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Mini-review

FLIP: A flop for execution signals

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ABSTRACT

Resistance to apoptosis is one of the established hallmarks of cancer cells. This is a function of an imbalance between the proteins that facilitate death execution and those that inhibit apoptosis or promote cell proliferation. The anti-apoptotic protein, FLICE inhibitory protein (FLIP), first identified as a viral protein, is over-expressed in a variety of human pathologies. Initial observations linked FLIP expression to inhibition of death receptor induced apoptosis, due to its structural homology to the cysteine protease, caspase-8. FLIP impedes full processing of pro-caspase-8 to its active form and its release to the cytosol, and by doing so blocks apoptotic signaling downstream of the membrane death initiating signaling complex (DISC). Recent observations have highlighted the complex regulation of this protein and its cross talk with diverse signaling networks and metabolic processes. As FLIP expression is directly associated with chemotherapy resistance, a better understanding of its genomic organization, gene transcription, as well as post-transcriptional regulation could yield novel targets with potential therapeutic implications against drug refractory cancers. In this short review, we provide a brief overview of the structural and functional biology of this somewhat complex protein with direct relevance to carcinogenesis.

1. Introduction

Escape from normal constraints on growth and proliferation, genomic instability, and resistance to apoptosis are among the hallmarks of cancer [1]. The latter is a function of up regulation of proteins that block apoptosis at different stages of the death execution program. Indeed, a tilt in the intracellular ratio of death promoting proteins and death inhibitory proteins in favor of the latter provides cells with a survival advantage resulting in aberrant proliferation and setting the stage for transformation. Therefore, the past three decades have seen a tremendous increase in interest in understanding the molecular mechanisms underlying the regulation of gene expression, as well as functional biology of the anti-apoptotic effector mechanisms/pathways operative in neoplasia. These efforts have provided insights into novel signaling networks and their regulatory nodes, particularly from the standpoint of genes/proteins that control cell fate decisions.

2. Apoptosis is a natural tumor suppressor mechanism

Deregulation of apoptotic machinery by suppression and/or decreased expression of pro-apoptotic molecules and/or increased levels of pro-survival proteins is an essential requirement for cancer initiation and progression [2]. Therefore, novel anti-cancer strategies include drugs that reactivate death signaling by specifically targeting anti-apoptotic proteins such as Bcl-2 [3], IAP family [4] and cFLIP (FLICE inhibitory protein [5].

Apoptotic cell death is classified into extrinsic and intrinsic pathways [6]. Extrinsic apoptotic pathway is triggered by specific receptor-ligand interaction at the cell surface leading to a cascade of signaling events resulting in death execution. In the classical receptor-mediated apoptosis model, such as via ligation of CD95 (Apo-1/Fas) receptor, a member of the Tumor Necrosis Factor (TNF) receptor superfamily, death signaling is triggered upon binding of the CD95 ligand (CD95L) to the death receptor [7], leading to the aggregation of proteins like FADD and procaspase-8 for the

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formation of the Death Initiating Signaling Complex (DISC) [8]. FADD is an adaptor protein that facilitates the recruitment of pro-caspase-8 to the death receptor complex. The formation of the DISC assembly results in the activation of caspase-8, which can then drive downstream signaling either via direct activation of executioner caspases, such as caspase 3 (Type I), or by engaging the mitochondrial amplification pathway via activation of pro-apoptotic members of the Bcl-2 family (Type II). The DISC assembly is regulated by the inhibitory protein cFLIP, which by dint of its homology to procaspase-8-via the Death Effector Domain (DED)-competes with procaspase-8 for binding to the adaptor protein FADD [9].

3. cFLIP promotes carcinogenesis by regulating apoptotic signaling

The cellular FLICE-inhibitory protein (cFLIP; also referred to as Casper, FLAME-1, CLARP) is the mammalian homolog of the viral cell death regulatory proteins v-FLIPs, and expressed in three different isoforms, namely $cFLIP_L$ (55 kDa), $cFLIP_R$ (24 kDa) and $cFLIP_s$ (26 kDa) [10,11]. It should be pointed out that the expression of the various isoforms is a function of alternative splicing of the cFLIP gene, which is transcribed under the same promoter. Of note, despite structural differences at the C-terminal end, all three isoforms have been shown to associate with the DISC via the DEDs [12]. Low levels of cFLIP have been reported in non-transformed cells, which serve to maintain the intricate physiological balance between cell survival and death [13]. In contrast, high levels of cFLIP (long or the short isoform) have been reported in a wide variety of pathological states including cancer, and its knockdown clearly sensitizes cells to drug-induced apoptosis [14].

Initial observations linked cFLIP to the regulation of receptor-induced apoptosis, however there is evidence to also implicate cFLIP in resistance to chemotherapy-induced cell death. Interestingly, $cFLIP_L$ is sufficient to block caspase-8 activation upon apoptosis induction in some cancers; however, other evidence indicates that $cFLIP_s$ is important in cells' resistance to TRAIL-induced apoptosis [14]. It should be pointed out that the main difference between $cFLIP_L$ and $cFLIP_s$ is the absence of the caspase-8 like domain in the short form of the protein [8]. It appears that an inverse relationship exists between the expression of cFLIP and cell sensitivity to apoptosis, and therefore therapeutic targeting of the two isoforms has potential therapeutic implications in drug refractory cancers [15].

4. Genome organization and regulation of cFLIP expression

The genome organization of cFLIP is quite complex as the gene is 50 Kb in length and spatially separated by 10 introns. The promoter region spans about 1.5 Kb upstream of the transcriptional start site and contains numerous transcription factors' binding sites [16]. Among the several possible transcription factors associated with the activation of cFLIP transcription are AP-1 (c-Fos and c-Jun), CREB, SP1, and NF-kB [10]. In addition, other transcription factor binding sites have been described that are associated with the down regulation of cFLIP transcription [17]. Presence of cis-acting elements within the 5′UTRs could also play a regulatory role in affecting transcription/translation of the gene [18]. In this regard, the 5′-UTR of the two isoforms of cFLIP possess small overlapping uORFs (upstream Open Reading Frames) encoding short peptides of about 16 and 24 amino acids that could perturb efficient translation of the gene. Thus, it is possible that this gene could be regulated at the transcriptional and/or at the translational level from its genetic organization.

4.1. Transcriptional control

The cellular expression of cFLIP is regulated at the transcriptional, translational, and post-translational levels [19]. The transcriptional control of cFLIP has been a subject of interest in cancer research for many years and studies have demonstrated that the isoforms of this protein are differentially regulated in cell- and signal-specific manners. As mentioned earlier, the cascade of events controlling the transcription of this anti-apoptotic gene is quite complex, due to the presence of a long promoter region containing binding sites for numerous transcription factors. A plethora of transcription factors induce cFLIP transcription such as NF-kB, P53, FoxO, CREB, AP-1 (cFos/c-Jun), NFATc2, EGR1, AR and SP1, while a host of others are involved in repression of its expression such as c-myc, FoxO3a, c-Fos, IRF5, and SP3 [16] (Fig. 1). More recently, a direct positive regulation of c-FLIP promoter by p63 (Np63) has been described in the epidermis [20]. There is also evidence that E2F1 down regulates cFLIPs in lung adenocarcinoma cell lines, leading to caspase-8 activation at the DISC [21]. In contrast, prostate apoptosis response factor -4 (Par-4) recruited onto the cFLIP promoter in the presence of androgens enhanced cFLIP expression in prostate cancer cells [22]. In other model systems, p53 has been shown to upregulate cFLIP [23], while c-myc binds to and represses transcription of the gene [24]. Similarly, c-fos has been shown to negatively regulate transcription of $cFLIP_L$ by direct binding to the putative promoter region of cFLIP [25]. The CREB family of transcription factors including the ATF1 and ATF2 are also candidates as the promoter region of cFLIP has putative CREB binding sites [26].

4.2. Post-transcriptional regulation of cFLIP expression

It should be pointed out that in some cell types, the amount of the cFLIP transcript does not directly correlate with the protein level, because of post-transcriptional events such as mRNA stability or mRNA decay [27]. Also, it has previously been reported that the half-lives of the cFLIP protein isoforms are very short, which could be one of the reasons for the high protein turnover [28]. Interestingly, there is convincing evidence that oxidative stress such as exposure to hydrogen peroxide (H_2O_2) specifically increases the ubiquitination of cFLIP protein for its degradation via the ubiquitin-mediated proteasomal degradation pathway [29]. This effect could be rescued by either the H_2O_2 scavenger, catalase [30], or the proteasome inhibitor, MG132 [31].

Recent evidence has also implicated miRNAs in the regulation of cFLIP expression. miRNAs are small non-coding, single-stranded RNAs that negatively or positively modulate gene expression at the post-transcriptional level through complementary base pairing at the 3′-UTR of the genes. In a model of Taxol-induced apoptosis, miR-512-3p overexpression amplified death signaling by inhibiting cFLIP expression in hepatocellular carcinoma cells [32]. In addition, Senescence-associated miRNAs (SA-miRNAs) have also been implicated in the regulation of cFLIP expression [33].

The cellular levels of cFLIP are also under indirect regulation by proteins that physically interact with cFLIP [34–36]. In this regard, a few interacting partners of cFLIP have been identified, and these interactions either facilitate or inhibit apoptotic signaling. Interacting proteins of $cFLIP_L$, like Daxx (a death domain-associated protein that has been implicated in promoting apoptosis and transcriptional regulation) block apoptosis by preventing Fas-induced JNK activation [37]; JNK activation is an important factor in proteasomal degradation of cFLIP, in particular $cFLIP_s$, which contains 2 critical residues in the C-terminal that are susceptible to ubiquitination [38]. In another report, using a yeast two-hybrid screen, the drosophila homolog of cFLIP, Casper, was shown to interact with NF-κB1 (p105), an atypical IκB molecule and

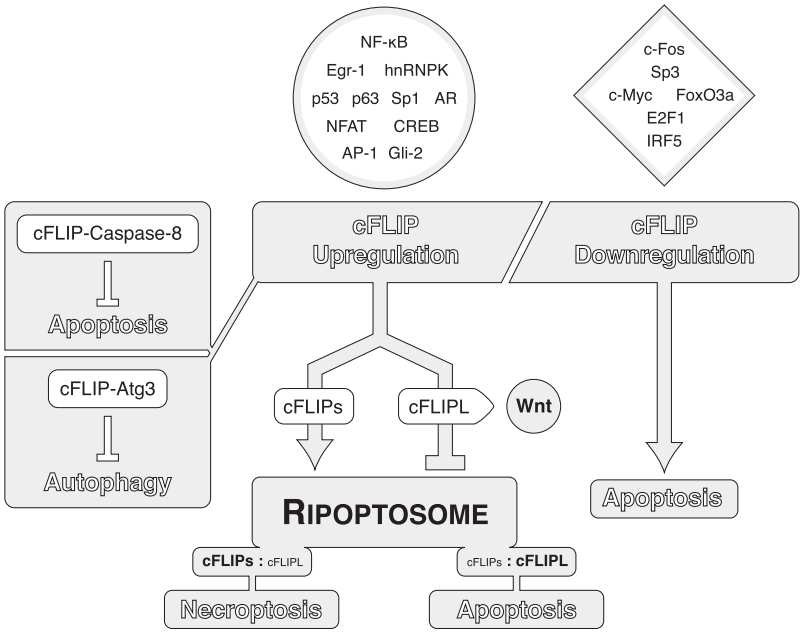

Fig. 1. A schematic representation of the various cell death regulatory activities of cFLIP isoforms. A plethora of transcription factors bind to the promoter region of cFLIP either inducing or repressing its gene transcription. cFLIP interacts with a number of cellular proteins and these interactions determine cell fate decisions via activating Wnt signaling, inhibiting autophagic flux, regulating RIP mediated necroptosis, and suppressing caspase 8-dependent apoptotic signaling.

precursor of NF-κB subunit p50, via an IκB-like domain (IκBγ) [34]. Other proteins, for example MKK7, have been reported to directly bind to $cFLIP_L$ and inhibit the JNK pathway [35]. Recently, Mind bomb 1 (Mib1), a multi-domain E3 ligase, has been shown to bind cFLIP and this interaction decreases the association of caspase-8 with cFLIP, thereby activating caspase-8 and induces cell death [36]. In neuronal differentiation, $cFLIP_L$ has been shown to play a critical role in neurotrophin-induced MAPK/ERK- and NF-κB-mediated control of neurite growth in developing neurons, and this involves interaction of $FLIP_L$ with TrkA and TrkB, two important receptor tyrosine kinases [39]. Interestingly, although cFLIP is under the transcriptional control of NF-kB, there is also evidence that $cFLIP_L$ functions upstream of NF-κB by interacting with proteins in the TNF-R signaling pathway, such as TRAF-1 and -2 (TNF-Receptor Associated Factor-1 and-2) and RIP (Receptor Interacting Protein) [40,41].

5. Cross talk between cell metabolism and cFLIP

Although, the major point of focus has been the death inhibitory activity of cFLIP, in particular its ability to inhibit death receptor-mediated signaling, recent evidence points to a complex network of pathways involved in the regulation of cFLIP as well as the effect of cFLIP expression on cells' metabolic activity. For example, the intricate crosstalk between NF-kB signaling and cFLIP expression underscores the importance of this protein in the workings of a "master" regulator of cell metabolism [42–44]; transcriptional targets of NF-kB are involved in a wide variety of physiological and pathological processes, such as inflammation, motility and invasion, and epithelial-mesenchymal transition [45]. A recent report demonstrated a novel mechanism of cFLIP-induced death inhibition involving autophagy [46], a critical pathway in cell metabolism. The expression of either cFLIP or vFLIP suppressed autophagic flux in cancer cells by blocking the binding of Atg3 to LC3, and thereby preventing LC3 conjugation; cFLIP-Atg3 complex prevented autophagy-associated cell death [46] (Fig. 1). In addition, inhibition of mTOR pathway by rapamycin suppressed polyribosome accumulation of cFLIP and sensitized glioblastoma cells to TRAIL-induced apoptosis [47]. Along similar lines, enhanced cFLIP-FADD interaction has been shown to increase cancer cells' metabolic activity by facilitating Wnt signaling through its inhibitory effect on the ubiquitination and degradation of β-catenin, thereby promoting cell cycle progression [48]. This effect on β-catenin signaling could also be attributed to cFLIP-mediated regulation of Gsk-3β activity, which prevents the nuclear translocation of β-catenin in the canonical Wnt pathway. To that end, an interaction between Akt/PKB and cFLIPL has been shown to amplify Akt signaling, which then inactivates Gsk-3β via its phosphorylation [49]. Among the many downstream targets of activated Akt is the FoxO family of transcription factors, and phosphorylation induced inactivation of FoxO3a is linked to an accumulation of reactive oxygen species (ROS) [50]via inability of FoxO3a to transcribe anti-oxidant genes, such as sestrin, catalase and MnSOD [51].

Interestingly, there is mounting evidence to implicate the cellular redox metabolism not only in the regulation of cFLIP levels, but also in the functional biology of this protein. An increase in intracellular ROS or reactive nitrogen species (RNS) has been shown to differentially regulate the cellular levels of cFLIP. For example, H_2O_2 promotes ubiquitination and proteasomal degradation of cFLIP, which could in part explain the pro-apoptotic activity of this particular ROS [29]. On the other hand, nitric oxide (NO)-mediated S-nitrosylation of cFLIP prevents its ubiquitination, thereby stabilizing its expression and anti-apoptotic activity [52]. These data are intriguing and somewhat corroborate our recent findings on the differential effects of intracellular H_2O_2 and O_2^- on cell fate signaling. Our work over the years has highlighted the pro-survival activity of an increased intracellular $O_2^- : H_2O_2$ ratio [53–59]. Not only did an increase in intracellular O_2^- inhibited death receptor signaling, but it was also associated with death resistance induced upon oncogenic Rac1 activation as well as overexpression of the anti-apoptotic protein Bcl-2 [60,61]. Interestingly, in the latter case, decreasing intracellular O_2^- restored the sensitivity of Bcl-2 over-expressing Type II cells to CD95-mediated apoptosis via robust activation of caspase-8 [60]. This indicates a switch from Type II (mitochondria-dependent) to Type I (mitochondria-independent) signaling and strongly implicates cFLIP in O_2^--induced

inhibition of caspase-8. Alternatively, amplification of TRAIL-induced apoptosis in human cancer cells upon exposure to small molecule compounds (LY294002 and LY303511) was mediated via a robust increase in intracellular H_2O_2, and one probable mechanism was the down-regulation of cFLIP [62,63]. These data beg the question whether cellular levels of cFLIP are differentially regulated by the two major ROS species? Interesting, in a recent communication, we demonstrated a very similar opposing effect of O_2^- and H_2O_2 on the promoter activity and transcription of the Na^+/H^+ exchange transporter gene, NHE-1 [64]. Could it be possible that the promoter regions of the two genes (and perhaps more) share binding sites for redox responsive/regulated transcription factors?

Although the apoptosis inhibitory activity of cFLIP isoforms has been well documented in death receptor signaling, recent evidence underscores the critical involvement of cFLIP in death signaling through the toll like receptor (TLR) pathways, in particular TLR3 [65]. Similar to the DISC complex, a functional complex termed "Ripoptosome" containing RIP1 as the core protein and including FADD, caspase-8/10, and cFLIP isoforms [66], has been implicated in death signaling downstream of TLR3 activation. The IAP family of proteins target RIP1 for ubiquitination and proteasomal degradation, but in the absence of IAP (antagonists) RIP1-mediated ripoptosome assembly is facilitated. RIP1 containing an active kinase domain phosphorylates RIP3 to activate necroptosis, whereas robust activation of caspase-8 upon an apoptotic stimulus inactivates this pathway by cleaving RIP1 and disassembling the ripoptosome [65]. Interestingly, the two isoforms of cFLIP have distinctly different effects on the outcome of RIP1-mediated death signaling (Fig. 1). cFLIPL-caspase-8 heterodimer retains enough enzymatic activity to disassemble the ripoptosome but not for full activation of caspase-8, and therefore blocks both apoptotic and necroptotic pathways [65]. On the other hand, cFLIPS-caspase-8 dimer is completely devoid of any proteolytic activity, which allows RIP1 accumulation and RIP1-mediated necroptosis [65]. These data provide a novel facet in the biology of this important death regulatory protein with tremendous therapeutic implications for a host of human pathologies.

6. Concluding remarks and unanswered questions

Overexpression of cFLIP isoforms is associated with resistance to death receptor and drug-induced apoptosis. Therefore, targeting cFLIP has been proposed as an attractive strategy for novel anticancer drug design. Thus, use of siRNA mediated gene silencing and identification of compounds with the ability to down-regulate cFLIP expression are attractive and logical options. In this regard, a number of small molecule compounds have been shown to sensitize cancer cells via their effect on the cellular levels of cFLIP. Nevertheless, a thorough understanding of the biology of this intriguing protein, which has close interlinks with a host of signaling networks and metabolic processes is imperative for targeted drug design. In this regard, an area less well understood is the relatively large and "busy" promoter region of cFLIP with binding sites for tens of transcription factors. How some of these transcription factors activate and others repress cFLIP transcription is not well understood. Another area of interest is the stability of the protein and the cellular events that regulate its ubiquitination and proteasomal degradation.

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