Bcl10 Is Involved in t(1;14)(p22;q32) of MALT B Cell Lymphoma and Mutated in Multiple Tumor Types

Tony G. Willis,* Dalal M. J. adayel,* Ming-Qing Du,3 Huaizheng Peng,3 Amanda R. Perry,* Munah Abdul-Rauf,* Helen Price,4 Lorraine Karran,* Olututuin Majekodunmi,* Ivona Wlodarska,1 Langxin Pan,3 Tim Crook,1 Rifat Hamoudi,2 Peter G. Isaacson,§ and Martin J. S. Dyer*#
*Academic Department of Haematology and Cytogenetics
†Cancer Gene Cloning Centre
‡Section of Cell Biology and Experimental Pathology
§Institute of Cancer Research
Sutton
Surrey, SM2 5NG
United Kingdom
³Department of Histopathology
University College London Medical School
University Street
London, WC1E 6J
United Kingdom
∥The Centre for Human Genetics and Flanders Institute of Biotechnology
University of Leuven
Belgium

Summary

MALT B cell lymphomas with t(1;14)(p22;q32) showed a recurrent breakpoint upstream of the promoter of a novel gene, Bcl10. Bcl10 is a cellular homolog of the equine herpesvirus-2 E10 gene: both contain an amino-terminal caspase recruitment domain (CARD) homologous to that found in several apoptotic molecules. Bcl10 and E10 activated NF-κB but caused apoptosis of 293 cells. Bcl10 expressed in a MALT lymphoma exhibited a frameshift mutation resulting in truncation distal to the CARD. Truncated Bcl10 activated NF-κB but did not induce apoptosis. Wild-type Bcl10 suppressed transformation, whereas mutant forms had lost this activity and displayed gain-of-function transforming activity. Similar mutations were detected in other tumor types, indicating that Bcl10 may be commonly involved in the pathogenesis of human malignancy.

Introduction

The lymphomas of mature B cells are a heterogeneous group of disorders that frequently exhibit chromosomal translocations targeted to the immunoglobulin (Ig) loci. These translocations may be specific for histologically defined subtypes of disease and involve genes controlling cell proliferation and apoptosis. Thus, translocations involving the Myc gene with the Ig loci are seen in nearly all cases of Burkitt’s lymphoma (Magrath, 1990). Similarly, Bcl2, which suppresses several forms of apoptosis, was cloned from the t(14;18)(q32;q21) found in follicular B cell lymphoma, whilst Ig translocations involving the Bcl1/Cyclin D1 gene on chromosome 11q13 are seen in mantle cell lymphoma (Tsujimoto et al., 1984; Vaandragter et al., 1996). Translocation to the Ig loci may result not only in deregulated expression of the incoming oncogene, due in part to the proximity of potent B cell transcriptional enhancers within the Ig loci (Jain et al., 1993), but also in mutations due to the action of the Ig somatic hypermutation mechanism (Rabbitts et al., 1983; Migliazza et al., 1995; Matolcsy et al., 1996). This mechanism may also induce mutations in normal B cells in nontranslocated genes (Shen et al., 1998).

B cell lymphomas of mucosa-associated lymphoid tissue (MALT lymphomas) are the commonest form of lymphoma arising in extranodal sites, in most cases arising in the gastric mucosa (Isaacs and Spencer, 1995). They may be either high or low grade. It is likely that most high-grade cases represent transformation of undetected low-grade disease (Peng et al., 1997). Low-grade MALT lymphoma is an indolent disease and is often preceded by either chronic inflammation or autoimmune disease such as Hashimoto’s thyroiditis in thyroid and chronic Helicobacter pylori infection in stomach. The etiological link between low-grade gastric MALT lymphoma and lymphoid reaction to H. pylori infection has been demonstrated by the regression of some cases with antibiotic therapy alone (Wotherspoon et al., 1993).

The molecular events leading to H. pylori-independent growth and high-grade transformation of MALT lymphoma are unknown. Cytogenetic studies of low-grade cases have identified abnormalities of chromosome 1p22, in particular translocation t(1;14)(p22;q32), as uncommon but recurrent events (Wotherspoon et al., 1992). Cases with 1p22 abnormalities were more aggressive than typical cases of low-grade disease and also grew in vitro without additional stimuli (Hussell et al., 1993 and M-Q. D. et al., unpublished observations).

We have cloned a t(1;14)(p22;q32) translocation breakpoint from a case of low-grade MALT lymphoma. This was a recurrent breakpoint adjacent to a novel gene, Bcl10, that exhibited an amino-terminal caspase recruitment domain, or CARD (Hofmann et al., 1997), similar to that found in apoptotic regulatory and effector proteins including RAIDD, (Duan and Dixit, 1997), RICK/RIP2/CARDIAK (Inohara et al., 1998; McCarthy et al., 1998; Thome et al., 1998), and ARC (Koseki et al., 1998). Unexpectedly, Bcl10 was proapoptotic in 293 cells. However, a MALT lymphoma with the t(1;14)(p22;q32) showed a Bcl10 frameshift mutation resulting in truncation beyond the CARD. Unlike the wild-type protein, truncated mutants failed to induce apoptosis and enhanced transformation by cooperating oncogenes. These data suggest that truncating mutations of Bcl10 may confer a survival benefit to MALT B cell lymphomas and may allow progression to antigen-independent proliferation. Truncating Bcl10 mutations similar or identical to those observed in the MALT lymphoma were seen in other tumor
Figure 1. Molecular Cloning of t(1;14)(p22;q32) Translocation Breakpoint in a Case of Low-Grade MALT Lymphoma

(A) Gel electrophoresis of LDI-PCR products from PstI-digested DNA together with schematic representations of the two rearrangements. LDI-PCR yielded products of 5.5 and 0.7 kb. Sequence analysis of these revealed a productive IgVDJ rearrangement on the shorter allele and novel sequences upstream of JH6 in the other. A single copy probe (0.27EX) was used to isolate cosmid clones that were mapped back to 1p22 by FISH, demonstrating that this allele represented the translocation breakpoint. Open box represents the region of identity with ESTs. Orientation of the open reading frame revealed a head-to-head configuration of Bcl10 and IgH. 

(B) Southern blot analysis of MALT lymphomas with t(1;14)(p22;q32). Left hand panel: Case G0186 (from which the breakpoint was cloned) showed biallelic IgJH rearrangement with one allele comigrating with rearranged Bcl10 probe 0.27EX, indicating that no artifacts had been introduced during LDI-PCR. Right hand panel: Case G0262 showed one IgJH rearrangement. This represented a productive VDJ rearrangement (data not shown); we assume that this case had undergone translocation to a downstream switch region rather than to IgH. This case showed rearrangements with the Bcl10 probe in multiple digests, indicating a clustering of breakpoints within the same region of the Bcl10 gene. "N" denotes normal and "T" tumor DNA samples. Arrowheads denote rearranged fragments.

(C) FISH image of MALT lymphoma case with t(1;14)(p22;q32). Cosmid Ig3/64 containing sequences for IgJH and Cm was labeled red, and CEPH YAC 929e1 containing Bcl10 was labeled green. The Bcl10 YAC was split by the translocation and colocalized with IgH on the derivative chromosome 14.

Results

Cloning of the t(1;14)(p22;q32) Breakpoint and Identification of Bcl10

Three cases of low-grade MALT lymphoma with t(1;14)(p22;q32) were studied. On the assumption that the translocation breakpoint involved the IgH locus directly, both IgH alleles from one case (G0186) were cloned using long-distance inverse PCR (LDI-PCR; Willis et al., 1997, 1998). Two products of 700 and 5500 base pairs (bp) were obtained (Figure 1A). The sequence of the 700 bp allele showed a productive and mutated VH3-20 rearrangement (data not shown), while the other allele contained sequences with no homologies to any known sequences beyond JH6. To confirm that this represented the translocation breakpoint, cosmids were isolated using single copy probes from this region and mapped back to chromosome 1p22 on normal metaphases by fluorescent in situ hybridization (FISH; data not shown). In case G0186, JH and 1p22 probes showed comigration of rearranged bands, indicating that no major artifacts had been introduced during the LDI-PCR (Figure 1B). Further FISH experiments showed a break within a derived Bcl10 YAC clone (Figure 1C), and Southern blots using 1p22 probes derived from the breakpoint showed rearrangement in all three cases, indicating that this was a recurrent breakpoint in MALT lymphoma (Figure 1B).

To identify the involved gene on chromosome 1p22, the 5.5 kb PstI fragment was sequenced and showed a 255 bp region 4.6 kb upstream of JH6 with 100% identity to EST clone # 1184534. Genomic sequences immediately 5' of this potential exon had features of a promoter site and contained many potential protein-binding sites (accession number AJ 006290). These data indicated a head-to-head configuration of the translocation (Figure 1A).

By a combination of DNA-database searching using dbEST (www.ncbi.nlm.nih.gov/dbEST) and reverse transcription PCR methods, the full-length coding sequence of this gene was determined (accession number...
**Bcl10 in Malignancy**

**Figure 2. Sequence of Bcl10**

(A) Nucleotide and predicted amino acid sequences of Bcl10. Exon/intron boundaries are indicated. Mononucleotide runs mutated in malignancies are underlined.

(B) Amino acid sequence alignment of human and mouse Bcl10 and the EHV-2 open reading frame E10. Bcl10 and E10 contain highly homologous CARDs but diverge thereafter. The published sequence of EHV-2 ORF E10 (accession U20824; Telford et al., 1995) contains a frameshift at codon 143, predicting a protein of 210 amino acids. The corrected sequence predicting a protein of 311 amino acids is shown. Black boxes indicate \( 50\% \) amino acid identity; gray shading indicates \( 50\% \) similarity through conservative amino acid substitutions.

(C) Amino acid alignment of CARD-containing proteins. The most homologous proteins to Bcl10 and E10 were RAIDD and procaspase-2.

---

AJ006288; Figure 2A). This was predicted to encode a 233 amino acid protein with a molecular weight of 26.2 kDa. Due to the direct involvement of this gene in three cases of MALT lymphoma with t(1;14)(p22;q32), we have termed this gene Bcl10 (B cell lymphoma/leukemia 10).

The mouse Bcl10 gene was also cloned and sequenced (AJ006289); this was 91% identical to the human gene (Figure 2B). BLAST searches revealed that Bcl10 contained a region with strong homology (47% identity and 24.7% similarity over 97 amino acids) to an EHV-2 open reading frame (ORF), E10; Bcl10 and E10 contain highly homologous CARDs but diverge thereafter. The published sequence of EHV-2 ORF E10 (accession U20824; Telford et al., 1995) contains a frameshift at codon 143, predicting a protein of 210 amino acids. The corrected sequence predicting a protein of 311 amino acids is shown. Black boxes indicate \( 50\% \) amino acid identity; gray shading indicates \( 50\% \) similarity through conservative amino acid substitutions.

Bcl10 Expression in Normal and Malignant Tissues

Bcl10, expressed as a transcript of 4.2 kb, was observed in all normal and malignant tissues examined (data not shown). To examine the expression in mature B cells, Northern blot, but all three cases also had weaker homologies with the amino-terminal CARD of RAIDD (25% identity and 25% similarity); however, all cases of B cell malignancy examined by Northern blot or by ISH expressed high amounts of Bcl10 irrespective of whether they exhibited t(1;14) (p22;q32), indicating that, like Bcl2 (Zutter et al., 1991) and Bcl6 (Cattoretti et al., 1995), mechanisms other than...
BC110 Induces Apoptosis and NF-kB Activation
By analogy with the t(14;18)(q32;q21) involving the BC12 gene in follicular B cell non-Hodgkin lymphoma, and from the predicted functions of E10 (Hofmann et al., 1997), we anticipated that both BC110 and E10 proteins would be antioncoprotein. However, both induced apoptosis when transfected into 293 cells (Figure 4C) and induced activation of NF-kB (Figures 4D and 4E); comparable apoptosis was seen when BC110 was transfected into other cell lines, including COS-7 and HeLa (data not shown). BC110-induced apoptosis was inhibited by both Z-Val-DEVD-fmk and by cotransfection of CRM1 (Figures 4C and 4G). Comparison with other apoptotic mediators, such as TNFR1, indicated that BC110 and E10 were only weakly proapoptotic (Figure 4C). Furthermore, IL3-dependent BA/F3 B cell lines stably expressing exogenous BC110 were isolated, and unlike BC12, expression of BC110 did not promote survival in the absence of IL3 and did not protect significantly from apoptosis induced by a combination of TNFα and cycloheximide (data not shown).

BC110 Mutations in MALT Lymphoma with t(1;14)(p22;q32)
Given that wild-type BC110 induced apoptosis, we examined case G0186 from which the translocation breakpoint was cloned for mutations within the BC110 ORF. Using RT-PCR and DNA sequencing of cloned PCR products, 18 clones were sequenced. Two were normal, while 16 showed mutations within the coding region of BC110. Insertion of an additional thymidine into a mononucleotide run of seven consecutive thymines (nucleotides 493 to 499) was observed in 10/16 mutated clones. In three clones (designated M106), this was the only mutation present. The consequence of this frameshift mutation was to induce premature termination of the ORF, resulting in a predicted protein of 168 amino acids (Figure 5A). In the most heavily mutated clone (M114), there were four other mutations, including a deletion of 11 amino acids (116-126) due to loss of a splice acceptor site at the boundary of the third coding exon (Figure 5B and see below), as well as two other point mutations within the CARD and another at codon 100. Most of the point mutations in this case of MALT lymphoma were A→G transitions consistent with IgVH gene somatic hypermutation. No mutations were detected in Bax and the TGFβ-RII genes that also contain mononucleotide repeats and are frequently mutated in diseases that exhibit microsatellite mutator phenotype (data not shown).

Truncated BC110 Retains NF-kB Activation but Does Not Induce Apoptosis
To assess any possible differences between the wild-type and truncated BC110 mutants, both M106 and M114 were transfected into 293 cells. Both M106 and M114 activated NF-kB to a level comparable to that seen with the wild-type protein (Figure 4E). However, neither mutant clone exhibited significant apoptosis in this assay (Figures 4C and 4G). These data indicate that the full-length BC110 molecule was necessary for the apoptotic function but not NF-kB activation. Cotransfection of either M106 or M114 BC110 mutants with wild-type BC110 was also performed; expression of either truncated mutant did not significantly abrogate wild-type BC110-induced apoptosis (Figure 4G).

Mutation Activates a Transforming Function in BC110
Some proteins that induce apoptosis function as suppressors of transformation (Eliyahu et al., 1989; Yin et al., 1997). The observation that BC110 induced apoptosis raised the possibility that it might also possess transformation suppressor properties. To address this issue, the effect of BC110 expression was determined in the primary rat embryo fibroblast (REF) system. In this assay, nuclear oncoproteins such as adenovirus E1a, papillomavirus E7, and some mutant p53 proteins cooperate with an activated ras oncogene to induce morphological transformation (Crook et al., 1994). Such transformation is suppressed efficiently by some human tumor suppressor proteins such as wild-type p53. We therefore performed transfections in which primary REFs received a transforming combination of oncoproteins (e.g., E1a + ras, HPVE7 + ras, mutant p53 + ras) together with plasmids encoding either wild-type BC110, E10, or tumor-derived BC110 mutants. Transfection of each cooperating pair of oncogenes, in the absence of exogenous BC110 expression, generated numerous transformed colonies that could be readily propagated as cell lines. Cotransfection of wild-type BC110 significantly reduced the numbers of transformed colonies generated by each pair of cooperating oncogenes (Table 1). In contrast, cotransfection with either of two tumor-derived mutants, M106 or M114, or with E10, markedly enhanced the
Bcl10 in Malignancy

Figure 4. Bcl10 and E10 Induce Apoptosis and Activate NF-κB

Apoptosis assay: 293 cells were transfected with 1 μg of the indicated expression constructs and 0.25 μg of pcDNA3.1 β-gal. Forty-eight hours after transfection, cells were stained with X-gal and at least 300 blue cells counted per well.

(A) Photomicrograph of cells transfected with pcDNA3.1.

(B) Photomicrograph of cells transfected with pcDNA3.1 Bcl10 showing cells with apoptotic morphology.

(C) Apoptosis is induced by Bcl10 and E10 but not by the carboxy-terminal region of Bcl10 or by tumor-associated truncation mutants. Data (± SD) are shown as the percentage of morphologically apoptotic cells for three independent transfections. Where indicated, 25 μM z-VAD-fmk was added 6 hr post-transfection.

(D and E) NF-κB reporter assay: 293 cells were transfected with the indicated amounts of the expression construct, 0.25 μg pTK-cSPAP reporter with (filled boxes) or without (open boxes) the NF-κB response element, and 0.25 μg of pcDNA3.1 β-gal, to a total of 1.5 μg of DNA. Forty-eight hours after transfection, absorbance at 405 nm relative to vector-transfected cells was measured. Results ± SD for experiments performed in triplicate.

(F) Western blot analysis of transfected 293 cells. Cell lysates from cells transfected with the indicated plasmids were immunoblotted with monoclonal antibody to myc.

(G) Truncated Bcl10 does not significantly inhibit wild-type Bcl10-induced apoptosis. 293 cells were transfected with wild-type Bcl10 in the presence of either M106 or M114 mutant clones and apoptosis assessed. Comparable levels of expression of the wild-type and mutant proteins were observed as shown in the Western blot.

We therefore sought Bcl10 mutations in a panel of fresh lymphoma cases and in cell lines derived from various human malignancies. A panel of archival lymphoma cases were studied by PCR-SSCP, and in some cases, the abnormal SSCP bands were excised, reamplified, and sequenced. PCR-SSCP analysis of a panel of 135 cases of B cell and 20 cases of T cell lineage lymphoma of various histological subtypes revealed abnormal migrating bands in 70 (45%); abnormal PCR-SSCP bands were observed in all lymphoma subtypes (M. J. S. D. et al., in preparation). A representative gel along with the derived sequences from one case of follicular B cell lymphoma that showed two deletions within the coding region of exon 3 is shown in Figure 6B. The sequence abnormalities in ten cases of MALT and follicular non-Hodgkin lymphoma in which the abnormal PCR-SSCP band was excised, successfully reamplified, and sequenced are shown in Table 2. Truncating Bcl10 mutations were detected in both subtypes of lymphoma in the absence of 1p22 chromosomal translocation.

Subsequently, 87 cell lines derived from patients with other forms of malignancy were examined either by sequencing cDNA clones (n = 6) or by genomic PCR of

number of transformed colonies in each case and caused the transformed colonies to appear earlier. Moreover, cotransfection of either Bcl10 mutant with ras alone revealed that each mutant had itself acquired a weak but reproducible ability to cooperate with ras.

Bcl10 Mutations Are Found in Multiple Human Tumor Types

No RNA samples were available from the other two cases of MALT lymphoma with t(1;14)(p22;q32). To search for Bcl10 mutations in these cases, the genomic sequence and the exon/intron structure of the Bcl10 coding region were determined and PCR primers designed to amplify the three coding exons from paraffin sections. Mutations in all three cases with t(1;14)(p22; q32) were sought by PCR-SSCP. All showed abnormal migrating bands consistent with mutation; different sections from different histological blocks from the same tumor showed different bands, indicating that in all three cases Bcl10 mutation was ongoing (Figure 6A).

Chromosome 1p22 is also a common site of deletions and translocations in subtypes of lymphoma other than MALT, as well as a wide range of solid tumors (Tilly et al., 1994; Mertens et al., 1997; Thangavelu et al., 1997). We therefore sought Bcl10 mutations in a panel of fresh lymphoma cases and in cell lines derived from various human malignancies. A panel of archival lymphoma cases were studied by PCR-SSCP, and in some cases, the abnormal SSCP bands were excised, reamplified, and sequenced. PCR-SSCP analysis of a panel of 135 cases of B cell and 20 cases of T cell lineage lymphoma of various histological subtypes revealed abnormal migrating bands in 70 (45%); abnormal PCR-SSCP bands were observed in all lymphoma subtypes (M. J. S. D. et al., in preparation). A representative gel along with the derived sequences from one case of follicular B cell lymphoma that showed two deletions within the coding region of exon 3 is shown in Figure 6B. The sequence abnormalities in ten cases of MALT and follicular non-Hodgkin lymphoma in which the abnormal PCR-SSCP band was excised, successfully reamplified, and sequenced are shown in Table 2. Truncating Bcl10 mutations were detected in both subtypes of lymphoma in the absence of 1p22 chromosomal translocation.

Subsequently, 87 cell lines derived from patients with other forms of malignancy were examined either by sequencing cDNA clones (n = 6) or by genomic PCR of
Figure 6. PCR-SSCP Analysis of Bcl10 Mutations
(A) Ongoing mutations in a case of MALT lymphoma with t(1;14)(p22;q32). Exons 2.2 (left panel) and 3.2 (right panel) of a case of MALT lymphoma with t(1;14)(p22;q32) (case G0262) were amplified from different sections taken from different blocks of the same tumor and subject to SSCP. "N" denotes normal DNA, lanes 1-5 different samples from the same tumor. Arrowheads denote abnormalities in MALT lymphoma mutants M106 and M114 in abnormal bands; note that these are only present in some tumor cell lines M25, Mero82 (mesothelioma), and Tera2 (teratoma) was samples and not all. (B) Two internal Bcl10 deletions in a case of follicular B cell lymphoma (case G0109). A PCR-SSCP gel showing two normal controls (A) and case G0109 with abnormally migrating bands is shown. Abnormal bands labeled "a" and "b" were excised, reamplified, and sequenced. The sequences are shown in the right-hand panel. Band "a" represented a 17 bp deletion, whereas band "b" represented a 28 bp deletion occurring within coding exon 3 and resulting in frameshifts within the Bcl10 coding sequence with predicted protein products of 176 and 169 amino acids, respectively.

Table 1. Effect of Bcl10 Expression on Transformation of Primary REFs

<table>
<thead>
<tr>
<th>Vector</th>
<th>Wild-Type</th>
<th>E10</th>
<th>M106</th>
<th>M114</th>
</tr>
</thead>
<tbody>
<tr>
<td>ras</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>ras + pLTRp53cG</td>
<td>12</td>
<td>1</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>ras + E1a</td>
<td>16</td>
<td>1</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>ras + p53 234C</td>
<td>6</td>
<td>0</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
<tr>
<td>ras + E7</td>
<td>10</td>
<td>0</td>
<td>&gt;50</td>
<td>&gt;20</td>
</tr>
</tbody>
</table>

Data shown are number of transformed colonies per plate following transfection with ras and the indicated cooperating oncogenes, with or without cotransfection with E10 or wild-type/mutant Bcl10. Data shown are typical of three transfections. Wild-type Bcl10 suppressed transformation relative to vector, while E10 and both tumor-derived mutants, M106 and M114, enhanced transformation by cooperating oncogenes. The mutants showed weak transforming activity with ras alone.
Table 2. Bcl10 Mutations in Primary NHL and in Cell Lines Derived from Lymphoid and Solid Tumors

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Case No. or Cell Line</th>
<th>Alteration</th>
<th>Coding Exon</th>
<th>Codon</th>
<th>Mutation Type</th>
<th>Predicted Protein Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphoid Tumors</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MALT lymphoma</td>
<td>G0186 M106(^1)</td>
<td>499insT</td>
<td>3</td>
<td>167</td>
<td>Truncation</td>
<td>168 aa</td>
</tr>
<tr>
<td></td>
<td>M114(^1)</td>
<td>499insT</td>
<td>3</td>
<td>167</td>
<td>Truncation</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>94A → G</td>
<td>2</td>
<td>32</td>
<td>Ile → Val</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>145A → G</td>
<td>2</td>
<td>49</td>
<td>Arg → Gly</td>
<td>157 aa</td>
</tr>
<tr>
<td></td>
<td></td>
<td>298A → T</td>
<td>2</td>
<td>100</td>
<td>Thr → Ser</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G0193</td>
<td>163insA</td>
<td>2</td>
<td>55</td>
<td>Truncation</td>
<td>61 aa</td>
</tr>
<tr>
<td></td>
<td>G0123</td>
<td>345delA</td>
<td>2</td>
<td>115</td>
<td>Truncation</td>
<td>116 aa</td>
</tr>
<tr>
<td></td>
<td>G0524</td>
<td>345delA</td>
<td>2</td>
<td>115</td>
<td>Truncation</td>
<td>116 aa</td>
</tr>
<tr>
<td></td>
<td></td>
<td>145A → G</td>
<td>2</td>
<td>49</td>
<td>Arg → Gly</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>298A → T</td>
<td>2</td>
<td>100</td>
<td>Thr → Ser</td>
<td></td>
</tr>
<tr>
<td>Follicular lymphoma</td>
<td>G3018</td>
<td>428insTT</td>
<td>3</td>
<td>143</td>
<td>Truncation</td>
<td>147 aa</td>
</tr>
<tr>
<td></td>
<td>G3009</td>
<td>231ins A</td>
<td>2</td>
<td>77</td>
<td></td>
<td>96 aa</td>
</tr>
<tr>
<td></td>
<td>G0109a(^2)</td>
<td>525delI7</td>
<td>3</td>
<td>175-181</td>
<td>Truncation</td>
<td>176 aa</td>
</tr>
<tr>
<td></td>
<td></td>
<td>508delI8</td>
<td>3</td>
<td>170-180</td>
<td>Truncation</td>
<td>169 aa</td>
</tr>
<tr>
<td></td>
<td>G3008</td>
<td>410delA</td>
<td>3</td>
<td>137</td>
<td>Truncation</td>
<td>146 aa</td>
</tr>
<tr>
<td></td>
<td>G0206</td>
<td>398insT</td>
<td>3</td>
<td>133</td>
<td>Truncation</td>
<td>139 aa</td>
</tr>
<tr>
<td></td>
<td>G3023</td>
<td>637delI3</td>
<td>3</td>
<td>210</td>
<td>Del Glu</td>
<td>232 aa</td>
</tr>
<tr>
<td>T-ALL</td>
<td>Molt-4</td>
<td>136insA</td>
<td>2</td>
<td>46</td>
<td>Truncation</td>
<td>48 aa</td>
</tr>
<tr>
<td></td>
<td>Hut 78</td>
<td>428deff</td>
<td>3</td>
<td>143</td>
<td>Truncation</td>
<td>146 aa</td>
</tr>
<tr>
<td>Solid Tumors</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colonic carcinoma</td>
<td>Lovo</td>
<td>136insA</td>
<td>2</td>
<td>46</td>
<td>Truncation</td>
<td>48 aa</td>
</tr>
<tr>
<td></td>
<td>LS513</td>
<td>delT</td>
<td>1/2</td>
<td>intron</td>
<td>Splice aberration</td>
<td></td>
</tr>
<tr>
<td>Mesothelioma</td>
<td>M25 a(^3)</td>
<td>346delI6</td>
<td>3</td>
<td>116-120</td>
<td>Splice aberration</td>
<td>141 aa</td>
</tr>
<tr>
<td></td>
<td></td>
<td>499insT</td>
<td>3</td>
<td>167</td>
<td>Truncation</td>
<td>168 aa</td>
</tr>
<tr>
<td></td>
<td></td>
<td>238G → A</td>
<td>2</td>
<td>80</td>
<td>Asp → Asn</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M41 a(^3)</td>
<td>136delI7</td>
<td>2</td>
<td>43</td>
<td>Truncation</td>
<td>69 aa</td>
</tr>
<tr>
<td></td>
<td></td>
<td>155C → T</td>
<td>2</td>
<td>52</td>
<td>Thr → Ile</td>
<td>Full length</td>
</tr>
<tr>
<td></td>
<td></td>
<td>499insT</td>
<td>3</td>
<td>167</td>
<td>Truncation</td>
<td>168 aa</td>
</tr>
<tr>
<td>Germ cell tumor</td>
<td>Tera 1</td>
<td>172C → G</td>
<td>2</td>
<td>58</td>
<td>Arg → Gly</td>
<td>Full length</td>
</tr>
<tr>
<td></td>
<td></td>
<td>653C → T</td>
<td>3</td>
<td>218</td>
<td>Ser → Phe</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tera 2 a(^3)</td>
<td>499insT</td>
<td>3</td>
<td>167</td>
<td>Truncation</td>
<td>168 aa</td>
</tr>
<tr>
<td></td>
<td></td>
<td>274C → T</td>
<td>2</td>
<td>91</td>
<td>Truncation</td>
<td>91 aa</td>
</tr>
<tr>
<td></td>
<td></td>
<td>56G → A</td>
<td>2</td>
<td>20</td>
<td>Ala → Thr</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>172C → T</td>
<td>2</td>
<td>58</td>
<td>Truncation</td>
<td>57 aa</td>
</tr>
</tbody>
</table>

Cell lines denoted in italics.
1 Mutants M106 and M114; see Figure 5C.
2 Two independent deletions within coding exon 3 were identified in this tumor (see Figure 6B).
3 cDNA clones containing independent mutations (designated "a" and "b") were identified from these cell lines.
4 See Figure 5B.

Discussion

We report the cloning of an apoptotic regulatory gene, Bcl10, from its direct involvement in the t(1;14)(p22;q32) of low-grade MALT lymphoma. From cytogenetic, FISH, Southern blot, and PCR experiments, breakpoints within the S' region of Bcl10 were recurrent but present in only a low percentage of cases (M-Q. D. et al., unpublished observations). The Bcl10/IgH translocations left the coding region of Bcl10 intact. By analogy with Ig translocations that break either S' or within the promoter regions of other genes such as Myc, Bcl2, and Bcl6, it would be anticipated that the t(1;14)(p22;q32) would result in either deregulated overexpression of Bcl10 due to the proximity of the Ig transcriptional enhancers and/or loss of normal regulatory controls. In situ hybridization showed high-level expression in all three MALT lymphomas with the t(1;14)(p22;q32), but comparable levels of expression were also seen in other lymphomas that lacked the translocation. In this regard, Bcl10 may be similar to Bcl2 and Bcl6 where high-level expression may be seen in many B cell lymphomas irrespective of whether or not they exhibit Ig translocation (Zutter et al., 1991; Cattoretti et al., 1995).

Bcl10, like a number of proteins involved in the control and execution of apoptosis, contained a CARD (Hoffmann et al., 1997). This domain has been shown to consist of six tightly packed antiparallel alpha helices and is similar in structure to the "Death Domain" (Chou et al., 1998). CARDs have been found in both pro- and antiapoptotic proteins and mediate homotypic CARD-protein interactions between structurally related proteins. The CARD of RIP2/CARDIAK was sufficient for the apoptotic actions of this molecule (McCarthy et al., 1998) as well as the NF-κB and JNK activation (Thome et al., 1998). However, CARD proteins may have functions other than apoptosis; the prodomain of caspase-2 contains a CARD that allows nuclear translocation (Colussi et al., 1998).

Bcl10 is the first CARD protein to be implicated in malignancy. Its involvement is unusual in two regards. First, there is at present no evidence to suggest that t(1;14)(p22;q32). Otherwise, there was no apparent clustering of the point mutations.
overexpression or deregulated expression of the wild-type molecule might confer any survival benefit. Overexpression of Bcl10 in 293 cells resulted in apoptosis, although the extent and rate at which apoptosis was induced was much less and slower than with death-signaling molecules such as TNFR1. Consistent with this was the efficient suppression of transformation of REFs by wild-type Bcl10. In contrast, although E10 induced a comparable degree of apoptosis as Bcl10 in 293 cells, in the REF assay it behaved like the Bcl10 mutants and markedly enhanced the number of transformed colonies. The biochemical reasons underlying this difference between wild-type Bcl10 and E10 are not known, but B cell proliferation induced by E10 might allow EHV-2 viral persistence.

Second, Bcl10 appears to be unusually susceptible to mutation. These mutations principally consisted of either nucleotide insertion or deletion resulting in protein truncation, in most cases distal to the CARD. Interestingly, the truncating mutation common to 10/16 mutated Bcl10 clones observed in the MALT lymphoma with the t(1;14)(p22;q32), insertion T 499, was also detected in both mesothelioma and male germ cell tumor cell lines. All three cases of MALT lymphoma with t(1;14)(p22;q32) exhibited Bcl10 mutation, and it appeared that this process was ongoing. Ongoing mutation is a feature of productively rearranged IgVH genes after a B cell has encountered antigen, resulting in affinity maturation of antibody, but has also been seen in other genes in B cell malignancies (Albert et al., 1994; Gutierrez et al., 1998). Many of the Bcl10 mutations could be ascribed to the Ig somatic hypermutation mechanism resulting in nucleotide transitions, and the function of the t(1;14)(p22;q32) chromosomal translocation may be to place the Bcl10 gene under the direct influence of this mechanism. Detection of Bcl10 abnormalities was hindered by ongoing mutation, and we have found it necessary to sequence individual cDNA or genomic clones; the frequency of Bcl10 mutations may be significantly underestimated by sequencing PCR reactions directly (T. G. W. et al., unpublished observations). The possible functional significance of the ongoing Bcl10 mutations are not clear. Both Bcl10 mutants cloned from the MALT lymphoma with t(1;14)(p22;q32) appeared to have similar properties in all assays.

The functional consequences of the Bcl10 truncations distal to the CARD were the loss of the pro-apoptotic functions with retention of NF-κB activation. Expression of NF-κB has been associated with suppression of apoptosis and prolongation of survival in many systems; NF-κB activation also appears to be an essential component of several transformation pathways (Wu et al., 1996; Mayo et al., 1997; Reuther et al., 1998; Van Antwerp et al., 1998). The dissociation of NF-κB activation and pro-apoptotic functions in the truncated forms of Bcl10 was consistent with their marked enhancement of transformation by cooperating oncogenes. Whether other Bcl10 truncating mutations that fall within the CARD have the same phenotype is not yet known.

Transdominant inhibition by tumor-associated mutants of wild-type Bcl10-induced apoptosis represents a basis for the selection of Bcl10 mutants in cancer. However, coexpression of truncated mutants did not substantially affect the proapoptotic activity of wild-type Bcl10 under the conditions of our assay. While the possibility of transdominant inhibition cannot be excluded, it seems likely that selection is for the loss of apoptotic activity and concomitant gain of proliferative function in the truncated mutants rather than for abrogation of wild-type activity. In this regard, the mutants display some functional similarity to some p53 mutants, which, although unable to inhibit transdominantly the apoptotic activity of the wild-type protein, are nevertheless frequently selected in a wide range of human cancers, presumably as a result of their proliferative activities (Gualberto et al., 1998).

Finally, Bcl10 abnormalities were shown to be present in several subtypes of lymphoma other than MALT. In follicular lymphoma, abnormalities of chromosome 1p22 have been associated with rapid progression and a poor prognosis (Tilly et al., 1994). However, Bcl10 mutation did not appear to be limited to malignancies of the lymphoid lineages. Chromosome 1p22 has been shown to be consistently deleted in several other tumor types, including 80% of mesothelioma (Lee et al., 1996) and 40% of male germ cell tumors (Mathew et al., 1994). So far, our analysis of these diseases has been restricted to derived cell lines. However, given the high frequency of Bcl10 mutations in these, but not other cell lines, it seems unlikely that Bcl10 is mutated solely as a consequence of prolonged in vitro culture. Bcl10 may be the target gene for the chromosome 1p22 deletions in a wide range of human malignancy. Both the compromised proapoptotic activity and gain of transforming activity of human tumor-derived Bcl10 mutants provide a mechanistic basis for their frequent selection in a wide range of cancers.

Experimental Procedures

Cloning of t(1;14)(p22;q32) Translocation Breakpoint

LDI-PCR was performed on high-molecular-weight DNA from a MALT lymphoma with t(1;14)(p22;q32) as described (Willis et al., 1997, 1998): 400 ng of DNA was digested with PstI (GIBCO-BRL, Gaithersburg, MD) in a volume of 30 μl and then ligated at 15°C overnight in a total volume of 500 μl with 5 U of T4 DNA ligase (GIBCO-BRL). Ligated DNA was purified using a Qiaquick column (Qiagen, Hilden, Germany), and 10 ng DNA was amplified with rTh polymerase (GeneAmp, XL; Perkin-Elmer, Foster City, CA) using primers for IgH and realigned using nested primers. Following amplification, the PCR reaction was run on a 0.8% agarose gel and the two bands excised. These were purified and A-tailed with 5 U T4 DNA ligase. Sequencing was performed on an Applied Biosystems model 377 sequencer using AmpliTaq DNA polymerase, FS (Perkin-Elmer). Sequences were analyzed against the NCBI GenBank EST database using the BLAST program. cDNA clones corresponding to positive ESTs were obtained from the Human Genome Mapping Project Resource Centre (Cambridge, UK) and sequenced. Southern blot, fluorescence in situ hybridization, and Northern blot analysis were performed as described (Willis et al., 1997, 1998).

In Situ Hybridization

In situ hybridization for Bcl10 mRNA was carried out as described (Pan et al., 1997). Briefly, fragments of paraffin sections were dewaxed in xylene, washed in ethanol, digested with proteinase K, fixed in 4% paraformaldehyde/PBS, and hybridized at 50°C for 16-18
hr with digoxigenin-11-UTP (Boehringer Mannheim, Lewes, UK) labeled Bcl10 antisense RNA probe. The hybridized sections were washed, incubated twice in alkaline phosphatase-conjugated sheep antidigoxigenin for 1 hr, and visualized with 5-bromo-4-chloro-3-indolyl phosphate/nitro-blue tetrazolium.

Expression Vectors

Expression clones were amplified by PCR, cloned into the TA cloning vector pCR2.1 TOPO, and then subcloned in frame into the expression vector pDNA3.1/Myc-His (Invitrogen). The open reading frame of Bcl10 was amplified from a Bcl10 CDNA clone using primers 5′-CCATCTCAGATCTCCACCATG-3′ and 5′-AAAGTGGTCGAGCTTGGT AACAGTA-3′, and the open reading frame for E10 was amplified from EHV-2 DNA using primers 5′-CCTCCAGAACAGCATGTATGGCG-3′ and 5′-CAGCAGATATCCCTCCT-3′. Both were subcloned into pDNA3.1/Myc-His. The carboxy terminus of Bcl10 (amino acids 101-233) and mutant MALT lymphoma cDNA clones 106 and 114 AGAAAAAGGGCTGGAAA-3′ were amplified from a MALT lymphoma cDNA clone using primers 5′-GGAACCCGGAAGAAGCGCCATCTCC-3′ and 5′-GATCTCTTCTGGCTCTGCAGACTC-3′ (anticipated product size 187 bp).

Expression in 293 cells

A total of 105 cells/well were seeded on 6-well (35 mm) dishes. Cells were transfected the following day by calcium phosphate coprecipitation. Stable G418-resistant transfectants of the mouse L3-dependent pro-B cell line BA/F3 were generated by electroporation of 106 cells in 400 ml of serum-free medium and mixed with 20 μg pcDNA3.1/V5-His plasmid with or without Bcl10 insert at 250 V and 900 μF. Cells were selected with 1.2 mg/ml G418, seeded at 0.5 cells per well in 96 well plates, and screened for exogenous Bcl10 expression.

Apoptosis Assays

293, COS-7, and HeLa cells were cotransfected with 1 μg of expression construct and 0.25 μg pcDNA3.1/β-galactosidase. Where indicated, the caspase inhibitor z-VAD-fmk (25 μM) was added 6 hr posttransfection. After 48 hr, cells were washed with phosphate-buffered saline (PBS), fixed with 0.5% glutaraldehyde, washed again in PBS, and stained with X-gal (50 mM Tris HCl [pH 8], 2.5 mM potassium ferricyanide, 15 mM NaCl, 1 mM MgCl2, 0.5 mg/ml X-gal) for 4 hr. At least 300 β-galactosidase-positive cells were counted for each transfection (n = 3) and identified morphologically as apoptotic or nonapoptotic.

NF-κB Reporter Assay

293 cells were transfected in phenol-free DMEM with 1 μg (except where indicated) of expression construct and 0.25 μg of a secreted placental alkaline phosphatase reporter construct pTK-CSPAP containing a minimal HSV tyrosine kinase promoter with or without four copies of the NF-κB response element GGAAGTCCC. In wells exposed to 40 μl of medium, 102.5 μg NaOH, and absorbance was then measured at 405 nm for each transfection (n = 3).

Western Blotting Analysis

293 cells (106) were lysed by sonication in 1% SDS, 100 mM Tris (pH 6.8), 0.14 M β-mercaptoethanol, 20% glycerol, and 0.025% bromophenol blue. Proteins were separated using 12% polyacrylamide gel, electrotransferred onto nitrocellulose paper, and incubated with 1:4000 monclonal mouse anti-myc antibody (Invitrogen) followed by 1:1000 sheep anti-mouse Ig horse- radish peroxidase-linked, whole antibody (Amersham). After washing, samples were developed using ECL Western blotting detection reagents (Amersham).

REF Transformation Assays

Primary REFs (BioWhittaker, Wokingham, UK) were maintained in DMEM plus 10% FCS. Subconfluent 90 mm dishes were transfected by the calcium phosphate method as described (Crook et al., 1994).

Following transfection, cells were grown in DMEM/10% FCS in the presence of 500 μg/ml G418 sulfate (GIBCO). Morphologically transformed colonies were counted after 3 weeks. The following plasmids were used in the REF assays: pLTRp53C (Eliyahu et al., 1985), pcB6 16E7 (Parker et al., 1996), pcB6 p53 234C (Parker et al., 1996), and pCE (encodes adenovirus E1a) (Schneider et al., 1987).

Cloning and Sequencing of Bcl10 Genomic Fragment

One hundred nanograms of normal human genomic DNA was amplified with rTth polymerase using primers for the Bcl10 open reading frame 5′-CCTCCCTCTCCTCCCTCCCTTACCC-3′ and 5′-CATTAAATATTAAAAAAGCAATAAGT-3′ in a 50 μl reaction at 95°C for 1 min followed by 35 cycles of 95°C for 15 s and 68°C for 10 min. A 10 kb product was subcloned into pCR2.1 TOPO, and automated DNA sequencing was performed using the primers 5′-AAACATCAAGT AGAAAAAGGGCTGGAAA-3′, 5′-ATTTCAGCCTTTTCTTACTGAT GTT-3′, 5′-GAGTGGTTCGTTGGCCCATCCT-3′, and those described above.

PCR-SSCP Studies

DNA samples from frozen and paraffin-embedded tissue blocks from 155 cases of lymphoma from the Department of Histopathology, University College were studied. The full coding sequence of the Bcl10 gene was amplified by five different PCR reactions (two reactions for exons 2 and 3) using the following primer sets: Exon 1, 5′-GGACCGGGAGAACGGCACCTTCC-3′ and 5′-GATCCTCTTCTGGCTCTGCAGACTC-3′ (anticipated product size 187 bp); Exon 2.1, 5′-AAGACCTGCCAATTAATGTCAGCT-3′ and 5′-AAGTATGCTTAC AATTTCACCGG-3′ (200 bp); Exon 2.2, 5′-CCTGAAAGAATTTCT TGTCGAAACA-3′ and 5′-AAAAAGCATTATCATTACAAAAATACCCT-3′ (244 bp); Exon 3.1, 5′-TTAAAAGTTCAAGATTGACGAG-3′ and 5′-CTAGAACCGAAATTCGAAAGAAG-3′ (263 bp); Exon 3.2, 5′-GGAGATCCGACCGACGCC-3′ and 5′-CATTAAAAATAGAGGGAAATAAAGCTG-3′ (257 bp). PCR was performed on a thermal cycler (Hybaid, Teddington, UK) using a “hot start” followed by a “touch-down” program. For SSCP analysis, PCR products (2 μl) were mixed with 4 μl sequencing loading buffer, denatured, and separated on Genphor electrophoresis system (Pharmacia-Amersham, Amersham, UK) under 15 W constant power for 2 to 3 hr at 5°C and then visualized by silver staining. In some cases, the abnormal bands were excised from the gel and reamplified prior to sequencing in both directions.

Bcl10 cDNA and Genomic DNA Sequencing

The Bcl10 ORF was amplified from reverse-transcribed poly(A+) mRNA using primers 5′-GGAACCCGGAAGAAGCGCCATCTCC-3′ and 5′-CATTAAAAATAGAGGGAAATAAAGCTG-3′ and cloned into pCR2.1. In MALT lymphoma case G0186, two separate RT-PCR amplifications were performed and individual clones sequenced; both reactions contained wild-type and mutant Bcl10 clones. Sequencing of the Bcl10-coding region from cell lines and cases with abnormally migrating SSCP bands was performed on amplified or cloned genomic PCR products using dihydrofolamine DNA polymerase (PerkinElmer) according to manufacturer instructions.

Acknowledgments

We acknowledge the assistance of Dr. Tim Doss and Rocio Hassan with the Southern blots. We thank Dr. A. J. Davison (Glasgow, Scotland) for providing EHV-2 DNA; Drs. Marjan Versnel (Dept. of Immunology, Erasmus University, Rotterdam, The Netherlands) and Sue Eccles (ICR) for kindly providing mesothelioma cell lines; Drs. A. Thiry and F. Andrien (Liege, Belgium) for kindly providing patient material; Dr. Stuart Farrow (Glaxo-Wellcome, Stevenage, UK) for kindly providing the NF-κB reporter constructs; Professor Mike Stratton (ICR) for tumor cell line DNA samples; Maurizio Valeri and Jacky Cordell (ICR) for their help with cell culture; and Rachel Jackson, Samantha Dibley, and Bina Desai (ICR) for their help with automated DNA sequencing. We thank the HGM Research Centre, Hinxton Hall, Cambridge for providing clones and computing facilities, and Dr. Gabriel Nunez (Dept. of Pathology, University of Michigan Medical School, Ann Arbor, MI) for helpful discussions and
for providing the TNFR-1 and CrmA constructs. This study was supported by grants from the Leukaemia Research Fund, the Kay Kendall Leukaemia Fund, and the Cancer Research Campaign. T. C. is a Leopold Muller Fellow, supported by Breakthrough Breast Cancer.

Received October 15, 1998; revised November 30, 1998.

References


