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*J Immunol* 2004; 172:1561-1566; doi: 10.4049/jimmunol.172.3.1561

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Phosphatidylinositol 3-Kinase Is a Determinant of Responsiveness to B Cell Antigen Receptor-Mediated Epstein-Barr Virus Activation

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B cell Ag receptor (BCR) cross-linking with anti-Ig Abs efficiently induces activation of latently infected EBV in some B cell lines, but not in others. The present study was aimed at defining the molecular mechanisms that determine the response to BCR-mediated EBV activation. Comparison of Burkitt’s lymphoma-derived Akata, Mutu-I, and Daudi cells, which are representative responders and nonresponders to BCR-mediated EBV activation, respectively, indicated that three signaling pathways, phosphatidylinositol 3-kinase (PI3K), extracellular signal-regulated kinase (ERK), and p38 mitogen-activated protein kinase (MAPK), were activated in anti-Ig-treated Akata and Mutu-I cells. However, in anti-Ig-treated Daudi cells PI3K was not activated, ERK was faintly activated, and p38 MAPK was constitutively phosphorylated irrespective of anti-Ig treatment. Restoration of PI3K activity with insulin-like growth factor 1 restored ERK and p38 MAPK pathways, and was accompanied by EBV activation in anti-Ig-treated Daudi cells. In contrast, a specific inhibitor for PI3K, wortmannin, inhibited EBV activation by anti-Ig Abs in Akata and Mutu-I cells. Transfection assays in EBV-negative Daudi cells revealed that PI3K activated a promoter for BZLF1, which is a switch of EBV activation from a latent infection, in the absence of other EBV products suggesting that the BZLF promoter was a target of BCR signaling, and that PI3K was important for BCR-mediated BZLF1 activation. These results indicate that the absence of PI3K impedes the progression of signals through the BCR and becomes a determinant of unresponsiveness to BCR-mediated EBV activation. The Journal of Immunology, 2004, 172: 1561–1566.

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Materials and Methods

Cell lines and cell culture

All cells were maintained in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FBS (Invitrogen, Groningen, The Netherlands), penicillin (40 U/ml), and streptomycin (50 μg/ml). Akata (11), Mutu-I (17), Daudi (18), Sav-I, Kem-I, and Oku-I are EBV-positive BL cell lines. EBV-negative Daudi cell clones were isolated from parental Daudi cells by the limiting dilution method (19).

Reagents

F(ab')2 fragments of rabbit anti-human IgM and IgG polyclonal Abs (DAKO, Copenhagen, Denmark) were used for BCR cross-linking. Abs used for immunoblot analysis of BCR signaling were polyclonal rabbit Abs against phospho-extracellular signal-regulated kinase (ERK) MAPK, phospho-p38 MAPK (BioSource International, Camarillo, CA), ERK, Akt, and phospho-Akt (Cell Signaling Technology, Beverly, MA), and mouse mAbs against phospho-extracellular signal-regulated kinase (ERK) MAPK, phospho-Akt (Cell Signaling Technology), and mouse mAbs against β-actin (Sigma-Aldrich) and EBV BZLF1 protein (Dako) were used for analysis of EBV activation. A specific inhibitor of PI3K, wortmannin (Sigma-Aldrich), and recombinant human insulin-like growth factor 1 (IGF-1) (R&D Systems, Minneapolis, MN) were used for inhibition and activation of PI3K, respectively. FITC-conjugated anti-human CD19 mouse mAb (Beckman Coulther, Brea, CA) was used for flow cytometry.

Immunoblot analysis

Cells (1.5 × 106 cells per sample) were treated with anti-IgG or IgM Abs (7.5 μg/ml) for 24 h. Then they were lysed in 200 μl lysis buffer (50 mM Tris-HCL (pH 7.5), 150 mM NaCl, 50 mM NaF, 5 mM EDTA, 1% (v/v) Triton X-100, 10% glycerol, 1 mM NaVO4, 1 mM PMSF, 2 μg/ml peptatin, 2 μg/ml aprotinin, and 2 μg/ml leupeptin). Lysates were subsequently denatured, resolved by 8%–10% SDS-PAGE, and electrotransferred to nitrocellulose membranes. Membranes were reacted with the primary Abs; anti-phospho ERK and anti-phospho-p38 (0.5 μg/ml), anti-ERK (1:2,000), anti-phospho MAPK (1:2,500), anti-Akt and anti-phospho-Akt (1:1,000), and anti-BZLF1 (1:40), followed by treatment with a HRP-conjugated anti-rabbit or anti-mouse IgG Ab (1:1,000, Amersham Pharmacia Biotech, Piscataway, NJ). Membranes were visualized with an ECL kit (Amersham Pharmacia Biotech). Membranes were reacted with the primary Abs; anti-phospho ERK and anti-phospho-p38 (0.5 μg/ml), anti-ERK (1:2,000), anti-phospho MAPK (1:2,500), anti-Akt and anti-phospho-Akt (1:1,000), and anti-BZLF1 (1:40), followed by treatment with a HRP-conjugated anti-rabbit or anti-mouse IgG Ab (1:2,000–1:10,000) (Amersham Pharmacia Biotech). Membranes were visualized with an ECL kit (Amersham Pharmacia Biotech).

Immunofluorescence assay

Cells were smeared on a glass slide and fixed in acetone for 10 min. Fixed cells were incubated with an anti-BHRF1 mouse mAb (C844–1:20) at 37°C for 1 h. After washing with PBS, cells were stained with an FITC-conjugated anti-mouse IgG Ab (1:200) (DAKO) for 30 min followed by washing with PBS.

RT-PCR analysis

RT-PCR was conducted as previously described (19). Primer pairs used for detection of B cell adaptor for phosphorylidyinositol 3-kinase (BCAP) were 5'-CAACATGCTCAATCCCGATC-3' and 5'-CTGCCACCTTTGTTGAGATGC-3'.

Reporter plasmid construction

The promoter region of the BZLF1 gene from −222 to +8 was amplified by PCR using the primers; 5'-CAACATGCTCAATCCCGATC-3' and 5'-GGAGAGTCTGGTGCAATGTTAGTG-3'. The PCR product was digested with MluI and BglII and then subcloned upstream of the luciferase gene of PGV-basic vector 2 (PGV-B2; Wako, Osaka, Japan).

Transfections and promoter analysis

Transfections were performed using lipofectamine reagent (Invitrogen) according to the manufacturer’s protocol. Transfected cells were cultured in 6-well plates for 24 h and further incubated for 24 h with addition of anti-Ig Abs and/or other reagents. Luciferase activities were determined using the dual-luciferase reporter assay system (Promega). To normalize luciferase activities, activities of the PGV reporter plasmid were divided by those of the pRL vector driven by the thymidine kinase gene promoter of HSV, which was cotransfected as an internal control. Reproducibility of results was confirmed by three independent transfections and each transfection was done in duplicate. Values were expressed as the means ± SE of three experiments.

Results

**BCR-mediated EBV activation in BL cell lines**

We studied six type I BL cell lines, Akata, Daudi, Mutu-I, Sav-I, Kem-I, and Oku-I. First, we examined the expression of membrane Ig on these cells by flow cytometry. The results indicated that the Akata cell line was positive for IgG, and other cell lines were positive for IgM. The level of Ig expression was not different among cell lines (data not shown). Then cells were incubated in a medium containing 7.5 μg/ml of anti-IgG (for Akata cells) or IgM (for Daudi, Mutu-I, Sav-I, Kem-I, and Oku-I cells) for 24 h, and EBV activation was assessed by expression of an EBV early protein, BHRF1, by the indirect immunofluorescence assay. As shown in Table I and Fig. 1, anti-Ig treatment induced BHRF1 in 66% of Akata cells, in ~4% of Mutu-I and Sav-I cells, and in none of the Daudi, Kem-I, and Oku-I cells. Spontaneous BHRF1 expression without anti-Ig treatment was < 0.1% in all cell lines. Thus, we selected Akata, Mutu-I, and Daudi cells to study biochemical differences that determine the response to BCR-mediated EBV activation.

**BCR-mediated activation of tyrosine kinases in Akata and Daudi cells**

We examined activation of tyrosine kinases in anti-Ig-treated Akata and Daudi cells. Immunoblot analysis with phosphorylated tyrosine-specific Abs demonstrated the rapid appearance of phosphorylated tyrosines, peaking 5 min after addition of anti-Ig, to a similar degree in both cell lines (Fig. 2). A similar activation of tyrosine kinases was also observed in anti-Ig-treated Mutu-I cells (data not shown).

**BCR-mediated activation of PI3K, ERK, and p38 MAPK pathways in Akata, Mutu-I, and Daudi cells**

The three main signal transduction pathways that have been established from the BCR in response to cross-linking with anti-Ig Abs are illustrated in Fig. 3A (8, 9). We studied whether these three pathways were activated after BCR cross-linking. As shown in Fig. 3B, immunoblot analysis with phosphorylated protein-specific Abs for Akt, ERK, and p38 MAPK, which are all known targets of BCR signal transduction, demonstrated the appearance of phosphorylated forms in anti-Ig-treated Akata and Mutu-I cells. In contrast, in anti-Ig-treated Daudi cells, phospho-Akt was not detected, phospho-ERK was faintly detected, and p38 MAPK was constitutively phosphorylated irrespective of anti-Ig treatment.

**EBV activation in Daudi cells by simultaneous treatment with anti-Ig Abs and IGF-1**

Because BCR-mediated signaling did not induce the PI3K pathway in Daudi cells, we attempted to activate the PI3K pathway by

| Table I. Effects of IGF-1 and anti-Ig Abs on EBV activation in type I BL cell lines |
|-------------------------------|----------|-----------|
| Cells                        | Percent BHRF1-Positive Cells* |
| Untreated                  | Anti-Ig | IGF-1 | Anti-Ig + IGF-1 |
| Akata                      | <0.1    | 66.3 ± 3.4 | Not done |
| Daudi                      | <0.1    | <0.1    | 12.3 ± 2.2 |
| Mutu-I                     | <0.1    | 4.2 ± 1.1 | <0.1    | 11.3 ± 3.5 |
| Sav-I                      | <0.1    | 3.5 ± 0.9 | <0.1    | 7.6 ± 0.9 |
| Kem-I                      | <0.1    | <0.1    | <0.1    | 1.0 ± 0.1 |
| Oku-I                      | <0.1    | <0.1    | <0.1    | <0.1    |

*Cells were treated with IGF-1 (50 ng/ml) and/or anti-Ig Abs (7.5 μg/ml) for 24 h. EBV activation was assessed by expression of an EBV early protein, BHRF1, by the indirect immunofluorescence method. Results were shown as means ± SE of three separate experiments.
IGF-1, which activated the PI3K pathway independent of signaling through BCR (20). As shown in Fig. 4A, IGF-1 alone had a substantial effect on the phosphorylation of Akt, and simultaneous treatment with IGF-1 and anti-Ig Abs greatly increased the phosphorylation of Akt. The immunofluorescence assay indicated that IGF-1 alone could not induce BHRF1, but the simultaneous treatment with IGF-1 and anti-Ig Abs induced BHRF1 in 12% of Daudi cells (Table I, Fig. 4B). EBV activation by the simultaneous treatment was further confirmed by detection of an EBV lytic protein, BZLF1 (13), by immunoblot analysis (Fig. 4C).

Other BL cell lines were also examined to determine the effect of the simultaneous treatment with IGF-1 and anti-Ig Abs on EBV activation. A substantial increase of BHRF1-positive cells was observed in Mutu-I, Sav-I, and Kem-I cells, but not in Oku-I cells (Table I).

To identify the BCR-associated PI3K signaling defect in Daudi cells, possible defects in the PI3K adaptors, CD19 and BCAP, were examined. As shown in Fig. 5, CD19 and BCAP were equally expressed in both Akata and Daudi cells.

**Effect of IGF-1 on ERK and p38 MAPK pathways in Daudi cells**

Although three main pathways involved in BCR-mediated signal transduction were impaired in Daudi cells, restoration of the PI3K pathway by IGF-1 made Daudi cells responsive to BCR-mediated EBV activation. Therefore, we studied whether the other two signaling pathways, ERK and p38 MAPK, were also restored by IGF-1 treatment in Daudi cells. As shown in Fig. 6, IGF-1 alone had a little or no effect on the phosphorylation of ERK and p38 MAPK, but the simultaneous treatment with IGF-1 and anti-Ig Abs greatly increased their phosphorylation in Daudi cells, suggesting that PI3K influenced the activation of ERK and p38 MAPK pathways.

**Role of PI3K on BCR-mediated EBV activation in Akata and Mutu-I cells**

Studies on Daudi cells suggested that PI3K was critical for BCR-mediated EBV activation. Therefore, we examined the effect of a PI3K inhibitor on BCR-mediated EBV activation in Akata and Mutu-I cells. The cells were treated with anti-Ig Abs in the presence of wortmannin (0.2 μM), a PI3K inhibitor, for 24 h. Wortmannin treatment did not give any effect on cell viability. Immunoblot analysis indicated that wortmannin treatment inhibited the phosphorylation of Akt by anti-Ig Abs (Fig. 7A). Further studies on EBV expression indicated that expression of BHRF1 and BZLF1 was also inhibited (Fig. 7, B and C), suggesting the importance of PI3K in BCR-mediated EBV activation.

**FIGURE 2.** Phosphorylation of tyrosine kinases by BCR cross-linking in Akata and Daudi cells. Cells were treated with anti-Ig Abs for various times. The phosphorylated tyrosines were detected by immunoblot analysis with phosphorylated tyrosine-specific Abs.

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**FIGURE 1.** EBV activation by BCR cross-linking in BL cell lines. Cells were treated with anti-Ig Abs for 24 h. EBV activation was assessed by expression of an EBV early protein, BHRF1, by the indirect immunofluorescence method.

**FIGURE 3.** A, Diagram of pathways activated upon BCR cross-linking (8, 9). PI(3), phosphatidylinositol triphosphate; BLNK, B cell linker protein; IP(3), inositol triphosphate; JNK, c-jun N-terminal kinase. B, Activation of PI3K and MAPK pathways by BCR cross-linking in Akata, Mutu-I, and Daudi cells. Cells were treated with anti-Ig Abs for 30 min. The phosphorylated forms of Akt, ERK, and p38 MAPK were detected by immunoblot analysis with phosphorylated protein-specific Abs.
Effect of PI3K on activation of the BZLF1 promoter

BZLF1 is the switch for EBV activation from a latent infection and the first-transcribed EBV gene after BCR cross-linking (21, 22). We studied whether PI3K activates the transcription from the BZLF1 promoter in the absence of other EBV products. A BZLF1 promoter-luciferase gene plasmid was transfected into EBV-negative Daudi cells, and cells were then treated with IGF-1 and anti-Ig Abs for 24 h. EBV activation was assessed by expression of an EBV early protein, BHRF1, by the indirect immunofluorescence method. C, EBV activation. Cells were treated with IGF-1 and anti-Ig Abs for 24 h. EBV activation was assessed by expression of an EBV immediate early protein, BZLF1, by the immunoblot method.

Discussion

In EBV-transformed LCLs, LMP2A is always expressed at a high level, and inhibits BCR-mediated signal transduction (14–16). Therefore, LCLs are unresponsive to EBV activation by anti-Ig Abs. The high-level expression of LMP2A in LCLs is caused by transactivation of the EBNA2 protein (23). In contrast, BL cells that have type I latency are negative for EBNA2 expression and express little or no LMP2A. The analysis of peripheral blood lymphocyte by PCR showed that only EBNA1 and LMP2A were expressed in EBV latency in vivo (24–27). Although the level of
LMP2A expression in peripheral lymphocytes has not been measured quantitatively, the absence of EBNA2 expression suggests a low level LMP2A expression in these cells. Therefore, BL cells with type I latency are likely to represent in vivo latency.

Although BL cells with type I latency are free from inhibition of signal transduction by LMP2A, many BL cell lines are not susceptible for EBV activation by anti-Ig Abs. The present study was aimed at defining the molecular mechanisms that determine the response to BCR-mediated EBV activation. The comparison of Akata, Mutu-I, and Daudi cells, which are representative responders and nonresponders to BCR-mediated EBV activation, respectively, indicated that three main signaling pathways, PI3K, ERK, and p38 MAPK, were impaired in Daudi cells, and that restoration of PI3K restored ERK and p38 MAPK pathways, which was accompanied by EBV activation. Furthermore, a specific inhibitor for PI3K, wortmannin, inhibited EBV activation in anti-Ig-treated Akata and Mutu-I cells. These results indicated that the absence of PI3K impedes the progression of signals through the BCR, acting as a determinant of unresponsiveness to BCR-mediated EBV activation.

However, the degree of EBV activation after restoration of PI3K was not striking in all BL cell lines examined as compared with that in Akata cells. In particular, Oku-I cells were completely unresponsive to EBV activation after dual treatment with IGF-1 and anti-Ig Abs. These results suggested the existence of other cellular or viral factors influencing BCR-mediated EBV activation.

The present results also indicated the existence of cross-talk among the three signaling pathways, PI3K, ERK, and p38 MAPK, because restoration of PI3K by IGF-1, which activated PI3K-independent of BCR-mediated signaling, was accompanied by restoration of ERK and p38 MAPK activation in anti-Ig-treated Daudi cells. This is consistent with recent reports that PI3K activates the phosphorylation of ERK and p38 MAPK (28, 29).

We have previously shown that a specific inhibitor for PKC and a calmodulin antagonist inhibit BCR-mediated EBV activation in Akata cells (30), suggesting the requirement of PKC and Ca2+/calmodulin pathways for BCR-mediated EBV activation. Other reports indicated that inhibitors for MAPK (ERK and p38) blocked BCR-mediated EBV activation in Akata cells (31, 32). More recently, Darr et al. (33) reported that an inhibitor of PI3K blocked anti-Ig-induced EBV activation in Akata cells. These results suggest that all signaling pathways, PKC, Ca2+/calmodulin, PI3K, and MAPK (ERK and p38), must be activated for efficient EBV activation by BCR cross-linking.

Another aspect that may influence the response to BCR cross-linking is the Ig class; Akata cells express membrane IgG, and other cells express IgM. A recent report has described that IgG-containing BCR transmits a signal distinct from that of IgM- or IgD-containing BCR, although all three use the same signal-transduction component (34).

In conclusion, we demonstrated that PI3K is important for BCR-mediated EBV activation in BL cells that may represent in vivo latency as discussed above. Clarification of the mechanism, which regulates PI3K activity, would provide an insight into the molecular basis of maintenance and disruption of EBV latency in B-lymphocytes.
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