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Nuclear localization of c-FLIP-L and its regulation of AP-1 activity

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ABSTRACT

Cellular FLICE-like inhibitory protein (c-FLIP-L), similar in structure to caspase-8, is capable of blocking Fas- or other death receptors (DR)-mediated apoptosis through association with FADD in the DISC. Recent studies have implicated the function of c-FLIP-L in T-cell proliferation, but the exact mechanism underlying this process remains to be elucidated. In this report, we showed for the first time that c-FLIP-L was present in both the cytoplasm and nucleus of cells, but was more abundantly distributed in the nucleus. The putative NLS signal locates within the p12 region of caspase-like domain. Furthermore, c-FLIP's export to cytoplasm membrane was dependent on apoptotic stimulation, while it rapidly translocated to the nucleus in response to proliferative stimuli. To gain insights into the possible function of c-FLIP-L in the nucleus, we found c-FLIP-L could activate the AP-1 transcriptional activity independent of MAPK activation. In sum, our findings describe a novel function of c-FLIP-L involved in AP-1 activation and cell proliferation.

1. Introduction

Apoptosis, or programmed cell death, is critical for tissue homeostasis in multicellular organisms. It plays an important role in many physiological processes, especially in development and in the immune system. Death receptors belonging to the tumor necrosis factor receptor family (e.g., Fas/CD95, TNFR1, TRAIL-R1, TRAIL-R2, TRAMP/DR3, and DR6) play an important role in apoptosis (Nagata, 1997; Locksley et al., 2001). Activation of Fas receptor by Fas ligand triggers a complex cascade of intracellular events that require Fas-associated death domain (FADD/Mort1) adaptor protein and the formation of death-inducing signaling complex (DISC), leading to caspase-8 activation and apoptosis (Muzio et al., 1996). FLIP is an anti-apoptotic molecule that inhibits the death cascade by all known death receptors (Hu et al., 1997; Schneider et al., 1997; Krueger et al., 2001a,b; Shu et al., 1997; Irmler et al., 1997).

Mammalian cells express two c-FLIP splice variants, a short form, c-FLIP-S, and a long form, c-FLIP-L (Irmler et al., 1997; Thome et al., 1997). c-FLIP-L contains two death effector domains and a caspase-like domain bearing significant homology to caspase-8. c-FLIP-L lacks the critical Cys and His residues which locate in the catalytic domain of caspase-8, rendering c-FLIP-L enzymatically inert (Irmler et al., 1997; Thome et al., 1997). Owing to its structural homology to caspase-8, c-FLIP-L acts as dominant-negative inhibitor of caspase-8 by preventing the process and release of active caspase-8 at the level of the DISC (Micheau et al., 2002; Krueger et al., 2001a,b). As such, c-FLIP-L and c-FLIP-S can bind to the adaptor protein FADD, compete with caspase-8 recruitment and thus block the death receptors-mediated apoptotic pathways via protein–protein interactions.

In addition to its anti-apoptotic function, recent studies have implicated c-FLIP-L's involvement in T-cell proliferation. Overexpression of c-FLIP-L in Jurkat T cells increased ERK and NF-κB activities upon T-cell receptor (TCR) ligation (Kataoka et al., 2000). c-FLIP-transgenic T cells displayed increased proliferation when stimulated with low concentration of anti-CD3 (Lens et al., 2002). Furthermore, mice conditionally lacking c-FLIP in T lymphocytes display severe defects in the development of mature T cells, and Rag chimeric mutant mice (rcFLIP−/−) T cells are impaired in proliferation in response to TCR stimulation (Zhang and He, 2005; Chau et al., 2005). In addition, c-FLIP-L has also been reported to inhibit p38 MAPK and NF-κB activation in other cell types than primary T cells (Grambihler et al., 2003; Bannerman et al., 2004; Kreuz et al., 2004). c-FLIP-L-mediated T-cell proliferation is reported not mediated through the MAPK/AP-1 pathway (Zhang et al., 2008). Thereby, the exact function of c-FLIP in T cells proliferation remains elusive.

Interestingly, c-FLIP$^{-/-}$ mice die during embryogenesis with a cardiac defect similar to that observed in FADD$^{-/-}$ and caspase-8$^{-/-}$ mice (Yeh et al., 2000; Zhang et al., 1998; Varfolomeev et al., 1998). Indeed, these proteins are not only required for the Fas-induced cell death of T cells, but also are involved in T cells proliferation induced by TCR engagement (Tibbetts et al., 2003). Recent evidence indicates that FADD primarily resides in the nucleus and appears

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to shuttle between nucleus and cytoplasm (Gomez-Angelats and Cidlowski, 2003; Screaton et al., 2003; Osborn et al., 2007). FADD may translocate to cytoplasm in response to apoptotic insults that lead to cell death via DISC formation, or alternatively, in the nucleus to regulate cell cycle progression (Hua et al., 2003; Zhang et al., 2004). Given the dual function of FADD in cell apoptosis and proliferation, we hypothesize that FLIP could mimic the effect of FADD and couple T cells proliferation with apoptosis via altering its cellular distribution.

To test this hypothesis, we provided experimental evidences in this study in support of the nuclear presence of c-FLIP-L. In an effort to clarify the role of nuclear c-FLIP-L, we found that c-FLIP could transport to nucleus upon mitogenic stimuli and overexpression of c-FLIP-L could activate AP-1 transcriptional activity independently of ERK and p38 activation in 293T cells. All those data suggest a novel nuclear function of c-FLIP-L in cell proliferation.

2. Materials and methods

2.1. Antibodies and reagents

Antibodies were obtained from the following sources: mouse monoclonal anti-tubulin (TU-02), anti-c-Jun (D), anti-fos (sc-52), anti-FLIP (H-202) and anti-HA (F-7) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Cy3-conjugated anti-rabbit antibody was from Sigma Chemical Co. (St. Louis, MO, USA). Anti-CD69-FITC, anti-Fas, anti-CD3 and anti-CD28 were from BD Pharmingen (Palo Alto, CA, USA), anti-phospho-Thr202/Tyr204 ERK, anti-phospho-Thr180/Tyr182 p38 MAPK, anti-phospho-Thr183/Tyr185 JNK, and polyclonal anti-FLIP (#3210) were from Cell Signaling (Beverly, MA). Other antibodies were used as follows: anti-lamin B (ZYMED, CA, USA), protease inhibitor cocktail (Sigma), PHA (Sigma). Recombinant TRAIL was prepared by our lab.

2.2. Plasmid constructs

pAS2-FLIP-L vector containing the full length of c-FLIP-L was kindly provided by Dr. JK Zhang (Kimmel Cancer Center, USA). The GFP-tagged c-FLIP deletion mutants were constructed by PCR and in-frame cloned into the EcoR I and BamH I sites with the N-terminal GFP tag in pEGFP-C1. The PCR primers were used as followed: c-FLIP (1-172) (5′-CCGAATTCATGGCCCAGAGCCCTGTG-3′) and (5′-CCGGATCCTCACTTTGTGTTCAAGTCTATTCTGTG-3′); c-FLIP (173-376): (5′-CCGAATCCATCCAGAAGTACACCCAG-3′) and (5′-CCGGATCCTCATACCTCCAGGCTGCTATC-3′); c-FLIP (377-480): (5′-CCGAATTCGTAGATGGGCCATCAATA-3′) and (5′-CCGGATCCTCACGTAAGAGCCAGGATG-3′). The pRK5-HA-tagged c-FLIP-L was a kind gift from Dr. Zhen Xing (Department of Molecular and Cell Biology, UC Berkeley). 4× AP-1 luciferase reporter is provided by Dr. Nancy H Colburn (National Cancer Institute, Frederick, MD).

2.3. Cell culture and transfection

293T cells and mouse melanoma B16F10 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5 g/l glucose and 10% calf serum in an atmosphere of 5% CO_2 at 37 °C. Jurkat A3, HeLa were grown in RPMI 1640 culture medium supplemented with 10% heat-inactivated fetal calf serum. All media was supplemented with 1 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

For transient transfections, 5×10^5 cells per ml were plated in 6-well plates. 4 μg of plasmid was transfected with Lipofectamine ™ 2000 (Invitrogen) according to the manufacturer's instruction. After 48 h, transfected cells were harvested and lysates were prepared for Western blot analysis.

2.4. Western blot

Cells were washed in PBS and lysed in a buffer containing 20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 10% glycerol, 0.5% NP-40 and protease inhibitors. After measurement of protein concentration, lysates were separated by SDS-PAGE, electrotransferred to PVDF membranes. The resultant membrane was blocked with 5% milk in TBST for 1 h and incubated with primary antibody at 4 °C overnight, then HRP-conjugated second antibody for 1 h at room temperature. The immune blots were developed using enhanced chemiluminescence (ECL) system (Amersham Bio-sciences).

2.5. RNA interference

c-FLIP siRNA oligonucleotides were synthesized by GenePharma Co. (Shanghai) based on previously described report (Day et al., 2008; Galligan et al., 2005). For transfection, 100 nM of siRNA was mixed with lipofectamine 2000 transfection reagent (invitrogen) according to the manufacturer's instructions. The cells were incubated with the siRNA-lipofectamine 2000 complexes for 48 h before analysis.

DNA oligonucleotides designed to express short hairpin RNA (shRNA) specific for human c-FLIP (5′-GATCCGAAAGAGG-TAAGCTGTCTGTCTTCAAGAGAGACAGACAGCTTACCTCTTTCTTTTTTG-GAAA-3′) were inserted into the pRNAT-U6.1/Neo vector (GenScript, USA). Cells were transfected with shRNA construct using lipofectamine 2000 (Invitrogen).

2.6. Luciferase assay

Cells seeded in 24-well plates were transiently transfected with 4× AP-1luciferase reporter plasmids (0.2 μg) together with *Renilla* Luciferase reporter vector pRL-null (0.1 μg), and pRK5-HA-FLIP-L (0.1–0.4 μg). The total mass of DNA for each transfection was kept constant by adding empty vector pRK5. After 24 h transfection, luciferase assays were preformed using the Dual Luciferase Kit (Promega) following the manufacturer's instructions.

2.7. Nuclear extracts and electrophoresis mobility shift assay (EMSA)

To prepare nuclear protein extracts, cells were suspended in cold hypotonic buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF) for 15 min on ice. Then, 0.2% (final concentration) NP-40 was added and tubes were vigorously vortexed for 10 s. The cytoplasmic fractions were collected by centrifugation for 30 s at 12,000 × *g*. The pellet was washed twice and re-suspended in cold buffer C (20 mM HEPES pH 7.9, 400 mM NaCl, 0.5 mM DTT, 1.5 mM MgCl2, 25% glycerol, 0.2 mM EDTA, 0.5 mM PMSF) for 30 min on ice. Nuclear extracts were prepared after centrifugation. Protein concentrations were determined using Bardford's method.

Nuclear extracts (2 μg) were incubated with γ-^{32}P-labeled oligonucleotide probe in binding buffer containing 100 ng/μl poly (dI:dC), 40% glycerol, 10 mM Tris and 100 mM NaCl for 1 h on ice. The oligonucleotides for AP-1 binding were: 5′-CGC TTG ATG AGT CAG CCG GAA-3′. DNA-protein binding complexes were separated on 4% polyacrylamide gel in 0.5× TBE at room temperature, dried and visualized by autoradiography and phosphorimager. In competition assays, a 30-fold molar excess of the unlabeled oligonucleotide was added.

2.8. Immunofluorescence

Cells were fixed with 4% paraformaldehyde for 30 min at room temperature, washed twice with PBS, then permeablized with 0.5% Triton X-100 in PBS for 15 min and blocked with 3% BSA-PBS