DNA Damage–Induced Bcl-xL Deamidation Is Mediated by NHE-1 Antiport Regulated Intracellular pH

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The pro-survival protein Bcl-xL is critical for the resistance of tumour cells to DNA damage. We have previously demonstrated, using a mouse cancer model, that oncogenic tyrosine kinase inhibition of DNA damage–induced Bcl-xL deamidation tightly correlates with T cell transformation in vivo, although the pathway to Bcl-xL deamidation remains unknown and its functional consequences unclear. We show here that rBcl-xL deamidation generates an iso-Asp52/iso-Asp66 species that is unable to sequester pro-apoptotic BH3-only proteins such as Bim and Puma. DNA damage in thymocytes results in increased expression of the NHE-1 Na/H antiport, an event both necessary and sufficient for subsequent intracellular alkalisation, Bcl-xL deamidation, and apoptosis. In murine thymocytes and tumour cells expressing an oncogenic tyrosine kinase, this DNA damage–induced cascade is blocked. Enforced intracellular alkalisation mimics the effects of DNA damage in murine tumour cells and human B-lineage chronic lymphocytic leukaemia cells, thereby causing Bcl-xL deamidation and increased apoptosis. Our results define a signalling pathway leading from DNA damage to up-regulation of the NHE-1 antiport, to intracellular alkalisation to Bcl-xL deamidation, to apoptosis, representing the first example, to our knowledge, of how deamidation of internal asparagine residues can be regulated in a protein in vivo. Our findings also suggest novel approaches to cancer therapy.

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Introduction

The deamidation of internal asparaginyl and glutaminyl protein residues has attracted increasing attention over the past decade as a modification leading to significant changes in protein function [1,2]. The protein deamidation rates of more than 18,000 proteins have been computed, containing 230,000 individual asparaginyl residues, generating Asn half-lives of less than 1 d to 50 y or more [3,4]. Protein deamidation has broad biological implications, ranging from changes in the specificity of antigen presentation [5], to modifications in eye lens proteins [6], to the activation of RhoA by cytotoxic necrotizing factor [7], to aging [1], to name but a few examples.

The deamidation of Gln proceeds both enzymatically and nonenzymatically in physiological systems, whereas only the nonenzymatic deamidation of internal Asn residues has been reported, involving conversion to Iso-Asp:Asp in a ratio of about 3:1, with the precise ratio depending on the environment of the Asn residue [1,8]. Deamidation of both Gln and Asn residues in vitro can be greatly accelerated by exposure to either acid or alkaline pH, with minima in the range pH 4–6. Until recently, it was assumed that Asn protein deamidation rates in vivo were set up by a “fixed clock” that was defined only by the primary, secondary, and tertiary structures of proteins that specified the half-life of the particular Asn residue in question. However, this view has been radically changed by the recent observation that DNA damage induces the relatively rapid deamidation of the pro-survival protein Bcl-xL in an osteosarcoma cell line system [9], indicating that the deamidation “clock”, far from being fixed, is a dynamic process that can be regulated in vivo by biologically critical events. Bcl-xL deamidation in response to DNA damage occurs at two internal Asn residues (Asn52 and Asn66), causing a characteristic retardation on SDS-polyacrylamide gel electrophoresis (PAGE) gels [9–12]. Initial work from the Weintraub laboratory suggested that when Asn52 and Asn66 are both mutated to Asp, then Bcl-xL loses its ability to bind to the BH3-only pro-apoptotic protein Bim, thereby providing a putative linkage between DNA damage and apoptosis [9]. However, a secondary mutation was later identified, which, when corrected, enabled the N52D/N56D Bcl-xL to bind Bim, casting doubt on this interpretation [13].

Using a different model system, we have previously implicated the oncogene-mediated inhibition of DNA damage-induced Bcl-xL deamidation in the transformation of murine thymocytes [14,15]. Our transgenic mouse model of T cell lymphoma was generated by crossing mice lacking...
expression of the CD45 tyrosine phosphatase with a line expressing a nononcogenic level of the mutant lck<sup>F505</sup> tyrosine kinase [16]. All the CD45<sup>+/−</sup>lck<sup>F505</sup> progeny develop aggressive T cell lymphomas at the early CD4<sup>+</sup>CD8<sup>+</sup> stage of thymic development, typically at 5–12 wk of age. The absence of CD45-mediated dephosphorylation results in hyperphosphorylation of positive regulatory p56<sup>lck</sup> pTyr-394, causing hyperactivation of the kinase and triggering oncogenesis [15]. The model enables the investigation of the earliest oncogenic events in primary tumourigenic thymocytes. Inhibition of DNA repair in CD45<sup>−/−</sup>lck<sup>F505</sup> mice leads to DNA damage, genomic instability, and chromosomal aberrations detectable in primary CD4<sup>+</sup>CD8<sup>+</sup> thymocytes before transformation.

Despite a normal p53 response, DNA damage–induced apoptosis is suppressed in tumourigenic thymocytes, correlating with the inhibition of Bcl-x<sub>L</sub> deamidation, the preservation of Bcl-x<sub>L</sub> binding to Bim, and the inhibition of cytochrome c release and the apoptotic caspase execution cascade. Therefore, we proposed that Bcl-x<sub>L</sub> deamidation is a critical switch in oncogenic kinase–induced T cell transformation, and we suggested that Bcl-x<sub>L</sub> deamidation to an Iso-Asp<sup>52</sup>/Iso-Asp<sup>66</sup>, Preventing Sequestration of Bim and Puma

Neither in the osteosarcoma cell line work [9] nor in our own work based on primary thymocytes [15] has there been any indication as to how DNA damage might induce Bcl-x<sub>L</sub> deamidation. Neither have there been previous reports in the literature showing how protein Asn deamidation in general might be regulated in vivo; we address here this question. We confirm that Bcl-x<sub>L</sub> deamidation does indeed destroy its ability to sequester pro-apoptotic proteins such as Bim and Puma, thereby establishing a clear molecular link between DNA damage, Bcl-x<sub>L</sub> deamidation, and apoptosis. Surprisingly, DNA damage–triggered deamidation in primary wild-type cells is mediated not enzymatically, but by intracellular alkalinisation caused by increased expression of the NHE-1 Na<sup>+</sup>/H<sup>+</sup> exchanger (antiport), events blocked by expression of the oncogenic tyrosine kinase (OTK). In the case of either murine or human cancer cells, enforced alkalinisation triggers Bcl-x<sub>L</sub> deamidation, crippling its ability to provide protection from the pro-apoptotic consequences of DNA damage, thereby indicating possible novel approaches to cancer therapy.

Results
DNA Damage–Induced Bcl-x<sub>L</sub> Deamidation Does Not Depend on Mitochondrial Apoptosis

An important consideration is whether DNA damage–induced Bcl-x<sub>L</sub> deamidation in murine thymocytes is a cause or consequence of thymic apoptosis. Figure 1 shows that whereas the addition of the caspase inhibitor Z-VAD-fmk, as expected, inhibited DNA damage–induced apoptosis in murine thymocytes (Figure 1A), no inhibition of DNA damage–induced Bcl-x<sub>L</sub> deamidation was observed in cell aliquots taken from the same thymic cultures (Figure 1B). It is known that in the absence of Bax and Bak, BH3-only proteins are unable to induce apoptosis [17]. We therefore used short hairpin RNA (shRNA) to deplete Bax and Bak from CD4<sup>+</sup>CD8<sup>+</sup> (double-negative, DN) thymocytes, confirmed that depletion was sufficient to block caspase 9 cleavage (Figure S1A), and showed that DNA damage–induced Bcl-x<sub>L</sub> deamidation proceeded normally in the absence of Bax and Bak (Figure 1C). We also showed that Bcl-x<sub>L</sub> deamidation was clearly detectable within 3–6 h after the instigation of DNA damage, and proceeded in parallel with increased apoptosis (Figure S1B and S1C). These results show that Bcl-x<sub>L</sub> deamidation is not caused by mitochondrial apoptosis and are consistent with a role for deamidation upstream of the apoptotic executor pathway.
plays a major role in DNA-damage triggered apoptosis [19,20], we also showed that both Puma and Bim are found in Bcl-xL immunoprecipitates from etoposide treated CD45^+/CD8^+/LckF505^+ thymocytes, whereas sequestration is ablated in wild-type cells, correlating with Bcl-xL deamidation (Figure 2B). A comparable result was obtained when Puma immunoprecipitates were blotted for Bcl-xL (Figure S2A). Therefore, deamidated Bcl-xL appears unable to sequester BH3-only proteins.

To confirm the results using intact thymocytes, we carried out in vitro biochemical experiments. Recombinant purified His-tagged Bcl-xL was exposed to alkaline conditions to cause partial deamidation and separated by anion-exchange chromatography into three peaks (Figure 2C, peaks A, B and C). Mass spectrometric analysis revealed an increase of 1 Da for peak B relative to peak A, and a further increase of 1 Da for peak C relative to peak B (Figure 2C). On SDS-PAGE gels, peak A Bcl-xL migrated slightly faster than the more acidic peaks B and C (Figure 2D), reproducing the characteristic profile of N52/N66 Bcl-xL and its deamidated versions found in our cellular studies (Figure 1B). It has already been demonstrated that these migratory shifts are not caused by phosphorylation [9,12]. In fact, deamidation of a single Asn increases protein mass by 1 Da, at the same time increasing its net negative charge, confirming that the shifts are due to deamidation. Importantly, when the three species of rBcl-xL were tested for their ability to bind to Bim in wild-type thymic lysates, only peak A bound Bim effectively, whereas binding to peak B rBcl-xL was reduced by 88% ± 2% and completely ablated using peak C rBcl-xL (Figure 2D, upper panel). Figure 2E shows that the Asp^52/Asp^66 version of Bcl-xL, or the Ala^52/Ala^66 version that cannot be deamidated, does still bind both Bim and Puma, consistent with the correction published by the Weintraub laboratory [13]. We therefore determined whether rBcl-xL Asn^52 and Asn^66 convert mainly to Asp or to iso-Asp upon alkali treatment. Consistent with previous results [8], Figure 2F and Figure S3 show that the ratios of iso-Asp/Asp conversion for Asn^52 and Asn^66 are 10:1 and 5:1, respectively. Kinetic analysis revealed that deamid-
tion of Asn<sup>66</sup> to iso-Asp is much faster than for Asn<sup>52</sup> (unpublished data).

Taken together, our results show that conversion of Bcl-x<sub>L</sub> Asn<sup>52</sup> and Asn<sup>66</sup> to iso-Asp, but not Asp, prevents sequestration of BH3-only proteins. Peak B represents rBcl-x<sub>L</sub> deamidated at either Asn<sup>52</sup> or Asn<sup>66</sup>, whereas peak C is deamidated at both sites (Figure 2C and 2D). Deamidation to iso-Asp causes greater perturbations of protein structure than conversion to Asp [1,8], presumably explaining the loss of BH3-only protein binding.

**DNA Damage–Induced Bcl-x<sub>L</sub> Deamidation and Apoptosis Is Mediated by Intracellular Alkalisation**

Until now, the in vivo mechanism for the deamidation of internal protein Asn residues has not been described for any protein. Because protein Asn deamidation is accelerated by...
increased pH in vitro, we investigated intracellular pH change (pHi) as a possible regulatory mechanism in thymocytes. Figure 3A shows that after DNA damage, the pHi of live wild-type CD4\(^+\)CD8\(^+\) thymocytes increased to 7.55, whereas no change was observed in pretumourigenic cells. But is that increase sufficient to cause Bcl-xL deamidation? To address this question, we incubated wild-type thymocytes in the pH range of 7.2–8.0 for 20 h in the presence of the Na\(^+\) ionophore monensin to ensure complete equilibration of pHi and extracellular pH (pHe), and to neutralize acidic intracellular compartments [21], and we then assessed the extent of Bcl-xL deamidation. Figure 3B shows that whereas only 22.5% ± 3.2% was deamidated at pH 7.2, this increased to 56.1% ± 3.8% at pH 7.6 and 67.0% ± 4.5% at pH 8.0. Therefore, a rise in pHi, comparable with that observed after DNA damage (Figure 3A) is sufficient to cause substantial deamidation. Furthermore, the addition of Z-VAD-fmk to thymic cultures following DNA damage did not inhibit their alkalisation (Fig 3C), showing that the rise in pHi is not downstream of casapse activation. To investigate Bcl-xL deamidation, pHi, and apoptosis in parallel, we manipulated pHi values artificially by incubating cells at varying pHi values in the absence of monensin. The left panel of Figure 3D shows that when DNA damage was induced in wild-type thymocytes, Bcl-xL deamidation could be largely prevented by artificially maintaining the pHi at 7.1 (value shown in Figure 3E, left panel), thereby reducing the percentage of apoptotic CD4\(^+\)CD8\(^+\) thymocytes by 2-fold relative to those incubated at physiological pH (Figure 3F, left panel). Conversely, Figure 3D (right panel) shows that the resistance to Bcl-xL deamidation observed in DNA-damaged pretumourigenic thymocytes could be completely overcome by artificially increasing the pHi to 7.55 or above (Figure 3E, right panel), correlating with a 2-fold increase in the percentage of apoptotic CD4\(^+\)CD8\(^+\) thymocytes relative to those incubated at physiological pH (Figure 3F, right panel). Interestingly, enforced alkalisation alone in the absence of DNA damage caused a marked increase in Bcl-xL deamidation in the OTK expressing thymocytes (Figure 3D, right panel), with a concomitant increase in apoptosis (Figure 3F, right panel), albeit at a level lower than with DNA damage, perhaps reflecting the somewhat lower pHi values achieved under these conditions (Figure 3E, right panel).

We considered that the tight correlation between pHi, Bcl-xL deamidation, and apoptosis might nevertheless be coincidental and that enforced alkalisation might be inducing apoptosis by a mechanism independent of Bcl-xL deamidation. Mutant Bcl-xL Ala\(^{52}\)/Ala\(^{66}\) or Asp\(^{52}\)/Asp\(^{66}\), both of which sequester BH3-only proteins (Figure 2E), were therefore over-expressed in wild-type CD4\(^+\)CD8\(^+\) thymocytes by retroviral transduction prior to enforced alkalisation by incubation in media at pH 8.0 or 8.5. Figure 3G (middle panel) shows that, as expected, the Ala\(^{52}\)/Ala\(^{66}\) mutant migrates as the lower nondeamidated version of Bcl-xL, whereas Asp\(^{52}\)/Asp\(^{66}\) migrates as the more negatively charged deamidated version. Interestingly, in the cells expressing these mutant forms of Bcl-xL, the apoptosis induced by enforced alkalisation was reduced 4-fold compared to cells transduced with empty vector, or more than 2-fold in comparison with the wild-type protein (Figure 3G, right panel), which of course undergoes deamidation in response to alkali treatment. These results show that Bcl-xL in a version able to sequester BH3-only proteins protects thymocytes from an enforced increase in pH, Nevertheless, protection was not absolute, suggesting that Bcl-xL may not be the only mechanism protecting cells from apoptosis triggered by alkalisation. As a further control, we have confirmed that Bcl-xL isolated from wild-type thymocytes exposed to a high pH buffer can no longer sequester Bim (Figure S2B), thereby mimicking the effects of DNA damage (Figure 2A).

Taken overall, these results demonstrate that intracellular alkalisation following DNA damage is both necessary and sufficient for nonenzymatic Bcl-xL deamidation, that the oncogenic suppression of Bcl-xL deamidation in pretumourigenic thymocytes is caused by inhibition of alkalisation, and that versions of Bcl-xL competent for BH3-only protein sequestration are sufficient per se to protect cells from apoptosis at alkaline pH.

DNA Damage–Induced Alkalisation, Bcl-xL Deamidation, and Apoptosis are Mediated by Increased NHE-1 Antiport Expression

We next investigated the molecular mechanisms leading from DNA damage to the regulation of pHi and subsequent Bcl-xL deamidation. Figure 4A shows that de novo protein synthesis is essential for Bcl-xL deamidation following DNA damage in wild-type thymocytes. Because the NHE-1 Na/H antiport is a well-established regulator of pHi [22] and has previously been implicated in the regulation of thymic apoptosis [23], we measured its expression in wild-type thymocytes after DNA damage and found that the NHE-1 level increased 2.5-fold within 5 h, whereas this increase was completely suppressed in pretumourigenic thymocytes (Figure 4B). No inhibition of increased NHE-1 expression in wild-type thymocytes was observed following addition of the Z-VAD-fmk caspase inhibitor (Figure S4A) nor following depletion of Bax and Bak from the cells (Figure S4B). We therefore carried out a further series of experiments to demonstrate that there was a direct causal linkage between the regulation of NHE-1 expression, pHi, Bcl-xL deamidation, and apoptosis. Given that the OTK blocks DNA-damage induced NHE-1 expression in pretumourigenic thymocytes, this provides a powerful system for examining the consequences of experimentally enforcing NHE-1 expression in these cells by retroviral transduction. As Figure 4C illustrates (upper panel), an enforced 2-fold–3-fold increase in NHE-1 expression in pretumourigenic thymocytes, without DNA damage, restored Bcl-xL deamidation to a level comparable to that observed in a retrovirotransduced wild-type control in five separate experiments, thereby bypassing the OTK-mediated inhibition in deamidation. Overexpression of NHE-1 increased both pHi and apoptosis to comparable levels in both pretumourigenic and wild-type thymocytes (Figure 4C, lower panels). These results suggest that increased NHE-1 expression per se is sufficient to cause increased pHi, Bcl-xL deamidation and apoptosis. To address this question further, we used the selective NHE-1 inhibitor 5-(N,N'-dimethyl)-amiloride (DMA) to block the actions of the antiport following its increased expression on thymocytes upon DNA damage. Figure 4D shows that DMA prevented the alkalisation of wild-type thymocytes following DNA damage (top left panel), their apoptosis (top right panel), and Bcl-xL,
Bcl-xL Deamidation Regulation by Intracellular pH

**A** CD4+CD8+ subset

**B** C57BL/6 thymocytes

**C** (-) z-VAD, (+) z-VAD

**D** pHe, Etop, Bcl-xL, tubulin

**E** C57BL/6, CD45−/−LckF505

**F** C57BL/6, CD45−/−LckF505

**G** vector, 7.2, 8.0, 8.5, 24h, 48h
**Figure 3.** DNA Damage Causes Intracellular Alkalisation and Subsequent Bcl-xL Deamidation

[A] Intracellular alkalisation occurs following DNA damage in wild-type but not in pretumourigenic CD45$^{-/}$Lck$^{F505}$ thymocytes. Cells were treated with etoposide (Etopo) for 20 h or exposed to 5 Gy of irradiation (IR) and then maintained in culture for 20 h. pH$_i$ was measured using SNARF in FACS in the gated live CD4$^+$CD8$^-$ subset. The histograms represent mean values ± SD (n = 5).

[B] Enforced intracellular thymic alkalisation causes Bcl-xL deamidation. Wild-type thymocytes were maintained in RPMI-1640/10% bovine fetal calf serum buffered at the indicated pH with Tris-HCl for 20 h in the presence of 20 μM monensin prior to lysis and immunoblotting for Bcl-xL. To minimize any deamidation produced during the gel-running process, the resolving gel buffer was adjusted to pH 8.0 in this experiment. The mean ratio of the lower band (native Bcl-xL) or upper band (deamidated Bcl-xL) to the total (upper plus lower bands) is shown in the graph (lower panel). The error bars represent SD (n = 3). Note that deamidation becomes prominent at pH 7.5.

(C) Aliquots of the cells from Figure 1A incubated in the presence or absence of Z-VAD-fmk (200 μM) were analysed for pH$_i$. The histograms represent mean values ± SD (n = 3).

(D) Wild-type or CD45$^{-/}$Lck$^{F505}$ pretumourigenic thymocytes were cultured for 24 h in media at the pH shown without monensin, with or without etoposide, and then analysed for Bcl-xL deamidation by immunoblotting. The upper and lower bands were quantified and the percentage of upper bands in total Bcl-xL calculated. The percentages shown below each lane are means ± SD (n = 5).

(E) Aliquots of cells used in (D) were assessed for pH$_i$ by FACS. The histograms show the pH$_i$ of live gated CD4$^+$CD8$^-$ thymocytes from five independent experiments ± SD. The pH$_i$ values refer to the pH values of the extracellular media.

(F) Apoptosis of aliquots of the cells from (D) was analysed by FACS. The histogram shows the sub-G1 peak (% of CD4$^+$CD8$^-$ thymocytes from five independent experiments ± SD. The pHe values refer to the pH values of the extracellular media.

(G) Wild-type (wt), N52A-N66A (AA), N52D-N66D (DD) Bcl-xL, and empty vector were retrovirally transduced into thymocytes. GFP-positive cells were FACS sorted (left panel) and cultured in media with the pHe shown for 24 h or 48 h, then processed for immunoblotting with Bcl-xL antibody (middle panel). Note that 8 x 10$^6$ and 1 x 10$^6$ cell equivalents were loaded per lane for the empty vector (lanes 1–3) and Bcl-xL (lanes 4–12) transfectants, respectively, such that the endogenous Bcl-xL is invisible in lanes 4–12. The histogram (right panel) shows mean apoptosis (sub-G1) values ± SD generated from five independent experiments.

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Enforced Alkalisation Causes Increased Bcl-xL Deamidation and Apoptosis in Murine and Human Cancer Cells

The experiments illustrated in Figures 1–5 were all carried out on wild-type or primary pretumourigenic CD45$^{-/}$Lck$^{F505}$ thymocytes. Signalling pathways can be markedly different in fully transformed cells compared to their pretransformed counterparts. We therefore wondered whether CD45$^{-/}$Lck$^{F505}$ T cell tumour cells, which develop from CD4$^+$CD8$^-$ thymocytes [16], might display a comparable set of properties. Figure S7 shows that this was indeed the case: murine tumour cells resistant to genotoxic insult at physiological pH$_i$ values can be sensitised to die by enforced alkalisation leading to Bcl-xL deamidation. Furthermore, a modest rise in pH$_i$ following incubation in a mildly alkaline buffer produces levels of Bcl-xL deamidation and apoptosis in murine tumour cells comparable to those observed by adding a DNA damaging reagent to wild-type thymocytes incubated at physiological pH$_i$.

Chronic lymphocytic leukaemia (CLL) is the most common adult haematological malignancy in the Western world and, like many cancers, is characterised by the development of drug resistance. We therefore determined whether genotoxic treatment in vitro of primary human B lineage CLL (B-CLL) cells might cause increased NHE-1, alkalisation, Bcl-xL deamidation, and apoptosis, as in primary murine thymocytes (Figures 3–5), or whether this might be inhibited, as with the murine cancer cells (Figure S7). In addition, we examined the consequences for these parameters of incubating cancer cells in alkaline pH buffers. To perform these investigations, we divided each sample of patient cancer cells into nine aliquots that were either untreated, subjected to γ irradiation, or exposed to etoposide, followed by incubation at pH 7.2, pH 8.0, or pH 8.5 for 24 h. Each aliquot was then further subdivided into three samples to measure pH$_i$, Bcl-xL deamidation, and apoptosis. As expected, exposure of cells to mildly alkaline buffers generated pH$_i$ values that displayed some variation between samples from different patients within a narrow range. The 18 values per patient obtained from 10 different patients, the mean values calculated for each pH$_i$ value considered separately, and representative...
Bcl-xL deamidation results from a single patient are illustrated in Figure 6A, Figure S8A, and Figure S8B, respectively. Interestingly, unlike the murine tumour cells expressing an OTK, the B-CLL cells behaved somewhat more like wild-type thymocytes in that DNA damage at physiological pH caused a mean increase of pH_i of 0.22 units, an 8% increase in Bcl-xL deamidation, and an 18% increase in the number of cells undergoing apoptosis (Figure 6A and Figure S8A), compared to the higher thymocyte values of 0.45 pH_i units, 40% increase, and 37% increase, respectively (Figure 3). The human cancer cell values for these parameters were greatly increased at alkaline pH_i, generating tight correlations between increasing pH_i, Bcl-xL deamidation, and apoptosis (r values shown in Figure 6A). Thus, a mean increased pH_i of 0.5 correlated with 1.7-fold and 2.4-fold increases in Bcl-xL deamidation and apoptosis, respectively. It is also striking that enforced intracellular alkalinisation alone (by 0.3 pH_i units), in the absence of experimentally induced DNA damage, was itself sufficient to increase Bcl-xL deamidation and apoptosis by 1.5-fold and 1.8-fold, respectively. This point is further illustrated by the gray shaded area shown in Figure 6A, which encompasses the overlap in sub-G1.
(apoptosis) values that were obtained either by DNA damage at 
physiological pH or by enforced alkalinisation without DNA 
damage. Conversely, incubation of B-CLL cells at lower pH 
inhibited DNA damage–induced Bcl-xL deamidation and 
apoptosis (Figure 6B). Therefore with respect to enforced 
changes in pHi, the B-CLL cells behaved in a comparable way 
to both murine thymocytes and tumour cells. A small increase 
in pHi induced by incubation in alkaline buffer in the absence 
of induced DNA damage generated as much, if not more, Bcl-
xL deamidation and apoptosis as that triggered by genotoxic 
attack at physiological pH.

NHE-1 expression in response to DNA damage was 
investigated in a further six B-CLL patients. Figure 6C shows 
by immunoblotting (right panel) that there was some 
variation between patients, but that in all cases (left panel), 
etoposide caused increased NHE-1 expression by 3 h, 
achieving optimal values by 6–9 h ranging from 1.9-fold– 
2.6-fold over basal levels. These increases correlate with the 
observed increases in Bcl-xL deamidation and apoptosis in 
patients’ cells (Figure 6A) and at the 2.6-fold level, at least, are 
comparable with the increases observed in wild-type thymo-
cytes (Figure 4B). Furthermore, DNA damage–induced Bcl-xL 
deamidation in B-CLL cells was prevented by addition of 
either cycloheximide (CHX) (Figure S8C) or DMA (Figure 
S8D), establishing a possible linkage between DNA damage, 
NHE-1 function, and Bcl-xL deamidation in human cancer 
cells.

Discussion

It has previously been suggested that Bcl-xL deamidation is 
critical in the signalling pathway that leads from DNA 
damage to apoptosis [9]. This interpretation was based to a 
large degree on the observation that N52D/N66D Bcl-xL, one 
of the species generated by deamidation, can no longer exert 
anti-apoptotic activity nor sequester the pro-apoptotic 
protein Bim. However, a secondary mutation in the N52D/
N66D Bcl-xL construct was later discovered, which, when 
corrected, restored binding, thereby casting doubt on the 
initial interpretation of the physiological significance of Bcl-
xL deamidation [13]. We now propose that the initial finding 
was correct, but for the wrong reason. Our results indicate 
that the major Bcl-xL species generated by deamidation in 
situ is not Asp52/Asp66 but iso-Asp52/iso-Asp66, which is 
consistent with the well-established biochemistry of Asn 
deamidation [1], and that this species is unable to sequester 
Bim or Puma (Figure 2 and Figure S2). The introduction of 
iso-Asp into the disordered loop in which these residues are 
located is expected to cause greater conformational change 
than Asp, because of the redirection of the peptide backbone 
through β carboxyl groups, as indicated by the known 
structural and functional changes that occur in proteins 
upon conversion of Asn to iso-Asp residues [26,27]. The 
structural importance of protein iso-Asp residues is likewise 
underlined by the expression of the putative repair enzyme
L-isooaspartate O-methyltransferase which converts iso-Asp to Asp residues: its deletion has striking effects on protein functions [28–30]. Furthermore, comparison of the crystal structures of native rat Bcl-xL with its deamidated version has revealed significant differences [10]; the structural implications of introducing iso-Asp residues into the disordered loop environment of Asn52/Asn66 merits further work.

We have identified critical elements in the signalling pathway leading from DNA damage to Bcl-xL deamidation in thymocytes and have shown, as Figure 7A illustrates, that deamidation is induced upon DNA damage by up-regulation of the NHE-1 antiport and consequent intracellular alkalinisation (Figures 3–5). To the best of our knowledge, this represents the first description of a molecular mechanism for the regulation of protein internal Asn deamidation in cells. Our results are consistent with the failure, until now, to identify genes encoding internal protein Asn deamidases [1]. The regulation of NHE-1 antiport function is complex,

Figure 6. DNA Damage Induces NHE-1 Expression, and Enforced Alkalinisation Promotes Apoptosis of Human B-CLL cells

(A) Enforced alkalinisation of cancer cells from patients (n = 10) with B-CLL causes Bcl-xL deamidation and associated cell death. Treatment with etoposide (Etop) in vitro further amplifies cell death. Patients' cells (PBMC, in the range 85%–95% CD19+ B220+) were incubated at pHv values of 7.2, 8.0, or 8.5, and the pH values were monitored by SNARF-1 staining using flow cytometry. Apoptosis was evaluated by measurement of sub-G1 peaks using flow cytometry. The data shows pooled results from ten patients via 30 values per treatment condition: due to identical values, some symbols overlap. The correlation coefficients (r) of deamidation or sub-G1 versus pHv are shown for each treatment. The p value (significance) for each correlation is shown in parentheses. The correlation coefficients of sub-G1 versus deamidation are r = 0.92 (p < 0.0001) for untreated cells and r = 0.87 (p < 0.0001) for etoposide treated cells.

(B) Purified PBMC from B-CLL patients were cultured for 24 h in media at the pH shown, with/without etoposide for 48 h, and then analysed for Bcl-xL deamidation by immunoblotting (left panel). The upper and lower bands were quantified and the upper deamidated Bcl-xL band was expressed as a percentage of total Bcl-xL. The percentages shown below each lane are means ± SD (n = 4). The same cell aliquots cultured in RPMI/10% FCS for 24 h or 48 h were analysed for apoptosis by sub-G1 staining (right panel).

(C) Assessment of NHE-1 expression in B-CLL patients' samples following exposure to etoposide for the times shown. Representative immunoblotting results are shown for three patients in the right panel and the values for six patients (normalized for tubulin loading) are graphed in the left panel. doi:10.1371/journal.pbio.0050001.g006
In pretumourigenic thymocytes expressing an OTK, the DNA damage–induced rise in NHE-1 expression is blocked, preventing alkalinisation, Bcl-xL deamidation, and apoptosis.

(iii) Enforced alkalinisation of murine tumour cells, or human B-CLL cells, causes Bcl-xL deamidation and subsequent apoptosis, even in the absence of external genotoxic attack.

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The resistance to genotoxic attack by CD45F/ItkF murine tumour cells correlates, as in their pretumourigenic counterparts, with the inhibition of DNA damage–induced NHE-1 antiport expression, alkalinisation, Bcl-xL deamidation, and apoptosis (Figure S7), which is an apparent example of "oncogene addiction", whereby oncogene expression continues to be important for survival [36]. By contrast, DNA damage of human B-CLL cells, which should not express OTKs, triggered increased NHE-1 expression and apoptosis, achieving levels comparable with wild-type thymocytes (Figure 6C). However, enforced alkalinisation of either the murine (Figure S7) or human (Figure 6) cancer cells triggered significant increases in Bcl-xL deamidation and apoptosis, even in the absence of genotoxic attack (Figure 7C). In the case of the B-CLL cells, we cannot yet exclude the possibility that the tight correlation observed between these events does not reflect causal efficacy, and further work will be necessary to elucidate this point. In any event, the key issue for cancer cell therapy in this context is not whether inhibition of Bcl-xL deamidation, resistance to DNA damage induced apoptosis, and oncogenesis, suggesting that the consequent accumulation of DNA-damaged thymocytes is critical in the transforming process [14,15]. It therefore seems conceivable that the OTK-induced inhibition of NHE-1 is likewise important in thymic transformation, and further in vivo work will be necessary to investigate this possibility.

The striking blockade in DNA damage–induced NHE-1 expression, a mechanism that is under active investigation. We have previously demonstrated in pretumourigenic thymocytes expressing an OTK, the DNA damage–induced rise in NHE-1 expression is blocked, preventing alkalinisation, Bcl-xL deamidation, and apoptosis.

The direct role played by the deamidation of Bcl-xL to its iso-Asp52/iso-Asp66 version in the signalling pathway from DNA damage to apoptosis is supported by the finding that either the N52D/N66D or N52A/N66A Bcl-xL mutants, which still bind BH3-only proteins (Figure 2E), protect thymocytes from dying upon enforced intracellular alkalinisation (Figure 3G). An alternative hypothesis involves the generation of new BH3-only family members as a consequence of alkalinisation, which compete for binding to Bcl-xL, thereby displacing Bim and Puma. However, such a hypothesis does not explain why the Bcl-xL mutants that still bind BH3-only proteins retain their anti-apoptotic potency at high pH (Figure 3G).

The striking blockade in DNA damage–induced NHE-1 expression, alkalinisation, Bcl-xL deamidation, and apoptosis noted in CD45F/ItkF murine tumourigenic thymocytes (Figures 3 and 4), together with the reversal of this blockade by enforced expression of NHE-1 (Figure 4C), provide strong support for the model illustrated in Figure 7B. The oncogenic hyperactive p56Lck-Y505F tyrosine kinase [15] must inhibit one or more steps on the pathway from DNA damage to increased NHE-1 expression, a mechanism that is under active investigation. We have previously demonstrated in pretumourigenic thymocytes expressing an OTK, the DNA damage–induced rise in NHE-1 expression is blocked, preventing alkalinisation, Bcl-xL deamidation, and apoptosis.
TNF-α-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis by antitumor drugs [40]. Furthermore, Bcl-xL deamidation is inhibited in hepatocellular carcinomas, which are highly resistant to genotoxic treatments [11]. Our findings therefore have potential relevance to cancer therapy, where-by enforced alkalinisation, perhaps by amplification of NHE-1 expression, would promote Bcl-xL deamidation, thereby triggering apoptosis.

The pioneering work of Warburg [41] established that tumours display acidic extracellular pH, although more than half a century passed before it was clearly established that the intracellular pH of tumour cells is comparable with normal cells [42]. Warburg's legacy has included intermittent interest in the possibility of pH manipulation as a means to cancer therapy. Our findings not only establish that protein deamidation can be regulated by intracellular pH change in vivo, but they also suggest that strategies for pH manipulation in antineoplastic therapy can continue to receive attention, albeit for reasons different from those envisaged by Warburg.

Materials and Methods

Mice. All mice were bred and housed in specific pathogen-free conditions in the animal facility at The Babraham Institute, Cambridge, United Kingdom. The p55TRAIL (TRAIL-Fc) transgenic mice [43] and the CD45<sup>−</sup>CD3<sup>+</sup> and CD45<sup>−</sup>CD3<sup>+</sup> mice have been previously described [16].

Reagents and antibodies. Etoposide, CHX, DMA, PI, monensin, nigericin, and goat-anti-rat immunoglobulin-agarose were from Sigma (St. Louis, Missouri, United States); protein A- and B- sepharose and protein G- sepharose from Amersham (Epscopa, Sweden); SNAP-25 was from Molecular Probes (Eugene, Oregon, United States); Z-VAD-fmk was from Santa Cruz Biotechnology. The DNA was sequenced and the sequence for human Bcl-xL was obtained from the MRC gene service (Cambridge, United Kingdom). The p56Lck-F505 (PLGF-A) transgenic mice [43] and the p56Lck-F505 (PLGF-A) transgenic mice [43] were added to C57BL/6 thymocyte lysates for 2 h at 4°C. The intracellular pH of tumour cells is comparable with normal cells half a century passed before it was clearly established that the intracellular pH of tumour cells is comparable with normal cells [42]. Warburg's legacy has included intermittent interest in the possibility of pH manipulation as a means to cancer therapy. Our findings not only establish that protein deamidation can be regulated by intracellular pH change in vivo, but they also suggest that strategies for pH manipulation in antineoplastic therapy can continue to receive attention, albeit for reasons different from those envisaged by Warburg.

DNA damage treatments. Freshly isolated thymocytes were irradiated with 10 Gy using a cesium source or treated with etoposide in DMSO at a concentration of 25 μM for murine cells, or 50 μM for R-CLL cells, for the times indicated. Carrier DMSO was added to control cells.

Immunoblotting and immunoprecipitation. Cells were lysed in 50 mM HEPES (pH 7.2), 130 mM NaCl, 1 mM EDTA, 0.2% NP-40, and complete protease inhibitors were included by standard Laemmli's SDS-PAGE (pH 8.8) unless otherwise stated. For immunoprecipitations: rat Bim antibody (Oncogene, San Diego, California, United States) was coated to goat-anti-rat immunoglobulin-agarose; rabbit Puma antibody was coated to protein A-sepharose; mouse NHE-1 antibody was coated to goat-anti-rabbit immunoglobulin-agarose; rabbit Bcl-xL antibody was coated to goat-anti-rabbit immunoglobulin-agarose. Lysates were precleared with the appropriate agarose. Quantification of immunoblots was carried out using a phosphorimager (Fuji FLA3000, http://www.fujifilm.com).

Intracellular pH measurement. Intracellular pH was measured using a standard ratiometric method with a pH-sensitive fluorophore SNARF-1 by flow cytometry [44]. Briefly, cells in phosphate-buffered saline (PBS) were loaded with 10 μM SNARF-1 for 40 min at 37°C, followed by washing and incubation in PBS at room temperature for 30 min prior to measurement of pH. pH calibration was carried out using standard pH 6.0 and pH 8.0 buffers, with 10 μM nigrosin were analysed using Flowjo software to obtain the ratio based on the FI3/FI2 channels. It should be noted that SNARF-1 measurements provide the average pH<sub>i</sub> of the intracellular environment in a cell population including, presumably, the contribution of acidified intracellular compartments. However, even if such compartments contribute significantly to the mean pH<sub>i</sub> values measured, in this work, it is the change in pH<sub>i</sub> that is most important. This point was also addressed by neutralising acidic compartments using monensin in some experiments.

Measurement of apoptosis. Cells were stained with 20 μg/ml PI (with 50 μg/ml RNase A) and analysed by flow cytometry, gating on the CD4<sup>+</sup>CD8<sup>+</sup> subset as necessary. The sub-G1 peak was quantitated as a measure of apoptosis. In addition, apoptosis was measured using the Annexin-V-Fluos Staining Kit (Roche) according to the protocol provided. To measure the percentage of dead cells, PI was used at 0.5 μg/ml.

Generation of Bcl-x<sub>L</sub> mutants. Mouse Bcl-x<sub>L</sub> cDNA was kindly provided by S. Korsmeyer (Howard Hughes Medical Institute, Harvard Medical School, Boston, Massachusetts, United States). N55 and N52D-2D-3D mutants were made using the Quick-Change Site-Directed Mutagenesis Kit from Stratagene (La Jolla, California, United States) according to the instructions provided. The sequences of the constructs were confirmed by DNA sequencing.

Retroviral gene knockdown and overexpression. Murine CD1d<sup>+</sup> T cells were prepared and cultured in the presence of interleukin-4 (IL-4) and PdBu as described [15]. The Suppressor-Retro kit was purchased from Imgenex (San Diego, California, United States), and NHE-1 shRNA sequences were designed using the siRNA
tool” from the company’s website. Five selected sequences were cloned into pSuppressorRetro. The sequence of NHE-1 shRNA2 is 5'-GAAACAAAGGGCGTCCATACAC-3' . Retroviral production and infection were performed according to the protocol provided. For overexpression, NHE-1 or Bcl-xL (wild-type, N52A-N66A, and N52D-N66D) DNA were amplified with AccuPrimer P5S DNA polymerase (Invitrogen), and cloned into Xhol and EcoR1 sites of the multiple cloning sites of the MigRI vector [45] upstream of an internal entry site followed by enhanced green fluorescent protein (EGFP). The sequences of the inserts were verified by DNA sequencing. The plasmids were transfected into qN cells using Lipofectamine (Invitrogen). Viral infection of CD4+ CD8+ thymocytes was performed by spinoculation (1,200 g for 90 min at 30 °C). To achieve high efficiency of gene transduction, the infection was repeated every 24 h for 2–3 d. GFP-positive cells were sorted by flow cytometry using a FACsAria.

Bax: Bak double knockdown. The SureSilencing shRNA kit for Bax and Bak was purchased from SuperArray (Frederick, Maryland, United States). One plasmid from each kit was screened out for the best gene ablation efficiency by transient transfection. The shRNA sequence for Bax is TCAGAGATGTCACCAAGAA, and the shRNA sequence for Bak is GGCGCTAGCACTTGTGTGTGTT. To enrich the cells transfected with both plasmids which express GFP, the GFP sequence in the shRNA:Bak plasmid was replaced by DsRed using the Smal restriction site before GFP and the AgeI restriction site after GFP. ShRNA:Bax-GFP and shRNA:Bak-DsRed were cotransfected into primary thymocytes using the Amxa mouse T cell nuclear factor kit (Amxa Biosystems, Koeln, Germany). Cells positive for both GFP and DsRed were sorted by flow cytometry and used for subsequent experiments.

B-CLL patients’ cell purification. B-CLL donor peripheral blood was centrifuged through lymphoprep (Axis-Shield PoC, Oslo, Norway), and the interphase peripheral blood mononuclear cells (PBMCs) were harvested for subsequent experiments. The purity of PBMCs was routinely checked by staining with antibodies CD3-Cy5, CD19-Fitc, and B220-PE and was analysed by flow cytometry.

Statistics. The Pearson coefficient of correlation (SPSS package, Chicago, Illinois, United States) was used to analyse the correlation between variables within the same group of data.

Supporting information

Figure S1. DNA Damage–Induced Bcl-xL Deamidation Correlates with the Kinetics of Thymic Apoptosis

(A) The membrane from Figure 1C was stripped and reprobed with caspase-9 antibody. Cleavage of caspase-9 following DNA damage was inhibited in Bax/Bak knock-down thymocytes. (B) Wild-type thymocytes were cultured in RPMI-1640/10% bovine fetal calf serum with 10 μg/ml etoposide for the times shown, and aliquots of cells from each time point were stained with 7-AAD and analysed by flow cytometry to estimate the percentage of cells undergoing apoptosis (sub-G1 peak expressed as a % of total cells).

Figure S2. Deamidation Disrupts the Sequestration of BH3-Only Proteins by Bcl-xL

(A) Puma binds to the native but not deamidated form of Bcl-xL. Either wild-type (1.5 × 10^6), lanes 3 and 4) or premuformic CD45^−/−Lck^+/+ thymocytes (1.5 × 10^6, lanes 5 and 6) were treated as in Figure 2A, and cells were lysed and subjected to immunoprecipitation with Puma antibody, followed by blotting with either Bcl-xL or Puma antibodies. Lane 1 is a wild-type thymocyte whole cell lysates (WCLs) control to facilitate comparison of native and deamidated forms of Bcl-xL. Lane 2 demonstrates the light chain of the Puma antibody used for immunoprecipitation.

(B) Deamidated Bcl-xL from alkali treated thymocytes no longer binds to Bim. Wild-type thymocytes were incubated in neutral (pH 7.0) or alkaline (pH 9.0) buffer at 37 °C for 24 h. Bim was immunoprecipitated from WCLs and WCL samples. Bim immunoprecipitates and Bim-depleted lysates were then separated and immunoblotted for either Bcl-xL or Bim.

Figure S3. The Asp and iso-Asp Forms of Bcl-xL Chymotryptic Peptides 1 and 2 Were Identified by Spiking an Aliquot of a Digestion Mixture with Asp- or iso-Asp–Containing Synthetic Peptides Before LC-MS

Peptides SDVEENRTEAPEGTESEMETPSAINGNPSW (peptide 1) and HLADSPAVNGTGHSSSL (peptide 2) and the corresponding deamidated forms, which contain the putative deamidation sites N52 and N66, respectively, were generated by digestion of rBcl-xL with chymotrypsin. The chromatographic conditions used for the separation of the peptides in the LC-MS analyses were optimised so as to resolve the Asn, Asp, and iso-Asp forms of peptides 1 and 2. The Asp and iso-Asp forms of the two peptides were identified by spiking an aliquot of a digestion mixture with Asp- or iso-Asp–containing synthetic peptides prior to LC-MS as shown. The chromatograms show LC-MS analyses at time point 72 h of the rBcl-xL base treatment.

Figure S4. DNA Damage–Induced NHE-1 Up-Regulation Is Mitochondrial Apoptosis–Independent

(A) Aliquots of the cells from Figure 1A incubated in the presence or absence of Z-VAD-fmk (200 μM) were analysed for the expression of NHE-1 and tubulin (as loading control) by immunoblotting.

(B) Aliquots of the cells from Figure 1C were analysed for the expression of NHE-1 by immunoblotting. Tubulin was reprobed as loading control.

Figure S5. Thymocytes Treated with DNA or Transduced with NHE-1 RNA-vector Display a Survival Advantage In Vitro Following DNA Damage

(A) Purified double-negative (DN) thymocytes treated with without DNA, etoposide, or irradiation were cultured in vitro. At 24 h, 48 h, or 72 h, an aliquot of cells was analysed by PI staining (0.5 μg/ml) using flow cytometry; PI-positive cells represent dead cells.

(B) Purified DN thymocytes transduced with NHE-1 shRNA2 or empty vector were treated with or without etoposide and irradiation and then cultured in vitro. At 24 h, 48 h, or 72 h, an aliquot of cells was analysed as in (A).
damage are both inhibited in CD45+ Lck505 tumour cells, pH(upper panel) and apoptosis (lower panel) were analysed as in Figure 3A and Figure 1A. (D) DNA damage causes up-regulation of NHE-1 in wild-type but not in CD45+ Lck505 tumour cells. Wild-type thymocytes or CD45+ Lck505 tumour cells were either treated with etoposide (Etop) for 5 h or exposed to 5 Gy of irradiation and then maintained in culture for 5 h, followed by immunoblotting for NHE-1 or tubulin. The histogram shows the quantification of NHE-1 expression from five independent experiments SD. Lane 3 was defined as 1(*). (E) Aliquots of the cells used for (D) were assessed for pHi . The side, and analysed for Bcl-xL deamidation by immunoblotting. The percentage deamidation was calculated as in Figure 1B. (F) Aliquots of the cells used for (D) were assessed for apoptosis. The histograms represent mean values SD (n = 3). (A) shows that Bcl-xL deamidation following DNA damage was suppressed in primary tumour cells to the same extent as in pretumourigenic thymocytes 24 h after inducing DNA damage, although after 48 h, the inhibition of deamidation was somewhat less (68.1% ± 3.2% inhibition in tumour cells compared to 96.2% ± 3.8% in pretumourigenic thymocytes, unpublished data). Likewise, alkalisation (B, upper panel), apoptosis (B, lower panel) and increased NHE-1 expression (C) were all suppressed in tumour cells to near normal extent, as in pretumourigenic thymocytes. Furthermore, in the absence of monensin, extracellular buffers at pH 8.0–8.5 forced pH values of 7.5–7.7 (E) triggering Bcl-xL deamidation (D) and apoptosis (F). It is particularly striking that incubation in buffer at pH 8.0, for example, which achieves a pH value of 7.43, triggers 66.4% and 36.6% levels of Bcl-xL deamidation and apoptosis, respectively, irrespective of whether, in addition, DNA damage was induced by etoposide or by γ irradiation. These results show that murine tumour cells resistant to genotoxic insult at physiological pH values can be sensitised to die by enforced alkalisation leading to Bcl-xL deamidation.

Figure S8. Inhibition of NHE-1 Synthesis by CHX or Inhibition of NHE-1 Function by DNA in B-CLL Cells Blocks DNA Damage–Induced Bcl-xL Deamidation

(A) Replotting of data from Figure 6A to show the absolute mean values SD (n = 10) for Bcl-xL deamidation (right panel) and apoptosis (left panel) obtained at each of the three extracellular pH values investigated. The numbers at the top of each bar represent the mean pH values measured in the cells incubated at the pH values shown. (B) A representative Bcl-xL Western blot from the B-CLL samples analysed in Figure 6A is shown. (C) B-CLL patients' PBMCs were treated with/without CHX, etoposide, and irradiation as in Figure 4A. 48 h later cells were subjected to immunoblotting for Bcl-xL. A representative blot from four independent experiments is shown. Tubulin was reprobed as loading control. (D) B-CLL patients' PBMCs were treated with/without DNA, etoposide and irradiation as in Figure 3D. 48 h later cells were subjected to immunoblotting for Bcl-xL. A representative blot from four independent experiments is shown. Tubulin was reprobed as loading control. Found at doi:10.1371/journal.pbio.0050091.s008 (1.2 MB TIF).

Accession Numbers

The GenBank (http://www.ncbi.nlm.nih.gov/Genbank) accession numbers for proteins discussed in this paper are: Bcl-xL (BC019307), Bim (NM009754), NHE-1 (BC052708), and Puma (U82987).

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Author contributions. RZ and DRA conceived and designed the experiments, RZ, DO, and TSS performed the experiments, RZ, DO, and TSS analyzed the data. GAF and ARG contributed reagents/materials/analysis tools. DRA wrote the paper.

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