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Isolation, characterisation and reconstitution of cell death signalling complexes

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ABSTRACT

Apoptosis and necroptosis are dependent on the formation/activation of distinct multi-protein complexes; these include the Death-Inducing Signalling Complex (DISC), apoptosome, piddosome, necrosome and ripoptosome. Despite intense research, the mechanisms that regulate assembly/function of several of these cell death signalling platforms remain to be elucidated. It is now increasingly evident that the composition and stoichiometry of components within these key signalling platforms not only determines the final signalling outcome but also the mode of cell death. Characterising these complexes can therefore provide new insights into how cell death is regulated and also how these cell death signalling platforms could potentially be targeted in the context of disease. Large multi-protein complexes can initially be separated according to their size by gel filtration or sucrose density gradient centrifugation followed by subsequent affinity-purification or immunoprecipitation. The advantage of combining these techniques is that you can assess the assembly of individual components into a complex and then assess the size and stoichiometric composition of the native functional signalling complex within a particular cell type. This, alongside reconstitution of a complex from its individual core components can therefore provide new insight into the mechanisms that regulate assembly/function of key multi-protein signalling complexes. Here, we describe the successful application of a range of methodologies that can be used to characterise the assembly of large multi-protein complexes such as the apoptosome, DISC and ripoptosome. Together with their subsequent purification and/or reconstitution, these approaches can provide novel insights into how cell death signalling platforms are regulated in both normal cell physiology and disease.
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1. Introduction

Many of the key signalling events which take place within the cell are initiated from, or carried out in, large multi-protein complexes. The well-known cell death mechanisms of apoptosis and necrosis are no exception and signalling complexes which can ultimately result in cellular demise include the apoptosome, Death Inducing Signalling Complex (DISC), necrosome and ripoptosome [1–3]. Typically, apoptosis is initiated through activation of either the intrinsic (mitochondrial) or extrinsic (death receptor) pathway. Central for activation of the intrinsic pathway is the Apaf-1/Caspase-9 apoptosome, a > 700 kDa complex formed following release of cytochrome *c* from mitochondria. By contrast, the extrinsic pathway is triggered by formation of the DISC; 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 apical caspase, procaspase-8, and additional modulator proteins such as c-FLIP. Although necrosis had long been considered to occur in a non-regulated fashion, the discovery of a programmed form of necrosis (necroptosis) led to the identification of the RIP1/RIP3/FADD/Caspase-8 necrosome complex which, under conditions where apoptosis is blocked, drives necroptosis downstream of death receptors. More recently an additional necroptosis-inducing complex termed the ripoptosome was identified. Intriguingly, unlike the necrosome, the ripoptosome is a ~2 MDa signalling platform which, depending on the balance of expression of c-FLIP isoforms, can switch between apoptotic and necroptotic cell death (reviewed in [4]).

It is now increasingly evident that the composition and stoichiometry of components within key cell death signalling platforms not only determines the final signalling outcome but also the mode of cell death. By analysing these complexes we can learn not only how cell death is regulated but also explore how cell death signalling platforms could potentially be targeted for therapeutic benefit. The successful application of a range of methodologies to enable characterisation of the assembly of large multi-protein complexes together with their subsequent purification and/or reconstitution can therefore provide novel insights into how cell death signalling platforms are regulated in both normal cell physiology and disease.

Large multi-protein complexes such as the DISC, apoptosome, and ripoptosome can be separated according to their size by gel filtration or sucrose density gradient centrifugation and further purified by subsequent affinity-purification or immunoprecipitation. By combining these methods, an indication of the size of

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the complex and the recruitment of individual constituents associated with the active complex can be determined, thus providing more precise and selective information on the complex itself. For example, this approach has been used to purify and characterise the active apoptosome complex [5,6] and more recently, we and others have used gel filtration to identify and characterise the ripoptosome [7,8]. Similarly, the complementary approach of sucrose density gradient centrifugation has been combined with immunoprecipitation/affinity purification to characterise using mass spectrometry both the active apoptosome complex and more recently the native TRAIL DISC [9,10]. Indeed, affinity purification of the DISC using biotin-labelled ligands [11], in tandem with DISC reconstitutions using tagged death receptors in cell lysates, not only provided novel insight into the mechanisms that regulate death receptor signalling in diverse cell types/upon different treatment regimes [12–14] but led to the identification of a DED chain assembly model and two-step activation mechanism for caspase-8 at the DISC [10,15].

Here, we outline the techniques and strategies that can be used to isolate, characterise and reconstitute cell death signalling complexes as illustrated in the above examples (Fig. 1), and where possible describe how the methods may be adapted according to the application.

2. Affinity purification of the TRAIL death-inducing signalling complex

In order to isolate and functionally characterise multi-protein cell death signalling complexes, technologies are required that enable purification of these complexes from different cellular paradigms. Methods that have been successfully employed to isolate

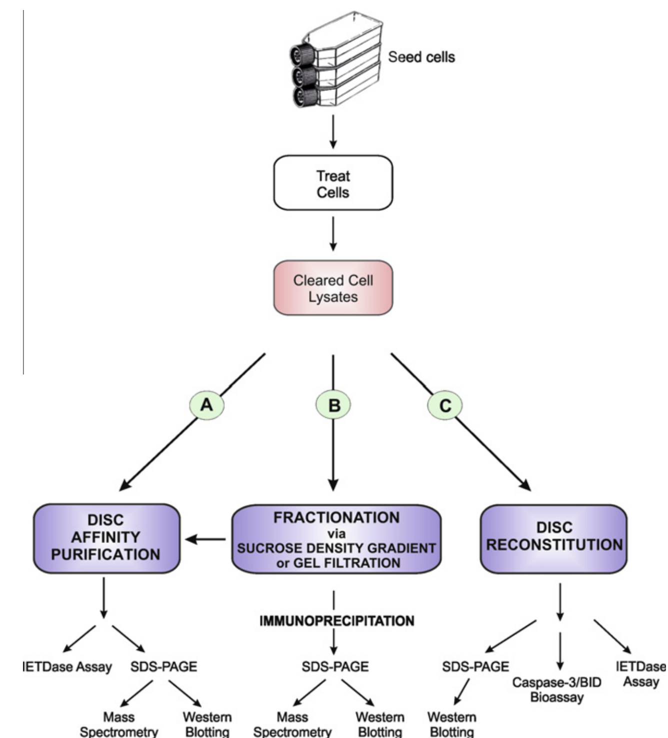

Fig. 1. Protocols for purification and characterisation of cell death signalling complexes. Cleared cell lysates from appropriately treated cells can be analysed by: (A) DISC affinity purification using biotin-labelled ligands; (B) size fractionation via gel filtration or sucrose density gradient centrifugation followed by purification of the complex via affinity pull-down or immunoprecipitation; (C) DISC reconstitution using GST-tagged intracellular domains of death receptors. Potential downstream applications are also summarized.

cell death signalling complexes such as the apoptosome, the DISC, and the ripoptosome, include immunoprecipitation and affinity purification. Immunoprecipitation relies on the use of an antibody that recognises the native form of a core component of the complex, whereas affinity purification instead takes advantage of the use of affinity labelled proteins to essentially act as 'bait' to isolate the complex of interest. Thus, for example, the apoptosome which is a soluble complex was successfully purified from cells using either an antibody to caspase-9 or a GST-tagged form of the apoptosome binding protein and key apoptosis regulator, XIAP [9]. More recently, the ripoptosome (another soluble complex) was initially isolated by immunoprecipitation using an antibody to one of its key components, namely caspase-8 [7,8] (see Section 3.1).

In the case of the DISC, which is a receptor-bound cell surface complex, we have successfully employed affinity purification *via* either biotin-labelled TRAIL or Strep-II-tagged TRAIL to isolate and characterise the TRAIL DISC from a range of transformed cell lines as well as primary tumour cells [10–13,16]. This approach relies on the ability of recombinant soluble TRAIL to bind the TRAIL death receptors, TRAIL-R1/TRAIL-R2 thereby triggering receptor aggregation and DISC formation *via* death-domain mediated recruitment of the bipartite adaptor molecule FADD. FADD in turn binds the apical procaspase-8 through its death-effector domain motifs. On binding to FADD, caspase-8 is activated through proximity-induced dimerization of adjacent procaspase-8 molecules which in turn leads to proteolytic cleavage and maximal caspase-8 activation [15,17,18]. Importantly, we and others have shown that analysis of TRAIL-R cell surface expression alone, in most cases, gives little/no indication of TRAIL sensitivity, thus the ability to isolate and characterise functional TRAIL-R signalling platforms can give new insight into those factors that might regulate sensitivity to TRAIL in a particular cell type.

DISC affinity purification can be performed directly on cleared cell lysates or TRAIL/TRAIL-R complexes can be initially characterised either by size exclusion chromatography or sucrose density gradient centrifugation (see Section 3.1) prior to affinity purification/DISC isolation (Fig. 2A). In this way, assembly of the TRAIL DISC from its individual components can be assessed in parallel with analysis of both its size and protein composition. Recently, this approach was successfully employed to purify the TRAIL DISC from haematopoetic tumour cells and when applied in combination with mass spectrometry led to the identification of a new stoichiometry for the TRAIL DISC [10].

2.1. Methodology

Fig. 2A summarises the main steps involved in affinity purification of the TRAIL DISC. Biotin-labelled TRAIL or Step-II-tagged TRAIL are generated as previously described [10,11]. Although the number of cells required for DISC analysis is dependent on cell type, in general $\sim 50 \times 10^6$ cells (e.g. BJAB cells at a concentration of 5×10^6/ml) are sufficient for each DISC isolation. Cells are initially treated with biotin-labelled/Strep-II-tagged TRAIL (500 ng/ml) at 4 °C (optional step to facilitate 'loading' of TRAIL-Rs with TRAIL) followed by the required time period at 37 °C (for optimal TRAIL DISC formation) and then immediately washed 2 times with ice-cold PBS. Cells are then lysed (3.5 ml lysis buffer/50×10^6 cells) for 30 min on ice in DISC lysis buffer (30 mM Tris/HCl (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100) containing Complete™ protease inhibitors (Roche, Sussex UK). Cleared lysates are subsequently generated by centrifugation at 15,000*g* for 30 min at 4 °C. Biotin-labelled/Strep-II-tagged TRAIL-bound complexes are precipitated by incubation of cleared cell lysates with 50 µl streptavidin–Sepharose™/Streptactin beads (magnetic M-280 streptavidin Dynabeads®) for 17 h at 4 °C. Beads are then washed with several volumes of fresh DISC lysis buffer, containing

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