Topic Introduction

Biochemical Analysis of Initiator Caspase-Activating Complexes: The Apoptosome and the Death-Inducing Signaling Complex

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Apoptosis is a highly regulated process that can be initiated by activation of death receptors or perturbation of mitochondria causing the release of apoptogenic proteins. This results in the activation of caspases, which are responsible for many of the biochemical and morphological changes associated with apoptosis. Caspases are normally inactive and require activation in a cascade emanating from an “initiator” or activating caspase, which in turn activates a downstream or “effector” caspase. Activation of initiator caspases is tightly regulated and requires the assembly of caspase-9 (via mitochondrial perturbation) or caspase-8/10 (via death receptor ligation) activating complexes, which are termed the apoptosome and the death-inducing signaling complex (DISC), respectively. These large multiprotein complexes can initially be separated according to size by gel filtration chromatography and subsequently analyzed by affinity purification or immunoprecipitation. The advantage of combining these techniques is one can first assess the assembly of individual components into a multiprotein complex, and then assess the size and composition of the native functional signaling platform within a particular cell type alongside a biochemical analysis of the enriched/purified complex. Here, we describe various methods currently used for characterization of the apoptosome and DISC.

Many key biological processes, including apoptosis, are initiated from or performed in large multiprotein complexes. Apoptosis signaling complexes that can initiate cell death include the apoptosome and the death-inducing signaling complex (DISC) (Bratton et al. 2000; Cain et al. 2002; MacFarlane 2003; Dickens et al. 2012b). Typically, apoptosis is triggered through activation of either the intrinsic (mitochondrial) or extrinsic (death receptor) pathway. Central for activation of the intrinsic pathway is the cytosolic Apaf-1/Caspase-9 apoptosome, a >700–1000-kDa complex formed following release of cytochrome *c* from mitochondria (Cain et al. 2002). In contrast, the extrinsic pathway is triggered by formation of the DISC at the plasma membrane; in this case, ligation of the death receptors CD95, TRAIL-R1, or TRAIL-R2 by their cognate ligands results in recruitment of the adaptor molecule FADD, the initiator caspase procaspase-8, and additional modulator proteins such as cFLIP (Dickens et al. 2012b).

It is now increasingly evident that the composition and stoichiometry of components within key cell death signaling platforms can determine not only the final signaling outcome but also the mode of cell death. By analyzing these complexes, we can learn how cell death is regulated as well as how key cell death signaling

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platforms like the apoptosome and DISC might be targeted for therapeutic benefit (MacFarlane 2009; Cain 2010). The successful application of a range of methodologies which couple characterization of complex assembly together with subsequent purification and biochemical analysis can therefore provide novel insights into how cell death signaling platforms are regulated in both normal cell physiology and disease.

Large multiprotein complexes such as the apoptosome and DISC can be separated according to their size by gel filtration chromatography and further purified by subsequent affinity purification or immunoprecipitation. By combining these methods, an indication of the size of the complex as well as the recruitment of individual components associated with the active complex can be determined, thus providing more precise and selective information on the complex itself. This combined approach has been used to purify and characterize the active apoptosome complex (Cain et al. 1999, 2000; Twiddy et al. 2006) and, more recently, we and others have used gel filtration to identify and characterize the ripoptosome (Feoktistova et al. 2011; Tenev et al. 2011). Intriguingly, this complex, depending on its protein composition, can switch between apoptotic and necrotic modes of cell death. Similarly, the complementary approach of sucrose density gradient centrifugation has been combined with immunoprecipitation/affinity purification to characterize both the active apoptosome complex, and, more recently, the native TRAIL DISC using mass spectrometry (Twiddy et al. 2004; Dickens et al. 2012a; Hughes et al. 2013). Indeed, affinity purification of the DISC using biotin-labeled ligands not only provided novel insights into the mechanisms that regulate death receptor signaling in diverse cell types and upon different treatment regimens (Harper et al. 2001, 2003a, 2003b; MacFarlane et al. 2002, 2005; Harper and MacFarlane 2008; Robinson et al. 2012), but also led us to propose a death effector domain (DED) chain DISC model and a crucial role for caspase-8 chain assembly in mediating apoptotic cell death (Dickens et al. 2012a).

ANALYZING THE APOPTOSOME AND DISC

In the accompanying protocols, we describe the various techniques and strategies we have developed over several years to successfully isolate and characterize two key initiator caspase-activating complexes, namely the apoptosome and DISC. The dATP-activation of caspases in cellular lysates has been used for many years as an *in vitro* model system for assembling the apoptosome complex and studying its characteristics, components, and activity (Cain et al. 1999, 2000, 2001; Beere et al. 2000; Freathy et al. 2000; Almond et al. 2001; Bratton et al. 2001; Thompson et al. 2001; Lademann et al. 2003; Twiddy et al. 2004, 2006; Hughes et al. 2013). The basis of the model, detailed in Protocol: **In Vitro Assembly and Analysis of the Apoptosome Complex** (Langlais et al. 2015), is production of a cell-free extract from a cell line of choice (e.g., the human monocytic tumor cell line THP.1) in which caspases can be processed and activated *in vitro* by treatment with dATP/MgCl₂ and cytochrome *c* (Liu et al. 1996; Li et al. 1997). As an alternative to apoptosome assembly based on cell lysates, a full *in vitro* reconstitution of the apoptosome is also feasible (Cain et al. 2001; Zou et al. 2003). In both cases, apoptosome assembly and caspase-dependent cleavage are analyzed using gel filtration in conjunction with western blotting and fluorimetric assays for effector caspase activity.

Our Protocol: **Activation, Isolation, and Analysis of the Death-Inducing Signaling Complex** (Hughes et al. 2015) is based on affinity purification using biotin-labeled antibodies and streptavidin beads for activation and capture of the native DISC complex. Triggering of receptor aggregation by the agonistic anti-CD95 antibodies (or the cognate CD95 ligand) leads to recruitment of the bipartite adaptor molecule FADD, which in turn binds the initiator caspase, procaspase-8, through its amino-terminal DED motifs (Peter and Krammer 2003; Dickens et al. 2012a; Schleich et al. 2012). On binding to FADD, procaspase-8 is activated through proximity-induced dimerization of adjacent procaspase-8 molecules, leading to proteolytic cleavage and maximal caspase-8 activation (Muzio et al. 1998; Boatright et al. 2003; Hughes et al. 2009). Activation of caspase-8 at the native DISC leads to cleavage and activation of downstream substrates, such as procaspase-3 and BID, amplification of the caspase cascade and subsequent apoptosis. Composition of the isolated DISC and caspase activation can be analyzed via western blot and fluorimetric assays for caspase-dependent cleavage.

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