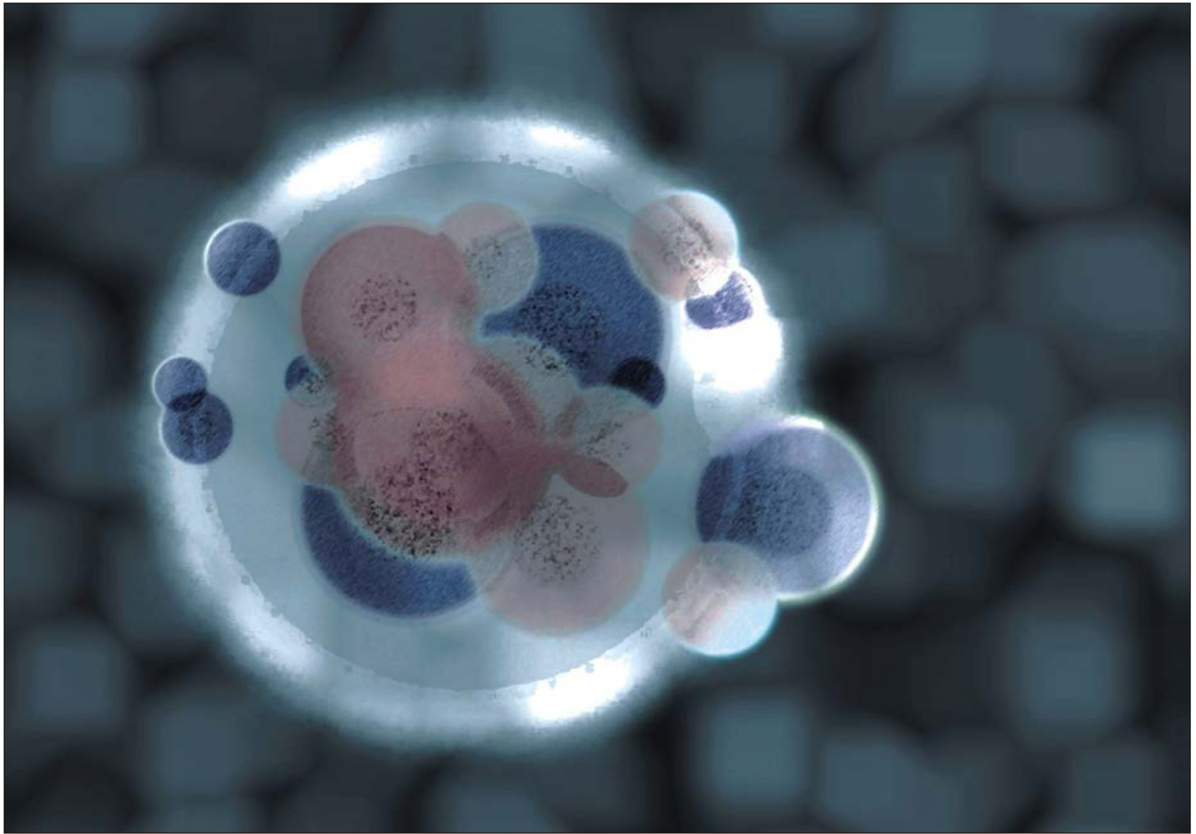

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Death Domain Interactions in Death Receptor Signaling

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Apoptosis is a form of programmed cell death that eliminates damaged, infected, or extraneous cells by the activation of a cell suicide program[1]. Induction of apoptosis is tightly regulated and disassembles the cell from within to avoid eliciting an inflammatory response. Apoptosis plays a critical role in the development and maintenance of multicellular orgamisms. In addition defective apoptosis is thought to be essential in the development of cancer[1] and is also involved in the genesis and treatment of other diseases[2]. For example, it is thought that cancer originates from cells receiving inappropriate growth signals as well as failing to receive or properly respond to appropriate signals to die while many cancer treatments work by inducing apoptosis. Therefore, there has been significant interest in the characterization of apoptotic pathways over the past 10-20 years.

There are two well-characterized pathways (and possibly several others) that regulate the induction of apoptosis[3]. Both of these pathways are depend upon a family of cysteine proteases termed caspases, which function to systematically dismantle the cell through their progressive proteolytic activity. Caspases fall into two main classes, initiator caspases and effector caspases. Initiator caspases can autoactivate and then subsequently cleave downstream substrates including effector caspases to activate the caspase cascade and cause the demise of the cell. The intrinsic apoptotic pathway is activated in response to cellular stress and relies upon disruption of the mitochondrion and release of cytochrome c. Upon release, cytochrome c promotes caspase 9 activation by forming a complex with APAF-1, procaspase 9 and dATP termed the apoptosome. In contrast, the extrinsic apoptotic pathway relies upon the engagement of a death receptor. There are currently 6 known death receptors including CD95 (FAS), TNF receptor 1 (TNFR-1), DR3/APO-3/TRAMP, TRAIL receptor 1/DR4, TRAIL-R2/DR5 and DR6. Death receptors are transmembrane receptors that activate apoptosis in addition to other signaling pathways in response to ligand binding. The activated receptor, which is a trimer[4], undergoes a conformational change facilitating the recruitment of proteins to the intracellular portion of the receptor, which is a death domain[5], a conserved approximately 80 amino acid domain that is characteristic of all members of the superfamily. The receptor death domain can bind to other death domain containing proteins to facilitate downstream signaling. While the initiator caspase for the intrinsic apoptotic pathway is thought to be caspase 9, the initiator caspase for the extrinsic apoptotic pathway is caspase 8 or sometimes a very similar caspase called caspase 10. While caspases 8 and 10 serve as the initiator caspases in response to death receptor activation, there is an amplification loop that is required for efficient death receptor induced death in certain cell types. This loop relies on cleavage of Bid[6], which translocates to the mitochondria to activate the intrinsic apoptotic pathway and caspase 9. Therefore although the two caspase activation pathways are separate, they can be linked in at least some cells via Bid. In both cases however the key regulatory event is the activation of caspase 8, which arises because of interactions between death domain-containing proteins. In this review, we have discussed what is known about how these interactions are regulated.

The death domain superfamily: The death domain superfamily is composed of a group of proteins possessing a Death Domain (DD) and two related domains called a Death Effector Domain (DED), or a Caspase Activation Recruitment Domain (CARD)[5]. These domains are highly related in that they are all composed of six antiparallel alpha helicies, but there are structural variations between domains within the family. For example the DD and the CARD rely on different helicies to

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facilitate interaction with other DD or CARD motifs. It has been shown that the CARD/CARD interactions of APAF-1 and procaspase 9 are mediated by helicies 2 and 3 of APAF-1, and helicies 1 and 4 of procaspase 9 which has been termed a type I interaction[7]. In contrast, DD/DD interactions between Pelle and Tube, two drosophila proteins important in degradation of the I κB homolog cactus rely on the loop regions between helices 1 and 2 and 4 and 5, as well as the loop between helices 5 and 6, which has been termed a type II interaction[8,9].

Death domains participate in homo and heterotypic interactions to promote downstream apoptotic signaling. The overall structure of death domains are similar however, the fold of the death domain varies and has been divided into two main types; subtype 1 and subtype 2. In subtype 1, helix 3 is antiparallel to helicies 2 and 4. In contrast, in subtype 2, helicies 2 and 4 are arranged parallel with one another, while helix 3 is nearly perpendicular to helicies 2 and 4.

DD-containing proteins propagate both pro-apoptotic and prosurvival signaling in response to ligand binding by binding to and recruiting other DD-containing proteins to multiprotein complexes. Signaling through death receptors requires an adaptor protein that is recruited to the receptor via its DD. The adaptor protein that is recruited to the receptor in response to Fas Ligand and TRAIL is FADD and recruitment of FADD to the Fas or TRAIL receptors (DR4 and DR5) is thought to be sufficient for the activation of caspase 8 and initiation of the apoptosis signal. FADD is also required for TNF dependent apoptosis, but another DD-containing protein, TRADD is recruited directly to the receptor. TRADD can then recruit either FADD to induce apoptosis or can recruit the DD- containing serine/threonine kinase Rip. Rip recruitment is required for Nuclear Factor κ B (NFκB) activation in response to TNF. As will be discussed later our ideas about these complexes have undergone recent revision and the situation now appears to be very complicated with different complexes in different locations having different effects. In addition, although we focus here on the activation of apoptosis through death domain interactions at death receptors, it should be remembered that death domain-containing proteins also have other effects. For example, the neurotrophin receptor p75 has a cytoplasmic death domain that resembles the TNFR1 death domain, but in contrast to TNFR1, Fas and the TRAIL receptors, apoptosis is activated not when the receptor binds to its ligand but instead when the ligand is withdrawn. The death domain of p75 has been classified into subtype 2 along with a number of other DD containing proteins including p100 NFκB, p105 NFκB, DAP kinase and myD88. Most of these proteins do not promote apoptosis upon overexpression and are often, though not always involved in inhibiting apoptosis. For example, both p100 and p105 are transcription factors of the Rel A family. Both proteins have a C-terminal DD, but only p100 has been implicated in the activation of apoptosis[10] and can activate a caspase 8-dependent apoptotic pathway and to sensitize cells to oncogenic RAS-induced apoptosis through as yet incompletely understood mechanisms.

The Fas death domain: The Fas death domain was the first death domain whose structure was solved using NMR and consists of six alpha helicies arranged antiparallel to one another[11]. Side chains of the hydrophobic residues form the core of the domain. Helicies 1 and 2 of the domain are located in the center while helicies 3 and 4 are on one side and 5 and 6 lie on the opposite side. When the structure was solved, the presence of a large number of charged residues on the surface of the domain suggested that electrostatic interactions mediate DD-DD interactions[11]. It was shown using deletion and mutagenic analysis that regions of the first cysteine rich domain of FAS led to disruption of receptor-receptor interaction and inhibited apoptotic induction in response to ligand[12]. Mutations in this region were also identified in patients with ALPS (Autoimmune lymphoproliferative syndrome) who are highly susceptible to lymphomas suggesting that Fas-mediated cell death achieved through interactions via these regions of the DD serves to remove the tumor cells. In addition, the role of FAS-induced death in the control of lymphocyte proliferation was identified by mutations in Fas in the lpr mouse[13]. This mouse strain serves as a model of human lupus as the mice develop similar symptoms with increasing age again suggesting that Fas signaling is important in controlling excess cell growth. In these mice a single point mutation (V238N) in the Fas DD is responsible for the phenotype. The V238N mutation greatly reduced self-association and binding to FADD compared to the wild-type protein. In addition, residues in the loop between helices 2 and 3 and within helix 3 blocked self-association.

The FADD death domain: The adaptor protein FADD is required for apoptotic signaling downstream of nearly all death receptors. The death domain of FADD is the most extensively studied of the DD-containing proteins. Like Fas, the FADD DD is composed of six alpha helicies arranged antiparallel to one another[14]. Again, helices 1 and 2 form the central portion of the domain, although in contrast to the Fas death domain, helices 3 and 6 are

located on one side while 4 and 5 are located on the other. Helix 6 is tightly packed against the interlocking helices while helix 3 is more loosely associated. Helix 2 is composed of primarily positively charged residues while helix 3 is largely negatively charged. Helix 1 and helix 6 are also negatively charged.

Extensive site directed mutagenesis has been performed on the FADD-DD. Initial characterization of important residues for mediating FADD-Fas interaction were charged residues localized primarily in helices 2 and 3. It was also suggested that the flexibility of helix 3 maximized contacts between interacting amino acids of helices 2 and 3[14]. However, more recent experiments have challenged these ideas and revealed that an expanded surface of the FADD-DD is actually important in binding to Fas. While the new data supports the previous view that helices 2 and 3 are required for the interaction, the new data revealed that residues in helices 1, 2, 3, 5 and 6 are also required for binding to Fas[15]. More surprisingly, in addition to the expansion of the binding surface to include more of the DD, data has recently been generated that indicates that residues within the DED of FADD (an entirely different domain of the protein) are also important in mediating the Fas-FADD interactions[16]. Using a modified reverse two-hybrid screen designed to identify random mutations in FADD that lost the ability to bind to Fas but retained binding to TRADD, it was found that mutations in residues that flank helix 5 of the DED abrogated Fas but not TRADD interaction. This established for the first time that the two domains do not function independently as was initially thought and that there is cooperativity in binding between the two domains.

FADD is also required for pro-apoptotic signaling in response to TNF Related Apoptosis Inducing Ligand (TRAIL). Again, FADD recruitment to the TRAIL receptors DR4 and DR5 was thought to be mediated by DD interactions as it has been shown that a dominant negative mutant of FADD that retains the DD but lacks the DED (DN-FADD) can be recruited to activated TRAIL receptors to inhibit TRAIL-induced death[17]. However, as is the case with Fas-FADD binding, more recent work has shown that regions in the DED are important for FADD-DR5 binding[18]. In fact the same DED-mutations that abrogated FADD-Fas binding also inhibit FADD-DR5 binding. Compensating mutations were then made to identify second site mutations that would restore binding and these mutations were also in the DED of FADD further establishing that the DED and the DD function in a cooperative manner to promote protein-protein interactions[18]. Together, these data show us that while the FADD DD is essential and, in the context of overexpression of the truncated DN-FADD molecule, sufficient for binding to the DDs of activated Fas or TRAIL receptors, the original view that the interaction occurs solely between the two death domains is not correct.

The TNFR1 death domain: The solution structure of the death domain of TNFR1 has been solved[19] in the context of a point mutant that inhibited self-association of the domain. Using TNFR1 R347A or R347K, which lies in helix 2, it has been shown that it too is composed of six alpha helicies and is structurally similar to the other death domains. However, the orientation of helicies 2, 3 and 4 are different than is found with Fas or FADD. While helices 2 and 4 are arranged parallel with one another, helix 3 is nearly perpendicular to helicies 2 and 4. In contrast, helix 3 is antiparallel to helicies 2 and 4 in the FADD and Fas death domains. The same orientation is seen in the neurotrophin receptor death domain. Other differences are also present. For example, helix 4 is longer in TNFR1 than in Fas or FADD, while helix 1 is considerably shorter than that of the other mammalian death domains. It has been shown the helices 2, 3 and 4 of TNFR1 are important for mediating both self-association and binding to TRADD[20]. Consistent with other death domains, data with the TNFR1 death domain indicated that homodimerization is inhibited at increasing salt concentrations supporting the idea that electrostatic interactions mediate self association as well as association with TRADD. In contrast to Fas death domain interactions where as discussed above, residues in all of the helicies mediate interactions, with the TNFR1 death domain helicies 2,3, and 4 are important in TNFR1-TRADD and TNFR1-TNFR1 interactions, but only the beginning of helix 3 is thought to be required for self-association and TRADD binding.

The TRADD death domain: The TNFR1 adaptor protein TRADD mediates signaling events downstream of the TNFR1 by binding to several death domain-containing proteins. Binding of TRADD to FADD or Rip promotes either apoptosis or NF□B activation in response to TNF. TRADD binds to FADD to promote caspase 8 dependent apoptosis. Based on our understanding of Fas signaling, this was originally thought to occur at the receptor- i.e. it was thought that the TNFR1 DD would bind TRADD (through its own DD) and that the TRADD DD would simultaneously interact with FADD through its DD. We now know that this idea is wrong. In fact the pro-apoptotic signaling events in response to TNFR1 stimulation occur in a cytoplasmic complex that contains TRADD, FADD and caspase-8 as well as RIP but is not associated with the receptor[21].

It has been suggested based on site directed mutagenesis studies that the binding surface required for FADD-Fas interaction is the same as that required for

FADD-TRADD binding[22]. These experiments suggested that helix 2 and helix 3 were key determinants of the interaction. However this work was performed with murine FADD and human TRADD and subtle differences between species could have caused important determinants of the interaction to be missed. Our understanding of FADD-TRADD binding is not nearly as comprehensive as that obtained studying FADD-Fas interactions and, unlike either Fas or TRAIL receptors there is as yet no evidence for a role of the FADD DED in the interaction with TRADD. Moreover, the structure of the TRADD death domain has been studied to a much lower resolution compared to the other death domain-containing proteins and all we really know is that as expected it is primarily alpha helical[23]. Alanine scanning mutagenesis of the death domain of TRADD has however been performed[24]. Through this work, it was suggested that the entire death domain probably mediates interaction with both the TNFR1 and FADD. It was not possible to subdivide the death domain into regions required for NFκB activation and regions required for induction of apoptosis suggesting that the interactions required for Rip and FADD binding may overlap[24]. To add to the complexity, because it is thought that a multimer of TRADD is required for interaction with the TNFR1, self-association of TRADD must also occur and the receptor bound complex probably involves simultaneous TRADD-TRADD, TRADD-TNFR1 and TRADD-RIP interactions while the pro-apoptotic cytoplasmic complex presumably involves combinations of TRADD-TRADD, TRADD-FADD and TRADD-RIP interactions. Since we do not know the stoichiometry of all these interactions some of them could be exclusive and some could occur simultaneously with other interactions.

Recent data from our lab adds an even greater level of complexity to the picture. Morgn *et al.*[25] found that TRADD is a nuclear shuttling protein suggesting that it might also bind to nuclear proteins. Moreover, we showed that an apoptotic pathway can activated by the accumulation of nuclear TRADD suggesting that these nuclear protein-protein interactions might promote apoptosis. Using a truncated protein that lacks the nuclear export signal but contains the DD and the nuclear localization signal, we showed that a TRADD molecule that is exclusively in the nucleus is a very effective inducer of apoptosis. However, the apoptotic pathway activated by expression of nuclear TRADD is not dependent upon interaction with FADD or activation of caspase 8 as is the case for death induced by the cytoplasmic TRADD-FADD-caspase-8 complex. Instead death induced by the nuclear protein involves the mitochondrial dependent apoptotic pathway and requires caspase 9 expression and catalytic activity (L.M.B. *et al.*, unpublished data). These data have led us to suggest that different protein-protein interactions mediate the cytoplasmic and nuclear apoptotic pathways activated by the adaptor protein TRADD. Thus our current picture of TRADD signaling is that TRADD can participate in at least three distinct complexes through its DD. In the first, which occurs at the cell membrane in association with the activated TNFR1 receptor, TRADD interacts with TNFR1, TRAF proteins and RIP and this leads primarily to anti-apoptotic signaling. After TNFR1 stimulation a second cytoplasmic complex involving TRADD interactions with FADD, caspase 8 and RIP leads to caspase 8-dependent apoptosis. Finally a third, nuclear complex that does not involve FADD or caspase 8 can also lead to apoptosis but this occurs through caspase 9.

Summary : Although it is now well established that death domain interactions are key regulators of apoptosis, there is still a long way to go before we completely understand how these interactions are regulated. The most extensively characterized of the death domain containing proteins in terms of protein-protein interactions is the FADD-DD. But as we learn more about the interactions of this protein the situation becomes more and more complicated. For example, studies initially characterized the important residues mediating FADD/Fas binding as lying in helicies 2 and 3 but recently the binding surface for has been extended to include regions within the DED as well as the loop between helicies 5 and 6 of the DD itself. These same regions have been shown to be important in binding to DR5 but different regions may be involved in binding to TRADD and TRADD itself can be involved in completely different apoptotic pathways that do not even involve FADD. Further structural and mutational analysis of these interactions will hopefully allow us to make better sense of these interactions and hopefully reach a stage where we can begin to consider adopting ways to modulate the interactions in a controlled way. When we reach this stage we hope to use this information design new therapeutic strategies to manipulate these responses. Since interactions between death domains are implicated in the development and treatment of cancer and other diseases, this could have important repercussions for human health.

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