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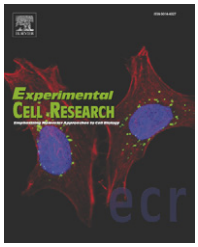

Research Article

TRIM39 is a MOAP-1-binding protein that stabilizes MOAP-1 through inhibition of its poly-ubiquitination process☆

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ABSTRACT

Bax, a multi-domain pro-apoptotic Bcl-2 family member, is a key regulator for the release of apoptogenic factors from mitochondria. MOAP-1, which was first isolated from a screen for Bax-associating proteins, interacts with Bax upon apoptotic induction. MOAP-1 is a short-lived protein that is constitutively degraded by the ubiquitin–proteasome system. Apoptotic stimuli upregulate MOAP-1 rapidly through inhibition of its poly-ubiquitination process. However, cellular factors that regulate the stability of MOAP-1 have not yet been identified. In this study, we report the identification of TRIM39 as a MOAP-1-binding protein. TRIM39 belongs to a family of proteins characterized by a *Tri*partite *M*otif (TRIM), consisting of RING domain, B-box and coiled-coil domain. Several TRIM family members are known to demonstrate E3 ubiquitin ligase activity. Surprisingly, TRIM39 significantly extends the half-life of MOAP-1 by inhibiting its poly-ubiquitination process. In agreement with its effect on enhancing MOAP-1 stability, TRIM39 sensitizes cells to etoposide-induced apoptosis. Conversely, knockdown of TRIM39 reduces the sensitivity of cells to etoposide-stimulated apoptosis. Furthermore, TRIM39 elevates the level of MOAP-1 in mitochondria and promotes cytochrome *c* release from isolated mitochondria stimulated by recombinant Bax. Together, these data suggest that TRIM39 can promote apoptosis signalling through stabilization of MOAP-1.

Introduction

Apoptosis is a physiological process in development and in maintenance of cellular homeostasis in multi-cellular organisms [1,2]. Impairment of apoptotic signalling has increasingly been recognized as a significant contributing factor to many human diseases, including cancer and autoimmunity [1,2].

Mitochondria are major cellular organelles involved in the signal transduction and biochemical execution of apoptosis [3]. Bcl-2 family members are central transducers of survival and apoptotic signals. They act at the mitochondria to regulate the permeability and integrity of the mitochondrial outer membranes, thereby controlling the efflux of apoptogenic factors [1,2,4]. The Bcl-2 family can be divided into three sub-families of pro-survival or pro-apoptotic molecules. The BH3-only proteins (Bim, Bad, Bid, Bik, Noxa, Puma and Hrk) serve as sentinels for the initiation of apoptosis by modulating the functions of multi-domain pro-survival (Bcl-2, Bcl-w, Mcl-1, Bcl-X_L and A1/Bfl-1) or pro-apoptotic members (Bax and Bak) [2,5,6]. Bax and Bak are critically involved in regulating the mitochondrial outer membrane permeability

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which is believed to be the essential step to committing cells to the irreversible execution phase of apoptosis [7,8]. De-regulation of Bax expression occurs in numerous human cancers and down-regulation of Bax has been reported in approximately one third of metastatic breast cancers [9]. Frameshift mutations in *Bax* has led to downregulation or loss of expression in various cancers that exhibit microsatellite instability [10,11]. In addition, accelerated tumour growth and reduced apoptosis are frequently observed in Bax-deficient mice, providing evidence that Bax could act as a tumour suppressor [12].

MOAP-1, *Mo*dulator of *Ap*optosis, initially named MAP-1, was first isolated from a yeast two-hybrid screen using Bax as bait [13]. Endogenous MOAP-1 is highly enriched in mitochondria. Apoptotic stimuli promote Bax translocation from cytosol to mitochondria and induce its association with MOAP-1 [14]. Furthermore, small interfering RNAs (siRNAs) that reduce MOAP-1 levels confer inhibition of Bax-mediated apoptosis, suggesting that MOAP-1 may act as an effector to facilitate apoptotic signalling of Bax in mitochondria [14]. In addition, MOAP-1 interacts with tumour suppressor RASSF1A upon activation of death receptors, together forming a complex with death receptors. This complex is essential for subsequent Bax conformational change and apoptosis [15,16]. Activated K-Ras has also been shown to enhance the RASSF1A-MOAP-1 interaction, resulting in Bax activation and cell death [17].

The role of MOAP-1 as a critical regulator in apoptotic signalling is underscored by the tight regulation of its expression level in mammalian cells [18]. MOAP-1 is a protein with a half-life of only about 25 min and it is constitutively degraded by the ubiquitin–proteasome system (UPS) [18]. Importantly, MOAP-1 is rapidly upregulated by multiple apoptotic stimuli in primary and tumour cell lines through inhibition of its poly-ubiquitination process [18]. Ubiquitination is a pivotal step to mark proteins for degradation by the proteasome. Under the sequential action of E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin ligase), ubiquitin is conjugated to substrate proteins [19]. A variety of mechanisms, including post-translational modifications, regulation by specific de-ubiquitination enzymes and association with a third negative regulatory partner, have been shown to affect protein ubiquitination and its subsequent degradation by the proteasome [19]. For instance, Mcl-1, an anti-apoptotic Bcl-2 family member, is a short-lived protein that is regulated by the UPS [20,21]. Degradation of Mcl-1 is mediated by two ubiquitin ligases, Mule and β-TrCP [22–24], while its stability can be enhanced by its association with TCTP (Translationally Controlled Tumour Protein) which blocks ubiquitination of Mcl-1, thereby reducing its degradation by the UPS [25]. As for MOAP-1, the underlying cellular mechanism and factors that serve to affect its stability have not yet been defined.

In this study, we report the cloning and characterization of a MOAP-1-interacting protein, TRIM39. TRIM39 is a member of the *Tri*partite *M*otif (TRIM) family. This tripartite motif consists of a RING domain, one or two zinc-binding domains known as B-box, and a coiled-coil domain [26]. The RING domain consists of a set of eight cysteine and histidine residues arranged with a defined order and spacing between the residues, which coordinate two zinc atoms [27]. The RING domain is present in a large number of E3 ubiquitin ligases and plays an important role in mediating the interaction of E3 ligases with E2 proteins and subsequent substrate ubiquitination [19]. Indeed, several TRIM family proteins demonstrate E3 activity *in vivo* [28–31]. TRIM39, also known as Ring Finger Protein 23 (RNF23), was first cloned from human and mouse testis cDNA libraries using degenerate primers that contained the conserved sequence of the RING domain [32]. *TRIM39* mRNA is highly expressed in the testis but also ubiquitously expressed in multiple tissues [32]. The *TRIM39* gene is located in the MHC class I region of genes within chromosome 6p21–23, together with at least seven other TRIM proteins [26,32,33]. Its function, however, remains unknown. Surprisingly, instead of promoting degradation of MOAP-1 as a putative E3 ligase, TRIM39 stabilized MOAP-1 by suppressing its poly-ubiquitination process. Furthermore, TRIM39 modulates the sensitivity of cells to etoposide-induced apoptosis and isolated mitochondria to Bax-mediated cytochrome *c* release, suggesting that TRIM39 may serve a role in facilitating apoptosis through stabilization of MOAP-1.

Materials and methods

Yeast two-hybrid screening

Yeast two-hybrid screening was carried out as previously described [13]. The C-terminal region of MOAP-1 which encompasses nucleotides 348–1053 was cloned in-frame with the Gal-4 DNA binding domain in pGBT9 vector and used as bait to screen an adult human brain library (BD Bioscience Clontech). 29 positive clones were isolated from approximately two million transformants and analysed by DNA sequencing.

Constructs and reagents

Full-length TRIM39 was cloned from SH-SY5Y cDNA by polymerase chain reaction (PCR), using the Expand High Fidelity PCR system (Roche Applied Science). The TRIM39 cDNA was subsequently subcloned into pXJ40HA or pXJ40Myc mammalian expression vectors [34]. Both HA and Myc epitope tags were positioned at the N-terminus of TRIM39. Expression plasmids for MOAP-1 full-length cDNA and pXJ40Myc-Ub were described previously [13,18]. Cycloheximide, MG132 (Z-Leu-Leu-Leu et al.), etoposide, staurosporine and thapsigargin, as well as anti-FLAG (M2), anti-Hsp60, anti-actin and anti-MOAP-1 (Atlas) antibodies were obtained from Sigma. Anti-Myc (9E10, A14), anti-HA (F7), anti-PARP (F2), anti-GFP (B2) and anti-Ubiquitin (P4D1) antibodies were obtained from Santa Cruz. Anti-PDI antibody was from Abcam (Cambridge, USA). Anti-mouse and anti-rabbit HRP-conjugated secondary antibodies were from GE Healthcare Amersham.

Cell culture and transfection

HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (HyClone Laboratories, USA) and 1% (v/v) antimycotic-antibiotic (Invitrogen). Lipofectamine 2000 (Invitrogen) and Opti-MEM (Invitrogen) were used for transfection of DNA plasmids into HEK293T cells, according to the manufacturer's instructions. EGFP-N1 (BD Bioscience Clontech) was used as a transfection control.

Indirect immunofluorescence and confocal microscopy analysis

Indirect immunofluorescence was carried out as described [14,35]. HEK293T cells were seeded on 12 mm glass coverslips. Transfected