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Hax-1 is rapidly degraded by the proteasome dependent on its PEST sequence

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Background: HS 1 associated protein X 1 (Hax 1), is a multifunctional protein that has sequence homology to Bcl 2 family members. *HAX 1* knockout animals reveal that it plays an essential protective role in the central nervous system against various stresses. Homozygous mutations in the *HAX 1* gene are associated with autosomal recessive forms of severe congenital neutropenia along with neurological symptoms. The protein level of Hax 1 has been shown to be regulated by cellular protease cleavage or by transcriptional suppression upon stimulation.

Results: Here, we report a novel post translational mechanism for regulation of Hax 1 levels in mammalian cells. We identified that PEST sequence, a sequence rich in proline, glutamic acid, serine and threonine, is responsible for its poly ubiquitination and rapid degradation. Hax 1 is conjugated by K48 linked ubiquitin chains and undergoes a fast turnover by the proteasome system. A deletion mutant of Hax 1 that lacks the PEST sequence is more resistant to the proteasomal degradation and exerts more protective effects against apoptotic stimuli than wild type Hax 1.

Conclusion: Our data indicate that Hax 1 is a short lived protein and that its PEST sequence dependent fast degradation by the proteasome may contribute to the rapid cellular responses upon different stimulations.

Keywords: Hax 1, Proteasome, Ubiquitin, PEST sequence, Bcl 2 family protein

Background

HS-1-associated protein X-1, Hax-1, is a 35 kDa protein with two Bcl-2 homology (BH) domains that was identified in a yeast two hybrid screen where it was found to interact with HS-1, a Src kinase substrate [1]. Hax-1 is ubiquitously expressed in most tissues and is reported to be localized in mitochondria as well as the endoplasmic reticulum (ER) and nuclear membrane [1-3]. Mutations identified in the human *HAX-1* gene have been shown to cause neutropenia and neurodevelopmental abnormalities [4-6]. Knockout *HAX-1* mice show increased apoptosis of neurons and postnatal lethality. [7]. Hax-1 is a multifunctional protein that plays roles in calcium homeostasis [8], cell migration [9] and apoptotic regulation [10,11]. It was reported that Hax-1 protects cells against various stimuli and has been shown to interact with a number of cellular and viral proteins to suppress their pro-death properties [12-15]. In addition, Hax-1 has been found to be up-regulated in breast cancer, lung cancer and melanoma [16], suggesting that it also has a role in oncogenesis.

A PEST sequence is a peptide sequence which is rich in proline (P), glutamic acid (E), serine (S), and threonine (T). It is known that the PEST sequence functions as a proteolytic signal to target proteins for degradation resulting in short intracellular half lives [17]. For example, the PEST sequence of NF-kappa B is responsible for its cleavage by calpain [18]. It was reported that c-myc, a protein with a PEST sequence, has a half-life shorter than one hour [17]. Notch 1, another short-lived protein, is ubiquitinated by an E3 ligase sel-10 and degraded by the proteasome dependent on its PEST sequence [19,20].

Hax-1 was predicted to contain a PEST sequence (aa 104–117) [1], however, it is still unknown whether this PEST sequence effects its turnover rate. In this study, we investigated the stability of Hax-1 in different cells and explored the role of the PEST sequence in its degradation and biological function.

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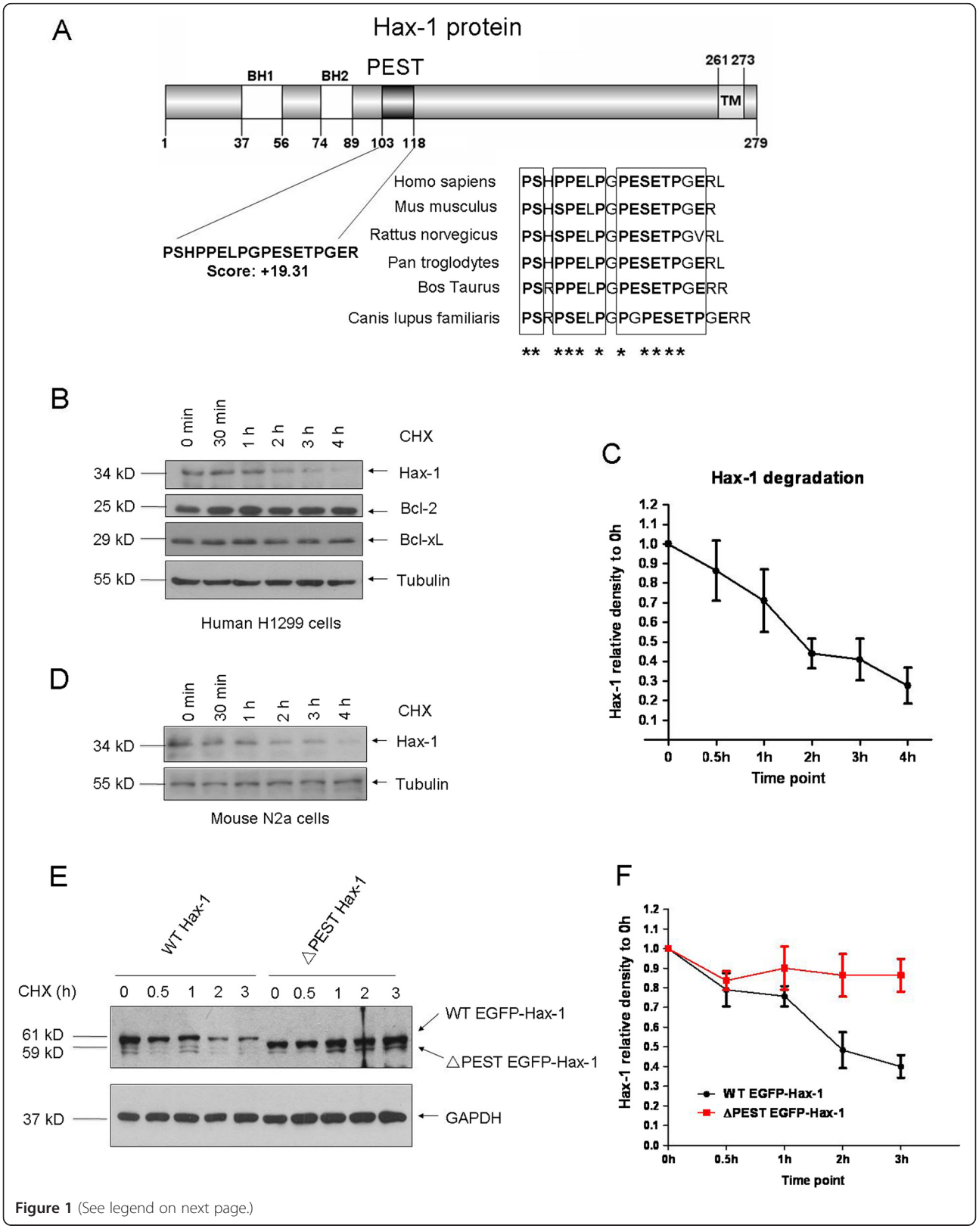

Figure 1 (See legend on next page.)

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Figure 1 Rapid degradation of Hax 1 is dependent on its PEST sequence. A. Schematic representation of a PEST sequence in Hax 1 protein. The PEST sequence was identified using Pestfind service on "emboss.bioinformatics.nl/cgi bin/emboss/pestfind". The PEST sequence in Hax 1 is conserved among different mammals. **B**. Chase time experiment of Hax 1 and other Bcl 2 proteins. H1299 cells treated with CHX (100 ug/ml) for different time points were harvested for immunoblot analysis using indicated antibodies. **C**. Data from three independent experiments in B were quantified. **D**. Similar experiments as B were carried out using mouse N2a cells. **E**. An EGFP tagged WT Hax 1 or ΔPEST Hax 1 was transiently transfected into H1299 cells. Forty eight hours later, CHX chase experiments were carried out. **F**. Quantitative analysis of data from E with three independent experiments.

Results

Rapid degradation of Hax-1

In addition to its BH domains and a trans-membrane domain, Hax-1 has a PEST sequence [1]. The PEST region in Hax-1 is highly conserved in mammalian animals (Figure 1A). We tested the degradation profile of Hax-1 using a cycloheximide (CHX) chase experiment in both human lung cancer cell line H1299 and mouse neuroblastoma cell line N2a. Hax-1 was found to have a much shorter half-life than other two pro-survival Bcl-2 family proteins, Bcl-2 and Bcl-xL (Figure 1B-D), suggesting that the Hax-1 protein is unstable and is rapidly degraded.

PEST sequence-dependent degradation of Hax-1

We next tested whether the PEST sequence in Hax-1 is responsible for its rapid degradation. A deletion mutant of Hax-1 was constructed in which the PEST sequence (aa 103–118) was deleted. The CHX chase experiments showed that the ΔPEST Hax-1 level remained largely unchanged up to 3 hours, whereas WT Hax-1 level rapidly decreased to < 50 % within 3 hours (Figure 1E and F), suggesting that the PEST sequence in Hax-1 is necessary for its rapid degradation.

Degradation of Hax-1 by the ubiquitin-proteasome pathway

Proteasome and autophagy systems are two main pathways for protein degradation. Here we tested which pathway is involved in the fast-turnover of Hax-1. Cells were treated with MG132, a proteasome inhibitor, or Bafilomycin A1, an autophagy inhibitor. The level of EGFP-Hax-1 increased in cells treated with MG132 for 3 hours (Figure 2A), whereas in cells treated with Bafilomycin A1 the protein level remained unchanged up to 18 hours (Figure 2B). These data suggest that Hax-1 is mainly degraded by the proteasome, but not by autophagy-lysosome pathway. A time-dependent increase in endogenous Hax-1 level was also observed in cells treated with MG132 (Figure 2C). We next examined the turnover of endogenous Hax-1 in the presence of MG132 using CHX chase experiments. In the presence of MG132, endogenous Hax-1 was not observed to be degraded within 4 hours, however, in the absence of MG132, it was rapidly degraded after two hours (Figure 2D).

Hax-1 conjugation with K48-linked ubiquitin chains is dependent on the PEST sequence

We have shown that Hax-1 is degraded by the proteasome. Usually, the proteasomal degradation process requires polyubiquitination of the substrates [21]. We therefore tested if Hax-1 is ubiquitinated and if yes, what kind of ubiquitin conjugation is involved in the degradation of Hax-1. Enhanced ubiquitination of Hax-1 was observed in the presence of MG132 than that in the absence of MG132 (Figure 3A) as revealed by co-immunoprecipitation experiments. Then, we examined the polyubiquitin of Hax-1 with two specific antibodies which recognize K48- or K63-linked ubiquitin, respectively. Increased polyubiquitination of Hax-1 was detected with an antibody specific to K48-linked polyubiquitin, but not with that to K63-linked polyubiquitin (Figure 3B), suggesting that Hax-1 is mainly conjugated by the K48-linked ubiquitin chains. We next evaluated if the PEST sequence affects Hax-1 polyubiquitination. We found that the deletion of the PEST sequence in Hax-1 greatly decreased its polyubiquitination (Figure 3C), suggesting that the PEST sequence in Hax-1 is necessary for its ubiquitination.

Increased degradation of Hax-1 during apoptosis

As Hax-1 is known to be an anti-apoptotic protein, we hypothesized whether its degradation is regulated under apoptosis. We transfected H1299 cells with EGFP-Hax-1 and treated them with DMSO or staurosporine (STS), an inducer of apoptosis. In the absence of MG132, the amounts of Hax-1 protein decreased with increasing concentration of STS, however, in the presence of MG132, the trend was largely attenuated (Figure 3D and E), suggesting an accelerated degradation of Hax-1 by the proteasome under apoptosis.

ΔPEST Hax-1 mutant attenuated STS-induced cell death

As overexpression of Hax-1 has been shown to have an anti-apoptotic effect and also regulates mitochondria membrane potential [10], we examined the effects of knockdown of Hax-1 on STS-induced apoptosis. The efficacy of the siRNA against Hax-1 was evaluated (Figure 4A). STS induced significantly higher level of apoptosis in those cells in which Hax-1 levels were knocked down as compared to control cells. This

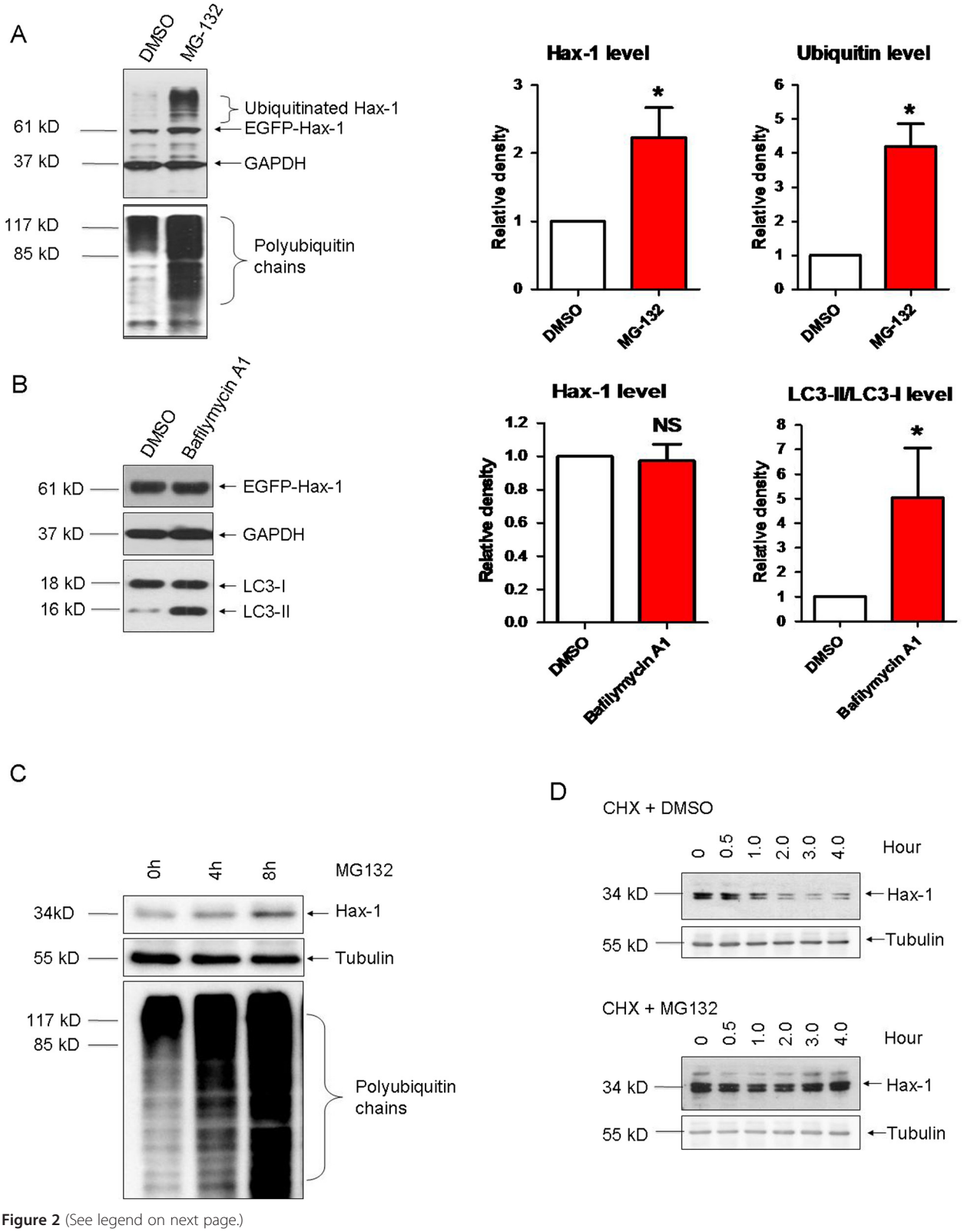

Figure 2 (See legend on next page.)