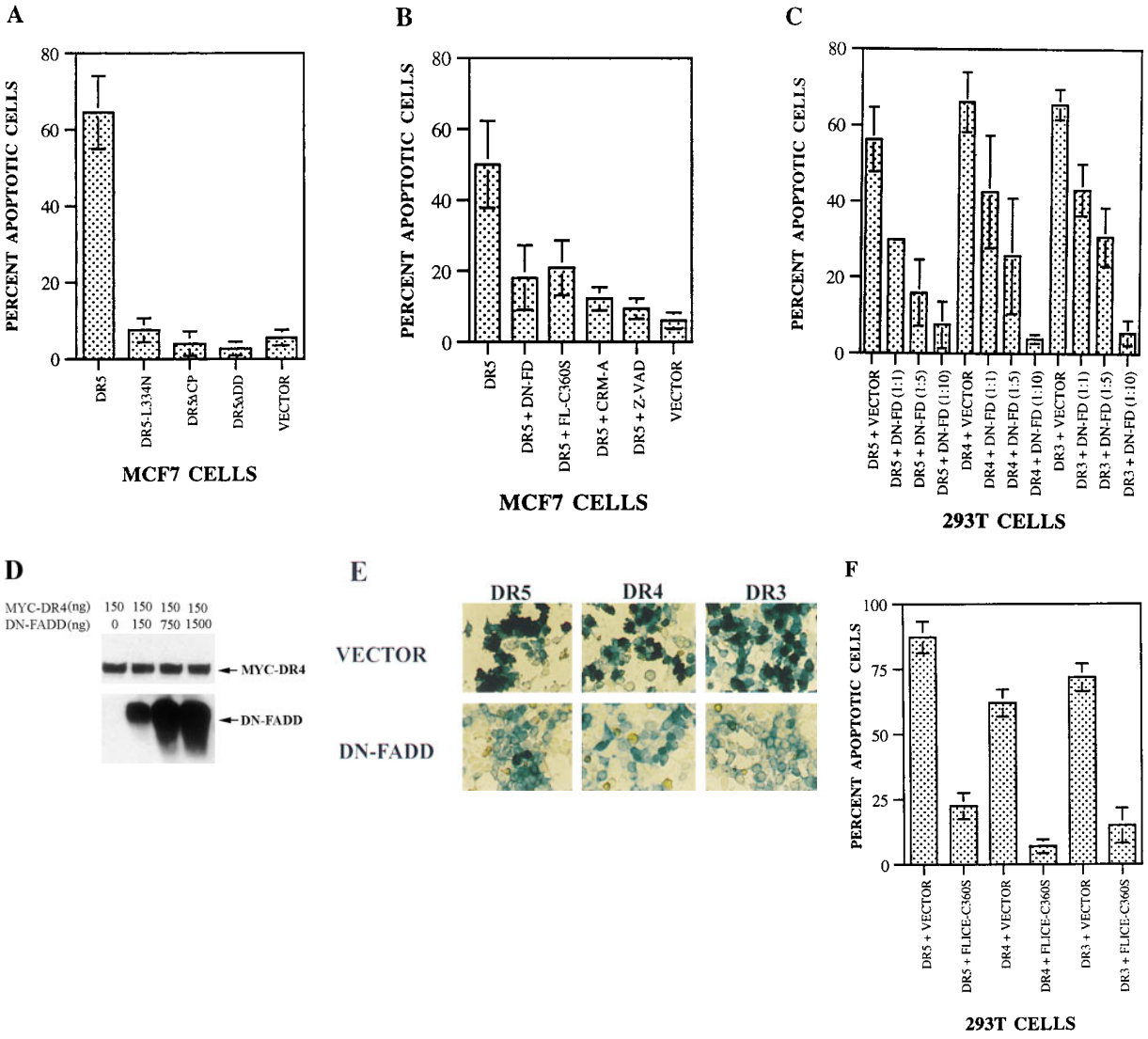

Figure 3. Induction of Apoptosis by DR5 Overexpression

(A) Induction of apoptosis in MCF7 cells by overexpression of either the full-length DR5 construct or various deletion or point mutant constructs. The data (mean ± standard deviation) are the percentage of round blue cells as a function of the total number of blue cells counted. A representative of three independent experiments performed in duplicate.

(B) Inhibition of apoptosis in MCF7 cells by dominant-negative FADD (DN-FD), dominant-negative FLICE/MACHα1 (FL-C360S), CrmA, and z-VAD-fmk (20 μM). A representative of three independent experiments performed in duplicate.

(C) Inhibition of DR5-, DR4-, and DR3-induced apoptosis by progressive, increasing amounts of DN-FADD (DN-FD) in 293T cells. Equal amounts (150 ng) of each receptor were transfected with 150 ng, 750 ng, or 1500 ng of DN-FADD. The total amount of DNA in each transfection was kept constant by adding vector plasmid DNA. A representative of two independent experiments performed in duplicate.

(D) Expression of DN-FADD and Myc-DR4 in 293T cells. Western analysis of 293T cells demonstrating the relative level of expression of the Myc-DR4 construct and progressive, increasing amounts of the DN-FADD construct using Myc and FADD MAbs, respectively.

(E) Phase-contrast photomicrograph of 293T cells transfected with either expression constructs containing DR5, DR4, or DR3 and pcDNA3 vector plasmid or DN-FADD. Receptor to DN-FADD (or vector control) ratio, 1:10. Cells were stained with X-Gal, which stains the transfected cells blue. Apoptotic cells have dark rounded appearance, show membrane blebs, and are lifting off from the plate. Original magnification, 400×.

(F) Inhibition of DR5, DR4, or DR3 induced apoptosis by dominant-negative FLICE (FLICE-C360S) in 293T cells. Receptors to FLICE-C360S ratio, 1:5. A representative of five experiments performed in duplicate.

its cytoplasmic tail, and mutagenesis studies revealed that the death domain is indeed responsible for the induction of apoptosis. Like other members of this family, a leucine-to-asparagine point mutation at the site of the *lpr* mutation in the mouse Fas receptor effectively abolished the ability of DR5 to induce apoptosis (Watanabe-Fukunaga et al., 1992; Itoh and Nagata, 1993; Tartaglia et al., 1993; Kitson et al., 1996). Several recent

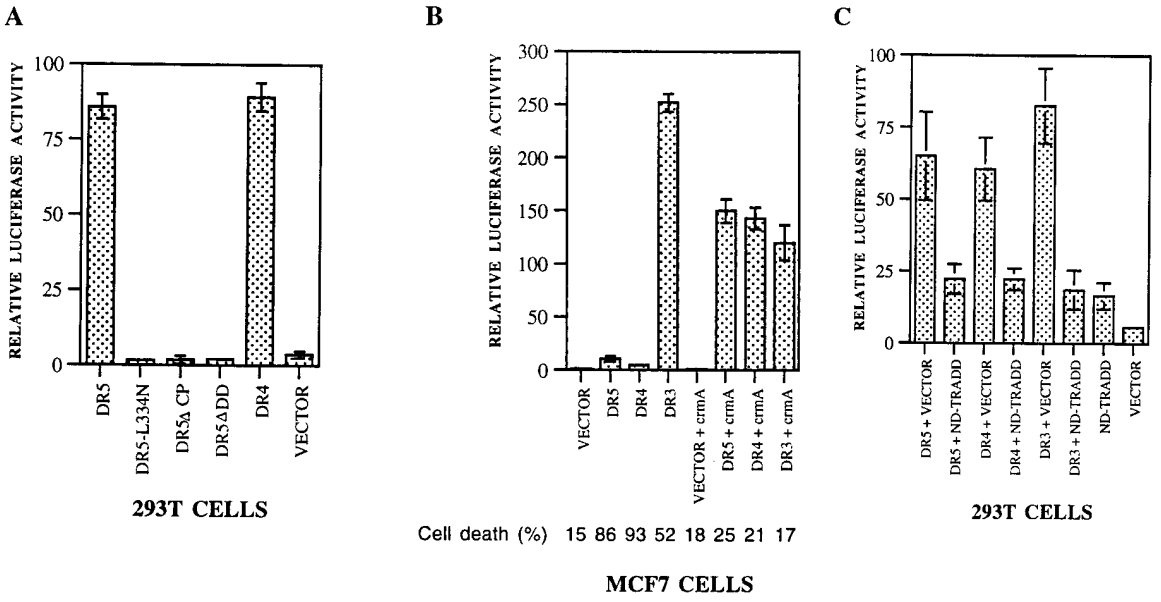

Figure 4. DR5 and DR4 Activate NF-κB by a TRADD-Dependent Pathway

(A) Activation of NF-κB by DR5 and DR4. 293T cells were transfected in duplicate with the indicated receptor constructs (0.5 μg) along with the NF-κB reporter construct (0.5 μg) (Berberich et al., 1994) and a *lacZ* reporter construct (0.2 μg) in a 24-well plate. Eighteen hours later, luciferase activity was measured from one of the wells using the luciferase assay reagent (Promega) and following the manufacturer's instructions. The cells in the other well were fixed with gluteraldehyde and stained with X-Gal to obtain the relative transfection efficiency. Results are from three independent representative experiments (mean ± standard deviation).

(B) Activation of NF-κB in the MCF7 cells. MCF7 cells were transfected in quadruplicate with the indicated receptor constructs (0.25 μg) along with the NF-κB reporter construct (0.25 μg) (Berberich et al., 1994) and a *lacZ* reporter construct (0.2 μg) either in the absence or presence of a crmA expression plasmid (0.25 μg). Twenty-four hours later, luciferase activity was measured from three wells as described above. The cells in the fourth well were fixed with gluteraldehyde and stained with X-Gal to obtain the relative transfection efficiency and to confirm the inhibition of cell death by CrmA. Results are from one of the two independent representative experiments (mean ± standard deviation).

(C) (ND-TRADD) an N-terminal deletion mutant of TRADD (0.75 μg) blocks NF-κB activation by DR5, DR4, and DR3 (0.15 μg each). Receptor to ND-TRADD ratio was 1:5. The total amount of plasmid was kept constant by adding empty vector DNA. Results (mean ± standard deviation) are from at least four independent experiments. Transfection and luciferase assay were performed as in Figure 4A.

studies have reported the occurrence of autoimmune disorders caused by point mutations in the death domain of the human Fas receptor that are capable of blocking the function of the wild-type receptor in a dominant-negative fashion (Fisher et al., 1995; Drappa et al., 1996; Sneller et al., 1997). Future studies should test whether point mutations in the death domain of DR5, similar to the L334N mutant generated in this study, occur naturally in the human population and whether they play a role in the pathogenesis of autoimmune disorders.

The high degree of sequence homology between the death domains of DR5 and DR4 initially led us to believe that DR5 mediates apoptosis by a FADD-independent pathway as well. One unexpected result of our study was the ability of DN-FADD to block apoptosis mediated by DR5 and DR4. While this manuscript was under preliminary review, three additional studies were published describing the signal transduction pathway utilized by DR4 or DR5 (Pan et al., 1997b; Sheridan et al., 1997; Walczak et al., 1997). Whereas two of the studies suggest that DR4 and DR5 mediate apoptosis by a FADD-independent pathway (Pan et al., 1997b; Sheridan et al., 1997), the third study agrees with our conclusion (Walczak et al., 1997). The discrepancy among the different studies may be explained by the choice of the cell lines and the relative level of expression of the transfected genes. The ability of DN-FADD to block apoptosis mediated by death domain receptors will depend on the level of expression of the endogenous FADD relative to the level of expression of the transfected DN-FADD construct. Therefore, different cell lines, depending on their level of expression of endogenous FADD and the relative transfection efficiency for the DN-FADD construct, will have a quantitative difference in the ability of DN-FADD to block apoptosis.

We conducted our initial experiments with DN-FADD in MCF7 cells and observed only a 2- to 3-fold reduction in apoptosis mediated by DR5, DR4, and DR3 (Figure 3B and data not shown). We chose 293T cells for our subsequent studies, based on their high transfection efficiency and their ability to replicate the transfected plasmid as an episome leading to high-level expression of the transfected genes. The importance of obtaining a sufficiently high-level expression of the transfected DN-FADD construct is clearly evident from Figure 3C, where increasing amounts of the DN-FADD construct led to a progressive decrease in the apoptosis mediated by all three death domain receptors. Notably, the other positive study demonstrating the involvement of FADD in the signaling by DR5 utilized EBNA-positive CV1 cells, which are also likely to lead to high level expression of the transfected DN-FADD construct (Walczak et al., 1997).

A related finding of our study was the ability of both DR5 and DR4 to activate the NF-κB pathway in 293T

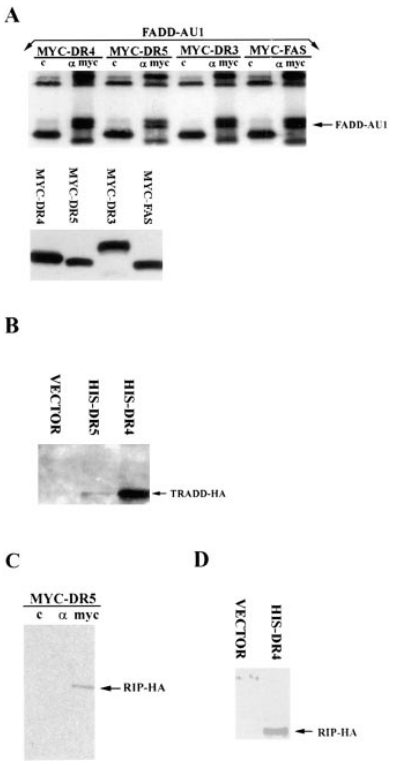

Figure 5. Interactions of DR5 and DR4 with FADD, TRADD, and RIP

(A) DR5 and DR4 interact directly with FADD. 293T cells were transfected with AU1 epitope-tagged FADD along with Myc-tagged DR5, DR4, DR3, or Fas/Apo-1. Myc-tagged receptors from the cell lysates were precipitated using control (Flag) beads (c) or Myc beads (α myc), and coprecipitating FADD was detected using a combination of FADD and AU1 antibodies (top). Western blot analysis on the total cell-lysate demonstrates the expression of the various tagged receptors (bottom).

(B) DR5 and DR4 interact directly with TRADD. 293T cells were transfected with HA epitope-tagged ND-TRADD along with 6X-His epitope-tagged DR5 or DR4. His-tagged receptors from the cell lysates were immunoprecipitated using a cocktail of three anti-His antibodies, and coprecipitating ND-TRADD was detected using a combination of TRADD and HA MAbs.

(C) DR5 interacts directly with RIP. 293T cells were transfected with HA epitope-tagged RIP along with Myc-tagged DR5. Myc-tagged receptor from the cell lysates was precipitated using control (Flag) beads (c) or Myc beads (α myc), and coprecipitating RIP-HA was detected using a HRP-conjugated HA MAb.

(D) DR4 interacts directly with RIP. 293T cells were transfected with HA epitope-tagged RIP along with 6X-His epitope-tagged DR4 or empty vector. His-tagged receptors from the cell lysates were precipitated using Ni-sepharose beads, and coprecipitating RIP-HA was detected using a combination of RIP and HA antibodies.

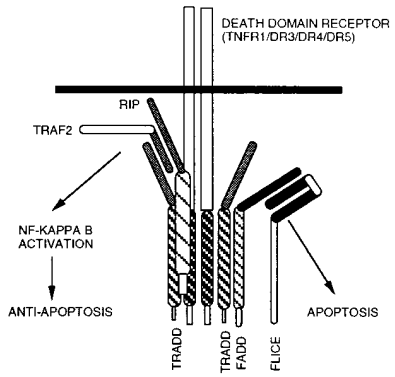

Figure 6. Signal Transduction Pathways Used by TNFR1, DR3, DR4, and DR5

Ligand-induced aggregation of the death domain–containing receptors TNFR1, DR3, DR4, and DR5 lead to recruitment of TRADD via death domain–death domain interaction. The death domain of TRADD then recruits FADD, which leads to apoptosis by the subsequent recruitment and activation of FLICE/MACH1. The death domains of the aggregated receptors can also recruit RIP directly or indirectly via their interactions with TRADD. While the C-terminal death domain of TRADD recruits RIP, its N-terminal domain recruits TRAF2. TRAF2 can also be recruited to the receptors via its interaction with RIP. Recruitment of TRAF2 and RIP leads to the activation of the NF-κB pathway, which may protect against cell death. Stripes, death domains; black, death effector domains; and gray, TRADD-RIP-TRAF2 interacting domains.

cells, a finding in agreement with a recently published report (Sheridan et al., 1997). Although a previous study failed to demonstrate effective NF-κB activation by DR4 in the 293 cells (Pan et al., 1997a), a careful examination reveals an approximately 10-fold induction of NF-κB by DR4 in that study as well. In an independent test involving treatment of the MCF7 cells with TRAIL or TNFα, Pan et al. (1997a) did not observe significant NF-κB activation by TRAIL either. This result is in agreement with the recent report by Sheridan et al. (1997) that also failed to demonstrate NF-κB induction by DR4 or DR5 in the MCF7 cells and concluded that this phenomenon is cell-type dependent. These negative results are probably secondary to the rapid induction of apoptosis by TRAIL or DR4/DR5 in the MCF7 cells, as demonstrated by the ability of both these receptors to activate NF-κB in the presence of CrmA in the present study (Figure 4B). Furthermore, the ability of DR4 and DR5 to activate NF-κB in the presence of CrmA suggests that they induce NF-κB expression independent of their ability to induce apoptosis.

An N-terminal deletion mutant of TRADD (ND-TRADD) is able to partially block NF-κB activation by DR5, DR4, and DR3. This deletion construct lacks the N-terminal 102 amino acids and therefore is missing a complete TRAF2-binding domain, but it does possess a complete death domain and probably works by competing with the endogenous full-length TRADD for binding to the death domains of the receptors (Figure 6). On being recruited to the aggregated death domain receptor complex, this construct will be unable to bind directly and recruit TRAF2. However, some TRAF2 might still be recruited to the aggregated death receptors via a RIP–TRAF2 interaction, which explains only partial inhibition of NF-κB by this construct (Figure 6). Thus, ND-TRADD possesses partial agonist activity for NF-κB activation, a conclusion also supported by the weak activation of NF-κB observed in our experiments involving the transfection of ND-TRADD construct alone (Figure 4C). A similar N-terminal deletion construct of TRADD (residues 25–312), which possesses an incomplete TRAF2 domain, has been previously shown to activate NF-κB partially while blocking TNFα–induced NF-κB activation (Park and Baichwal, 1996). Therefore, our result with ND-TRADD confirms the importance of the N-terminal

TRAF2 binding domain in the activation of NF-κB by the death domain–containing receptors (Park and Baichwal, 1996).

Finally, DR3, DR4, and DR5 were able to coimmunoprecipitate FADD. Our results with DR3 are in agreement with a previous report demonstrating its ability to immunoprecipitate FADD even in the absence of the cotransfected TRADD (Bodmer et al., 1997). However, two other studies could demonstrate only a weak coimmunoprecipitation or no coimmunoprecipitation of FADD with DR3 (Chinnaiyan et al., 1996a; Kitson et al., 1996). Similarly, in contrast to the present study, two recent studies failed to demonstrate any interaction between DR4/DR5 and FADD, TRADD, or RIP (Pan et al., 1997a, 1997b). This discrepancy among various studies might be due to the difference in the endogenous level of expression of other adapter proteins that might stabilize the transient and unstable interactions between the receptors and the adapter protein being tested. For example, TRADD has been reported to enhance the interaction between RIP and TNFR1 or DR3 (Chinnaiyan et al., 1996a; Hsu et al., 1996a), and we have observed similarly that the interaction between RIP and DR4 or DR5 can be enhanced by the coexpression of FADD (unpublished data). A difference in the conditions of cell lysis or immunoprecipitation might also contribute to this discrepancy.

In summary, both DR4 and DR5 resemble TNFR1 and DR3 in mediating FADD-dependent apoptosis and activating the NF-κB pathway. However, our ability to detect the activation of the NF-κB pathway may be hampered by the rapid induction of apoptosis by these receptors. Finally, the extent of NF-κB activation triggered by these receptors may be influenced by the susceptibility of their host cells to apoptosis and therefore may be cell-type and context- dependent.

Experimental Procedures

Cloning of DR5 cDNA and Sequence Analysis

Two EST clones (IMAGE Consortium clones 650744 and 664665) encoding a new member of the TNFR superfamily were identified by searching the database of expressed sequence tags (dbEST) for sequences sharing homology to the extracellular of human TNFR1 receptor. Initial attempts to obtain the plasmids from these clones failed. Therefore, based on the sequence of these EST clones, polymerase chain reaction primers were designed, and 5′ and 3′ RACE was performed on human fetal brain Marathon ready cDNA (Clontech) using the Marathon cDNA amplification kit (Clontech) and following the manufacturer's instructions. RACE fragments were cloned using a TA cloning kit (Invitrogen) and sequenced on an automated fluorescent sequencer to obtain the sequence of the full-length cDNA. Percentage identity and similarity scores were obtained by using the Gap program (Genetic Computer Group, Madison, WI). Hydrophobicity plots were generated by using the Pepplot program (Genetic Computer Group, Madison, WI).

Northern Blot Analysis

Northern blot analysis was performed by using human multiple-tissue Northern blot (Clontech). The blot was hybridized under high stringency conditions with a radiolabeled DR5-encoding cDNA probe according to the instructions of the manufacturer.

Expression Vectors

To construct Myc and 6X-His epitope-tagged receptors, amino acids 51-411 of DR5 were amplified using pfu polymerase (Stratagene) with a primer containing a BamHI site at the 5′ end and a SalI site at the 3′ end and then were ligated to a modified pSectag A vector (Invitrogen) containing a Myc or an MRG-6X-His epitope downstream of a murine Ig κ-chain signal peptide. Epitope-tagged versions of DR4 (amino acids 106-468), DR3 (amino acids 27-418), and Fas/Apo1 (amino acids 19-335) were constructed similarly except that the 5′ primer for amplifying Fas contained an EcoRI site. The DR5-L334N was generated by mutagenesis of the 6X-His-DR5 plasmid using the Quickchange kit (Stratagene), following the manufacturer's instructions. A FLICE-C360S mutant was constructed similarly. DR5ΔCP and DR5ΔDD lack 192 and 134 C-terminal amino acids, respectively, and were generated by creating a polymerase chain reaction generated XbaI site or by taking advantage of a naturally occurring SmaI site in the DR5 sequence, respectively. ND-TRADD-HA (lacking the N-terminal 102 amino acids) and RIP-HA plasmids were constructed by using custom primers designed to generate a C-terminal HA tag and using cDNAs derived from human breast and fetal spleen mRNA as template, respectively. The sequences of all the above constructs were confirmed by automated fluorescent sequencing.

Transient Transfection Assays

For transient transfection assays, 0.8–1 × 10^5 MCF7 or BHK cells were cotransfected with 1 μg of various test plasmids and 200 ng of a *lacZ* expression plasmid in a 24-well plate using 3 μl of Superfect (Qiagen) in Dulbelco's modified Eagle's medium supplemented with 10% fetal calf serum, following the manufacturer's instructions. After 3 hr, 0.5 ml of fresh Dulbelco's modified eagle's medium supplemented with 10% fetal calf serum was added. Twenty-four to 36 hr later, cells were fixed with gluteraldehyde (0.05% in phospate-buffered saline) and stained with 5-bromo-4-chloro-3-indoxyl-β-D-galactosidase (X-Gal). Cells were examined at high magnification under a phase-contrast microscope and apoptotic cells identified on the basis of their dark rounded appearance, membrane "blebs," and lifting off from the plate. 293T (1 × 10^5) cells were transfected by calcium phosphate precipitation in a 24-well plate.

Coimmunoprecipitation and Western Blot Analysis

Monoclonal antibodies against human FADD, TRADD, and RIP were obtained from Transduction Laboratories. Antibodies against the AU1 and HA epitope tags were obtained from Babco and Boehringer Mannheim, respectively. Myc beads and Flag beads were obtained from Santa Cruz Laboratories and IBI Kodak, respectively. For studying in vivo interaction between the receptors and various adapters, 2 × 10^6 293T cells were plated in a 10 mm plate and cotransfected the following day with 5 μg of various epitope-tagged receptor plasmids, 5 μg of various adapter plasmids, 2 μg of CrmA plasmid, and 0.5 μg of a green fluorescent protein (GFP) encoding plasmid (pEGFP-C1) (Clontech) by calcium phosphate coprecipitation. The following day cells were examined under a fluorescent microscope to ensure equal transfection efficiency as determined by the expression of the GFP. Twenty-four to 36 hr posttransfection, cells were lysed in 1 ml of lysis buffer containing 0.1% Triton X-100, 20 mm sodium phosphate (pH 7.4), 150 mm NaCl, and 1 EDTA free protease inhibitor tablet per 10 ml (Boehringer Mannheim). Cell lysate was precleared by incubation with Flag beads.

For immunoprecipitation, precleared cell lysate (500 μl) was incubated with 10 μl of Myc beads or control (Flag) beads for 1 hr at 4°C. Beads were washed twice with lysis buffer; twice with a wash buffer containing 1% Triton X-100, 20 mm sodium phosphate (pH 7.4), 500 mM NaCl, and again with lysis buffer. Bound proteins were eluted by boiling for 3 min in SDS loading buffer, separated by SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and analyzed by Western blot. For immunoprecipitation of MRG-6X-His tagged receptors, cells were lysed in a 1 ml of lysis buffer containing 1% Triton-X 100, 20 mm sodium phosphate (pH 7.4), 150 mm NaCl, and 1 EDTA free-protease inhibitor tablet per 10 ml. A cocktail of three monoclonal antibodies (MAbs) (MRG-4Xhis, Penta-His, and Tetra-His [Qiagen]) was used for immunoprecipitation. For precipitation using Ni-sepharose, cell lysates were incubated for 1 hr with 20 μl of Ni-sepharose beads precoated with 1% bovine serum albumin in a buffer containing 1% Triton-X 100, 20 mm sodium phosphate (pH 7.4), 500 mM NaCl, and EDTA-free protease inhibitor cocktail. Beads were washed extensively with the

above buffer supplemented with 20 mM imidazole and the bound protein subsequently eluted and analyzed by SDS-polyacrylamide gel electrophoresis and Western blot as described above.

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