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The VTLISFG motif in the BH1 domain plays a significant role in regulating the degradation of Mcl-1☆

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ABSTRACT

Mcl-1 is a member of the Bcl-2 family protein; its degradation is required for the initiation of apoptosis. The mechanism, however, is not yet clearly known. Previously, it was reported that Mcl-1 is degraded through the ubiquitination-mediated pathway and the PEST domain is the motif responsible for promoting this degradation. We found evidence that this may not be true. We generated several Mcl-1 deletion mutants and examined their effects on protein stability. Deletion of the PEST domain did not prevent the degradation of Mcl-1 during apoptosis. The BH1 domain, but not the PEST, BH3 or BH2 domain, exhibited a short half-life. A peptide named "F3" (VTLISFG) in the C-terminus of the BH1 domain appears to be critical for the rapid turnover of Mcl-1. Deletion of F3 from GFP-Mcl-1-ΔPEST retarded the degradation of this mutant. F3 appeared to be the minimum functional sequence of the degradation motif, since deletion of a single residue was sufficient to abrogate its short half-life. Fusion of F3 with p32 resulted in the degradation of p32 during UV-induced apoptosis, while wild type p32 was not affected. Taken together, these findings suggest that F3 (VTLISFG), instead of PEST, is the major motif responsible for the degradation of Mcl-1 during apoptosis.

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1. Introduction

Apoptosis is a tightly controlled process of cell suicide that is of fundamental importance in maintaining the normal physiological function of a biological organism. The Bcl-2 family proteins are known to play a crucial role in regulating the apoptotic progression. The Bcl-2 family proteins include both pro- and anti-apoptotic members. The ratio between these two subgroups plays an important role in determining the fate of cells [1–3].

Among the anti-apoptotic Bcl-2 family proteins, Mcl-1 has an unusual short half-life. Mcl-1 was originally identified in differentiating myeloid cells in 1993 [4]. The human MCL1 gene is located on chromosome 1q21. During the apoptotic process, the Mcl-1 protein level decreases dramatically in contrast to the other anti-apoptotic proteins Bcl-2 and Bcl-xL [5–7]. The down regulation of Mcl-1 is thought to result from a suppression of Mcl-1 synthesis as well as an enhancement of Mcl-1 protein degradation. The mRNA level of Mcl-1 decreases in response to various apoptotic stimuli such as UV irradiation and staurosporine [6]. In particular, during the apoptosis induced by DNA damage agents or hydrogen peroxide, transcription is blocked due to ubiquitination and this is subsequently followed by the degradation of RNA polymerase II [8–11].

The degradation of the Mcl-1 protein is believed to be mediated through the ubiquitination pathway, since applying the proteasome inhibitor MG132 is able to stabilize the protein level of Mcl-1 [6,7]. Moreover, Mcl-1 can be ubiquitinated at five lysines (5, 40, 136, 194 and 197) and a 482 KDa HECT-domain-containing ubiquitin ligase named Mule was identified as the E3 ubiquitin ligase [12]. Another E3 ligase, β-TrCP, which is a Skp1-CUL1-F box protein (SCF) family member, was also found to recognize the phosphorylated Mcl-1 mediated by GSK3 [13]. In addition, a deubiquitinylase called "ubiquitin-specific

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Abbreviations: β-TrCP, β-transducin repeat-containing protein; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma-2; BH domain, Bcl-2 homology domain; Bim, Bcl-2-interacting mediator; BSA, bovine serum albumin; Caspase, cysteine aspartase; CCD, charge-coupled device; EGFP, enhanced green fluorescent protein; EIF2, eukaryotic translation initiation factor 2; EYFP, enhanced yellow fluorescent protein; GCN2, general control nonrepressed 2; GSK-3β, glycogen synthase kinase-3β; HECT, homologous to E6-AP carboxylterminus; h, hour; HRP, horseradish peroxidase; kD, kilodaltons; Mcl-1, myeloid cell leukaemia sequence 1; MEM, minimum essential medium; Mule, Mcl-1 ubiquitin ligase E3; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; pDNA, plasmid DNA; PERK, PKR-like ER kinase; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; TM domain, transmembrane domain; UV, ultraviolet light.

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peptidase 9 X-linked" (USP9X) is reported to be a regulator of Mcl-1 degradation, as silencing of USP9X by RNA interference (RNAi) led to the loss of Mcl-1 at the protein but not mRNA level [14].

In addition to poly-ubiquitination, Mcl-1 may be cleaved by caspase-3 to disrupt the Mcl-1-Bim interaction, allowing the release of Bim to exert the pro-apoptotic function and lead to Bax activation and cytochrome *c* release [15,16]. On the other hand, a short form of Mcl-1, Mcl-1S/ΔTM, was generated by caspase-3 cleavage [15,17,18]. This short form resembles pro-apoptotic "BH3 only" proteins and promotes apoptosis. Therefore, the cleavage of Mcl-1 may contribute to the feed-forward amplification of apoptotic signals once caspase-3 is activated.

In addition to their role in down regulation, what is the functional domain that plays a crucial role in facilitating the degradation of Mcl-1? The Mcl-1 protein is comprised of 350 amino-acid residues and contains the BH domains 1–3. In the C-terminus, Mcl-1 contains a transmembrane (TM) domain that is involved in localization to the outer mitochondrial membrane [19]. In the N-terminus, Mcl-1 contains two PEST regions (rich in proline, glutamic acid, serine and threonine amino-acid residues), which are often found in rapidly turn-over proteins. Previously the PEST regions were suggested to be responsible for the short half-life of Mcl-1 [20]. This suggestion, however, was questioned by other investigators [21,22]. Thus, we would like to conduct a series of experiments to directly examine which domain is responsible for the degradation of Mcl-1 during apoptosis.

2. Materials and methods

2.1. Chemicals

Anti-GFP and anti-cdc2 monoclonal antibodies (sc-9996) were obtained from Santa Cruz Biotechnology, Inc. Anti-GFP polyclonal antibody (A-6455) was purchased from Molecular Probes. Anti-β-tubulin mouse monoclonal antibody (T4026) was from Sigma–Aldrich, Inc. MG132 (474781) was from Calbiochem.

2.2. Mammalian cell culture and gene transfection

HeLa cells, which were obtained from American Type Culture Collection (ATCC), were cultured in minimum essential medium (MEM) containing 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin in 5% CO_2 at 37 °C. The fusion genes were transfected into cells with Lipofectamine™ 2000 (Invitrogen) using the standard protocol provided by Invitrogen.

2.3. Apoptosis induction

UV irradiation was used as the inducer of apoptosis in this study. The light source was originated from the UV light equipped inside a biological safety cabinet. To induce apoptosis using UV irradiation, cells that were grown as a monolayer in a petri dish were washed with PBS once, covered with PBS and then exposed to UV light (300 mW) for 3 min. The PBS was then replaced with MEM.

2.4. Plasmid construction

The human Mcl-1 gene was kindly provided by Dr. Steven W. Edwards from the University of Liverpool [21]. Mcl-1 was amplified with primers: Forward 5′-CCGGAATTCCGATGTTTGGCCTCAAAAGAAACG-3′ and Reverse 5′-CGCG-GATCCCGCTATCTTATTAGATATGCCAAAC-3′. Then the amplified Mcl-1 was cloned into a pEGFP-C3 vector (Clontech) using EcoR I and BamH I (Roche).

The human P32 gene construct pYW59, encoding the Flag-tagged P32/TAP (1–282) gene, was kindly provided by Dr. S. Diane Hayward from the Johns Hopkins School of Medicine [23]. P32 was then cloned into a pEYFP-N1 vector (Clontech) with primers: Forward 5′-CCGCTCGAGATGCTGCCTCTGCTGCGCTG-3′ and Reverse 5′-GGAATTCCCTGGCTCT-TGACAAAACTCTTG-3′. The truncation mutants of GFP-Mcl-1 and F3 fused P32-YFP were generated by the same method as described above (Table 1).

2.5. Western blotting analysis

HeLa cells were cultured in 60 mm petri-dishes. Cells at different time points after UV treatment or gene expression were collected and lysed in NP-40 lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl and 1% NP-40) in the presence of 1 × protease inhibitor cocktail. Whole cell lysates (80–100 mg/lane) were separated on 10–12% SDS–PAGE and transferred onto a Hybond ECL nitrocellulose membrane (Amersham). After blocking, the membranes were incubated for 3 h at room temperature or overnight at 4 °C with antibodies at a dilution of 1:500 or 1:1000. Then, the membranes were washed three times with 1 × PBS with 0.1% of Tween-20 for 10 min, incubated with horseradish peroxidase-conjugated secondary antibody at a dilution of 1:5000 for 1 h, and ultimately developed using the ECL™ Western-blotting analysis system.

2.6. The living cell imaging system and image analysis

The GFP fusion protein over-expressed HeLa cells were examined under a fluorescent microscope equipped with a CCD camera. The image data acquired were further processed and analyzed by MetaMorph (Universal Imaging Corp.). Then the refined images were obtained using Confocal Assistant v4.0 (Bio-Rad) and Adobe Photoshop (Adobe Systems).

3. Results

3.1. It is the BH1-BH2 domain instead of the PEST domain that has a short half-life

In order to determine which domains of Mcl-1 are responsible for its rapid turnover, we generated a series of mutants by fusing GFP with the different functional domains of Mcl-1 (Fig. 1A).

It has been reported that the PEST domain, in which proline (P), glutamic acid (E), serine (S) and threonine (T) are enriched, is responsible for the short half-life of certain proteins [20]. However, researchers have also argued that the PEST domain is not responsible for the rapid turnover of Mcl-1 [21,22]. Therefore, we wanted to first test whether or not the PEST domain is responsible for its rapid turnover of Mcl-1. We examined the stability of two mutants, GFP-Mcl-1-ΔPEST and GFP-$PEST_{Mcl-1}$, by Western blotting analysis (see Fig. 1B). Surprisingly, the protein level of GFP-Mcl-1-ΔPEST was found to decrease rapidly, during UV-induced apoptosis, just like endogenous Mcl-1. The protein level of GFP-$PEST_{Mcl-1}$, on the other hand, remained largely unchanged. Fluorescence imaging also showed a high expression level of GFP-$PEST_{Mcl-1}$ (Fig. 2A). These findings suggest that the PEST domain is not responsible for the rapid turnover of Mcl-1.

Next, we examined the stability of other domains, including N-terminus (short N-terminus without PEST, Ns), BH3 and BH1-BH2. Interestingly, both the proportion of fluorescence-positive cells and the fluorescence intensity in the GFP-BH1-$BH2_{Mcl-1}$ over-expressed HeLa cells were much lower than the other mutants (Fig. 2A). Also, as shown by Western blotting analysis, the protein level of GFP-BH1-$BH2_{Mcl-1}$ in the HeLa cells decreased rapidly during UV-induced apoptosis, while GFP-$BH3_{Mcl-1}$ and GFP-Ns_{Mcl-1} did not (Fig. 1C). These results suggest that the BH1-BH2 domain is responsible for the rapid turnover of Mcl-1.

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