A Domain in TNF Receptors That Mediates Ligand-Independent Receptor Assembly and Signaling

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A conserved domain in the extracellular region of the 60- and 80-kilodalton tumor necrosis factor receptors (TNFRs) was identified that mediates specific ligand-independent assembly of receptor trimers. This pre–ligand-binding assembly domain (PLAD) is physically distinct from the domain that forms the major contacts with ligand, but is necessary and sufficient for the assembly of TNFR complexes that bind TNF-α and mediate signaling. Other members of the TNFR superfamily, including TRAIL receptor 1 and CD40, show similar homotypic association. Thus, TNFRs and related receptors appear to function as preformed complexes rather than as individual receptor subunits that oligomerize after ligand binding.

Tumor necrosis factor (TNF-α) is an important effector cytokine for immune responses and inflammation (*1*). TNF-α exerts its biological effects through two TNF receptors (TNFRs): a 60-kD receptor (p60) and an 80-kD receptor (p80). The TNFRs are the prototypes of a large family of cell surface receptors that are critical for lymphocyte development and function (*2*). Homotrimeric TNF-α is thought to recruit three receptor chains into a complex that juxtaposes the cytoplasmic domains (CDs). Subsequently, p60 recruits apoptosis-inducing and other proteins through a "death domain" in its cytoplasmic tail, whereas p80 induces inflammatory responses through a cytoplasmic TNFR-associated factor (TRAF)–binding domain (*3*). Signaling may also require loss of binding of cytosolic negative regulators such as the Silencer of Death Domain (SODD) protein (*4*). The extracellular domain (ECD) of both TNFRs contains three well-ordered cysteine-rich domains (CRD1, -2, and -3) that characterize the TNFR superfamily and a less conserved, membrane-proximal, fourth CRD (*5*). The ligand-binding pocket for TNF-α is mainly formed by CRD2 and CRD3 of the TNFRs (*5*). How CRD1 contributes to receptor function is unknown.

Because the first step in signaling by members of the TNFR superfamily is thought to be ligand-induced trimerization of the receptor (*6*), we attempted to identify trimer complexes using the thiol-cleavable, membrane-impermeant, chemical crosslinker 3,3′-dithiobis[sulfosuccinimidylpropionate] (DTSSP) (*7*). Indeed, complexes were found for p80 that exhibited molecular sizes approximately three times the unit size, consistent with glycosylated and nonglycosylated trimers (Fig. 1A). The p80 complexes were efficiently captured in the presence or absence of TNF-α (65 to 70% by densitometry). Despite the fact that most p60 resides in the Golgi apparatus and was inaccessible to the cross-linker (*8*), as much as 15 to 20% of the p60 chains were cross-linked as apparent trimers and discrete higher order complexes, whether or not TNF-α was added (Fig. 1A). Control experiments detected no endogenous TNF-α and no other proteins such as p80 cross-linked to the p60 complex (*9*). The complexes were reduced to monomers by cleaving the cross-linker with β-mercaptoethanol (Fig. 1A).

Because p60 and p80 chains apparently self-associate before ligand binding, we sought a domain that would mediate ligand-independent self-assembly. It is well established that the cytoplasmic death domain of p60 can self-associate and trigger apoptosis when overexpressed (*10*). However, because the preassembled complexes we observed were apparently nonsignaling, we hypothesized that the assembly domain resides outside of the cytoplasmic region. Indeed, the NH_2-terminal regions of the ECDs of p60 and p80 could specifically self-associate in a yeast two-hybrid interaction assay (*11*). In mammalian cells, a chimeric p60 receptor with the CD replaced by the green fluorescent protein (GFP) interacted strongly with a CD-deleted p60 (p60ΔCD-HA) but not with the TNFR-like herpesvirus receptor (HveAΔCD-HA) (Fig. 1B) (*12*). GFP alone failed to associate with p60ΔCD-HA (Fig. 1B). Homotypic interaction was also observed between full-length p80 and p80ΔCD-HA (Fig. 1C). However, removal of amino acids 10 through 54 of p80, overlapping CRD1, completely abrogated association with intact p80 (Fig. 1C). Self-association was eliminated by a similar deletion (amino acids 1 through 54) in p60 (*13*).

The importance of the NH_2-terminus of p80 (amino acids 10 through 54) was further illustrated by experiments in which it was appended to the p60 receptor. This chimeric receptor interacted with full-length p80 (Fig. 1D). Thus, this domain was sufficient to mediate specific association of a heterologous receptor. This association is ligand-independent because the chimera $p80_{10\text{-}54}60_{55\text{-}211}$(R1)-HA has two amino acids encoded by an Eco RI restriction site inserted at the junction of the p80 and p60 sequences that abolished TNF-α binding but permitted self-association (Fig. 1E). Thus, a distinct functional domain of the TNFR-ECD mediates self-assembly in the absence of ligand. Henceforth, we refer to this as the pre–ligand-binding assembly domain (PLAD).

The deletion of the PLAD from either p60 or p80 completely abrogated ligand binding (Table 1 and Fig. 1E) but was unlikely to disrupt the overall ECD structure (*14*). However, the addition of the PLAD from p80 enabled the PLAD-deleted p60 ($p80_{10\text{-}54}60_{55\text{-}211}$-HA) to bind TNF-α (Fig. 1E). Thus, efficient TNF-α binding by TNFRs depends on receptor self-assembly. Furthermore, two substitutions (*15*) in the PLAD that are not expected to disturb direct ligand contact, $Lys^{19}Tyr^{20} \rightarrow Ala^{19}Ala^{20}$ (KY19/20AA) and $Lys^{32} \rightarrow Ala^{32}$ (K32A) (*5*), abrogated self-association (Fig. 2A) and eliminated TNF-α binding (Table 1). Substitution of another residue within the PLAD, Q24A (*16*), did not affect self-association or TNF-α binding (Fig. 2A and Table 1). In contrast, two substitutions outside of the PLAD in the CRD2 ligand binding pocket, E57A and N66F, disrupted TNF-α binding but had little effect on receptor self-association (Table 1 and Fig. 2A). Association of a mutant receptor lacking the CD with the wild-type ECD correlated with its ability to dominantly interfere with p60-induced apoptosis, indicating that the mutant receptors enter into endogenous functional p60 receptor complexes via the PLAD (Table 1). Thus, the PLAD is physically distinct from the ligand contact domain but is nonetheless essential for efficient TNF-α binding and receptor function.

To confirm receptor self-interaction in living cells, we used a flow cytometric approach to analyze fluorescence resonance energy

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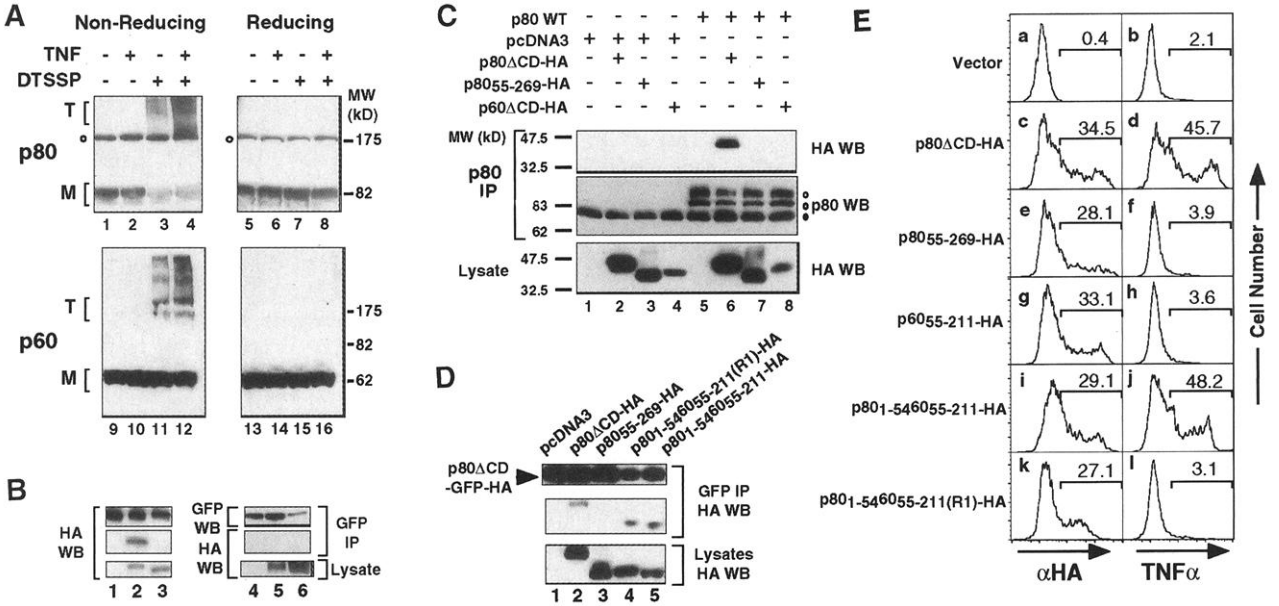

Fig. 1. Definition of the PLAD. (**A**) Trimeric TNFR complexes in the absence of ligand. H9 cells were treated as indicated and analyzed for p60 or p80 complexes on Western blot (WB). The position of monomers (M), trimers (T), and a nonspecific protein species (open circle) are shown. (**B**) Specific self-association of p60. 293T cells were transfected with p60ΔCD-GFP-HA (lanes 1 through 3) or pEGFP-N1 (lanes 4 through 6) and with pcDNA3 (lanes 1 and 4), p60ΔCD-HA (lanes 2 and 5), or HveAΔCD-HA (lanes 3 and 6). Immunoprecipitation (IP) (top two panels) and WB (bottom panel) are shown (*12*). (**C**) Specific self-association of p80. IP and WB were done as shown. The glycosylated and unglycosylated forms of p80 (open circles) and IgH (solid circle) are indicated. (**D**) The PLAD is necessary and sufficient for self-association. Cotransfection of p80ΔCD-GFP-HA (lanes 1 through 5) with the indicated plasmids is shown. IP and WB are shown. (**E**) The PLAD is required for TNF-α binding (*26*). The numbers shown are percentages of positive population compared to the vector-transfected control.

Table 1. Summary of the phenotypes of the p60ΔCD mutants (*16*).

	MAB225*	Clone 4.12†	TNF-α binding‡	Self-association§	Dominant interference‖
p60ΔCD	1	1	1	+	+
$p60_{55-211}$	0.87	0.02	0.01	NT	−
K19E	1.05	1.07	1.1	NT	+
KY19/20AA	0.59	0.14	0.03	−	−
Q24A	1.06	0.97	1.13	+	+
K32A	0.36	0.01	0.01	−	−
DT49/50AA	1.16	0.96	1.13	NT	+
E57A	1.68	0.04	0.02	+	+
T61A	1.42	1.28	1.35	NT	+
N66F	0.67	0.04	0.01	+	+
R77A	1.06	0.99	1.24	NT	+
W108T	1.32	1.11	1.25	NT	+
L112E	1.31	1.27	0.85	NT	+

*Staining of p60-specific monoclonal antibody clone MAB225 (R&D Systems). The values were normalized against the staining of the HA epitope tag by dividing the percentage of MAB225-positive cells by the percentage of HA-positive cells. †Staining with p60-specifc monoclonal antibody clone 4.12 (Zymed) was normalized against HA staining. ‡TNF-α binding was determined with a biotinylated form of TNF-α and normalized against HA staining (*26*). §Self-association was determined by immunoprecipitation assays in 293T transient transfections as described (*12*). NT, not tested. ‖Dominant interference was determined as described (*27*). Dominant inhibition by the p60ΔCD-HA mutants was at least 50% of p60ΔCD wild type (+). $p60_{55-211}$, KY19/20AA, and K32A did not confer any protection (<5%) against TNF-induced death (−) in all experiments. The antibodies and TNF-α binding to p60ΔCD are arbitrarily set at 1. Results are representative of three independent experiments.

transfer (FRET) (*17*) between receptor subunits fused at the COOH-terminus to either cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP) (*18*) as described in an accompanying paper [also see the protocol at *Science*'s STKE (www.stke.org/cgi/content/full/OC_sigtrans;2000/38/pl1)] (*19*). We found that there was energy transfer between p60ΔCD-CFP and p60ΔCD-YFP that increased substantially after the addition of TNF-α (Fig. 2B). This FRET was abolished by deletion of the PLAD or by the K32A mutation that prevented PLAD association (Fig. 2B). The p80ΔCD-CFP:p80ΔCD-YFP pair also yielded a strong FRET signal that increased with TNF-α addition (Fig. 2B). Controls using p60ΔCD-YFP as an acceptor for p80ΔCD-CFP or CFP-p80ΔCD (CFP fused to the NH_2-terminus of p80 ECD) as donor showed no FRET (Fig. 2B). Thus, the p60 and p80 chains are in close proximity to themselves in living cells, and ligand induces a change in the complexes that leads to tighter association of the CFP and YFP moieties in the cytoplasm. Furthermore, other members of the TNFR superfamily, including the ECDs of TRAIL receptor 1 (DR4), CD40 (Fig. 2, C and D), and Fas (*19*, *20*), all self-associate but do not interact with ECDs from heterologous receptors. Thus, self-assembly through the PLAD is a conserved feature of the TNFR superfamily.

The presence of PLAD-mediated pre-assembled TNFR complexes sheds new light on signaling by this large family of receptors, many of which are critical for lymphocyte function and homeostasis (*2*). Previously, ligand was thought to bring monomer receptor chains into apposition in threefold complexes that recruit cytoplasmic signal transduction proteins (*1*, *3*, *5*, *6*). It is now clear that p60 and p80 preassociate as oligomers on the cell surface and are only found as monomers if the PLAD is deleted. Cross-linking the endogenous p60 and p80 receptors suggests that trimers are a favored conformation. However, the p60 ECD crystallizes in the absence of ligand as parallel dimer (*21*), which suggests that further work will be needed to define the stoichiometry of cell-surface oligomers. The presorting of chains into homotypic complexes on the cell surface could promote the rapidity and specificity of response for the different receptors in the TNFR superfamily (*3*). Also, "receptor interference" in which, for example, a p80 chain (lacking a death domain) is recruited by TNF-α into a complex with p60 and causes dominant inhibition

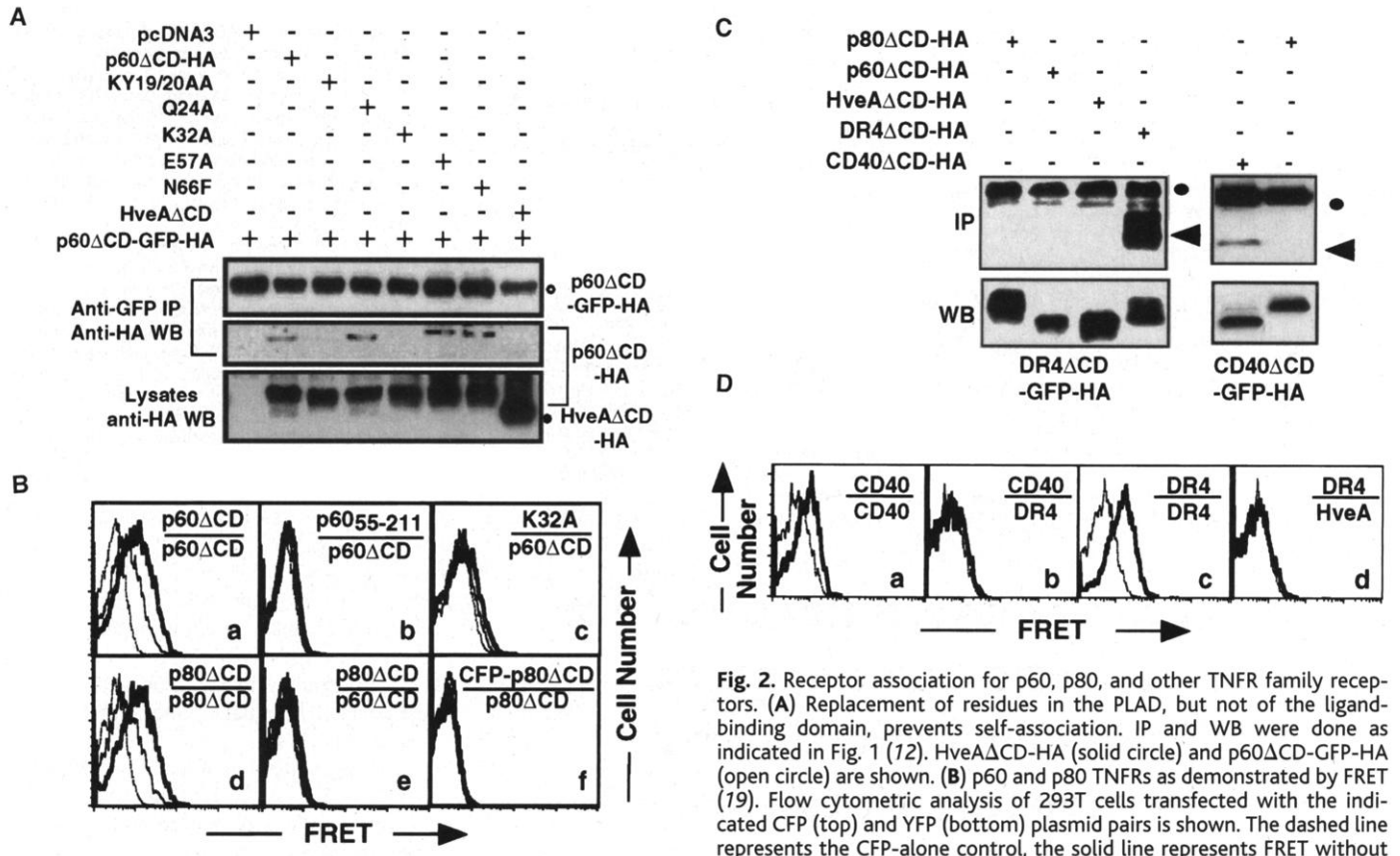

Fig. 2. Receptor association for p60, p80, and other TNFR family receptors. (**A**) Replacement of residues in the PLAD, but not of the ligand-binding domain, prevents self-association. IP and WB were done as indicated in Fig. 1 (*12*). HveAΔCD-HA (solid circle) and p60ΔCD-GFP-HA (open circle) are shown. (**B**) p60 and p80 TNFRs as demonstrated by FRET (*19*). Flow cytometric analysis of 293T cells transfected with the indicated CFP (top) and YFP (bottom) plasmid pairs is shown. The dashed line represents the CFP-alone control, the solid line represents FRET without TNF-α, and the thick line represents FRET with TNF-α added. (**C**) Self-association of CD40 and DR4. IP and WB were performed with antibodies to GFP and HA, respectively. The solid circles denote the GFP fusion proteins, and the arrowheads indicate the ΔCD protein in the immune complexes. (**D**) Specific receptor association of DR4 and CD40 as demonstrated by FRET. Transfections with the indicated CFP (top) and YFP (bottom) plasmid pairs were performed as in (B). The dashed lines represent background FRET with CFP alone, and the thick lines represent FRET in the presence of both CFP and YFP fusion proteins.

of apoptosis would be avoided (*22*, *23*). Preassembly has been described for other receptor families, notably interleukin-1 (IL-1) and IL-2 receptor, which are composed of heteromers of different polypeptides (*24*). The erythropoietin receptor dimers apparently undergo a scissors-type movement to accommodate ligand (*25*). In that case, self-association of the receptor chains occurs via the same amino acid contacts that are critical for ligand binding (*25*). By contrast, the TNFR superfamily uses a dedicated self-association domain distinct from the CRD2/3 ligand contact region. Identification of the PLAD could allow development of therapeutics that selectively inhibit the PLAD of individual TNFR-like receptors and thereby prevent signaling.

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12. The various truncations and mutations of p60, p80, HveA, DR4, and CD40 were generated by polymerase chain reaction (PCR) and sequenced. Briefly, the leader sequence and the first 10 amino acid residues from p80 were amplified so that the hemagglutinin (HA) epitope tag was included at the 3′ end to create a HA tag at the NH_2-terminus of the receptors. The PCR product was digested with Bam HI and Eco RI and cloned into pcDNA3. The PCR fragments containing the receptor fragments were then introduced into this plasmid using the Eco RI and Xho I sites. For the GFP/CFP/YFP chimeras, the fragments were amplified by PCR and introduced in-frame into the Xho I and Xba I sites of p60ΔCD-HA. 293T cells were transfected with Fugene 6 (Boehringer-Mannheim) as per the manufacturer's protocol. Cells were lysed in 150 mM NaCl, 20 mM tris-Cl (pH 7.5), 1 mM EDTA, 30 mM NaF, 2 mM β-glycerophosphate, 1 mM sodium orthovanadate, 5 mM iodoacetamide, 2 mM dithiothreitol, 1% Triton X-100, and protease inhibitors (Boehringer-Mannheim). After preclearing with protein G agarose beads (Boehringer-Mannheim) and normal mouse immunoglobulin G (IgG), proteins were immunoprecipitated from the lysates with 2 μg of anti-GFP and protein G agarose beads. Immune complexes were washed twice with lysis buffer containing 0.5 M NaCl and then three times with regular lysis buffer. Immune complexes were resolved on Tris/Glycine gels (Novex, San Diego, CA). Results shown are representative of five independent experiments. Transfection in Jurkat cells showed similar results.
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16. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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26. Receptor expression was monitored by staining with monoclonal antibodies to HA and p80 (clone MAB226, R&D Systems). TNF-α binding was determined with biotinylated TNF-α (NEN Life Sciences) and a secondary fluorescein-conjugated streptavidin label. Samples were analyzed on a FACScan flow cytometer. Results shown are representative of at least five experiments.
27. Fifteen micrograms of the corresponding plasmids were transfected into p80 Jurkat cells (*22*) by electroporation using a BTX Electro Cell Manipulator 600. After 9 to 24 hours, cells were stimulated with the indicated amount of TNF-α for 12 to 16 hours. Cells were then stained for HA expression and propidium iodide uptake. The number of HA-positive cells was scored under constant time, and percent inhibition of apoptosis was calculated by normalizing to the percentage of cell death induced in the HveAΔCD-HA–transfected samples. Results are representative of three experiments.
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Fas Preassociation Required for Apoptosis Signaling and Dominant Inhibition by Pathogenic Mutations

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Heterozygous mutations encoding abnormal forms of the death receptor Fas dominantly interfere with Fas-induced lymphocyte apoptosis in human autoimmune lymphoproliferative syndrome. This effect, rather than depending on ligand-induced receptor oligomerization, was found to stem from ligand-independent interaction of wild-type and mutant Fas receptors through a specific region in the extracellular domain. Preassociated Fas complexes were found in living cells by means of fluorescence resonance energy transfer between variants of green fluorescent protein. These results show that formation of preassociated receptor complexes is necessary for Fas signaling and dominant interference in human disease.

Fas (CD95 or APO-1) is a cell surface receptor that transduces apoptotic signals critical for immune homeostasis and tolerance (*1–3*). The Fas protein is a 317–amino acid type 1 transmembrane glycoprotein with three extracellular cysteine-rich domains (CRDs) that are characteristic of the tumor necrosis factor receptor (TNFR) superfamily. Both Fas and Fas ligand (FasL) are predicted to form trimers, with CRD2 and CRD3 forming the major contact surfaces for FasL (*4, 5*). The Fas cytoplasmic portion contains a death domain that rapidly recruits the adaptor molecule FADD (Fas-associated death domain protein) and the caspase-8 proenzyme after binding of FasL or agonistic antibodies, leading to caspase activation and apoptosis (*6–10*).

Patients with autoimmune lymphoproliferative syndrome (ALPS) type 1A have heterozygous germ line mutations in the *APT-1* Fas gene. Their lymphocytes are resistant to Fas-induced apoptosis, and transfection of the mutant allele causes dominant interference with apoptosis induced through Fas (*11–16*). This was thought to result from ligand-mediated crosslinking of wild-type and defective Fas chains into mixed trimer complexes. However, a mutation that causes an extracellular domain deletion of most of CRD2 (ALPS Pt 2, deletion of amino acids 52 to 96) as a result of altered RNA splicing shows no binding to agonistic antibodies or FasL, but still dominantly interferes with Fas-induced apoptosis almost as efficiently as does a death domain mutant [ALPS Pt 6, $Ala^{241} \rightarrow$ Asp (A241D)] (Fig. 1A) (*13, 17*). Control experiments showed equal cell surface expression of the wild-type and mutant Fas molecules (*18*). Thus, dominant interference cannot be explained by the conventional model of signaling by FasL-induced oligomerization of receptor monomers because, in this scheme, the Pt 2 mutant Fas molecule would not become part of a mixed receptor complex. We therefore tested for ligand-independent interactions between Pt 2 Fas and wild-type Fas. Both full-length and Pt 2 Fas coprecipitated with a Fas 1–210:GFP chimera in which green fluorescent protein (GFP) replaces the death domain (Fig. 1C). This interaction was specific, because the TNFR family receptors TNFR2/p80 and HveA did not interact with Fas (*1*).

We have found that TNFR superfamily members share a self-association domain in CRD1, termed the "pre-ligand assembly domain" (PLAD) (Fig. 1B) (*19*). To test whether Fas contains a functional PLAD, we constructed hemagglutinin (HA)-tagged NH_2-terminal Fas truncations (*20*). Deleting the first subdomain in CRD1 (amino acids 1 to 42) (*21*) substantially reduced ligand binding but did not prevent binding of the Fas monoclonal antibody (mAb) APO-1. Deleting the entire CRD1 (amino acids 1 to 66) abrogated binding of both FasL and Fas mAb (Fig. 1A). Both truncations eliminated coprecipitation with a differentially tagged Fas molecule and abrogated apoptosis signaling; this result indicates that the NH_2-terminus of Fas, including CRD1, functions as a PLAD (Fig. 1, C

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