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ORIGINAL ARTICLE

# Regulation of pri-microRNA *BIC* transcription and processing in Burkitt lymphoma

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**BIC is a primary microRNA (pri-miR-155) that can be processed to mature miR-155. In this study, we show the crucial involvement of protein kinase C (PKC) and nuclear factor-κB (NF-κB) in the regulation of *BIC* expression upon B-cell receptor triggering. Surprisingly, Northern blot analysis did not reveal any miR-155 expression upon induction of *BIC* expression in the Burkitt lymphoma-derived Ramos cell line, whereas other microRNAs were clearly detectable. Ectopic expression of *BIC* in Ramos and HEK293 cells resulted in miR-155 expression in HEK293, but not in Ramos cells, suggesting a specific block of *BIC* to miR-155 processing in Ramos. In line with the results obtained with Ramos, lack of miR-155 expression after induction of *BIC* expression was also observed in other Burkitt lymphoma cell lines, indicating a generic and specific blockade in the processing of *BIC* in Burkitt lymphoma. In contrast, induction of *BIC* expression in normal tonsillar B cells resulted in very high levels of miR-155 expression and induction of *BIC* expression in Hodgkin's lymphoma cell lines. It also resulted in elevated levels of miR-155. Our data provide evidence for two levels of regulation for mature miR-155 expression: one at the transcriptional level involving PKC and NF-κB, and one at the processing level. Burkitt lymphoma cells not only express low levels of $BIC_2$ but also prevent processing of *BIC* via an, as yet, unknown mechanism.**
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## Introduction

Recent evidence indicates that the regulation of gene expression by microRNAs (miRNAs), a family of non-protein coding $\sim 22$ nt RNAs, is an important mechanism in maintaining cellular and tissue homeostasis. Some miRNAs have been shown to be critically involved in biological processes as diverse as insulin secretion (Poy *et al.*, 2004), dendritic spine development (Schratt *et al.*, 2006) and hematopoiesis (Chen *et al.*, 2004). In addition, several reports have now shown that deregulated miRNA expression plays an important role in the development of human disease, especially cancer (Chen, 2005; Hammond, 2006).

miRNAs are initially transcribed as longer transcripts called primary miRNA (pri-miRNA), which are processed in the nucleus to $\sim 70$ nt precursor miRNA (pre-miRNA) by the microprocessor complex (Gregory *et al.*, 2004). The pre-miRNAs are transported to the cytoplasm by Exportin-5 (Lund *et al.*, 2004) where they are further processed to mature miRNAs by the RNase III dicer (Hutvagner *et al.*, 2001). Finally, miRNAs are loaded into RNA-induced silencing complex (RISC) to induce translational repression and/or degradation of the target gene transcripts (Hutvagner and Zamore, 2002).

A pri-miRNA that has been studied for several years is *BIC*, now known as pri-miR-155. The *BIC* gene was originally described as a common site of viral DNA integration in virally induced lymphomas in chicken (Clurman and Hayward 1989). The oncogenic potential of *BIC* was shown in lymphoma and leukemia and a possible collaboration with the oncogene c-myc was suggested (Tam *et al.*, 2002). In a miRNA cloning study of mouse tissues, Lagos-Quintana *et al.* (2002) identified that the small phylogenetic conserved region of the *BIC* transcript encodes miR-155. High expression of *BIC* and miR-155 was shown in various human B-cell neoplasms including Hodgkin's lymphoma, primary mediastinal B-cell lymphoma and diffuse large B-cell lymphoma (van den Berg *et al.*, 2003; Eis *et al.*, 2005; Kluiver *et al.*, 2005). In contrast, very low levels of *BIC* and miR-155 are present in Burkitt lymphoma (Kluiver *et al.*, 2006). Little is known about the expression of *BIC* and miR-155 in the normal lymphoid or hematopoietic system. No miR-155 was detected in sorted B cells, T cells, monocytes and granulocytes from peripheral blood samples (Ramkissoon *et al.*, 2006). Upregulated *BIC* expression was reported in T cells after incubation with

antibodies against CD3 and CD28 (Haasch *et al*., 2002). By RNA *in situ* hybridization (ISH), we have previously shown only a limited number of *BIC*-positive cells in tonsil and lymph node (van den Berg *et al*., 2003). *BIC*-positive cells were predominantly located within the germinal center and at least part of those cells were also positive for the B cell-specific marker CD20, confirming expression of *BIC* in germinal center B cells. Northern blotting confirmed expression of miR-155 in normal tonsil and lymph node (Kluiver *et al*., 2005).

The recently established importance of miRNA in cellular and tissue homeostasis implicates that pri-miRNA transcription and processing needs to be tightly regulated. The germinal center reaction is characterized by an antigen-driven activation of B cells in which B cell-receptor (BcR)-mediated signaling is instrumental to the transformation of naïve B cells to mature antibody producing B cells. We therefore hypothesized that the expression of miR-155 is controlled by BcR signaling. Indeed, BcR triggering of Burkitt lymphoma cell line Ramos resulted in upregulation of the pri-miRNA *BIC* (van den Berg *et al*., 2003). We have now investigated the molecular and cell biological nature of the BcR triggering-induced expression of *BIC* and demonstrate a critical involvement of protein kinase C (PKC) and nuclear factor-κB (NF-κB) in this process. Interestingly, the upregulation of *BIC* transcripts in Burkitt lymphoma cell lines does not result in increased miR-155 expression. This is in contrast to HEK293 cells and normal B cells, where upregulation of *BIC* does result in induction of miR-155. These results provide evidence that mature miR-155 expression is regulated at the *BIC*/pri-miR-155 transcriptional, as well as at the processing level.

## Results

### *Pri-miR-155/*BIC *expression is regulated by the BcR via PKC and NF-κB*

We have previously reported upregulation of *BIC* transcription upon BcR triggering, which was first detectable after 1.5 h and became full blown after 24 h of stimulation with anti-immunoglobin M (IgM) (van den Berg *et al*., 2003) (Figure 1). To elucidate the molecular pathway regulating *BIC* transcription, we studied downstream BcR signaling components. BcR signaling is known to involve activation of PKC and the transcription factor NF-κB. First, we studied the involvement of PKC in the regulation of *BIC* transcription by direct stimulation of PKC with phorbol 12-myristate 13-acetate (PMA) in combination with the $Ca^{2+}$ ionophore, ionomycin. Indeed, direct stimulation of PKC in Ramos cells using PMA/ionomycin resulted in a significant upregulation of *BIC* (Figure 1a). The specific involvement of PKC was further confirmed by treatment of Ramos cells with anti-IgM in the presence of the specific PKC inhibitor bisindolylmaleimide-I. After 24 h, induction of *BIC* expression upon BcR triggering was completely blocked by bisindolylmaleimide-I
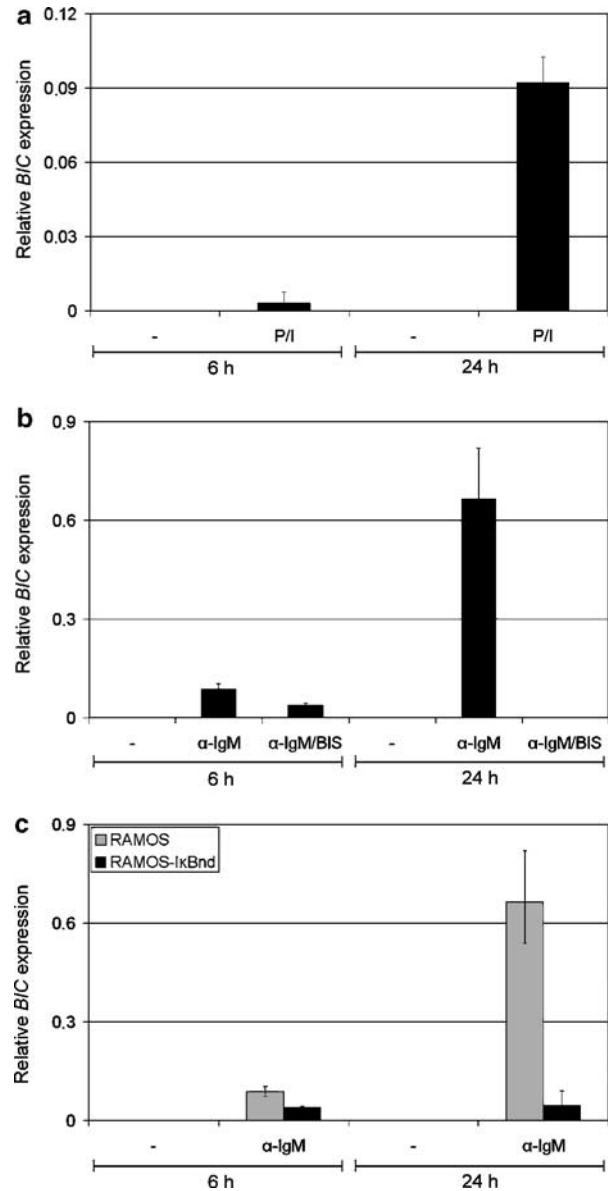


**Figure 1** The expression of *BIC* is regulated by the BCR via PKC and NF-κB. (**a**) Direct activation of PKC in Ramos cells with PMA/ionomycin ( + P/I) induces *BIC* expression. (**b**) The induction of *BIC* expression upon anti-IgM-induced BcR stimulation can be blocked using the PKC inhibitor bisindolylmaleimide-I (BIS). (**c**) The Ramos–IκBα$_{nd}$ cell line, overexpressing a non-degradable form of the natural NF-κB inhibitor IκB, cannot induce *BIC* expression at similar high levels as the parental Ramos cell line revealing involvement of NF-κB in the regulation of *BIC* expression.

(Figure 1b). No effect on anti-IgM-induced *BIC* expression was observed in dimethylsulfoxide (DMSO)-vehicle control cells, confirming specificity of the observed inhibition of *BIC* induction by bisindolylmaleimide-I (not shown).

The promoter region of *BIC* has been reported to contain a putative NF-κB binding site (van den Berg *et al*., 2003). Activation and nuclear translocation of the transcription factor NF-κB is known to occur
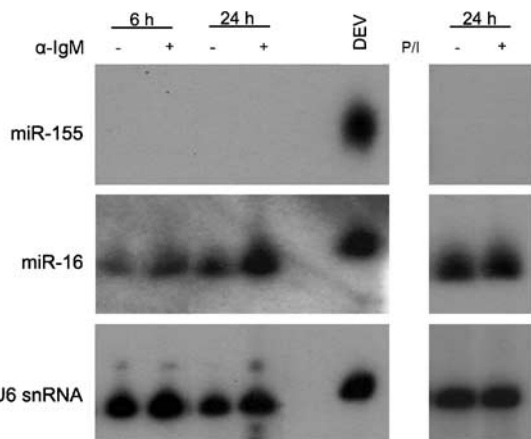
downstream from BcR triggering and activated PKC. Next, we investigated the involvement of NF-κB in BcR-induced *BIC* expression. To this end, we used a Ramos cell line overexpressing a non-degradable form of IκBα, the endogenous cellular inhibitor of NF-κB (Ramos-IκBα$_{nd}$). As reported previously (van den Berg *et al*., 2003), incomplete inhibition of *BIC* expression by IκBα overexpression was observed early, that is, 6 h after treatment with anti-IgM. However, as shown in Figure 1c, prolonged stimulation with anti-IgM, that is, upto 24 h, resulted in a further significant increase of the *BIC* levels in normal Ramos cells, whereas in Ramos-IκBα$_{nd}$ cells *BIC* expression remained at the level observed after treatment for 6 h. These results show that the PKC–NF-κB signaling axis is critically involved in the regulation of *BIC* transcription in Ramos cells.

*Expression of pri-miR-155/BIC does not result in miR-155 expression in Ramos*
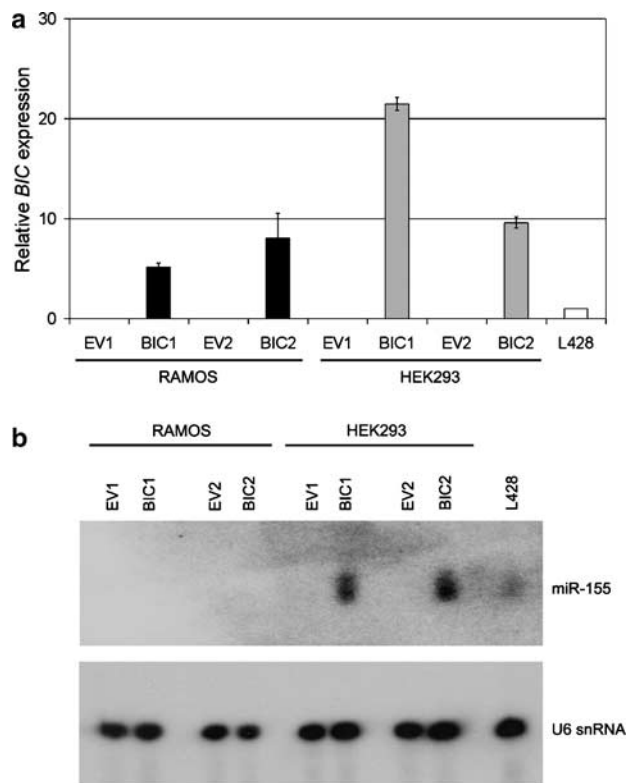
Next, we studied the processing of *BIC* into miR-155 upon treatment with anti-IgM or PMA/ionomycin using Northern blot analysis. Surprisingly, neither anti-IgM nor PMA/ionomycin induced upregulation of miR-155 expression (Figure 2). Also pre-miR-155 could not be detected, which may not be surprising given the fact that even in HL, which express significantly more *BIC*, pre-miR-155 is hardly detectable (data not shown). As a control, we analysed the expression of the more ubiquitously expressed miR-16. In contrast to miR-155, miR-16 was expressed in all treated and untreated samples. A slightly stronger miR-16 signal was noted after 24 h of treatment with anti-IgM.

To exclude the possibility that *BIC* expression levels in Ramos were too low to detect appreciable amounts of

miR-155, we generated two stable transfectants of Ramos using a construct containing the full-length *BIC* cDNA and empty vector (EV) controls. As an additional control, we also generated two stable *BIC* and EV transfectants of HEK293. Both parental HEK293 cells and Ramos cells did not express *BIC*. The stably transfected derivatives of HEK293 and Ramos showed significant expression of *BIC* RNA, which was at least 10-fold higher than the levels induced with anti-IgM treatment of Ramos (Figure 3a). Northern blot analysis revealed significant expression of miR-155 in HEK293 cells, whereas Ramos cells again failed to express miR-155 (Figure 3b) and pre-miR-155 (not shown), even at this high level of *BIC* expression. As a positive control for miR-155 expression, the classical HL cell line L428 is shown. Although *BIC* RNA levels in L428 are 5- to 7-fold lower than those in stably transfected Ramos cells, miR-155 could be detected by Northern blot analysis excluding the possibility that miR-155 detection in the Ramos sublines was missed owing to insufficient sensitivity (Figure 3a and b).





**Figure 3** Stably transfected Ramos and HEK293 cells differ in miR-155 processing capacity. (**a**) *BIC* qRT–PCR results for Ramos and HEK293 cell lines stably transfected *in duplo* with a construct containing the full-length *BIC* cDNA (*BIC*1/2), reveals high *BIC* expression in both Ramos and HEK293 cells. The empty vector controls (EV1/2) were negative for *BIC* expression. (**b**) Northern blot analysis for miR-155 in Ramos and HEK293 stable transfectants revealed lack of expression in Ramos cells whereas HEK293 cells are able to process *BIC* to miR-155. No signals were observed at the position of the pre-miR-155 molecule indicating that lack of miR-155 is not caused by accumulation of its precursor (data not shown). U6 snRNA is shown as a control for RNA quality and blotting procedure. HL cell line L428 is shown as a control of *BIC*- and miR-155-positive cell line.



**Figure 2** No miR-155 expression upon induction of *BIC* expression in Ramos. Northern blot analysis of Ramos cells treated with anti-IgM or PMA/ionomycin (P/I) shows that, despite the induction of *BIC*, no miR-155 is expressed. No signals were observed at the position of the pre-miR-155 molecule indicating that lack of miR-155 is not caused by accumulation of its precursor (data not shown). The HL cell line DEV is shown as a positive control for miR-155 expression. MiR-16 is shown as a control for miRNA expression in Ramos, note the slightly higher expression of miR-16 after 24 h of anti-IgM treatment. U6 snRNA is shown as a control for RNA quality and blotting procedure.
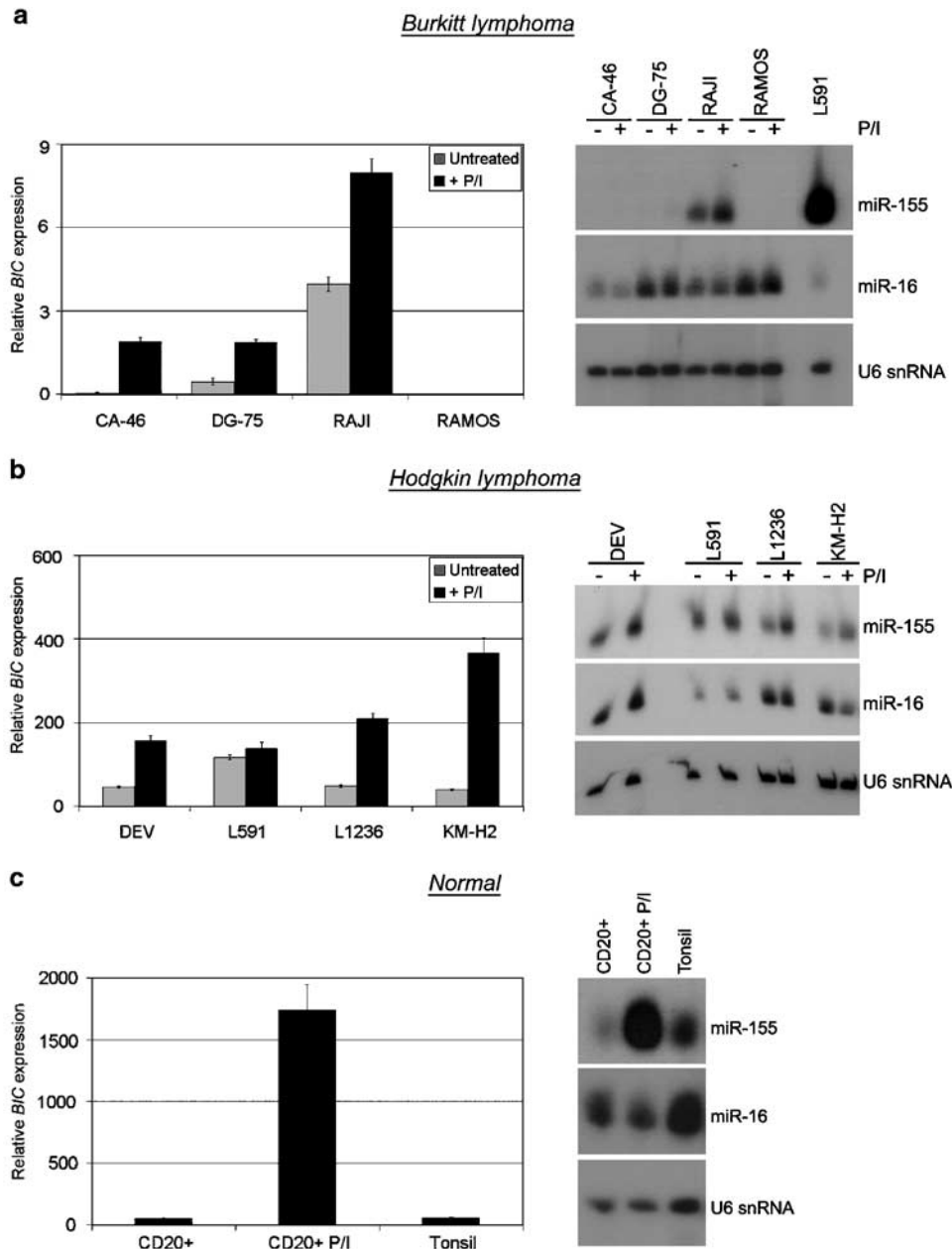
*MiR-155 processing in BL, HL and normal B cells*
To determine whether the defect in pri-miR-155/*BIC* processing is a general feature of Burkitt lymphoma, we additionally analysed Burkitt lymphoma cell lines CA-46 and DG-75 which, like Ramos, display no or relatively low levels of both *BIC* and miR-155. *BIC* expression was induced via direct PKC activation, using PMA/ionomycin. In this experimental setup, *BIC* and miR-155 expression levels were compared with the Epstein–Barr virus (EBV) latency type III-positive



**Figure 4** Hampered miR-155 processing in Burkitt lymphoma and successful miR-155 processing in HL and normal B cells. (**a**) Left panel: 24 h stimulation of Burkitt lymphoma (BL) cell lines with P/I results in upregulation of *BIC*. Right panel: Northern blot analysis of the same samples for miR-155 reveals that similar to Ramos, CA-46 and DG-75 also fail to process *BIC* to miR-155. EBV latency type III-positive Burkitt lymphoma cell line Raji, known to express *BIC* and miR-155, shows a slight upregulation of miR-155 upon P/I treatment in line with the twofold upregulation of *BIC* mRNA levels. MiR-16 is expressed in BL and levels are not affected by P/I treatment. HL cell line L591 is shown as a positive control for *BIC* and miR-155 expression. (**b**) Left panel: Hodgkin's lymphoma cell lines DEV, L1236 and KM-H2 that already express *BIC* show elevated *BIC* mRNA levels after 24 h P/I stimulation. Note that L591, that was almost unresponsive to P/I, was already expressing the highest levels of *BIC*. Right panel: HL cell lines DEV, L1236 and KM-H2 show increased miR-155 signals upon P/I treatment in line with upregulated *BIC* levels. In contrast, L591 showed an unchanged miR-155 signal in line with qRT–PCR for *BIC*. MiR-16 is shown for comparison; only KM-H2 shows a slightly lower signal after treatment. (**c**) Left panel: total tonsil cell suspension and isolated CD20 + B cells express *BIC* at moderate levels, stimulation of CD20 + cells with P/I results in massive upregulation of *BIC*. Right panel: Stimulated CD20 + cells show high levels of miR-155 in line with the massive upregulation of *BIC*. In contrast, unstimulated CD20 + cells and tonsil show much lower levels of miR-155. MiR-16 is elevated in tonsil compared with CD20 + cells. U6 snRNA is shown as a control for RNA quality and blotting procedure.

Burkitt lymphoma cell line Raji known to express both *BIC* and miR-155 (Kluiver *et al.*, 2006). Both CA-46 and DG-75 showed a several fold upregulation of *BIC* (Figure 4a, left panel). However, similar to Ramos, neither Ca-46 nor DG-75 contained detectable levels of miR-155 (Figure 4a, right panel). In contrast, *BIC* and miR-155 were clearly detectable in both the treated and untreated Raji cells. In line with the detected increase in *BIC* levels, a slight increase in miR-155 levels was detected in Raji cells upon PMA/ionomycin treatment. MiR-16 is expressed in all Burkitt lymphoma cell lines, and treatment with PMA/ionomycin did not appear to have an effect on miR-16 levels.

Next, we determined whether PMA/ionomycin stimulation does results in induction of *BIC* expression and elevated miR-155 levels in Hodgkin's lymphoma cell lines. PKC was directly activated using PMA/ionomycin, resulting in a several fold induction of *BIC* expression in 3/4 Hodgkin's lymphoma cell lines. Levels got up from ~3-fold in DEV to more than 9-fold in KM-H2. L591, having the highest intrinsic levels of *BIC*, did not show much increase in *BIC* expression in response to treatment with PMA/ionomycin (Figure 4b, left panel). Northern blot analysis revealed some induction of miR-155 expression after PMA/ionomycin treatment, especially in the cell lines showing a very strong induction of *BIC* expression, like KM-H2 and L1236. In line with the unresponsiveness to PMA/ionomycin with regard to *BIC* expression, L591 did not show a clear visually significant induction of miR-155 expression in response to treatment with PMA/ionomycin. Analysis of miR-16 showed expression in all HL cell lines without clear evidence that miR-16 expression was affected by PMA/ionomycin stimulation (Figure 4b, right panel).

To further study whether the apparently hampered *BIC* processing is specific for Burkitt lymphoma, we determined *BIC* and miR-155 expression levels in normal lymphoid tonsil tissue and sorted CD20 + tonsillar B cells. A moderate expression level of *BIC* was shown in both total tonsil and CD20 + B cells. Treatment with PMA/ionomycin strongly increased *BIC* expression in the CD20 + B cells (Figure 4c, left panel). In contrast to Burkitt lymphoma cell lines, upregulation of *BIC* in normal B cells did result in the induction of very high miR-155 levels, providing further evidence for a BL-specific block of miR-155 processing (Figure 4c, right panel).

*BIC processing is not hampered owing to ADAR editing*
More recent reports have indicated that miRNA processing may be regulated by editing of pri-miRNA sequences by adenosine deaminases acting on RNA (ADARs) (Luciano *et al.*, 2004; Yang *et al.*, 2006). ADARs can convert adenosines to inosines (A→G) in stem–loop sequences that may interfere with proper recognition and cleavage of pri-miRNA by the microprocessor complex. To evaluate whether ADAR editing might explain the observed differences in *BIC* to miR-155 processing, we analysed the miR-155 sequence

including 100 nt up- and downstream of *BIC* reverse transcriptase–polymerase chain reaction (RT–PCR) products from stably transfected Ramos cells and PMA/ionomycin-stimulated CA-46 cells and compared these with the miR-155 sequences obtained from stably transfected HEK293 cells and L1236 cells. Analysis of 30 clones per cell line revealed an A→G conversion in one clone, 56 nt downstream the miR-155 sequence in stably transfected Ramos cells, an A→G conversion in one clone 48 nt upstream the miR-155 sequence in stimulated CA-46 cells and an A→G conversion in two independent clones 82 nt upstream the miR-155 sequence in sequences from L1236 were detected. Thus, ADAR editing is not responsible for the lack of *BIC* processing to miR-155 in Burkitt lymphoma.

## Discussion

The Burkitt lymphoma derived cell line Ramos is widely used to study BcR signaling and has been invaluable to the current understanding of this signaling pathway. Burkitt lymphoma typically expresses very low levels of *BIC* (Kluiver *et al.*, 2006), although expression of *BIC* in pediatric Burkitt lymphoma is increased compared with *BIC* levels in pediatric leukemia (Metzler *et al.*, 2004). The expression of *BIC* RNA, however, can be significantly induced by activation of Burkitt lymphoma Ramos cells via BcR stimulation (van den Berg *et al.*, 2003). In this study, we show that the BcR-induced expression of pri-miR-155/*BIC* in Ramos critically involves PKC and the downstream transcription factor NF-κB. Blocking either PKC signaling or NF-κB activation abrogates induction of *BIC* expression.

As it is now known that *BIC* is processed to miR-155 (Eis *et al.*, 2005), we investigated whether induction of *BIC* expression resulted in induction of miR-155 expression. The results obtained with normal blood B cells and the Hodgkin's lymphoma cell lines show that, in general, increased levels of *BIC* are associated with increased levels of miR-155.

Surprisingly, Ramos cells did not express pre-miR-155 upon induction of *BIC* by BcR triggering or after direct PKC activation. Differential *BIC*/miR-155 expression suggests additional control in the level of miR-155 expression as reported earlier (Suh *et al.*, 2004; Eis *et al.*, 2005). In line with the results obtained in Ramos, Burkitt lymphoma cell lines CA-46 and DG-75 also showed no (CA-46) or very low (DG-75) basal expression of *BIC*, and lacked miR-155 expression after induction of *BIC* expression. This suggests presence of a common blockade in Burkitt lymphoma cell lines to process activation-induced *BIC* into mature miR-155. Further evidence for post-transcriptional control of miR-155 expression in Burkitt lymphoma was provided in stable transfectants of Ramos and HEK293 cells containing a full-length *BIC* cDNA construct, which showed similar *BIC* levels and absence of pre-miR-155 in both cell types, but presence of miR-155 in HEK293 cells and absence in Ramos.

Interestingly, in contrast to the above listed EBV-negative classical Burkitt lymphoma cell lines, the EBV latency type III-positive Burkitt lymphoma cell line Raji did show both increased basal levels of *BIC* and miR-155. In addition, Raji cells were found to upregulate miR-155 expression upon PKC-mediated induction of *BIC* RNA expression. This may be explained by Burkitt lymphoma's unusual EBV latency type III expression pattern, which has been shown to be associated with enhanced *BIC* and miR-155 expression (Jiang *et al.*, 2006; Kluiver *et al.*, 2006).

Together, this strongly indicates that Burkitt lymphoma cells are characterized by a block in the processing of *BIC* to miR-155, most likely already at an early step suggesting a second level of regulation for miR-155 expression. In addition, these results indicate that Burkitt lymphoma cell lines should be used with caution for the study of miR expression upon BcR triggering as a model for BcR signaling in general. Our results may be related to specific characteristics of the BcR triggering pathway in the germinal center reaction or to a specific pathophysiological characteristic of BL.

The molecular mechanism, responsible for the blockade in *BIC* to miR-155 processing in BL, remains to be elucidated and can only be speculated about. General factors necessary for miR processing, like components of the microprocessor complex or exportin-5 (Gregory *et al.*, 2004; Lund *et al.*, 2004) could be downregulated or absent. However, this seems unlikely because an unrelated miR (miR-16) is detected in Burkitt lymphoma cells. Differences in processing efficiency of miR-155 have been noted by others (Eis *et al.*, 2005), and were proposed to be due to nuclear export of *BIC* transcripts. This would result in the cytoplasmic degradation of *BIC* rather than proper processing to pre-miR-155 by the microprocessor complex. In HL and PTLD, RNA-ISH for *BIC* clearly shows a consistent nuclear staining in *BIC*-positive cells (van den Berg *et al.*, 2003; Kluiver *et al.*, 2005, 2006). Extrapolating these results to Burkitt lymphoma would argue against the degradation of *BIC* owing to nuclear export of *BIC* transcripts. Moreover, we observed a strong induction of *BIC* transcripts, which is not associated with accumulation of pre-miR-155, indicating that the first steps in pri-miRNA processing are blocked in Ramos cells. Evidence for regulation of pri-miRNA processing is also observed in other studies. Expression of pri-let-7a-1 in embryonic stem cells is not always accompanied by expression of mature let-7a-1 (Suh *et al.*, 2004). Other studies show that miRNA that are transcribed together from one cluster are not always expressed at similar levels. For instance, miR-221 and miR-222 form a cluster at Xp11, but only miR-221 is highly expressed in glioblastoma samples (Ciafre *et al.*, 2005). Comparison of miRNA profiles of CLL cases with normal CD5+ B cells revealed upregulation of two members of the miR-17-92 cluster at 13q31 and downregulation of two others (Calin *et al.*, 2004). Recently, Obernosterer *et al.* (2006), provided evidence for dicer-regulated expression of miR-138 in mouse embryos.

Several groups have reported that sequences outside the pre-miR hairpin sequence are important for miRNA processing (Lee *et al.*, 2003; Chen *et al.*, 2004; Zeng and Cullen, 2005). It might be that *BIC* transcripts in Burkitt lymphoma differ from those present in normal B cells or Hodgkin's lymphoma, for instance owing to mutations at the genomic DNA level in or closely flanking the stem–loop sequence or owing to alternative splicing, and therefore may not be processed. However, our experiments using Ramos and HEK293 stable transfectants expressing the full-length wild-type *BIC* cDNA sequence argues against this explanation.

A role for ADARs was described in modulation of miR processing and expression (Luciano *et al.*, 2004; Yang *et al.*, 2006). Pri-miR-142-specific adenosines within the stemloop were almost always edited by ADARs and *in vitro* assays showed that expression of mutated pri-miR-142 transcripts resulted in suppression of its processing to mature miR-142 (Yang *et al.*, 2006). Thus, specific editing of *BIC* in Burkitt lymphoma – and not in HEK293, HL and normal B cells – could result in the inability of the microprocessor complex to process *BIC* in BL. However, cloning of *BIC* transcripts and subsequent sequencing revealed no or very few A→G conversions both in cells that can (HEK293-*BIC* and L1236) and in cells that cannot (Ramos-*BIC* and PMA-Ionomycin activated CA-46) process *BIC* to miR-155.

In conclusion, we show that expression of *BIC* is regulated by the BcR signaling pathway in a PKC and NF-$\kappa$B-dependent manner. Interestingly, classical Burkitt lymphoma cell lines, in contrast to normal B cells, Hodgkin's lymphoma cell lines and HEK293 cells, are not able to process *BIC* to mature miR-155. This indicates two levels of regulation of mature miR-155 expression, one at the transcriptional and one at the processing level.

## Materials and methods

*Cell lines and tissues*
The Hodgkin's lymphoma cell lines L591, L428, KM-H2, L1236 and DEV, and the Burkitt lymphoma cell lines Ramos, Ramos–I$\kappa$B$\alpha_{nd}$ (van den Berg *et al.*, 2003), DG-75, CA-46 and Raji were cultured in RPMI-1640 medium (Cambrex Biosciences, Walkersville, MD, USA). HEK293 cells were cultured in Dulbecco's modified Eagle's medium (Cambrex Biosciences). Culture media were supplemented with ultraglutamine 1 (Cambrex Biosciences), 100 U/ml penicillin/streptomycin and 10% fetal calf serum (FCS) (Cambrex Biosciences) at 37°C in an atmosphere containing 5% $CO_2$. L428 was supplemented with 5% FCS and DEV was supplemented with 20% FCS. Cell suspensions were prepared from a tonsil taken from a patient during routine tonsillectomy. CD20+ B cells were isolated using CD20-PE (clone B-Ly1, DAKO, Copenhagen, Denmark) by fluorescence-activated cell sorting (MoFlo Cytomation, Fort Collins, CO, USA). All protocols for obtaining and studying human tissues and cells were approved by the institution's review board for human subject research.

*Constructs and generation of stable transfectants*
A vector containing the full-length *BIC* cDNA was purchased (RZPD, Berlin, Germany) and subcloned into the

pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA, USA). The insert was sequenced to confirm the correct *BIC* sequence. Ramos cells ($5 \times 10^6$) were transfected with $25 \mu g$ plasmid DNA using an electroporator (Genepulser, Biorad, Hercules CA, USA). For HEK293, $3 \times 10^6$ cells were transfected with $5 \mu g$ plasmid DNA using the AMAXA system (Amaxa Biosystems, Cologne, Germany) with buffer V and nucleofactor program A23. As a control, both cell lines were also transfected with the empty pcDNA3.1 vector. Two days after transfection, selection was started using geneticin (Invitrogen, Carlsbad, CA, USA) with final concentrations of 0.5 mg/ml for HEK293 and 1 mg/ml for Ramos cells.

### Stimulation/inhibition experiments

For all experiments cells were cultured 1 day before the start of the stimulation/inhibition experiment. The next day, cells were spread to $0.5 \times 10^6$ cells/ml in RPMI 5% FCS and incubated with $5 \mu g$/ml anti-IgM (Jackson Immuno Research, West Grove, PA, USA) or 1 ng/ml PMA and $0.5 \mu M$ ionomycin (both Sigma Aldrich, St Louis, MO, USA) for indicated time periods. The PKC inhibitor bisindolylmaleimide-I (Calbiochem, La Jolla, CA, USA) was dissolved in DMSO and used at a concentration of $1 \mu M$. All experiments were performed at least in triplicates. After treatment, cells were harvested and directly stored at $-20°C$ before RNA isolation.

### RNA isolation and Northern blotting

Total RNA was isolated using Trizol (Invitrogen) according to the manufacturer's protocol. RNA integrity was monitored using a 1% agarose gel. For Northern blotting $20 \mu g$ of good-quality total RNA was loaded on a 7.5 M ureum 12% PAA denaturing gel, and after electrophoresis transferred to Hybond N+ nylon membrane (Amersham, Freiburg, Germany). Membranes were cross-linked using ultraviolet light for 30 s at 1200 mJ/cm². Hybridizations with antisense starfire probes (IDT, Coralville, IA, USA) for miR-155 (5′-CCCCTATCACGATTAGCATTAA-3′) or miR-16 (5′-CGCCAATATTTACGTGCTGCTA-3′) were performed at 42°C, which is the optimal temperature for detection of miR-155, and at 55°C, which is the optimal detection for the pre-miR155, according to the instructions of the manufacturer. In general, no pre-miR155 signals were observed even at the most optimal hybridization temperature. Only in the Hodgkin's lymphoma cell lines expressing very high levels of miR-155, weak signals could be observed at the position of the pre-miR-155 after prolonged exposure of the membranes to Kodak XAR-5 (Sigma Chemical, St Louis, MO, USA) films

(data not shown). Figure 5 shows a schematic representation of *BIC*, the location of miR-155 and the probes used for analysis of *BIC* and miR-155 by ISH, Northern blotting and quantitative RT–PCR. After washing, membranes were exposed to Kodak XAR-5 films (Sigma Chemical, St Louis, MO, USA). Relative amounts of micro-RNA detected by Northern blotting, as present in the cell lines, were visually compared on the same blot only. As a control for RNA quality and blotting procedure, all membranes were hybridized with a U6 snRNA probe 5′-GCAGGGGCCATGCTAATCTTCTCT GTATCG-3′ (Pfeffer *et al.*, 2004).

### Quantitative RT–PCR

All RNA samples were DNase treated and checked for possible DNA contamination with primer sets that specifically amplify genomic DNA. The first-strand cDNA synthesis, primed with random primers, was performed using the protocol provided by the manufacturer (Life Technologies Inc., Gaithersburg, MD, USA). PCR was performed in qPCR core kit (Eurogentec, Seraing, Belgium) containing 200 nmol/l probe, and 900 nmol/l primers and 2 ng cDNA using the standard amplification procedure. All analyses were performed in triplicate. Hypoxanthine phosphoribosyltransferase 1 (HPRT) was used for normalization. Primers and probes used for *BIC* and HPRT were as reported previously (Specht *et al.*, 2001; van den Berg *et al.*, 2003). The relative amount of *BIC* was calculated by subtracting the average $C_T$ value for HPRT from the average $C_T$ value of *BIC* ($\Delta C_T$). Next, $\Delta\Delta C_T$ values were calculated by subtracting the $\Delta C_T$ of the common calibrator (HL cell line L428) from the $\Delta C_T$ of the sample. Finally, expression was defined as $2^{-\Delta\Delta C_T}$. The range of expression levels was determined by calculating the standard deviation (s.d.) of $\Delta C_T$, using the formula s.d. $(\Delta C_T) = \sqrt{[(s.d. - C_T BIC)^2 + (s.d. - C_T HPRT)^2]}$, followed by $2^{-[(\Delta\Delta C_T \pm s.d.(\Delta C_T)]}$.

### Determination of BIC editing by ADARs

To identify adenosine residues that underwent editing by ADARs, *BIC* RNA transcripts were sequenced. To this end, *BIC* RT–PCR products of Ramos cells stably transfected with the full-length *BIC* construct and of PMA/ionomycin-stimulated CA-46 cells were compared with *BIC* RT–PCR products of HEK293 cells stably transfected with the *BIC* construct and of L1236 cells. Primers used for RT–PCR were F: 5′-AACC TACCAGAGACCTTACC-3′ and R: 5′-ATGCTTCTTTGTC ATCCTCC-3′. The resulting 296 bp PCR products were cloned using the TOPO TA cloning system (Invitrogen). Per sample, 30 clones were sequenced using both forward and
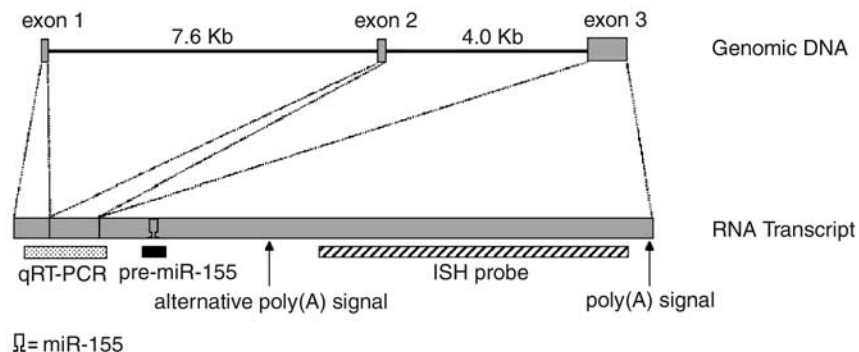


**Figure 5** Schematic representation of the *BIC* gene. Exons, genomic organization, primary RNA transcript and location of the pre-miR-155 sequence and the quantitative RT–PCR primer set and the ISH probe are denoted.

reverse M13 universal primers. The resulting sequences were analysed for A to G conversions in miR-155 and 100 nt up- and downstream regions, using Seqman software version 6.1 (DNASTAR, Madison, WI, USA).

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