COMMENTARY

The flip side of FLIP

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Two major pathways regulate apoptosis induction in mammalian cells. In the extrinsic pathway, apoptosis is induced through specialized surface receptors, whereas in the intrinsic pathway, apoptosis is induced from within the cell, mainly through activation of mitochondria. Shortly after the major mediators of the extrinsic apoptosis pathway, the initiator caspases-8 and -10, were identified, c-FLIP [FLICE-like inhibitory protein; FLICE is FADD (Fas-associated death domain protein)-like interleukin-1 β -converting enzyme], a catalytically inactive caspase-8/-10 homologue, was reported. Whether this structure acts as an inhibitor or promoter of the extrinsic apoptosis pathway has been the subject of much debate. In this issue of the *Biochemical Journal*, Boatright

et al. provide further evidence for the long splice form of c-FLIP (c-FLIP_L) being an activator of caspase-8/-10, and demonstrate that the resulting heterodimer is enzymically active with a substrate specificity identical with that of the caspase-8 homodimer. Our understanding of the regulators of the extrinsic apoptosis signalling pathway biochemically may provide the means to design drugs to correct the imbalance between apoptosis and proliferation, as found in many diseases.

Key words: apoptosis, caspase-8, death-inducing signalling complex (DISC), induced proximity, proliferation.

Apoptosis is a cellular response that regulates important processes such as tissue homeostasis, defence against certain pathogens and elimination of unwanted cells. In recent years, it has become clear that most, but not all, forms of apoptosis involve activation of a class of cysteine proteases, the caspases. All caspases contain at least three structural domains, an N-terminal prodomain and two active subunits with full activation involving proteolytic cleavage of the inactive zymogens. Caspases are activated in a hierarchical order. Initiator caspases cleave effector caspases which in turn degrade a number of intracellular protein substrates causing the typical biochemical responses of apoptosing cells. Two major signalling pathways ensure proper execution of apoptosis: the 'extrinsic pathway', which is initiated by surface structures of the death receptor family such as CD95, and the 'intrinsic pathway', which relies on mitochondrial activity through the release of cytochrome *c* and other mediators, causing the activation of downstream caspases. Specialized initiator caspases are located on top of both apoptosis signalling cascades. Caspases-8 and -10 are mediators of the extrinsic pathway, and caspase-9 initiates the intrinsic pathway. Both groups of initiator caspases carry distinct, but related, homophilic protein–protein interaction domains in their prodomains, and caspases-8 and -10 contain a tandem motif of DEDs (death effector domains), whereas caspase-9 contains a CARD (caspase-recruitment domain). In contrast, effector caspases, such as caspases-3 and -7, contain very small prodomains, and the removal of these domains results in their activation. Triggering of death receptors results in the recruitment of adaptor proteins such as FADD (Fas-associated death domain) and TRADD (tumour-necrosis-factor-receptor-associated death domain) which then recruit the initiator caspases-8 and -10 to form the DISC (death-inducing signalling complex). This recruitment step was initially believed to cause a local increase in initiator caspases that have intrinsic catalytic activity, causing proteolytic cleavage and activation of these caspases through a process widely referred to as 'induced proximity' [1].

Apoptosis is a highly regulated process, and various inhibitors of this process are known to interfere with many steps of the ex-

trinsic or the intrinsic pathway. Since various diseases are characterized by de-regulated apoptosis through death receptors, interest in identifying cellular inhibitors that were responsible for blocking the extrinsic pathway was very high. Shortly after the identification of caspase-8 and caspase-10, a structurally related protein was found through its homology with the prodomain of caspase-8/-10. This gene was cloned independently by nine different groups and therefore originally had eight different names [2]. I will refer to it as c-FLIP, for cellular FLICE inhibitory protein [FLICE (FADD-like interleukin-1 β -converting enzyme) was one of the original names for caspase-8]. c-FLIP comes in two variants, c-FLIP_s only contains two N-terminal DEDs very similar to the prodomains of caspase-8/-10. In contrast, the long splice form of c-FLIP (c-FLIP_L) is identical in length with caspase-8, also contains N-terminal tandem DEDs, but its caspase domain is altered, rendering it enzymically inactive. c-FLIP_L therefore has the classical structure of a dominant-negative inhibitor: an intact upstream protein–protein interaction domain linked to an inactive downstream enzymic domain. The idea of c-FLIP_L being an inhibitor of the extrinsic pathway was quickly accepted and supported by most data, and more notably by data on MEFs (mouse embryonic fibroblasts) from mice deficient for c-FLIP. c-FLIP^{-/-} MEFs are more sensitive to death-receptor-induced apoptosis [3].

However, c-FLIP_L seemed to have another side to it. First, it was expressed in virtually all cells irrespective of their sensitivity to death-receptor-mediated apoptosis. Secondly, the ratio of 1:100 between c-FLIP_L and procaspase-8 in cells seemed to be inconsistent with c-FLIP_L being an inhibitor of caspase-8. Thirdly, overexpression of c-FLIP_L in HEK-293 cells induced apoptosis probably through activation of caspase-8.

Recently, two reports demonstrated that c-FLIP_L is not only an inhibitor of apoptosis, but also an activator of procaspase-8. Using a designed dimerization system *in vitro* and *in vivo*, Chang et al. [4] established that the caspase-like domain of c-FLIP_L can activate caspase-8 through heterodimerization. Furthermore, controlled overexpression of c-FLIP_L, either transiently at different concentrations, or stably, in an inducible system, either sensitized cells

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Figure 1 Model of the different functions of c-FLIP_L

Shown is the CD95 DISC at different concentrations of c-FLIP_L. In the absence of c-FLIP_L (no FLIP), both procaspase-8 (C8) and procaspase-10 (C10) are recruited to the DISC through binding to the adaptor molecule FADD. This recruitment causes processing and activation of the initiator caspases through homodimerization, release of the active enzymes (heterotetrameric structures), subsequent cleavage of various intracellular caspase substrates and apoptosis. When c-FLIP_L is expressed at low levels (low FLIP), activation of caspase-8/-10 is accelerated due to the ability of c-FLIP_L to associate with caspase-8/-10 and its activity to form heterodimers more efficiently than caspases-8/-10 to form homodimers. At high concentrations of c-FLIP_L, caspase-8/-10 are still activated, but are not released any longer from the DISC. According to the model, DISC-tethered caspase-8/-10 has the same substrate specificity as active caspase subunits released into the cytosol. However, owing to their DISC-proximal location, these incompletely processed, but fully active, caspases cleave a different set of substrates such as themselves, RIP and c-FLIP_L. These cleavage events may be important in regulating apoptosis-independent processes such as proliferation. The inactive active site in the caspase domain of c-FLIP_L is labelled X.

to CD95-mediated apoptosis (at low c-FLIP_L expression levels) or blocked it (at high c-FLIP_L expression levels). By using stable c-FLIP transfectants, Micheau et al. [5] confirmed that c-FLIP_L promoted the initial processing step of caspase-8 in the DISC, but they concluded that this prevents the release of active caspase-8 subunits and apoptosis [5], allowing only DISC-proximal caspase-8 substrates to be cleaved, such as the RIP (receptor-interacting protein). These data raised the question of whether DISC-bound caspase-8 activated by c-FLIP_L had a different substrate specificity than the mature fully processed enzyme released from the DISC.

In this issue of the *Biochemical Journal*, Boatright et al. [6] have reported their detailed analysis of the enzymic properties of the c-FLIP_L caspase-8 heterodimer. They found that c-FLIP_L can induce the activity of caspase-8 regardless of whether the DED prodomain is present or not. Interestingly, heterodimer formation was favoured over caspase-8 homodimer formation, consistent with the finding that, despite its low expression levels in the cytosol, c-FLIP_L concentrations are much higher in the DISC [4]. For the first time, the enzymic activity of this heterodimer is demonstrated directly. Interestingly, its enzymic properties are similar to those of the caspase-8 homodimer. Using a positional scanning synthetic combinatorial library, Boatright et al. [6] go on to demonstrate that also the substrate specificity of the two enzymic structures is very similar, yet not identical. Whereas the caspase-8 homodimer prefers the sequence Leu-Glu-Thr-Asp, consistent with a previous analysis [7], the c-FLIP-caspase-8 heterodimer

seems to have a slight preference for the tetrapeptide Ile-Glu-Thr-Asp, the sequence of the most widely used caspase-8-selective inhibitor z-IETD-fmk which corresponds to the caspase-8 cleavage site in procaspase-3.

Despite some discrepancies, all three reports [4–6] conclude that c-FLIP_L is an efficient activator of caspases-8 and -10, and recognize that the initiator caspases-8 and -10 do not need to be fully processed to have full enzymic activity. In fact, the data suggest that the unprocessed proenzyme can be induced to be enzymically active. This was most efficiently accomplished by c-FLIP_L. This finding is reminiscent of the mechanism of activation of the other major initiator caspase, caspase-9. Biochemical data and structural analysis of caspase-9 revealed that the unprocessed enzyme can be fully active and even more striking is that only one active site in the caspase-9 homodimer is enzymically active [8]. The situation with caspase-8 seems to be similar; however, in this case, this specific structural property resulted in the development of the enzymically inactive activator c-FLIP_L, through heterodimerization, allowing for the regulation of initiator caspases in the DISC. What could be the purpose of this specific regulation? Why not just develop a specific cytosolic inhibitor for activated caspase-8? Members of the IAP (inhibitors of apoptosis) family are known to bind and block the activities of caspases-3, -7 and -9. It appears that a complete block of caspase-8 activity is not desirable, owing to alternative apoptosis-independent activities of caspase-8 as evidenced in caspase-8 deficient patients and mice [9,10]. Caspase-8 is required for proliferation and cell cycle

progression, especially in lymphoid cells. The report by Boatright et al. [6] now demonstrates that it is not a difference in substrate specificity that determines the function of caspase-8 by c-FLIP, but rather suggest that it is the localization of caspase-8 that determines functional outcomes (Figure 1). In the absence of c-FLIP, active caspase-8 is released from the DISC and mediates apoptosis if downstream signals are set on apoptosis execution. In contrast, if c-FLIP_L is expressed at a sufficient concentration, it will efficiently activate procaspase-8, but will not allow full processing to the mature enzyme. Active full-length caspase-8 and first cleavage intermediates may stay tethered to the DISC. It is this activity of caspase-8 that may explain its non-apoptotic activities. Future experiments will have to answer the questions of under what conditions caspase-8 activation by c-FLIP_L results in induction of apoptosis and under what conditions c-FLIP_L induces caspase-8 activation which results in induction of other pathways.

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