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Apoptosis Mediated by the TNF-Related Cytokine and Receptor Families

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Abstract T lymphocytes use several specialized mechanisms to induce apoptotic cell death. The tumor necrosis factor (TNF)-related family of membrane-anchored and secreted ligands represent a major mechanism regulating cell death and cell survival. These ligands also coordinate differentiation of tissue to defend against intracellular pathogens and regulate development of lymphoid tissue. Cellular responses are initiated by a corresponding family of specific receptors that includes two distinct TNFR (TNFR60 and TNFR80), Fas (CD95), CD40, p75NTF, and the recently identified lymphotoxin β-receptor (LTβR), among others. The MHC-encoded cytokines, TNF and LTα, form homomeric trimers, whereas LTβ assembles into heterotrimers with LTα, creating multimeric ligands with distinct receptor specificities. The signal transduction cascade is initiated by transmembrane aggregation (clustering) of receptor cytoplasmic domains induced by binding to their multivalent ligands. The TRAF family of Zn RING/finger proteins bind to TNFR80; CD40 and LTβR are involved in induction NFκB and cell survival. TNFR60 and Fas interact with several distinct cytosolic proteins sharing the "death domain" homology region. TNF binding to TNFR60 activates a serine protein kinase activity and phosphoproteins are recruited to the receptor forming a multicomponent signaling complex. Thus, TNFRs use diverse sets of signaling molecules to initiate and regulate cell death and survival pathways.

Key words: Death domain, ring finger, signal transduction, serine kinase, T lymphocytes

The immune system uses several different strategies to induce the death of cells infected with viruses or other types of parasites. Activated T cells and macrophages produce cytotoxic proteins, for example, lymphotoxin (LTα) and tumor necrosis factor (TNF), that induce cell death. The molecular cloning of these two proteins in 1984 revealed their structural similarity [1,2] and they are now recognized as the prototypes of a superfamily of immunoregulatory and effector molecules (Table I). Two distinct cell surface receptors of 60 kDa (TNFR60, CD120a) and 80 kDa (TNFR80, CD120b) bind both TNF and LTα. Molecular cloning of the TNFRs identified additional cognates of mammalian and viral origin that comprise the parallel family of receptors that mediate the biologic actions of this ligand family [3,4] (Fig. 1). Cell death initiated by the ligand–receptor complex has characteristics of apoptosis, including membrane blebbing, nuclear condensation, and DNA cleavage. This is distinguished from the necrosis type of cell death inflicted by the plasma complement system, or the granule-associated protein, perforin, present in cytotoxic T lymphocytes (CTL), both of which target the lipid bilayer and form nonselective ion pores inducing osmotic imbalance. However, granule-associated proteins of CTL and natural killer (NK) cells, particularly the esterases (granzymes), participate with perforin to activate the apoptotic cell death pathway [5], another distinct mechanism of activating apoptosis.

TNF is now recognized as a critical component orchestrating many different aspects of host inflammatory defenses including differentiative and proliferative activities as well the ability to induce cell death [6]. The dual identity of TNF as cachectin, a factor that induces wasting associated with chronic disease states, such as cancer, acquired immunodeficiency syndrome (AIDS), and malaria. This other side of TNF is a cornerstone of the concept of cytokine based disease processes. Other examples include the immune deficiency, hyper-IgM syndrome, caused

Abbreviations: CTL, cytotoxic T lymphocyte; LT, lymphotoxin; TNF, tumor necrosis factor; TNFR, TNF receptor.

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by mutations in the ligand for CD40, which results in a failure to switch immunoglobulin classes [7]. Mutations in Fas/Fas Ligand pair are responsible for the autoimmune-like syndromes of the *lpr* and *gld* mouse strains, respectively [8,9]. The stories emerging from virus strategies evolved to thwart the actions of TNF [10] provide evidence of the critical importance of this family in host defense. These include the poxvirus version of soluble TNF receptors, adenovirus E3 gene region proteins that block TNF-induced apoptosis, enhancement of HIV transcription by TNF via NFκB activation, and the Epstein-Barr virus LMP-1 oncogene that interacts directly with the signaling proteins associated with this receptor family.

Until recently, a clear role for LT in immune responses had not emerged; LTα was largely consigned as a redundant form of TNF based on a similar spectrum of activities and binding to the same two receptors as TNF. However, in most in vitro assays using human cell lines, including cytotoxicity, the potency of LT is greatly reduced (10- to 1,000-fold) compared to TNF [11]. In some assays LT behaves as a partial agonist; however, the low potency of LT is not directly attributable to differences in binding affinity to the two receptors [12]. Discovery of LT as a cell surface protein on activated T cells in a form distinct from secreted LT, and identification of a specific receptor for the surface LT, hinted at unique functions for this cytokine. On the cell surface, LT exists as a heteromeric complex with a 33-kDa subunit, called LTβ, and the 25-kDa LT subunit (because it is a subunit of a complex LT) is now referred to as LTα [13,14]. By contrast, classic secreted LT is a homotrimer of LTα subunit. Assembly of these different forms of LT occurs during biosynthesis, and not as a rebinding of secreted LTα to cell surface LTβ. A role distinct from TNF was convincingly demonstrated by genetic deletion (knockout) of the LTα gene, which results in a mouse with developmental deficiency of peripheral lymph organs [15].

TABLE I. Functions of the TNF-Related Ligands and Receptors

Receptor	Ligand	Function
TNFR60	TNF, LTα	Inflammation/host-defense; apoptosis
TNFR80	TNF, LTα	Inflammation/host-defense; cell survival
LTβR	LTαβ complex	Peripheral lymph node development; apoptosis
CD40	CD40 L	Ig class switching (hyper-IgM syndrome); cell survival
Fas	Fas L	Lymphoproliferation (*Ipr/gld*); apoptosis
CD27	CD27 L	Costimulation of T cells
CD30	CD30 L	Hodgkin's lymphoma marker; costimulation of T and B cells
OX40	OX40 L	Costimulation of T cells
4-1BB	4-1BB L	Costimulation of T cells
p75NTR	NGF, neurotrophins	Neuron survival/apoptosis

LTαβ AND TNF CYTOKINE LOCUS

The genes encoding TNF, LTα, and LTβ are tightly linked on human chromosome 6 (17 in the mouse) within the major histocompatibility complex (MHC) sandwiched between the class III (complement C2 and C4 genes) and HLA-B locus [14]. The exon–intron organization is similar for all three genes. Allelic differences in this MHC cytokine locus may be a factor in several pathophysiologic conditions, as exemplified by the recent identification of a TNF allele involved in fatal cerebral malaria [16]. The TNF-related ligands are structured as type II transmembrane proteins with the C-terminus on the exterior cell surface, a single transmembrane domain, and a short cytoplasmic tail. The secreted form of TNF is released by proteolytic processing of the membrane precursor by a cell surface metalloproteinase [17]. LTα is an exception; the homotrimer is exclusively secreted because it lacks a retained transmembrane domain. LTβ provides the transmembrane domain that stably anchors the LTαβ complex to the cell surface. There is no evidence that the LTαβ complex is secreted. This suggests that the mode of signal transmission will depend upon cell to cell contact and the effects of the ligand will be restricted to the individual target cell in intimate contact with the ligand-producing effector cell.

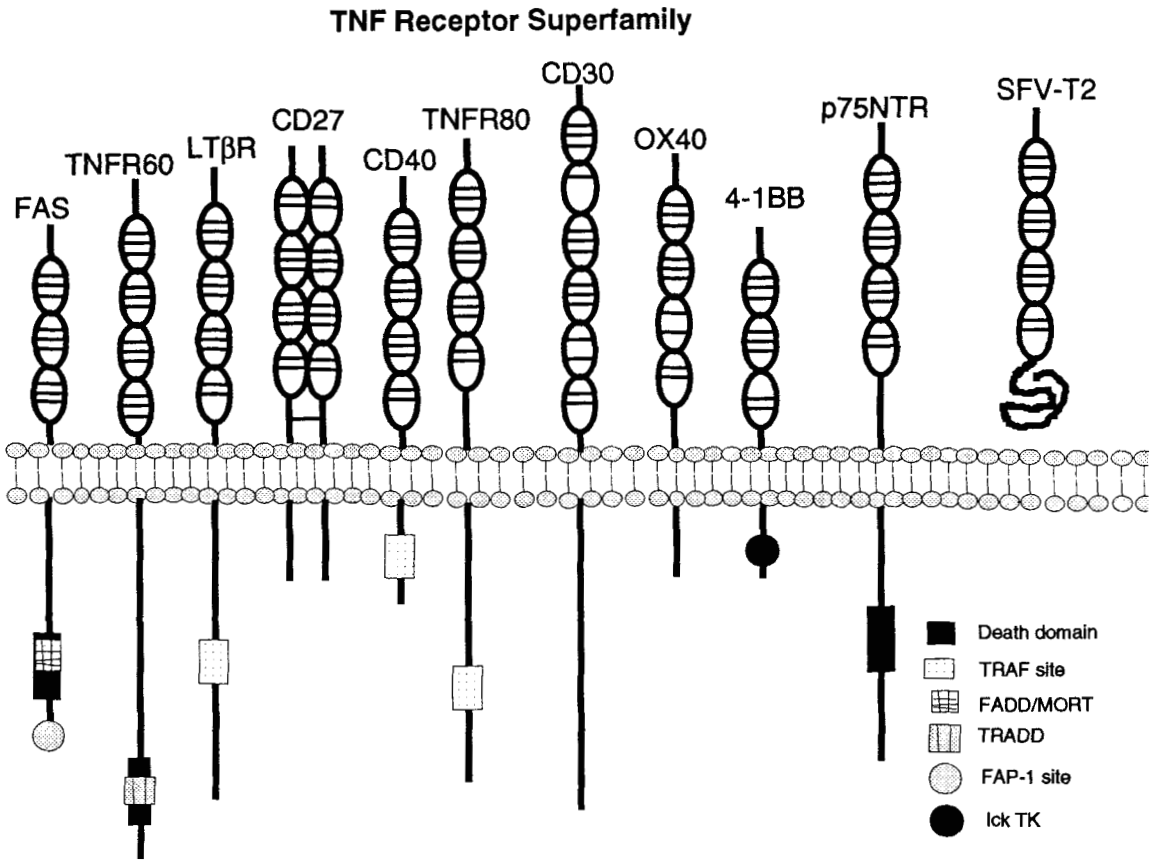

Fig. 1. The TNF receptor family. The extracellular cysteine-rich domains are represented by ovals and the horizontal bars denote disulfide bonds. The vertical bars below the membrane represent the cytoplasmic domains and, where known, functional binding sites for cytosolic proteins. p75NTR, low-affinity neurotrophin receptor. SFV-T2, shope fibroma virus T2 protein.

This mode of action is in contrast to the systemic effects mediated by secreted TNF and LT [6].

Activated T and B lymphocytes and interleukin-2 (IL-2)-treated NK cells produce surface LT, whereas TNF is produced by macrophages, T cells, and nonlymphoid cells [18]. Expression of LT and TNF is rapidly inducible by stimuli that characteristically activate T cells, but expression is transient, lasting only a few hours for TNF and somewhat longer for surface LT (~ 24 h). This pattern is dependent on transcription of TNF or both LTα and β genes [14]. Other ligands, such as CD40L and FasL, show a similar pattern of rapid, inducible expression in T cells but may vary in the duration of expression.

TRIMERS AND RECEPTOR BINDING SITES

LTα and TNF share a common structural motif as a β-sheet sandwich with the propensity to assemble into trimers. Members of this family share sequence homology in residues that form the β-sheet scaffold that allow the subunits to oligomerize [14]. The oligomeric is central to the ability to initiate cellular responses because the receptor binding sites on TNF and LTα are located at the interfaces of the adjacent subunits. Site-directed mutagenesis [19] and the crystal structure of the LTα-TNFR60 complex [20] have revealed that the loops connecting the a–a″ and d–e β-strands contain the major receptor contact residues. These loops are located on opposite sides of the individual monomers such that each binding site is a composite of adjacent subunits. Each homotrimer of TNF or LTα contains three equivalent binding sites and thus can aggregate (cluster) upto three receptors. Although TNF and LTα both bind to TNFR60 and TNFR80, there is little conservation in residues within these loops that clearly explains the overlapping specificity. However, site-directed mutagenesis of TNF has revealed subregions

within the a–a'' and d–e loops that discriminate between TNFR60 and TNFR80 binding [21].

The LTαβ complex presents a different picture of a composite binding site. Within a trimeric configuration, two types of LTαβ complexes can be formed with distinct subunit stoichiometry, an α1β2 and α2β1. Protein crosslinking studies indicate that the LTα1β2 complex is the most abundant form expressed on the surface of activated T cells [13]. However, this form does not contain subunit interfaces equivalent to that found in the LTα homotrimer and suggests that the LT α1β2 complex is not likely to bind to TNFR60 or TNFR80. Indeed, this was the case and spurred the search for an LTβ-specific receptor. To study membrane-anchored ligands soluble forms of the receptors were constructed as chimeras between the extracellular domain of the receptor and the Fc region of immunoglobin G (IgG) [22]. This provided a reagent that could utilize sensitive immunochemical assays coupled with the specificity for the ligand. In 1993, Marynen and coworkers identified a TNFR-related gene encoded on human chromosome 12p13 [23]. When this protein was constructed as a Fc chimera, it bound with high affinity to the LTα1β2 complex and was designated LTβR [24]. It was also shown that the minor form of surface LT, $LT\alpha_2\beta_1$ complex, bound to TNFR60 and TNFR80 and thus represented a third ligand for these two receptors.

RECEPTOR STRUCTURE

The family of TNF receptors are all type I transmembrane glycoproteins and are defined by a cysteine-rich extracellular domain [25]. The extracellular region is organized into a tightly knit core domain that is variably repeated among family members, four times for TNFRs. Each domain repeat contains three disulfide bonds; in some receptors the membrane proximal domain may have only two disulfide bonds. In TNFR60, the second and third repeats make the major contact with LTα subunits [20]. Even though they bind the same two ligands, TNFR60 and TNFR80 are as related to each other as they are to other receptors in the family. The LTβR is somewhat of a hybrid between TNFR60 and TNFR80 in the positioning of the cysteine residues, resembling TNFR60 in the first two domains and TNFR80 in the third and fourth repeats.

Tissue expression of the receptors is varied, and members fall into two general types: widely distributed receptors, such as TNFR60 or LTβR, and receptors with more tissue restricted expression, such as CD40 or p75NTR. Cell lines often coexpress both types of TNFR with TNFR80 predominant on lymphoid lineages and TNFR60 abundant on lines of epithelial origin. The receptor genes located on chromosome 1p36, TNFR80, 41BB, CD30, and OX40, are expressed as inducible proteins on the surface of activated T cells [26]. Interestingly LTβR is absent on T or B lymphocytes, indicating that LTαβ complex signals in a unidirectional (paracrine) fashion and not as an autocrine regulator like TNF.

Common functional relationships are seen in this receptor family although minimal sequence homology is found in their cytoplasmic domains. Several of the receptors share the ability to signal apoptosis or stimulate cell growth, or both. This is best illustrated by TNFR60 and Fas, both of which induce cell death in tumor cells or lymphocytes but also enhance cell proliferation. Fas or TNF can enhance T-cell growth as a coactivation signal along with signals provided by stimulation through the antigen receptor [27]. Fas and TNFR60 share limited homology over a short span in the cytoplasmic region referred to as the "death domain" [28]. In normal cells, TNF induces resistance to its own cytotoxic effect, which is blocked by actinomycin D or cycloheximide. The C-terminal region of Fas acts as a negative regulator of apoptosis, as deletion of this region in transfected cells causes enhanced cell death [29]. Although structurally similar, Fas and TNFR60 appear to induce cell death by distinct processes [30]. Also, Fas and TNF induce death in cells derived from different lineages; typically TNF is not active on lymphocytes, whereas Fas kills T cells extremely well if they are appropriately differentiated, and the time course of apoptosis induced by TNF is typically slower (8–12 h) than for Fas (2–4 h). Both Fas and TNFR60 require activation of cysteine protease in the ICE family and are blocked by the poxvirus serpin, Crm A. The ability to induce death and growth is also seen in other members of this family. LTβR, CD30, and p75NTR can signal apoptosis in selected cell lines, whereas CD40 prevents apoptosis of B lymphocytes in germinal centers; the chromosome 1-linked receptors, TNFR80, OX40, and

41BB, function as coactivating signals enhancing T-cell proliferation.

The role of TNFR80 in signaling cell death is controversial. Antibodies to individual TNFR have clearly demonstrated that TNFR60 is sufficient to induce cell death. By contrast, anti-TNFR80 antibodies, although not directly cytotoxic, can antagonize the cytotoxic action of TNF. Furthermore, mutations in TNF have been engineered, creating a ligand that binds selectively to TNFR60. The TNFR60 selective muteins are fully functional in cytotoxic assays on human target cells [21], further supporting the position that TNFR80 plays an auxiliary role in cell death. Tartaglia et al. [31] hypothesize that the auxiliary function of TNFR80 is to cooperate in the binding of TNF to TNFR60 (the ligand-passing hypothesis). Their evidence indicates TNF binding to TNFR80 has a relatively fast off-rate that creates a locally high TNF concentration at the cell surface, this in turn facilitates binding to TNFR60, which has a slow dissociation rate, helping to stabilize the actively signaling ligand–receptor complex. An alternate hypothesis, suggested by Higuchi and Aggarwal [32], is that TNFR80 may be responsible for the DNA fragmenting activity associated with TNF-induced apoptosis. However, other studies have indicated that TNFR80 is also a direct signaling receptor, including the demonstration that antibodies to TNFR80 costimulate T-cell proliferation and granulocyte–macrophage colony-stimulating factor (GM-CSF) production in T cells [33]. Genetic deletions of these receptors have further demonstrated the separation of function between TNFR60 and TNFR80 in vivo. Despite this apparent divergence in function, both receptors appear to initiate pathways that converge at the level of activating NFκB [34].

Genetic deletions of members of the TNF/LT locus have revealed an unexpected role of the LTαβ heterotrimer in the development of the immune system. Mice lacking TNFR60 show normal T- and B-cell development but are unable to mount an effective defense against *Listeria monocytogenes,* while they are also resistant to systemic shock induced by endotoxin [35,36]. Mice with deleted TNFR80 gene exhibit less profound immune dysfunction in host defense [37], even though in vitro studies suggested a potential role of TNFR80 in T- and B-cell function. Since these two receptors bind both TNF and LTα homotrimers it was entirely unexpected that a knockout of the LTα gene would cause a developmental abnormality in the peripheral lymphoid system. De Togni et al. [15] found that the LTα knockout mice completely lacked all peripheral lymph nodes and gut-associated lymphoid tissues (Peyer's Patches) but were competent in mounting antibody and cellular immune responses, including allogeneic-specific CTL. This result implicates the LTαβ complex and LTβR as key figures in the development of the peripheral lymphoid tissue, with the prediction that deletion of the LTβ or LTβR genes will produce mice with a similar lymph node-deficient phenotype. However, one could invoke an as yet undefined, specific receptor for LTα as an alternate hypothesis.

RECEPTOR SIGNALING

At the surface of the target cell, the initiating event in receptor-mediated cytotoxic pathway is binding of multiple receptors to the TNF trimer, leading to a spatially close transmembrane cluster of cytoplasmic domains. Juxtaposition of the membrane-anchored ligands and receptors on opposing cells provides the proper orientation for engagement of receptor and ligand during cell to cell contact. Extensive data support the notion that clustering of receptors by TNF is the initiating event: (1) biochemical and crystallographic studies clearly show that TNF and LTα are multivalent; (2), bivalent antibodies to TNFR60 or Fas mimic the action of the ligands, whereas monovalent Fab fragments are inhibitory; and (3), selected mutations in TNFR60 cytoplasmic domain function as dominant negative mutants in the presence of wild-type receptors suggesting that receptor complexes must form in order to initiate signaling [38].

For the TNF-related receptors, clustering of cytoplasmic regions is thought to provide a binding site(s) for cytoplasmic proteins that serve as the effector molecules that activate signaling pathways. The events following ligand-receptor binding and downstream processes involved in signal transduction pathway(s) remain incompletely understood due in part to the uniqueness of the cytoplasmic domains, which exhibit little homology to other known signaling mechanisms. The lack of intrinsic enzyme activity in these receptors suggests that signaling is accomplished by proteins that are recruited or activated after assembly of the ligand-receptor com-

plex. Using protein purification and the two-hybrid cloning system, two novel TNFR associated factors, TRAF1 and 2, have been identified that bind to TNFR80 [34]. TRAF2 contains an N-terminal RING and zinc finger motifs followed by a coiled-coil and a C-terminal TRAF domain that is sufficient for binding to receptor (Fig. 2). Homology with TRAF1 is found in the C-terminal "TRAF" domain. TRAF2 binds directly to TNFR80 via the TRAF domain, whereas TRAF1 binds indirectly by forming a heterocomplex with TRAF2. TRAF2 is broadly expressed, whereas TRAF1 is restricted to spleen and thymus.

TRAF3 was discovered as a CD40 binding protein (also known as LAP-1, CRAF-1, CAP-1, or CD40bp) [39,40] and independently by its ability to bind LMP-1, the transforming protein of Epstein Barr virus [41]. TRAF3 also binds LTβR and TNFR80. TRAF3 is similar in overall structure to TRAF2 and is also expressed by many cell types. TRAFs are strongly linked to signal transduction function: TRAF3 by its association with LMP-1, a dominant oncogene of Epstein-Barr virus, and mutation of CD40 at threonine 234 blocks TRAF3 binding and inhibits the growth and differentiation regulating activity of this receptor [40]. By contrast, over expression of TRAF2, but not TRAF1 or 3, leads directly to activation of NFκB [46]. It is expected that the number of TRAF family members will increase, at least, to match the numbers of receptors.

Recently, a distinct family of proteins with a common motif have emerged that associate with TNFR60 or Fas [43]. The common motif is homologous to the ~80-residue region TNFR60 and Fas known as the death domain. This region allows for self-aggregation of the receptor and interaction with other proteins containing a death domain [44]. Three cytosolic proteins contain homologous death domain sequences. TRADD (TNFR-associated Death Domain) binds to TNFR60, whereas FADD-1 (or MORT-1) binds specifically to Fas. The third is RIP (receptor interacting protein). RIP has a serine/tyrosine kinase domain and can bind to either Fas or TNFR60 in the presence of either FADD or TRADD, respectively. Overexpression of FADD/RIP, or TRADD will induce apoptosis directly [42].

A serine kinase activity and four phosphoproteins associated with TNF–TNFR60 complexes in the U937 monocytic leukemia line were recently identified [45]. Activation of the serine kinase activity occurs rapidly and dependent upon the concentration of TNF with optimal effects observed at ~100 pM. Serine phosphorylated proteins of 125, 97, 85, and 60 kDa were identified by immunoprecipitation with either anti-TNFR60 or anti-TNF antibodies from cells labeled with phosphate or by in vitro kinase

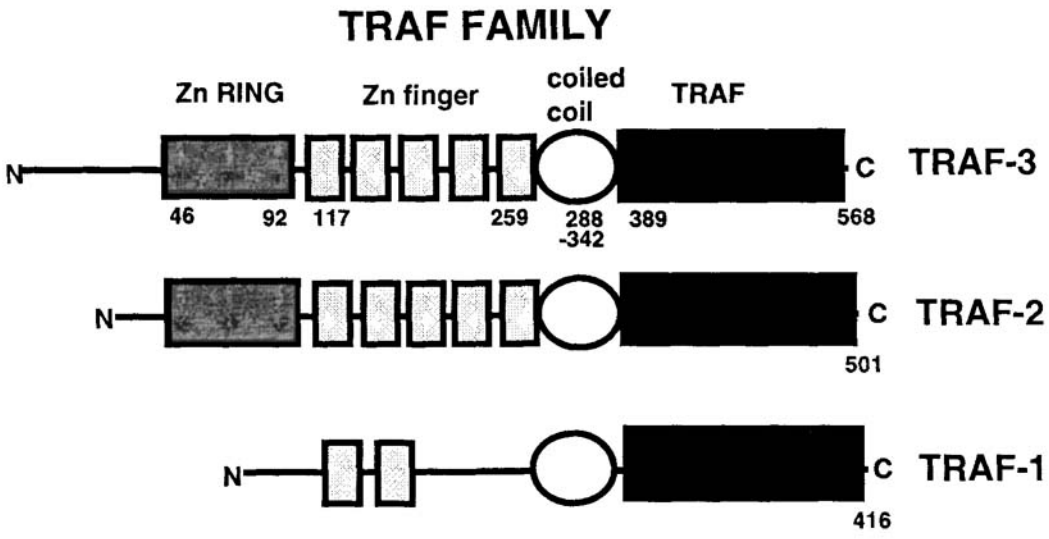

Fig. 2. TRAF family of signaling molecules. Human TRAF family of Zn RING finger proteins. For TRAF3, numbers mark the amino acid residues for each structural domain (see Mosialos et al. [41]). N, N-terminus; C, C-terminus.

assays with γ-$^{32}PO_4$-ATP as the phosphate donor. Only TNF treated cells showed the association between TNFR60 and serine kinase activity. The time course of kinase association as measured by phosphorylation was very rapid (1-min) and indicated that the 97-kDa substrate was recruited to the complex. Additionally, ^{35}S-methionine-labeled cells revealed additional unphosphorylated proteins associated with the TNFR60–ligand complexes. The TNFR60 associated kinase was inhibited by staurosporine, but not by protein kinase A or C inhibitors. Interestingly, staurosporine dramatically enhances the cytotoxic effect of TNF on the U937 cell line, suggesting the possibility that the TNFR60 serine kinase may be involved in signaling resistance to the cytotoxic action of TNF. It will be interesting to determine if TRADD is a substrate for this serine kinase and the relationship of RIP to the TNFR60-associated kinase.

The downstream processes initiated by ligand binding appears to involve classical and novel second messenger systems including GTP binding proteins, phospholipases, and various protein kinases [46]. A novel ceramide-generating pathway produced by TNF-dependent sphingomyelinases has recently been described [47]. Two types of sphingomyelinases with acidic and neutral pH optimums are activated via TNFR60. A proline-directed serine/threonine protein kinase and phospholipase A2 are activated by ceramide generated from the membrane-localized neutral sphingomyelinase. By contrast, NFκB is activated by the endosome-associated acidic sphingomyelinase. The two pathways are dependent on different regions of the TNFR60 cytoplasmic domains. Which enzyme is linked to apoptosis is unclear, since both generate ceramide indicating that compartmentalization of second messengers may be critical in the type of cellular response. Proteases related to ced-3 and ICE have been strongly implicated in Fas- and TNFR60-induced apoptosis [48].

Collectively, these results indicate that different receptors may use distinct sets of proteins to activate common downstream signaling pathways that lead to cell death. Connections to downstream pathways such as ceramide or ICE cysteine proteases will be important areas to explore. The Zn RING finger motif is found extensively in DNA binding proteins, such as transcription factors, enticing speculation of a direct link between the receptor to gene regulation. In addition, phosphatases such as FAP-1 provide negative regulation of apoptosis. What role these proteins play in signaling TNF responses remains to be elucidated. Determination of life or death pathways signaled by these receptors obviously depend on many factors in the intracellular environment, including the state of cellular differentiation and counteracting processes initiated by cellular parasites attempting to evade the immune response.

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