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Bcl-x_L deamidation is regulated by multiple ion transporters and is intramolecularly catalyzed

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ABSTRACT

In susceptible tumor cells, DNA-damaging antineoplastic agents induce an increase in intracellular pH during the premitochondrial stage of apoptosis. The rate of nonenzymatic deamidation of two asparagines in the anti-apoptotic protein Bcl-x_L is accelerated by this increase in pH. Deamidation of these asparagines is a signal for the degradation of Bcl-x_L, which is a component of the apoptotic response to DNA damage. It has previously been shown that the increase in pH is mediated by the ion transporter Na^+/H^+ exchanger 1 in some cells. Here we demonstrate that one or more additional ion transporters also have a role in the regulation of Bcl-x_L deamidation in at least some tumor cell lines and fibroblasts. As a second, independent finding, we report that there are histidines in close proximity to the Bcl-x_L deamidation sites that are highly conserved in land-dwelling species and we present evidence that deamidation of human Bcl-x_L is intramolecularly catalyzed in a manner that is dependent upon these histidines. Further, we present evidence that these histidines act as a pH-sensitive switch that enhances the effect of the increase in pH on the rate of Bcl-x_L deamidation. The conservation of such histidines implies that human Bcl-x_L is in essence "designed" to be deamidated, which provides further evidence that deamidation serves as a *bona fide* regulatory post-translational modification of Bcl-x_L.

1. Introduction

Asparagine deamidation is a common post-translational modification. It results in the formation of a succinimide intermediate that is subsequently hydrolyzed to either an isoaspartate or an aspartate, with isoaspartate as the major product. Notably, deamidation of an asparagine is a nonenzymatic reaction that requires nothing more than a water molecule to proceed [1]. We have previously demonstrated that the antiapoptotic Bcl-2 family member Bcl-x_L undergoes deamidation at Asn 52 and Asn 66 when susceptible tumor cells are treated with DNA-damaging antineoplastic agents [2]. We subsequently found that deamidation is a signal for Bcl-x_L degradation and that this is a component of cellular susceptibility to DNA-damaging antineoplastic agent-induced apoptosis [3].

The rate of asparagine deamidation is determined by the amino acid sequence surrounding the asparagine and environmental factors such as pH, ionic strength, and temperature [4]. DNA-damaging antineoplastic agents induce an increase in the intracellular pH of tumor cells during the premitochondrial phase of apoptosis and we and others have previously reported that an increase in pH is sufficient to cause an increase in the rate of Bcl-x_L deamidation [2,3,5,6]. An increase in pH facilitates deamidation because either the rate of base-catalyzed hydrolysis of the succinimide intermediate is increased under alkaline conditions [7] or alkaline conditions increase the rate of succinimide formation from a tetrahedral intermediate [8].

Intracellular pH is regulated by several bicarbonate transporters [9] and a family of transporters that raise intracellular pH by exchanging extracellular Na^+ for intracellular H^+, the Na^+/H^+ exchangers (NHEs) [10]. Ten NHE family members (NHE1 to NHE10) have been identified thus far [11]. It has been shown that one of these, NHE1, can mediate DNA damage-induced intracellular alkalization and, thereby, increase the rate of Bcl-x_L deamidation and death in some cells [6,12].

The NHE isoforms share ~20% to 60% amino acid identity [13], with all isoforms sharing a similar topology: an N-terminus of 12

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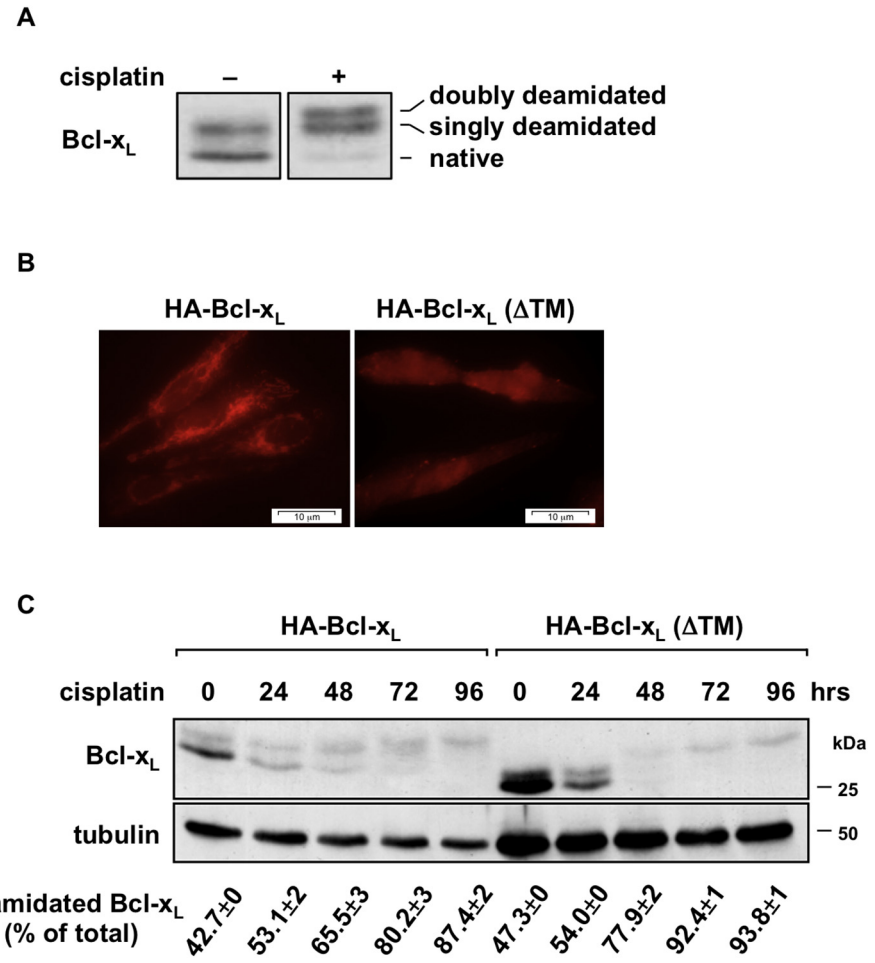

Fig. 1. DNA damage-induced Bcl-x_L deamidation is mediated by a general cellular process. (A) Illustration of the migration pattern of native and deamidated forms of Bcl-x_L on immunoblot. (B) Representative images of immunostaining of Bcl-x_L. HA-Bcl-x_L and HA-Bcl-x_L(ΔTM) was expressed in SAOS-2 cells ($n = 3$). Scale bars = 10 μm. (C) Immunoblots of HA-Bcl-x_L and HA-Bcl-x_L(ΔTM) from SAOS-2 cells that were treated with 10 μM cisplatin for the indicated times. The experiment was performed twice. A representative blot is shown. The deamidated (upper) and native (lower) forms of Bcl-x_L were quantified using ImageJ software and the deamidated percentage of total Bcl-x_L is shown below the blot as mean ± SD ($n = 2$). Molecular weight markers are indicated.

transmembrane α-helices that collectively function in ion exchange and a C-terminal cytoplasmic regulatory domain that modulates transport activity by the transmembrane domain [14]. Extracellular signals mediated by diverse classes of cell surface receptors regulate NHE activity through distinct signaling networks that converge to directly modify the C-terminal regulatory domain [14–16]. We noted that there are examples of multiple NHE proteins that are regulated in parallel in response to certain signals [17,18]. We therefore sought to determine whether other NHE family members besides NHE1 could have a role in regulating Bcl-x_L deamidation.

Additionally, we have previously shown that comparative sequence analysis of Bcl-x_L proteins across species and between Bcl-x_L and other Bcl-2 family members underscores the importance of deamidation in the regulation of cellular Bcl-x_L activity: (i) an asparagine is most susceptible to deamidation when it is followed by a glycine in a flexible region of a protein (glycine lowers steric hindrance facilitating the nucleophilic attack of the nitrogen on the asparagine side chain [19]), and two asparagine-glycine sequences are found in close proximity within a flexible region of human Bcl-x_L [3]; (ii) asparagine-glycine sequences are found in a similar position in every Bcl-x_L protein ortholog from the sponge to the human form that has been sequenced to date [3]; (iii) there is no other sequence similarity besides the glycines surrounding the asparagine sequences that occurs across all of the Bcl-x_L proteins; and (iv) there are no corresponding asparagine-glycine sequences in other Bcl-2 family members. Together these findings provided strong evidence that the presence of asparagine-glycine sequences in a specific domain *per se* is a conserved feature of Bcl-x_L. Consequently, because the only known function of asparagine-glycine sequences is asparagine deamidation, these findings provide strong evidence that asparagine deamidation is a conserved feature of Bcl-x_L and the extent of its conservation is evidence of its importance in the regulation of Bcl-x_L.

We now report that human Bcl-x_L contains histidines in close proximity to each of its two deamidation sites and that such histidines are conserved throughout the Bcl-x_L proteins of land-dwelling species. We thought this was notable because studies using model proteins demonstrate that histidines increase susceptibility to deamidation when in proximity to asparagine [19]. Indeed, we show here that deamidation of human Bcl-x_L is intramolecularly catalyzed in a manner that involves these highly conserved histidines and that treatment of susceptible tumor cells with DNA-damaging agents increases the catalytic activity.

2. Materials and methods

2.1. Cell culture

SAOS-2 osteosarcoma cells (ATCC HTB-85), MEFs, 3T3 (ATCC CRL-1658), C33A (ATCC HTB-31), and HeLa (ATCC CCL-2) were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS. Caco-2 (ATCC HTB-37) were maintained in MEMα supplemented with 10% FBS. HTB-9 cells (ATCC HTB-9) were cultured in RPMI with 10% FBS. $p53^{-/-}$ mice were a generous gift from L. Donehower (Baylor College of Medicine). $nhe1^{-/-}$ and $nhe2^{-/-}$ mice were a generous gift from J. Orlowski (McGill University). $nhe1^{+/+}/p53^{-/-}$, $nhe1^{-/-}/p53^{-/-}$, $nhe2^{+/+}/p53^{-/-}$, and $nhe2^{-/-}/p53^{-/-}$ MEFs were prepared from E13.5 embryos according to standard protocols. $bcl\text{-}x^{-/-}/p53^{-/-}$ MEFs were described previously [2]. Cells were treated with 100 μM DMA, 10 μM etoposide, and 10 μM cisplatin unless otherwise indicated.

Survival was quantified by the Cell Counting Kit-8 assay (CK04-11; Dojindo) in accordance with manufacturer's instructions. After