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Dual roles of FLIP in controlling caspase-8 and their implications on cell death and survival

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Cellular FLIP (cFLIP; cellular FLICE inhibitory protein) is a key regulator of caspase-8 activity, and its viral homolog (vFLIP) is found in various viruses. cFLIP inhibits the caspase-8 activation in death-inducing signaling complex (DISC) when its expression level is high as in cancer cells. In contrast, cFLIP can also activate the caspase-8 in its lower expression level and in lymphocyte in particular which results in the activation of NF-κB transcription factor. Recent biochemical studies on DISC has proposed that DED proteins in DISC, that are FADD, procaspase-8 & cFLIP, utilize two hydrophobic faces of their DEDs for establishing DED-DED network for DISC assembly. Regarding the NF-κB activation, the crystal structure of K13-vFLIP showed that two grooves in the N-terminal DED in FLIP are the sites where dimeric IKKγ binds. FLIP is associated with various cancers seemingly through the dual functions of inhibiting apoptosis and promoting proliferation, so it would an attractive anti-cancer therapeutic target at either mRNA or protein level.

INTRODUCTION

Apoptosis, one of the three major modalities of cell death, is characterized by shrinking of cells, chromatin condensation, nuclear fragmentation, and plasma membrane blebbing (Kerr et al., 1972; Kroemer and Levine, 2008; Thome and Tschopp, 2001). Once the apoptosis is initiated in a cell, the apoptotic cell is engulfed by neighboring cells within 24 hours (Kroemer and Levine, 2008; Wyllie et al., 1980). The dysfunctional apoptosis is a hallmark of neurodegenerative diseases and cancers (Hanahan and Weinberg; Mattson, 2000). Particularly relevant to cancer, chemotheraphy relies on the induction of apoptosis in cancer cells (Wajant, 2003), and the cancers with alterations to block the apoptosis signaling are resistant to chemotheraphy (Fesik, 2005). For these reasons, apoptosis has been an attractive target point for the therapeutic intervention against cancer (Hanahan and Weinberg, 2011).

Caspases are cysteine proteases which play a central role of executing the cell death through apoptosis. Caspases are expressed as inactive zymogens called procaspases, and then, on the initiation of the apoptosis signaling, they are cleaved to form the active proteases (Hengartner, 2000). The caspases can be categorized into initiator caspases and effector caspases (Shi, 2004). Initiator caspases (or apical caspases; caspase-8 & -9) are activated through autocatalytic cleavage on their activation platforms formed in response to the apoptosis signals. The most studied examples are caspase-8 for extrinsic apoptosis and caspase-9 for intrinsic apoptosis. The caspase-8 is activated in death-inducing signaling complex (DISC) whose major components are Fas, FADD and cFLIP (Chinnaiyan et al., 1996; Dickens et al., 2012; Kischkel et al., 1995; Majkut et al., 2014; Muzio et al., 1996). On the other hand, the caspase-9 is activated in apoptosome composed of Apaf-1 and cytochrom C (Shi, 2004). The activated initiator caspases then activate effector caspases (or executioner caspases; caspase-3 & -7), which execute the cell death by degrading more than 280 cellular proteins (Fischer et al., 2003).

In extrinsic apoptosis, death signal is given externally by death ligands including Fas ligand (FasL) and TRAIL.(Debatin and Krammer, 2004) The binding of the trimeric death ligand to the death receptor induces the formation of DISC where procaspase-8 is recruited and activated (Chinnaiyan et al., 1996; Kischkel et al., 1995; Muzio et al., 1996). Death receptors are a subfamily of the TNF receptor superfamily, and eight human death receptors have been identified; Fas (also known as Apo-1 and CD95), TNF-R1, DR-3 (Apo-3, TRAMP, WSL-1, LARD), TRAIL-R1 (DR-4), TRAIL-R2 (DR-5), DR-6, EDA-R and NGF-R (French and Tschopp, 2003). The assembly of DISC is accomplished by two different homotypic interactions of death domain (DD) and death effecter domain (DED) that are commonly members of death domain superfamily. These interactions are, firstly, DD-DD interaction between Fas and Fadd, and secondly, DED-DED interaction between Fadd, procaspase-8, and cFLIP (Boldin et al., 1996; Chinnaiyan et al., 1995; Kischkel et al., 1995; Muzio et al., 1996; Yang). Among these DISC component proteins, I will review the function and the structure of FLIP (cFLIP and its viral homolog, vFLIP) which acts in the signaling

pathways of cell death and survival.

DUAL ROLES OF FLIP IN REGULATING CASPASE-8 ACTIVITY

FLIP was initially identified from the homology to procaspase-8 in that they commonly have two DEDs in tandem, and its inhibitory function in extrinsic apoptosis was established (Bertin et al., 1997; Hu et al., 1997a; Irmler et al., 1997; Thome et al., 1997). FLIP was firstly found in virus and its human cellular homologs are identified soon after. So the human cellular one is called as cellular FLIP or cFLIP, and the viral homolog is called viral FLIP or vFLIP (Figure 1). Among at least 13 splice variants of cFLIP (Djerbi et al., 2001), three isoforms of cFLIP have been most studied: one long isoform $cFLIP_L$, and two short ones, $cFLIP_S$ and $cFLIP_R$ (Djerbi et al., 2001; Golks et al., 2005; Hu et al., 1997b; Irmler et al., 1997) (Figure 1). The long isoform $cFLIP_L$ has two DED's in N-terminus and the following caspase-like domain without a protease activity. The domain organization of $cFLIP_L$ is very similar to procaspase-8/-10 except that the C-terminal protease-like domain lacks the activity of the caspase. On the other hand, two other isoforms, $cFLIP_S$ and $cFLIP_R$, are composed of only tandem DEDs so that they are much shorter than the long isoform $cFLIP_L$.

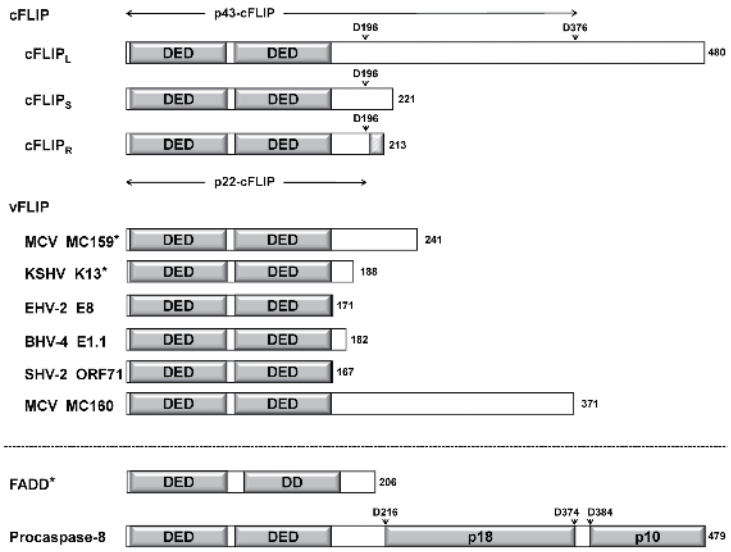

FIGURE 1 | cFLIP and vFLIP. Proteolytic cleavage sites are denoted by arrows with residue numbers, and asterisks indicate the proteins of which three dimensional structures were experimentally determined. Shaded region in C-terminus of $cFLIP_R$ is its unique sequence which is not included in the other two isoforms.

Short isoforms of cFLIP ($cFLIP_S$ and $cFLIP_R$) are solid inhibitors of caspase-8 activation in DISC (Golks et al., 2005; Yu and Shi, 2008). However, $cFLIP_L$ has been shown to act in two opposite directions for caspase-8 activation depending on the expression level and the cell types (Goltsev et al., 1997; Han et al., 1997; Inohara et al., 1997; Shu et al., 1997; Yeh et al., 2000) (Figure 2). At high expression level, as widely observed in most types of tumor cells, $cFLIP_L$ acts as an inhibitor of caspase-8 activation (Chang et al., 2002; Scaffidi et al., 1999; Yu and Shi, 2008). At lower expression level, which is as ~ 1% of procaspase-8, $cFLIP_L$ enhances the caspase-8 activation (Scaffidi et al., 1999; Yu and Shi, 2008). The activated caspase or procaspase-8 can cleave cFLIP at Asp198 and Asp376 by caspase-8 which produces p22-cFLIP and p43-cFLIP, respectively (Figure 1 & 2). These cleaved N-terminal fragments of cFLIP acts in NF-κB signaling for the lymphocyte proliferation. p22-cFLIP binds to IKKγ, the regulatory subunit of IKK complex, and thereby consequently activating NF-κB (Golks et al., 2006) (Bagneris et al., 2008). p43-cFLIP was shown to bind TRAF2 and the C-terminal fragment to RIP1 (Yu and Shi, 2008). This issue will be discussed in detail below.

STRUCTURAL FEATURES OF FLIP

NMR structure of FADD DED revealed that DED also adopts a hexa-helical bundle fold commonly observed in other DD superfamily members such as DD (Eberstadt et al., 1998) (Figure 3A). However, for the tandem DEDs of cFLIP, its atomic-detail structure has not been reported yet mainly because of its aggregating tendency hampering the structural analysis trials. Instead of cFLIP, the crystal structures of its two viral homologs were determined: MC159 from a poxvirus *Molluscum contagiosum* virus (MCV) and K13 from Kaposi's sarcoma herpes virus (KSHV) (Yang et al., 2005) (Bagneris et al., 2008) (Figure 3C). The crystal structure of MC159-vFLIP showed how two DEDs interact with each other and provided the basis for understanding the DED-DED network in the assembly of DISC (Dickens et al., 2012; Majkut et al., 2014; Yang, 2015; Yang et al., 2005). In the crystal structure of MC159-vFLIP, the homotypic DED-DED interaction is established by the hydrophobic contact between H2-H5 face of N-terminal DED (N-DED hereafter) and H1-H4 face of C-terminal DED (C-DED) (Figure 3B). This is quite contrasting to CARD-CARD interaction which is mainly electrostatic between H2-H3 face and H1-H4 face (Qin et al., 1999) (Yang, 2008). One more notable feature of MC159-vFLIP structure is that the N-DED deviates significantly from the canonical hexa-helical bundle fold which was conserved in C-DED. The N-DED lacks a helix corresponding to the helix 3 (H3) of the canonical fold and resultantly a DED uses H2-H5 face (not H2-H3 face for CARD or DD) for binding to other DED (Yang, 2008). The H2-H5 face must be the characteristic element of DED-DED interaction, since the CARD-CARD and in DD-DD interactions commonly involve the alternative H2-H3 face (Qin et al., 1999; Wang et al., 2010; Yang, 2008). In addition to the hydrophobic faces, one more characteristic of DED domain is its RxDL sequence motif in the beginning of helix 6 which is highly conserved among DED proteins (Tibbetts et al., 2003). The RxDL motif is essential for the self-association of FADD and the resultant apoptosis signaling

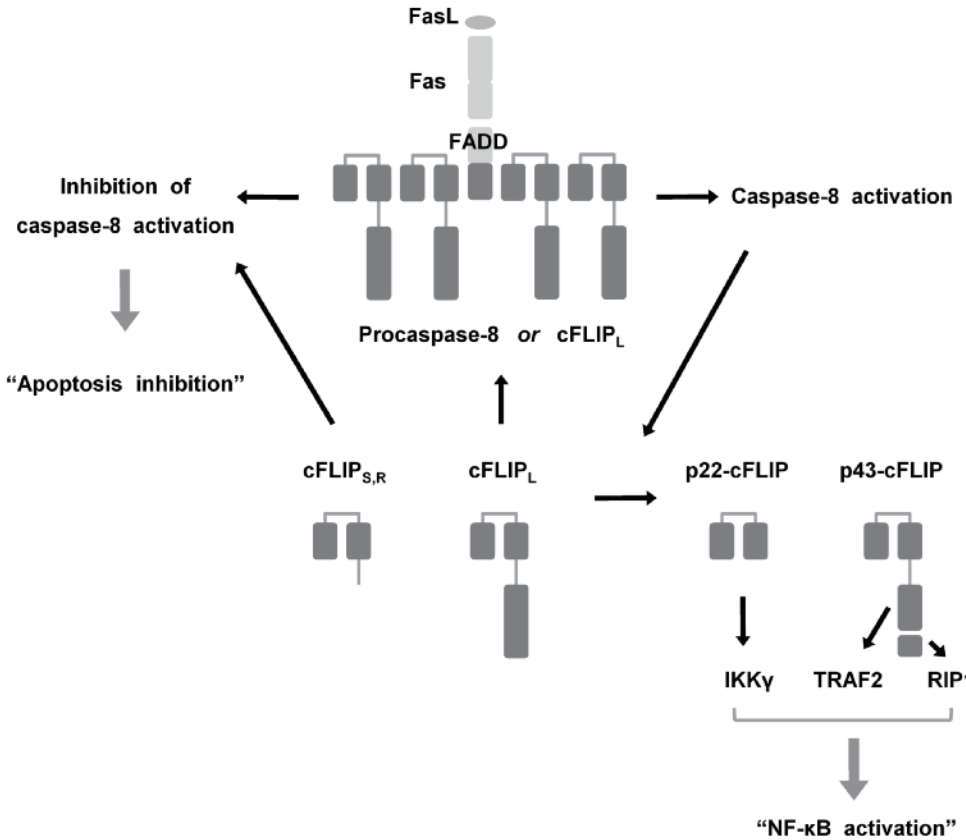

FIGURE 2 | Dual roles of cFLIP in regulating caspase-8 activity. Short isoforms of cFLIP ($cFLIP_S$ & $cFLIP_R$) inhibit caspase-8 activation, and thereby blocks the extrinsic apoptosis. In contrast, the long isoform, $cFLIP_L$ can act in two opposite directions for caspase-8 activation. With these dual ways of action, FLIP can be involved both in apoptosis signaling and NF-κB pathway depending on conditions and cell types.

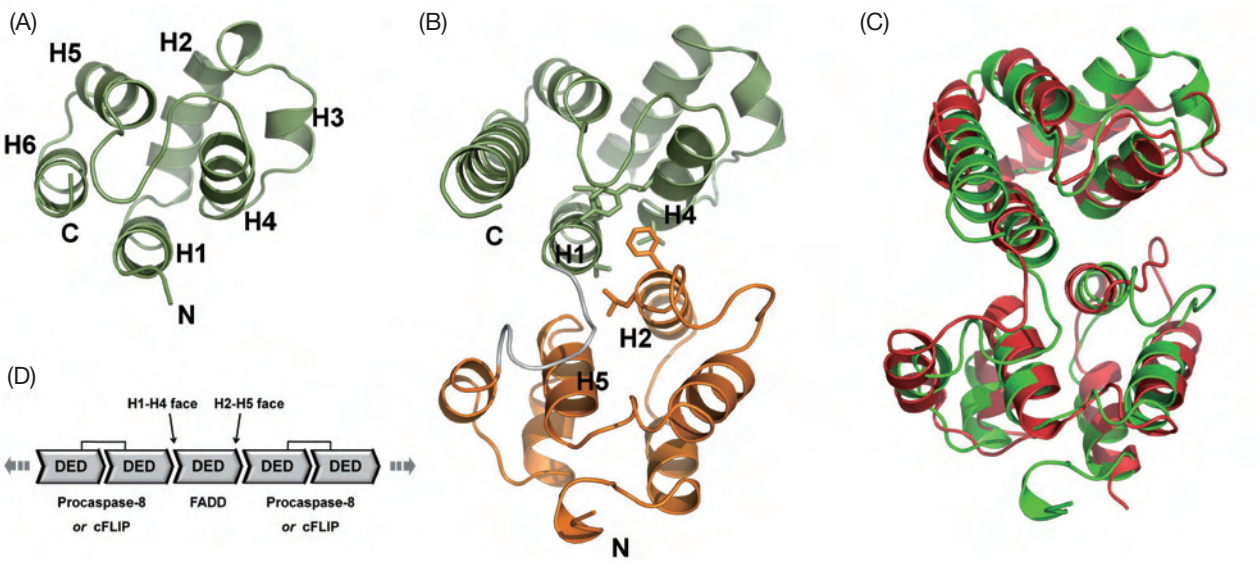

FIGURE 3 | Structure of vFLIP. (A) NMR structure of FADD DED (Protein Data Bank, PDB, accession code 1A1W). (B) Crystal structure of MC159-vFLIP (PDB 2BBR). (C) Superposition of two crystal structures of vFLIP: MC159-vFLIP in green and K13-vFLIP (PDB 3CL3) in purple. (D) DED chain model for the DED-DED network in death-inducing signaling complex.

(Muppidi et al., 2006), and also for the antiapoptotic activity of MC159-vFLIP (Garvey et al., 2002). Commonly for both DEDs of MC159-vFLIP, the arginine residue in the RxDL motif (Arg66 in N-DED & Arg166 in C-DED) interacts with two negatively charged residues; an aspartate in the same sequence motif (Asp71 in N-DED & Asp168 in C-DED) and a glutamate in helix 2 (Glu24 in N-DED & Glu111 in C-DED) (Yang, 2008). The interaction between the three charged residues seems to contribute to the fold stability by holding the two helices, H2 and H6, together in their proper positions (Yang, 2008; Yang et al., 2005). This notion is supported by NMR study on R72A mutant of Fadd DED in which its NMR peaks exchange broaden and none of the DED resonances are visible even though its circular dichroism spectra is the same as its wild type (Carrington et al., 2006).

FLIP IN DISC ASSEMBLY AND EXTRINSIC APOPTOSIS

Since procaspase-8 and cFLIP also have tandem DEDs just as vFLIP, the crystal structure of MC159-vFLIP was used as a reference for the homology modeling studies on their tandem DEDs (Dickens et al., 2012; Majkut et al., 2014). Recent biochemical studies based on the homology models have provided new insights into the roles of cFLIP for the assembly of DISC in the extrinsic apoptosis signaling. The homology models of cFLIP and procaspase-8 are very similar to MC159-vFLIP structure in that the H1-H4 face and H2-H5 face are exposed for possible further inter-molecular DED-DED interaction (Dickens et al., 2012; Majkut et al., 2014). In the intra-molecular DED-DED interaction observed in MC159-vFLIP, the two hydrophobic faces, H2-H5 face and H1-H4 face, are responsible for the interaction (Yang et al., 2005). Since each DED has both the faces, one remaining face for each DED is not involved in the intra-molecular DED-DED interaction. Therefore, in any structure of tandem DEDs of procaspase-8 or FLIP, one face is exposed and available for inter-molecular DED-DED interaction. This is the basic idea to postulate the DED-DED chain network model in the recent studies (Dickens et al., 2012; Majkut et al., 2014). The recently proposed DED chain model for DED-DED interaction network in DISC argue that the inter-molecular DED-DED interaction between FADD, procaspase-8 and cFLIP would be established through the same way of the intra-molecular DED-DED interaction in tandem DEDs of vFLIP (Figure 3D). This notion was supported by the mutational studies on the interface residues in H2-H5 face (F25 in FADD, F122 and L123 in DED of procaspase-8, and F114 of cFLIP) (Dickens et al.; Majkut et al.), which showed decrease of DISC assembly and their recruitment there (Majkut et al.). In addition, it was shown for the stoichiometry of DISC that two tandem-DED-containing proteins, that are caspase-8 and cFLIP, exist in a larger amount in a sum than the single-DED-containing FADD. However, cFLIP exists in DISC in a comparatively small amount, which confirms the previous reports that cFLIP promotes the caspase-8 activation when the level is low (Scaffidi et al., 1999). More importantly, the substoichiometric existence of FADD relative to caspase-8 in DISC suggests that the self-association of procaspase-8/cFLIP molecules accounts for the substantial part of DED-DED network in DISC.

FLIP IN NF-κB SIGNALING FOR LYMPHOCYTE PROLIFERATION

$cFLIP_L$, when expressed in a high level as in cancer cells, inhibits the caspase-8 activation in extrinsic apoptosis as discussed above. In contrast, $cFLIP_L$ has been reported to promote the caspase-8 activation especially in lymphocytes where its expression level is lower (Chaudhary et al., 2000; Golks et al., 2006; Hu et al., 2000; Kataoka et al., 2000; Kataoka and Tschopp, 2004). The caspase-8 activation in lymphocytes leads to its survival and proliferation by specifically activating NF-κB signaling (Misra et al., 2007; Su et al., 2005). $cFLIP_L$ requires to be cleaved into p43-FLIP and p22-FLIP for activating NF-κB.

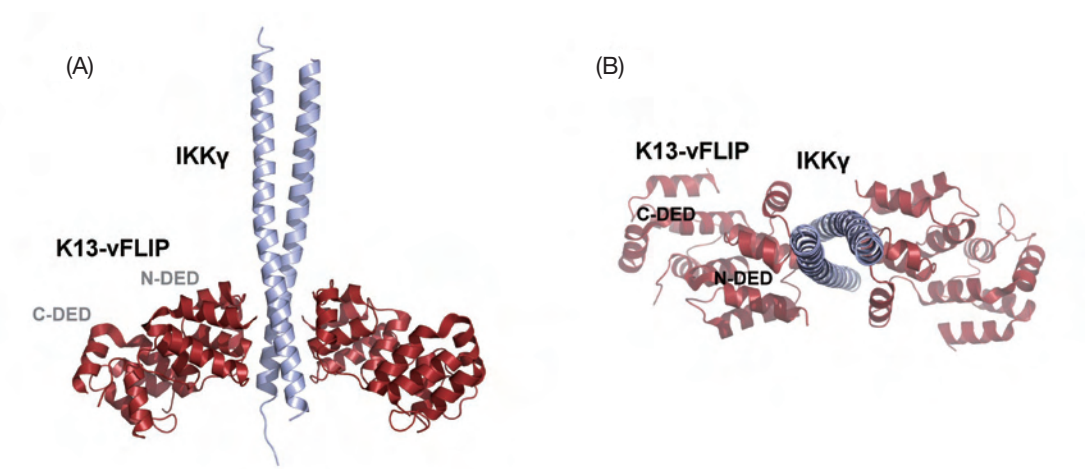

FIGURE 4 | Structure of K13-vFLIP bound to IKKγ. (A) Side view and (B) top view. IKKγ is in blue color and K13-vFLIP in red.

p43-FLIP is produced from the cleavage of $cFLIP_L$ at Asp376 by fully active mature caspase-8, and p22-FLIP is from the cleavage at Asp196 (Golks et al., 2006; Kataoka and Tschopp, 2004). In addition, the p22-FLIP can be generated also from $c\text{-}FLIP_S$ and $c\text{-}FLIP_R$ (Golks et al., 2006; Yu and Shi, 2008). Both the cleaved fragment of cFLIP can activate NF-κB through direct binding to different components of NF-κB signaling pathway: p43-FLIP to TRAF2 (Kataoka and Tschopp, 2004) and p22-FLIP to IKKγ (Bagneris et al., 2008; Golks et al., 2006). It should be noted that caspase-8 is essential for NF-κB activation in T, B and NK cells (Chun et al., 2002; Su et al., 2005). When the NF-κB activation by cFLIP is not under control, certain lymphoproliferative disorders can be developed. For example, Kaposi's sarcoma herpes virus (KSHV) not only infects the endothelial cells to cause Kaposi's sarcoma (Wang et al., 2004), but also infects B cells to contribute to primary effusion lymphoma (PEL), muticentric Castleman's disease (MCD), and associated plasmablastic lymphoma (Bagneris et al., 2008; Cesarman et al., 1995; Dupin et al., 2000; Gessain et al., 1996; Soulier et al., 1995). One of the key players for the establishment of these diseases by KSHV infection is K13-vFLIP which mimics the cellular p22-FLIP. The crystal structure of K13-vFLIP in complex with IKKγ was able to show how FLIP binds to IKKγ in atomic detail (Bagneris et al., 2008) (Figure 4). In this structure, the long helical dimer of HLX2 region of IKKγ binds to two molecules of K13-vFLIP. The two helices of IKKγ dimer are positioned into two adjacent vertical clefts on each N-DED of K13-vFLIP. Even though each IKKγ molecule contacts both of two vFLIP molecules, each IKKγ significantly leans to one vFLIP molecule. In other words, each vFLIP molecule binds to both of two IKKγ molecules at the same time through its two binding clefts (cleft 1&2; Figure 4B), but the vFLIP molecule contacts more tightly to one IKKγ than the other one. As seen in Figure 4, the inter-protein contacts are mediated mainly by the cleft 1. Through these contacts, K13-vFLIP seems to stabilize the conformation of IKKγ as in the crystal structure which would be optimal for recruiting IKKβ and/or IKKα kinases for phosphorylation of themselves and then IκB. This may be how FLIP (K13-vFLIP or p22-cFLIP) can promote the activation of NF-κB (Bagneris et al., 2008).

CONCLUDING REMARKS

cFLIP is often overexpressed in various types of cancer such as colorectal carcinoma, gastric carcinoma, pancreatic carcinoma, Hodgkin's lymphoma, B cell chronic lymphocytic leukemia, melanoma, ovarian carcinoma, cervical carcinoma, bladder urothelial carcinoma, and prostate carcinoma (Yang, 2008). The c-FLIP is one of the main causes for the resistance, and it is also well known that the down-regulation of cFLIP can sensitize resistant cancer cells (Yang, 2008). These proliferative and apoptosis-resistant characters of cancer cells should be acquired at least partly by the dual functions of cFLIP, that are firstly to inhibit the extrinsic apoptosis and secondly to promote the NF-κB activation as discussed above. The numerous clinical and mechanistic studies commonly argue that c-FLIP should be an attractive therapeutic target against the cancers which especially show an elevated expression of cFLIP. Therefore, all the basic knowledge on the biochemistry and the structure of cFLIP reviewed here must be the valuable groundwork for the future attempts to develop the inhibitors of cFLIP.

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AUTHOR INFORMATION

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