Phosphatidylinositol 3-Kinase/Akt Activity Regulates c-FLIP Expression in Tumor Cells*

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The capase-8 homologue FLICE-inhibitory protein (FLIP) functions as a capase-8 dominant negative, blocking apoptosis induced by the oligomerization of the adapter protein FADD/MORT-1. FLIP expression correlates with resistance to apoptosis induced by various members of the tumor necrosis factor family such as TRAIL. Furthermore, forced expression of FLIP renders cells resistant to Fas-mediated apoptosis. Although FLIP expression is regulated primarily by MEK1 activity in activated T cells, the oncogenic signaling pathways that regulate FLIP expression in tumor cells are largely unknown. In this report, we examined the roles of the MAP kinase and phosphatidylinositol (PI) 3-kinase signaling pathways in the regulation of FLIP expression in tumor cells. We observed that the MEK1 inhibitor PD98059 reduced FLIP levels in only 2 of 11 tumor cell lines tested. In contrast, disruption of the PI 3-kinase pathway with the specific inhibitor LY294002 reduced Akt phosphorylation in the same cell lines evaluated. The introduction of a dominant negative Akt adenoviral construct also consistently reduced FLIP expression as well as the phosphorylation of the Akt target glycogen synthase kinase-3. In addition, infection of the same cell lines with a constitutively active Akt adenovirus increased FLIP expression and the phosphorylation of GSK-3. These data add FLIP to the growing list of apoptosis inhibitors in which expression or function is regulated by the PI 3-kinase-Akt pathway.

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‡ The abbreviations used are: TNF, tumor necrosis factor; DD, death domain; FADD, Fas-associated death domain; FLICE, FADD-homologous ICE-like protease; FLIP, FLICE-inhibitory protein; ERK, extracellular signal-regulated kinase; p-ERK, phosphorylated ERK; p-Akt, phosphorylated Akt; GSK-3α/β, glycogen synthase kinase 3α/β; HA, hemagglutinin; MAP, mitogen-activated protein; MEK, MAP/ERK kinase; PI, phosphatidylinositol; CREB, cAMP-response element-binding protein; RT-PCR, reverse transcriptase-polymerase chain reaction; FCS, fetal calf serum; DN-Akt, dominant negative Akt adenoviral construct; cIAP, cellular inhibitor of apoptosis protein; TRAIL, TNF-related apoptosis-inducing ligand.
PI 3-Kinase/Akt Regulates FLIP Expression

In the ability of FLIP to act as a competitive inhibitor of the binding of caspase-8 to FADD, it has also been shown to induce the activation of NF-κB via TNF receptor-1 through TRADD, TRAF-2, RIP, NIK, and IKK (27), thus providing a second anti-apoptotic mechanism for FLIP.

Although initially described as a viral product (28), a cellular analogue of FLIP has since been discovered, and high levels are commonly found in tumors (18, 29). In one study in melanoma cells, the levels of FLIP were found to correlate inversely with susceptibility to apoptosis induced by exposure to TRAIL (30). However, other studies have failed to identify a linkage between FLIP expression and sensitivity to Fas or TRAIL (7).

Very little is known about the signaling pathways or transcription elements that control the expression of FLIP. In activated T cells, FLIP expression has been shown to be dependent on the ERK/MAP kinase pathway because the addition of a dominant active MKK1 (MEK-1) induces FLIP in 293T and Jurkat cells (31). The molecular basis for the high constitutive levels of FLIP observed in many tumor cell lines is unknown.

PI 3-kinase has been shown to protect cells from apoptosis in a caspase-dependent manner (32–34). PI 3-kinase catalyzes the phosphorylation of phosphoinositol-4 phosphate and phosphoinositol-4,5 phosphate at the 3 position. Kinases such as PDK1 and pkBAkt bind to these phosphorylated intermediates via their pleckstrin homology domain. PDK1 in turn phosphorylates and activates pkBAkt (35), which then phosphorylates several proteins that have been implicated in the control of cell survival (32, 34, 36–39). The phosphorylation of procaspase-9 by Akt, for example, inactivates this protease, blocking the propagation of death signals originating in the mitochondria (39). Likewise, Akt-mediated phosphorylation of Bad results in the binding of this pro-apoptotic Bcl-2 family member to 14-3-3, thus blocking its activity, reducing the levels of expression of pro-apoptotic proteins such as Fas ligand (46). These data illustrate the diversity of downstream effectors through which the PI 3-kinase/Akt signaling pathway affects cell survival.

The ability of a constitutively active MEK1 to induce FLIP expression in T cell lines and the multiplicity of anti-apoptotic proteins in which expression and/or function are regulated through the PI 3-kinase pathway suggest that the constitutive expression of FLIP by tumor cells might be attributable to the activation of either the Ras-Raf-MEK-ERK and/or PI 3-kinase pathways. To test these hypotheses, we carried out a series of studies with drugs and a dominant negative kinase that inhibit these pathways. The results of our studies clearly implicate the PI 3-kinase/Akt pathway as the predominant regulator of FLIP expression in tumor cells.

RESULTS AND DISCUSSION

**FLIP Expression Is Dependent on MEK Activation in Two Colon Cancer Cell Lines but Not in Other Tumor Cell Lines Examined**—The human colon carcinoma cell lines HT29, clone A, and DLD-1, the prostate carcinoma cell lines DU145 and PC-3, the breast cancer cell lines MCF7, MDA-MB231, and T47D, the renal carcinoma cell lines 786-0 and 769-P, and the melanoma cell line G-361 all constitutively express FLIP (Fig. 1). Because of prior reports linking FLIP expression in T cells to the activation of MEK1 (31), we sought to determine the extent to which the MAP kinase pathway contributed to the constitutive expression of FLIP in tumor cells. To examine the role of the Ras-Raf-MEK-ERK pathway in FLIP expression, tumor cells were first cultured overnight in medium containing 1% FCS and then placed in medium containing 5% FCS with or without the MEK inhibitor PD98059. As shown in Fig. 1A, ERK phosphorylation was readily detectable in both the HT29 and clone A colon carcinoma cell lines after 15 min of serum starvation (zero time point). The readdition of serum transiently enhanced ERK phosphorylation in clone A and, to a lesser extent, in HT29 cells. In both cell lines, the addition of PD98059 (50 μM) for even 15 min completely eliminated ERK phosphorylation as determined by Western blot analysis. The FLIP level declined with time in HT29 but not Clone A cells. Similar studies with all of the other cell lines listed above except the colon carcinoma DLD-1 demonstrated that PD98059, at concentrations sufficient to suppress ERK phosphorylation (50 μM), had no effect on FLIP mRNA expression.
on FLIP levels (Fig. 1B). The DLD-1 cells were similar to the HT29 cells in that their FLIP levels declined, albeit modestly, in response to PD98059. Thus, of the 11 tumor cell lines studied, HT29 and DLD-1 were the only cells in which FLIP expression appeared to be linked to sustained MEK activity.

**FLIP Expression Is Dependent on PI 3-Kinase Activity**—To assess the role of the PI 3-kinase pathway in the regulation of FLIP levels, four of the tumor cell lines were cultured in fresh medium containing or lacking the PI 3-kinase inhibitor LY294002. As shown in Fig. 2, the addition of fresh medium increased the levels of p-Akt to a variable extent in each cell line examined, and this increase was blocked by 50 μM LY294002. In each of the tumor cell lines tested, LY294002 down-modulated FLIP expression. In the case of the HT29 cells, this suppressive effect was apparent within 6 h (not shown), whereas in Clone A, MCF7, and DU145 cells, no significant decrement was observed until 24 h. To determine whether the suppressive effect of LY294002 on FLIP protein levels was associated with a decrement in the level of FLIP transcripts, FLIP mRNA levels were analyzed by RT-PCR at various time points after placing the cells in either control medium or medium containing 50 μM LY294002. As shown in Fig. 3, the addition of LY294002 resulted in a decline in the level of FLIP mRNA levels. The extent of inhibition at 6 h ranged from 50% of the control for the DU145 and MCF7 cells to as much as 70 and 85% for the Clone A and HT29 cells, respectively, as determined by densitometry. In Clone A, MCF7, and DU145 cells, the FLIP mRNA levels were reduced after only 3 h of exposure to LY294002.

**FLIP Expression Is Dependent upon pkB(Akt) Activity**—To determine the role of pkB(Akt) in FLIP expression, tumor cells were infected with an adenoviral construct containing an HA-tagged dominant negative form of pkB/Akt. A similar adenovirus containing a β-galactosidase insert was used as a negative control. For the HT29, Clone A, and MCF7 cell lines, infection was carried out with a multiplicity of 300 and an overnight incubation followed by the addition of fresh medium and an additional 24 h of incubation. In each case, infection of the tumor cells was confirmed by Western blot analysis using an HA-specific antibody to detect the HA-tagged dominant negative pkB/Akt (Fig. 4A). To confirm that this DN-Akt virus actually inhibited the activation of endogenous Akt, we first carried out Western blot analyses using commercially available anti-phospho-Akt antibodies as we had done previously in experiments with LY294002 (Fig. 2). These studies were largely unsuccessful...
because of unexpected cross-reactivity of the antibodies with the DN-Akt virus. To avoid this confounding artifact, we chose a downstream Akt target, glycogen synthase kinase-3, as an indicator of Akt activity in our cell lines (50). As shown in Fig. 4, GSK-3 phosphorylation was reduced in cells infected with the DN-Akt virus but not in the uninfected control cells or cells harboring the β-galactosidase virus. The extent of inhibition was variable, ranging from almost complete inhibition in the HT29, Clone A, and DU145 cells to 40% for the MCF7 cells, as determined by densitometry. These data indicate that the DN-Akt virus effectively blocks the activity of endogenous Akt. Infection with the DN-Akt virus down-regulated FLIP expression in all of the tumor cell lines tested (Fig. 4A). This finding corroborates the results of studies with LY294002 implicating the PI 3-kinase/Akt pathway as a key regulator of FLIP expression in tumor cells. To verify that the down-regulation of FLIP was not a general effect of adenoviral infection and to further verify the influence of activated Akt on FLIP expression, the same cell lines were infected with an adenovirus containing a constitutively active Akt insert. As shown in Fig. 4B, infection of each cell line with the constitutively active Akt adenovirus resulted in increased phosphorylation of GSK-3. FLIP expression in each cell line was also increased. In addition to confirming a direct link between Akt activity and FLIP expression, these experiments confirm that the adenovirus was not responsible for observed changes in FLIP expression.

The mechanism by which the PI 3-kinase/Akt pathway regulates FLIP expression is yet to be determined. As mentioned previously, Akt has been shown to phosphorylate several transcription factors (e.g. CREB, NF-κB, Forkhead), many of which have been implicated in the expression of genes whose protein products regulate susceptibility to apoptosis. CREB, for example, plays a major role in T cell survival (51) and is phosphorylated by Akt at the same site phosphorylated by its structural ple, plays a major role in T cell survival (51) and is phospho-

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