Biochemical and Biophysical Research Communications 383 (2009) 32–36

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

BimL upregulation induced by BCR cross-linking in BL41 Burkitt's lymphoma results from a splicing mechanism of the BimEL mRNA

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ARTICLE INFO

Article history:
Received 16 March 2009
Available online 28 March 2009

Keywords:
Apoptosis
Bim
Isoforms
Intron retention

ABSTRACT

B lymphocyte receptor-mediated apoptosis is associated with increased expression of the BimL isoform of Bim. The mechanisms involved in the regulation of BimL protein expression are still unknown. We report that BimL expression following BCR activation is not associated with a specific increase of BimL mRNA but rather to the intron retention structure of the BimEL mRNA. Indeed, expression of a BimEL cDNA leads in Hela cells leads to the production of both BimEL and BimL proteins. Mutation of the intron-splicing GT sequence present in the exon 3 results in the production of only BimEL protein. Ectopic expression of BimEL cDNA resulted in a large increase of BimL expression upon BCR-stimulation, whereas cells transfected with the GT/AA mutated form of BimEL only produced BimEL proteins upon BCR-activation. These data showed that BimL expression induced by BCR activation may result from the splicing of BimEL mRNA independently of Bim promoter regulation.

Introduction

Apoptosis is central to the development and maintenance of homeostasis within all multicellular organisms. Defects in the control of this mechanism have been implicated as a cause or are characteristic of a variety of diseases including cancer and autoimmunity. The BH3-only proteins are regulators of the balance between the anti-apoptotic and pro-apoptotic members of the Bcl-2 family, and molecules mimicking their function can effectively induce apoptosis in cancer cells [1]. One of these BH3-only proteins, Bim, is a key regulator of apoptosis induced by various stimuli including serum withdrawal, glucocorticoid exposure and antigen-receptors activation [2–5]. Bim is expressed in diverse tissues such as epithelial, neuronal, germinal, lymphoid, myeloid and fibroblastic cells [6]. However, work with Bim-deficient mice shows that Bim is essential for the control and development of lymphocyte homeostasis [7]. The expression of Bim is subject to various regulations both at the transcriptional and post-transcriptional levels. Transcriptional regulation of Bim has been reported in serum-deprived neuronal cells [8,9], haematopoietic cells [10,11] and in epithelial and hepatocytes cells stimulated with TGFβ [12,13]. More recently various studies showed that Bim is also regulated by miRNA, in particularly the miR17-92 cluster, which is amplified, and over expressed in various cancers [14,15]. At least 18 Bim isoforms are produced from the Bim gene [16–18]. However, only three of these proteins – BimEL, BimL and BimS – are abundant in cells. These isoforms have different apoptotic properties: the shorter BimS and BimL isoforms are more apoptotic than the BimEL protein. Bim EL mRNA differs from BimL mRNA by the presence of the 168 nucleotide-long exon 3 which possesses the GT and CAG sequences at its boundaries characteristic of an intron [19].

Phosphorylation of the serine 69 in BimEL, encoded by exon 3, by the MAPKinases ERK and JNK leads to the degradation of BimEL through the proteasome pathway [20–23]. We previously reported that activation of the B cell receptor in Burkitt's lymphoma cells induced apoptosis; this apoptosis was dependent on Bim and associated with both ERK-mediated proteasome-dependent degradation of BimEL and a large increase of BimL protein abundance [20]. The mechanisms involved in the regulation of BimL protein expression, in this context, are still unknown. However, a better understanding of the mechanisms involved in regulating the expression of Bim isoforms is essential to better understand Bim proapoptotic activity.

Here, we show that BimL expression upon BCR activation is associated with the intron retention structure of the BimEL mRNA. We found that BCR-mediated up-regulation of BimL protein in B cells may be dependent on BimEL mRNA expression independently of regulation of the Bim promoter.

Materials and methods

Reagents

The murine DA44 ab (anti-human IgM, IgG1) was obtained from hybridoma cell lines (American Type Culture Collection (ATCC),

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doi:10.1016/j.bbrc.2009.03.123

Rockville, MD) and was purified from ascitic fluids on protein A-Sepharose columns (Pharmacia, Uppsala, Sweden). The F(ab′)2 of goat anti-mouse IgG (cross-linker ab (CL)) was obtained from Immunotech (Marseille, France).

Antibodies

Anti-Bim and anti-c-Myc 9E10 antibodies were from Epitomics (Burlingame, CA, USA) and Sigma (St. Louis, MO, USA), respectively. Anti-tubulin (clone TU-02) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

Cell lines

The Burkitt's lymphoma cell line BL41 was provided by Dr. S. Sharma (Brown University, RI, USA) and the CA46 cell line was obtained from the ATCC. Both cell lines were cultured as described previously [24]. HeLa cells (American Type Culture Collection) were cultured in Dulbecco's minimal essential medium supplemented with 10% foetal bovine serum, 100 U/mL penicillin, 100 mg/mL streptomycin, sodium pyruvate and nonessential amino acids (Invitrogen Life Technologies, Strasbourg, France.).

Western blot analysis

Cells were lysed in 20 mM Tris (pH 7.4) and 0.5% SDS in the presence of 10 U of Benzonase (Merck Eurolab) for 5 min at room temperature and then boiled for 3 min. Aliquots of the supernatants were used for protein determination (microBCA protein assay; Pierce). Cell lysates were subjected to SDS–PAGE, and the proteins were then electrophoretically transferred onto nitrocellulose filters. The filters were probed with specific antibodies (Ab). Ab binding was detected by incubation with sheep anti-mouse or anti-rabbit IgG HRP-conjugated Abs and chemiluminescence (West-Pico; Pierce). Images were captured using a DDC camera (LAS-1000; Fuji).

Cell transfection

Stable mutants of CA46 cells (CA46 overexpressed BimEL wt and mutated GT*) were obtained by transfection with pcDNA6b vector coding for BimEL wild-type and BimEL mutated at the 5′ GT donor site of exon 3. Transfections were performed by electroporation at 0.24 V and 960 μF using a Bio-Rad apparatus. Stable transfectants were selected by incubating the cells with 30 μg/mL blasticidin (Invitrogen Life Technologies) for approximately 3–4 weeks. Stable clonal transfectants were isolated from blasticidin-resistant cells by limiting dilution. Recombinant BimEL protein was assayed in the various clones by Western blotting with the anti-c-Myc Ab 9E10.

Transient transfections of HeLa cells with empty pcDNA6b vector and the vector coding for BimEL wild-type, BimL wild-type and BimEL mutated at the 5′ GT donor site of exon 3 were performed using Lipofectamine 2000 transfection reagent (Invitrogen Life Technologies) and a standard manufacturer's protocol.

All constructs were checked by sequencing.

RNA extraction, RT-PCR and sequencing

RNA extraction. Total cellular RNA was isolated using the RNeasy kit (Quiagen) according to the manufacturer's instructions.

RT-PCR. The first-strand cDNA was generated by reverse transcription of 5 μg of total RNA using oligo(dT) nucleotides. PCR was performed with the primers: forward 5′-TGATGTAAGTTCTG AGTGTG-3′, reverse 5′-ACGTAACAGTCGTAAGATAA-3′ for endogenous Bim; forward 5′-TGATGTAAGTTCTGAGTGTG-3′, reverse 5′-CCGGTATGCATATTCAGATCCTC-3′ for myc-tagged Bim; and forward 5′-GGTGAAGGTCGGAGTCAACGGA-3′, reverse 5′-GAGGGATC TCGCTCCTGGAAGA-5′ for GAPDH. PCR products were separated by electrophoresis in 2% agarose gels and were visualized by ethidium bromide staining.

Mutations. Mutations were created using the QuikChange Site-Directed Mutagenesis kit from Stratagene according the manufacturer's instructions (Quiagen).

Sequencing. cDNA was produced by RT-PCR, subjected to electrophoresis and extracted from the agarose gel with the QIAquick Gel Extraction kit (Quiagen, Courtaboeuf, France) by a standard manufacturer's protocol. Sequencing was performed by Genome Express (Meylan, France).

Results and discussion

BCR-mediated BimL protein production is not associated with a specific upregulation of BimL mRNA in the Burkitt's cell line BL41

We previously described an original pathway of regulation of BimEL and BimL protein expression during apoptosis induced in Burkitt's cell lines by BCR cross-linking [20]. BimEL, highly abundant in healthy cells, is rapidly downregulated by an ERK-dependent proteasome pathway after 5–6 h of BCR stimulation (Fig. 1A). This downregulation of BimEL protein is associated with the upregulation of BimL protein expression within 2 h of stimulation. We next investigated the mechanisms controlling BimL expression. First, we compared the amounts of transcripts for the two Bim isoforms during BCR stimulation (Fig. 1B). BimEL and BimL transcripts were both rapidly but only moderately increased after 8 h of BCR stimulation; the kinetics for the two mRNAs were similar. We determined the relative mRNA concentrations and found that the BimEL and BimL mRNA were increased in the same proportion such that the ratio between them remained unchanged following stimulation (Fig. 1C). These results showed that the increase in BimL protein abundance mediated by activation of the BCR in BL41 cells is not a consequence of changes in the ratio between BimEL/BimL mRNAs.

BimEL mRNA is an intron retention form of BimL mRNA

It has been reported that the BimEL exon 3 in the murine Bim gene is in fact a facultative intron. This sequence possesses the characteristic donor GT and acceptor CAG sites of introns in 5′ and 3′ positions, respectively (Fig. 2A) [19]. Splicing of this exon 3 gives rise to the mRNA from which BimL protein is produced with a specific junction "GCCACAAGACAGGAG" between the exon 2 and exon 4 (Fig. 2A). Therefore, we investigated whether modification of this conditional splicing, independently of Bim promoter regulation, is involved in the upregulation of BimL protein observed in Burkitt's cell lines following BCR stimulation. We first verified that BimEL mRNA was sufficient for the production of both BimEL and BimL proteins. For this, we inserted cDNA coding for BimEL into a pcDNA6b vector between a CMV promoter and a C-terminal-fused Myc tag (pcDNA6b BimEL wt); the construct was used for transient transfection of HeLa cells. Both myc-tagged BimEL protein and myc-tagged BimL protein were detected in transfected cells (Fig. 2B). Transfection with an empty vector did not result in Bim protein production, and confirmed that the lower band was specific to the insert. In addition, BimEL cDNA expression was also associated with the production of a second RNA with an apparent MW similar to that of BimL mRNA (transcribed from a control plasmid containing a tagged-myc BimL insert). To test whether this additional RNA corresponded to BimL, we isolated this lower band by gel extraction and subjected it to RT-PCR, with

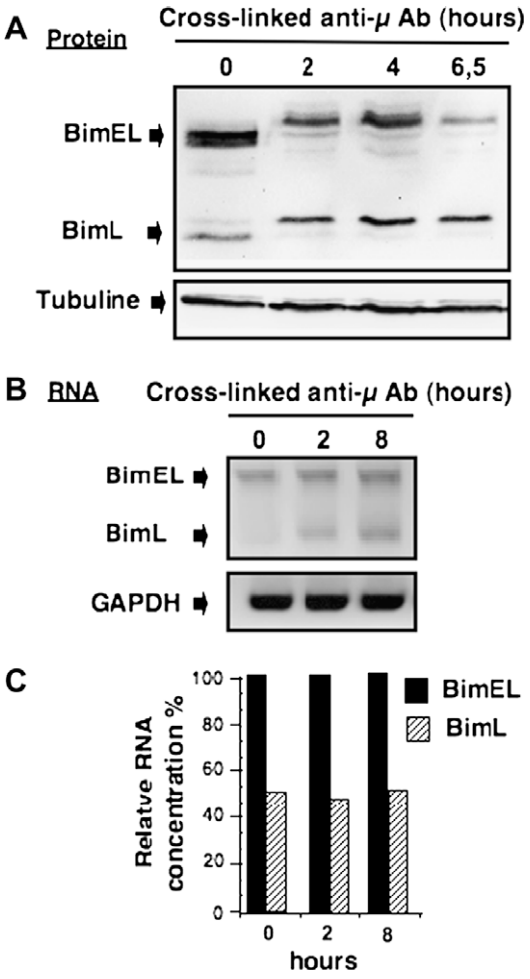

Fig. 1. BimL expression following BCR stimulation is not associated with specific upregulation of BimL mRNA in Burkitt's cells lines. Burkitt's lymphoma cells (cell line BL41) were treated with cross-linked DA44 Ab (anti-μAb) for various times. (A) Whole extracts were subjected to SDS–PAGE and Bim isoforms were detected by immunoblotting with an anti-Bim Ab. (B) mRNA specific for BimEL and BimL were analysed by RT-PCR and separated by gel electrophoresis. Tubulin protein and GAPDH mRNA were used as loading controls for immunoblotting (A) and RT PCR (B), respectively. (C) Graphic representation of the relative concentrations of the BimEL and BimL mRNAs following BCR stimulation.

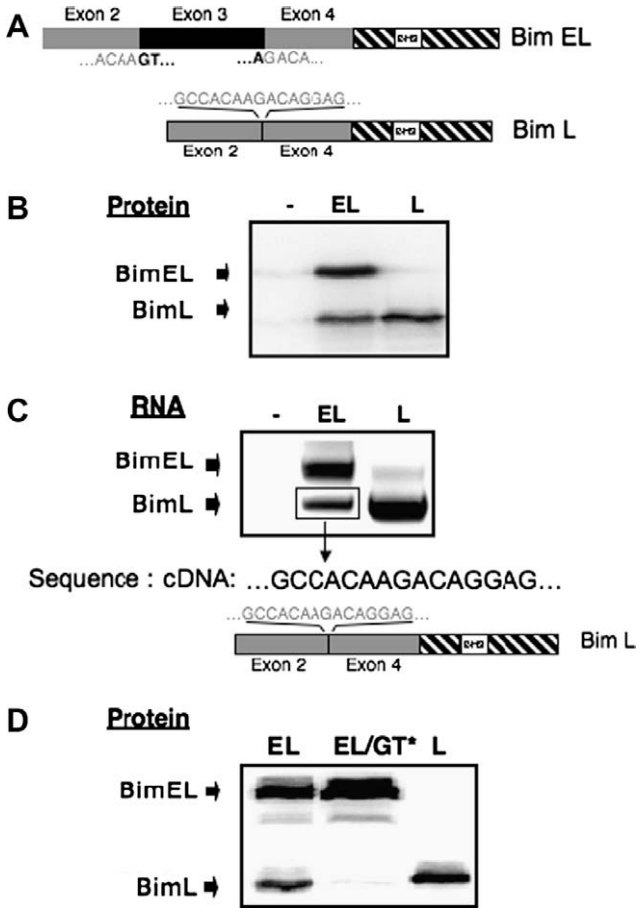

Fig. 2. BimEL mRNA is an intron retention form of BimL mRNA. (A) Schematic representation of the organisation of BimEL and BimL mRNA. (B–D) HeLa cells were transfected for 24 h with pcDNA6b BimEL, pcDNA6b BimL, pcDNA6b BimEL mutated at the 5′ GT donor site of exon 3, or empty pcDNA6b vector. (B and D) Exogenous Bim proteins were detected by immunoblotting with an anti-Myc 9E10 Ab. Tubulin protein was used as loading control for immunoblotting. (C) cDNA corresponding to BimL was extracted from agarose gel after RT-PCR before sequencing.

primers spanning coding exon 2 and Myc tag sequences. We sequenced the resulting cDNA and found the sequence "GCCACAAGACAGGAG" corresponding to the junction between exon 2 and exon 4 in BimL RNA (Fig. 2C). This demonstrated that the lower RNA band produced from BimEL cDNA was indeed mRNA for BimL. To confirm these observations, we prepared a mutated BimEL construct in which the 5′ GT of exon 3 was mutated to two cytosines (pcDNA6b BimEL GT/CC*). We then compared the BimEL and BimL protein production after transient transfection of HeLa cells with the two BimEL vectors (pcDNA6b BimEL wt and pcDNA6b BimEL GT/CC*). The mutation of the 5′ donor GT site did not affect the production of BimEL, but abolished BimL protein production (Fig. 2D).

These findings show that BimEL mRNA is a pre-mRNA form of BimL mRNA and that conditional splicing of BimEL mRNA can lead to the production of BimL mRNA and consequently BimL protein independently of any regulation of the Bim promoter.

BimL expression induced by BCR activation in Burkitt's cell lines results from a splicing mechanism of the BimEL mRNA

The experiments described above suggest that BCR stimulation in Burkitt's cell lines might increase conditional splicing of the BimEL mRNA to give more BimL mRNA resulting in greater production of the BimL protein. To test this possibility, we stably transfected Burkitt's cell line CA46 with the wild-type BimEL cDNA construct and the mutated GT/CC* BimEL construct. CA46 cells do not express endogenous BimEL or BimL either when untreated (Fig. 3A) or after BCR stimulation; however, they do express a functional B cell receptor (data not shown). We isolated one clone that strongly expressed recombinant BimEL following transfection with each the wild-type construct (Fig. 3B) and the mutated construct (Fig. 3C). Interestingly, this ectopic expression of wild-type BimEL cDNA in CA46 cells did not lead to production of BimL protein in unstimulated cells. The isolated clones (CA46 cells over-expressing wild-type BimEL-myc or mutated BimEL-myc GT/CC*) were stimulated with cross-linked DA44 Ab and immunoblotting with an anti-Myc Ab was used to test for recombinant Bim protein isoforms. BCR ligation in CA46 expressing BimEL-myc wt induced significant production of BimL protein. (Fig. 3B). Mutation of the 5′ donor GT site of BimEL exon 3 was sufficient to abolish this upregulation of BimL protein induced by BCR ligation in CA46 cells over-expressing mutated BimEL-myc GT/CC* (Fig. 3C).

Alternative splicing is a major source of protein heterogeneity, and numerous studies have demonstrated that it is frequent in the human transcriptome [25–27]. Alternative splicing is observed for at least half of all human genes, and this is probably an underestimate due to the difficulty of detecting splicing variants of

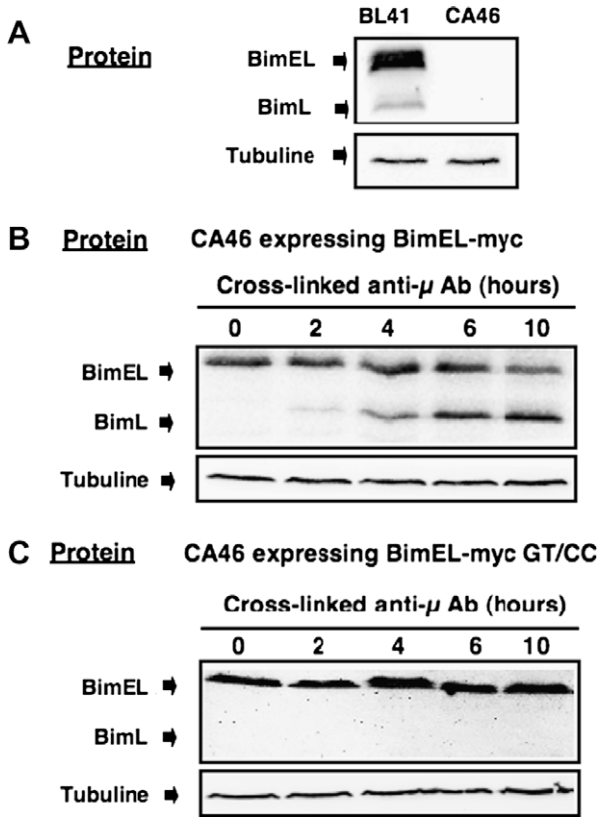

Fig. 3. BimL expression induced by BCR ligation in Burkitt's cells lines results from splicing of the BimEL mRNA. (A) BimEL and BimL proteins in BL41 and CA46 Burkitt's lymphoma cells were studied. (B) CA46 cells over-expressing wild-type BimEL-myc and (C) CA46 cells over-expressing mutated BimEL-myc GT/CC* were stimulated with cross-linked DA44 Ab (anti-μAb) for various times. The Bim protein isoforms were detected by immunoblotting with an anti-Myc 9E10 Ab. Tubulin protein was used as a loading control.

weakly expressed genes [28,29]. Four major types of alternative splicing have been described (exon skipping, alternative 3- splice or 5-splice site, and intron retention), of which intron retention is certainly the least studied [30]. The biological consequences of intron retention are still poorly documented. For instance, in *Drosophila,* retention of the third intron of the P-element transcript is associated with blocking of transposition in somatic cells [31]. Intron retention is also responsible for truncated forms of the Ret tyrosine kinase that are enriched in familial and sporadic pheochromocytomas [32]. Our data suggest that intron retention is also a physiological mechanism regulating the expression of the pro-apoptotic BimL isoform; this regulation may contribute to the control of BCR-mediated apoptosis that is critical for the control and elimination of auto-reactive B cells. Obtaining mice deficient for the BimL isoform as well as screening patients with autoimmune diseases for mutations in the 5′ part of exon 2 should help elucidate this mechanism associated with the biological activity of Bim.

In conclusion, our study shows that BimL expression induced by BCR activation (1) may result from the splicing of BimEL mRNA independently of Bim promoter regulation and (2) is related to an intron retention mechanism. This unusual mechanism of regulation of the pro-apoptotic BimL protein triggered by BCR activation may be important for Bim-mediated control of B lymphocyte homeostasis and autoimmune diseases.

Acknowledgments

This work was supported by INSERM and Grants from the Association pour la Recherche sur le Cancer (ARC), Agence Nationale de la Recherche (ANR) and Pole de Compétitivité-Medicen (projet Hexocan). C. Clybouw and A. Hadji hold fellowships from Ministère de la Recherche, Fédération de la Recherche Médicale (FRM) and ARC, respectively.

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