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Epstein-Barr Virus Latent Membrane Protein 2A Blocks Calcium Mobilization in B Lymphocytes

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LMP2A is expressed in latent Epstein-Barr virus (EBV) infection and interacts with LMP1 and members of the *src* tyrosine kinase family in the plasma membrane. Since tyrosine kinases mediate receptor-induced changes in intracellular free calcium, the effect of LMP2A on receptor-mediated intracellular calcium mobilization was evaluated by stably expressing LMP2A in an EBV-negative Burkitt tumor cell line (BJAB) or in LMP1-converted BJAB cells. LMP2A significantly blocked calcium mobilization following class II, CD19, or immunoglobulin M cross-linking. LMP2A effects were partially reversed in LMP1-converted cell lines. These results are compatible with LMP2A acting in latent B-lymphocyte infection to downmodulate LMP1 effects on cell growth or to inhibit induction of lytic EBV infection in specific human tissues following receptor ligation.

Epstein-Barr virus (EBV) is a herpesvirus which causes infectious mononucleosis in normal adolescents and B-lymphoproliferative disorders in immunocompromised hosts (28, 40). EBV has been etiologically linked with B-lymphocyte and epithelial cell malignancies, including African Burkitt's lymphoma, Hodgkin's disease, and nasopharyngeal carcinoma (2, 6, 18, 21, 23, 28, 40). B lymphocytes become latently infected and growth transformed by EBV. Latent infection involves the expression of a restricted set of latency-associated gene products which are presumed to mediate latent infection or growth transformation. This set includes six nuclear proteins (EBNAs), two small RNAs (EBERs), and two integral membrane proteins (LMP1 and LMP2) (28).

The LMP2 gene is simultaneously transcribed under control of two promoters separated by 3 kb (30, 49). Both transcripts are spliced into nearly identical mRNAs which differ only in their first exons. The LMP2A mRNA first exon encodes 119 amino acids, while the LMP2B mRNA first exon is noncoding. The LMP2A predicted primary amino acid sequence includes the 119 cytoplasmic amino acids encoded by the LMP2A unique first exon, 12 putative hydrophobic transmembrane domains, and a 27-amino-acid carboxyterminal domain (33). LMP2B translation initiates at the beginning of the second exon common to both LMP2A and LMP2B. LMP2B therefore consists of the 12 putative hydrophobic transmembrane domains and the 27-amino-acid carboxy terminus.

LMP2A has been shown to colocalize with LMP1 patches in B-lymphocyte plasma membranes, to undergo serine and tyrosine phosphorylation, and to associate with members of the *src* tyrosine kinase family (4, 33, 34). LMP1 has transforming effects on rodent fibroblasts, activating effects in human B lymphocytes, and is essential for B-lymphocyte growth transformation (1, 27, 51–54). LMP2A associates with the *lyn* and *fyn src* family kinases which interact with, and are activated by, the immunoglobulin receptor complex (4, 7). LMP2A is a substrate for these tyrosine kinases (33). The amino-terminal domain of LMP2A is likely to be essential for tyrosine kinase association, since a mutant LMP2A consisting of only the amino terminus and first two transmembrane domains associates with B-lymphocyte *src* tyrosine kinases (4, 33).

Because of the association of LMP2A with *lyn* and *fyn* and with LMP1, which provides constitutive activation in B cells, LMP2A is likely to affect signal transduction. Since a rise in intracellular free calcium is a consequence of receptor activation through associated tyrosine kinases, we investigated the effect of LMP2A or both LMP2A and LMP1 on receptor-activated calcium mobilization.

MATERIALS AND METHODS

Cell lines and cell culture. BJAB is an EBV-negative Burkitt lymphoma cell line (39). BJAB cell lines were maintained in RPMI 1640 media supplemented with 10% inactivated fetal bovine serum and gentamicin at 2 μg/ml. BJAB cell lines expressing LMP1 under the control of the human metallothionein type II promoter (BJMTLMP-11 and BJMTLMP-15) and the vector control cell lines (BJgpt-1 and BJgpt-2) were previously described (22, 54). BJAB mycophenolic acid vector control or LMP1-converted BJAB cells were electroporated with pMP2LMP2A (34), which expresses LMP2A from the murine leukemia virus long terminal repeat of pBAMHYGRO, or with pBAMHYGRO vector alone (57) and selected in media containing 400 μg of hygromycin per ml. All clones were also maintained in 3 μg of mycophenolic acid, 10 μg of hypoxanthine, and 160 μg of xanthine per ml. LMP2A and LMP1 expression was evaluated by immunofluorescence and Western immunoblotting, as described below.

Flow cytometric analysis. For direct immunofluorescence staining, 10^6 cells were washed three times in phosphate-buffered saline (PBS) containing 1.0% inactivated fetal bovine serum. Cells were then stained in staining buffer (PBS, 1.0% serum, 0.01% sodium azide) with freshly diluted dichlorotriazinylaminofluorescein-conjugated anti-immunoglobulin M (IgM) added (Jackson Immunologicals) for 30 min, washed three times, and kept on ice until analyzed. Cells were also stained with anti-HLA-DR (DAKO), anti-CD19 (DAKO), or anti-CD22 (DAKO) freshly diluted in staining buffer for 30 min. These cells were washed three times, and freshly diluted dichlorotriazinylaminofluorescein-

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conjugated goat anti-mouse IgG (Jackson Immunologicals) was used as a secondary antibody. After being thoroughly washed, cells were kept on ice until analyzed on a FACSCAN flow cytometer (Becton-Dickinson).

Calcium analysis. A total of 2×10^6 cells were loaded with 1 μM Indo-1 (Molecular Probes) in 0.5 ml of RPMI complete media for 20 min at 37°C. Loaded cells were then brought up to 2.5 ml with HEPES buffer, pH 7.2 (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], 145 mM NaCl, 5 mM KCl, 0.5 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM glucose) and transferred to 2.0-ml Nunc cryovials. All calcium experiments were done with cells in these vials maintained at 37°C. The flow rate was maintained at 900 cells per s throughout experiments. Baseline calcium levels were established for 1 min prior to the addition of the appropriate antibody. Cells were stimulated with 10 μg of goat anti-human IgM (Southern Biotechnology Associates, Inc.) per ml, and the anti-IgM response data were collected for 6 min. For CD19 and class II, the appropriate antibody (DAKO) was added at a final concentration of 10 μg/ml and allowed to incubate for 2 min. Goat anti-mouse IgG (Sigma) at 200 μg/ml was then added to cross-link the primary antibody, and data were collected for 6 min. Baseline absolute intracellular calcium levels were determined for all cell lines analyzed, by using ionophore and ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA) as previously described (47, 48). Data were collected and analyzed on an EPICS 750 series flow cytometer (Coulter Electronics) as previously described (13, 47, 48). Briefly, data analysis was performed with MDADS analysis system software. The percentage of cells responding to the indicated antibody treatment was calculated by integration and subtraction of the background events. The percentage of cells responding to antibody treatment in a time slice representing 10,000 cells was evaluated at the point of maximal response. A time slice representing 10,000 unstimulated cells was used to determine the background events, which were then subtracted from the percentage of cells responding. The percentage of change in intracellular calcium was determined by comparing the mean fluorescence of the cells before and after antibody treatment.

Additional techniques and reagents. Cell transfections by electroporation, crude membrane preparation, and Western transfer were performed as previously described (34, 37). LMP2A immunoblots were performed on crude membrane preparations resuspended and heated to 70°C in 2.5 volumes of cracking buffer (250 mM Tris [pH 6.8], 5% sodium dodecyl sulfate, 8 M urea, 10% β-mercaptoethanol) and separated on a 10% denaturing polyacrylamide gel. Polyclonal rabbit antibody against LMP2A (34) and S12 monoclonal antibody directed against LMP1 (38) were used for immunofluorescence and immunoblots as previously described (34).

RESULTS

Expression of LMP2A in B lymphoblasts. To assess the effect of LMP2A on membrane-activated calcium mobilization, LMP2A was stably expressed in an EBV-negative Burkitt lymphoma cell line, BJAB. The potential interaction between LMP2A and LMP1 was investigated by expression of LMP2A in BJAB cells that had been converted to LMP1 expression by single-gene transfer (22, 54). Eleven hygromycin-resistant pMP2LMP2A-transfected BJAB clones and four pMP2LMP2A superconverted LMP1 clones, which by immunofluorescence were comparable in their level and homogeneity of LMP2A expression to EBV-infected lymphoblastoid cell lines (data not shown), were derived. Additionally, nine vector controls and seven LMP1-converted clones were derived in parallel as controls. LMP1 expression in all LMP1-converted cell lines was also comparable to LMP1 expression in lymphoblastoid cell lines as determined by immunofluorescence (data not shown). The accuracy of immunofluorescence in assessing LMP2A and LMP1 levels was confirmed by Western blot for LMP2A and LMP1 in representative cell lines (Fig. 1). One exception was the LMP2A.2 clone, which was similar to other LMP2A clones in immunofluorescence but consistently had less LMP2A by immunoblot (Fig. 1).

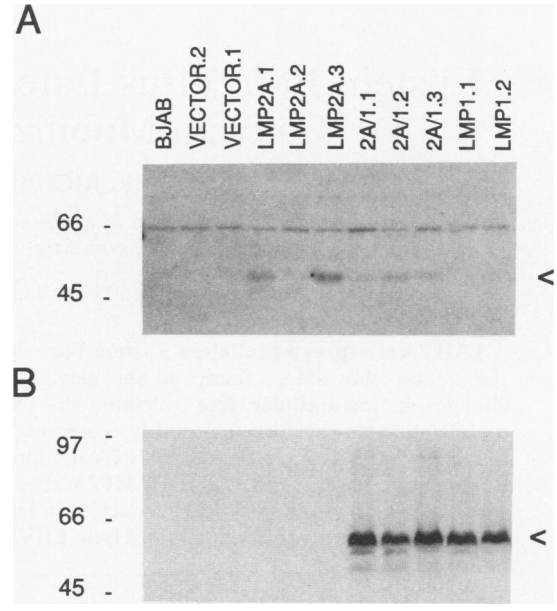

FIG. 1. LMP1 and LMP2A in BJAB clones as assayed by immunoblot. Membrane proteins from 10^6 cells were separated on 10% denaturing polyacrylamide gels, transferred to nitrocellulose, and reacted with either rabbit anti-LMP2A antibodies which detect the 54-kDa LMP2A protein (A) or mouse anti-LMP1 monoclonal antibodies which detect the 60-kDa LMP1 protein (B). BJAB is an EBV-negative human B-lymphoma cell line, VECTOR.1 and .2 are BJAB control cell lines which were transfected with the pBAMHY-GRO vector only, LMP2A.1 to .3 are BJAB cell lines stably expressing LMP2A, LMP1.1 and .2 are BJAB cell lines stably expressing LMP1, and 2A/1.1 to .3 are BJAB cell lines stably expressing both LMP2A and LMP1. Size standards (in kilodaltons) are indicated to the left.

MHC class II, CD19, CD22, and IgM are expressed on the surface of LMP2A- and LMP2A/LMP1-converted BJAB clones. Major histocompatibility complex (MHC) class II, CD19, and IgM are among the B-lymphocyte surface molecules which induce a transient rise in intracellular free calcium when ligated with bivalent specific antibody. As a control for subsequent studies on the effect of LMP2A in receptor-mediated changes in intracellular free calcium, surface expression of these proteins on pMP2LMP2A-transfected clones was evaluated by direct immunofluorescence staining and fluorescence-activated cell sorter (FACS) analysis. CD22 surface expression was also evaluated, since CD22 can affect anti-immunoglobulin-induced intracellular calcium mobilization (45). Class II, CD19, CD22, and IgM were detected on all clones of each type of transfectant (Table 1). Class II and CD22 expression were similar on

TABLE 1. Summary of flow cytometric analysis of cell surface receptor expression on BJAB stable transfectants

Cell line	Mean fluorescence of stained cells[a]			
	Class II	CD19	CD22	IgM
BJAB[b]	1,296	375	65	154
VECTOR.1	592	396	101	100
VECTOR.2	712	436	86	196
Avg of 6 clones	846	370	73	168
LMP2A.1	858	181	86	37
LMP2A.2	866	524	84	35
LMP2A.3	1,821	421	137	32
Avg of 6 clones	1,032	400	101	38
2A/1.1	921	155	50	22
2A/1.2	1,139	178	72	23
2A/1.3	1,275	104	75	26
Avg of 4 clones	1,111	143	68	30
LMP1.1	837	313	95	314
LMP1.2	834	342	38	45
LMP1.3	828	317	61	78
Avg of 6 clones	834	299	62	113

[a] Live cells were stained with anti-class II, -CD19, -CD22, or -IgM antibody as described in Materials and Methods. Five thousand events were collected on a FACSCAN flow cytometer (Becton-Dickinson).

[b] BJAB background mean fluorescence of cells processed for FACS analysis in the absence of anti-class II, anti-CD19, anti-CD22, or anti-IgM antibody was 2 to 4.

vector controls, LMP2A-converted, LMP1-converted, and LMP2A-superconverted LMP1 clones. CD19 levels were also comparable on vector controls and on LMP2A-converted and LMP1-converted cell lines. However, CD19 expression on all four LMP2A-superconverted LMP1 clones was approximately 40% of those of six vector controls.

Surface IgM was detected on all clones at levels significantly above background (Table 1). However, each of the six LMP2A clones and the four LMP2A-superconverted LMP1 clones expressed IgM at only 20% of the average level of six vector controls. One vector control had surface IgM levels similar to those of LMP2A cell lines. Figure 2 displays the results of flow cytometric analysis done on representative BJAB clones. BJAB and vector controls stain very positive for surface IgM, with mean fluorescence ranging from 100 to 196 (background mean fluorescence of 3 to 4 was obtained for cells processed for FACS analysis in the absence of anti-IgM antibody). LMP2A-converted cell lines, or LMP2A-superconverted LMP1 cell lines, displayed significant but reduced IgM levels, with a mean fluorescence of 22 to 37 (Fig. 2, LMP2A.1-3 and 2A/1.1-3). IgM expression on the six LMP1 cell lines was more variable than on the other transfectants. The mean fluorescence for representative LMP1 clones ranged from 45 to 314 (Fig. 2, LMP1.1-3).

LMP2A blocks calcium mobilization through multiple cell surface receptors. BJAB cell lines expressing LMP2A, LMP1, LMP2A and LMP1, or vector controls were treated with antibodies to human class II or CD19 and their intracellular free calcium levels were monitored by flow cytometry in three independent experiments with each cell line. Seven LMP1-converted cell lines and nine vector controls exhibited similar changes in intracellular calcium after anti-class II or anti-CD19 treatment. Eleven LMP2A-converted and four LMP2A-superconverted LMP1 clones all repeatedly displayed diminished calcium mobilization in response to anti-class II or anti-CD19 antibody compared with those of seven LMP1-converted and nine vector control cell lines. This was evident in both percentage of cells responding and in the intracellular free calcium change among responding cells.

The percentage of cells responding to antibody treatment with a change in intracellular calcium and the percentage of change in intracellular calcium levels among those cells at the point of maximal change are presented in Table 2 for three representative clones of each type. LMP2A convertants had reduced responses to anti-MHC class II or anti-CD19 compared with those of vector controls and LMP1 convertants. The percentage of cells responding (1 to 9% versus 18 to 71% for class II and 2 to 3% versus 14 to 30% for CD19) and the percentage of change in intracellular calcium levels (5 to 14% versus 17 to 105% for class II and 5 to 8% versus 20 to 48% for CD19) were reduced in LMP2A clones compared with those in vector or LMP1-converted controls. LMP2A-superconverted LMP1 clones were also reduced in the number of cells responding as well as the degree to which responding cells mobilized calcium compared with those of vector or LMP1-converted controls (10 to 31% cells responding to class II antibody with a 6 to 28% change in intracellular calcium, 2 to 7% responding to CD19 antibody with a 6 to 14% change in intracellular calcium). However, the LMP2A effect on the class II antibody-induced calcium release in LMP2A-superconverted LMP1 clones was not as great as in cell lines expressing only the LMP2A protein (Table 2).

The 11 LMP2A- and 4 LMP2A-superconverted LMP1 clones were each analyzed in five independent experiments and had consistently diminished calcium mobilizations in response to treatment with anti-IgM compared with those of 9 vector controls and 7 LMP1 cell lines. Experiments with three representative cell lines are presented in Fig. 3. BJAB and vector control cell lines responded to anti-IgM treatment with similar calcium mobilizations. The responses were rapid and transient, with return to baseline calcium levels by 2 min after antibody addition. LMP1-converted cell lines were more variable in their responses; LMP1.1 cells displayed a reduced calcium mobilization, whereas LMP1.2 and LMP1.3 had equivalent or greater responses than the controls (Fig. 3). Four additional LMP1 cell lines tested exhibited a similar range of variability (data not shown). LMP1-expressing cell lines responded to anti-IgM treatment as rapidly as the controls but characteristically returned to baseline calcium levels more slowly than the cell lines expressing no LMP1 in several independent experiments with each LMP1 cell line (Fig. 3, BJAB, VECTOR.1 and .2, and LMP2A.1 to .3). In contrast, most cells in the LMP2A.1 to .3 populations have no discernible change in intracellular free calcium following anti-IgM treatment. Interestingly, LMP2A-superconverted LMP1 cell lines (2A/1.1 to .3) have diminished calcium mobilizations compared with those of vector control and LMP1 cell lines but greater responses than LMP2A-converted clones (LMP2A.1 to .3).

The percentage of cells responding to anti-IgM treatment with a change in intracellular calcium and the percentage of change in intracellular calcium levels among those cells at the point of maximal change are presented in Table 2. For BJAB and vector controls, 74 to 93% of the cells responded with a 180 to 251% change in mean intracellular calcium levels. Similarly, 57 to 78% of LMP1 converted cells responded to anti-IgM and with substantial changes (110 to 237%) in intracellular free calcium. However, only 11 to 36% of LMP2A-converted cells responded, and among those cells, intracellular calcium increased by only 19 to 57%. LMP2A-superconverted LMP1 cell lines, 2A/1.1 to .3, also

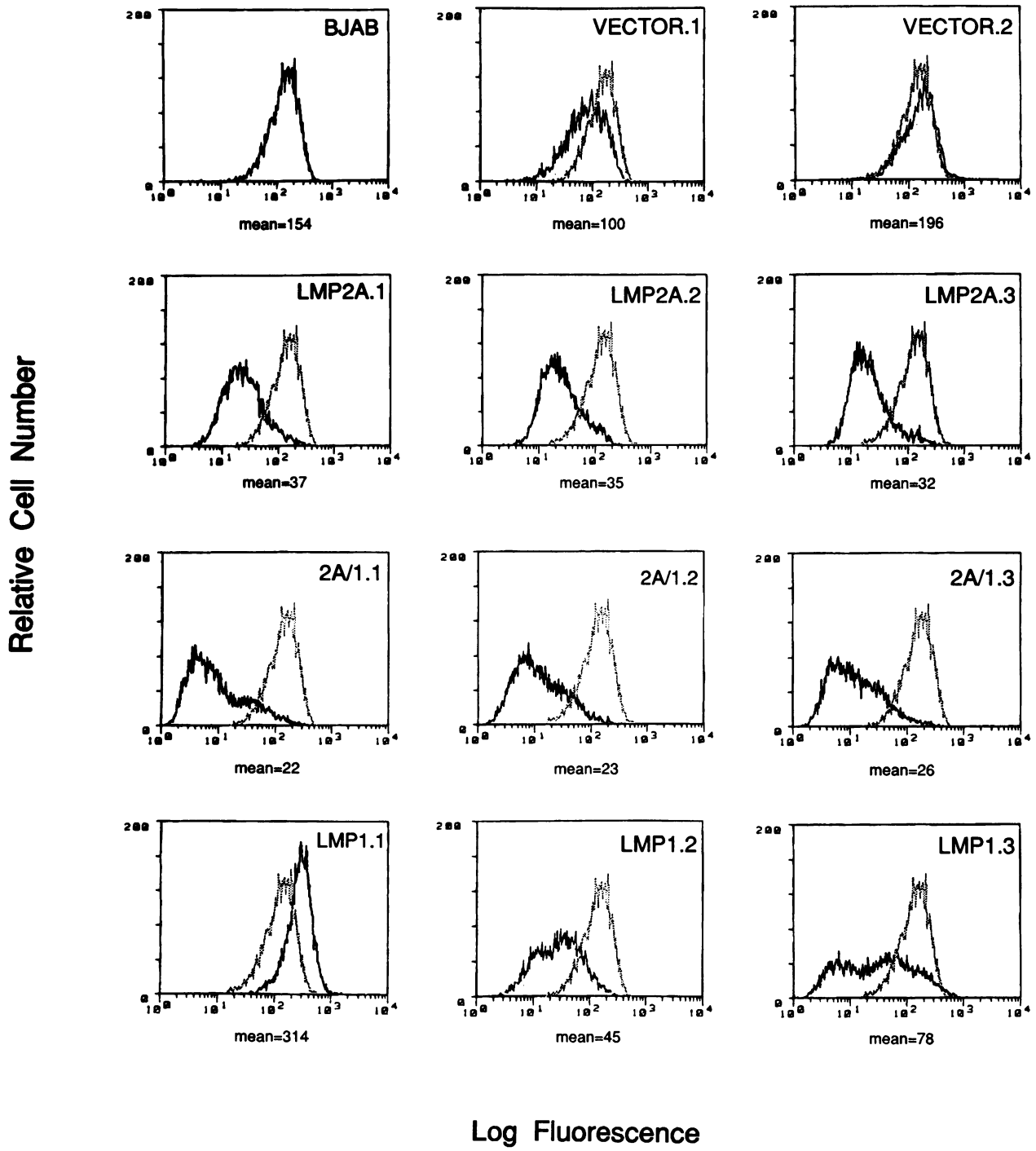

FIG. 2. Flow cytometric analysis of surface IgM expression on BJAB cell lines. Live cells were stained for the expression of surface IgM as described in detail in Materials and Methods. Five thousand events were collected on a FACSCAN flow cytometer (Becton-Dickinson). For reference, each histogram has been overlaid with the BJAB histogram in grey. The mean fluorescence of the labeled cell line is indicated directly below the appropriate histogram.

responded less frequently (38 to 41%) than vector controls, but cells which responded displayed larger changes (88 to 116%) in intracellular calcium than the LMP2A-converted clones.

The lack of response by LMP2A cell lines was unlikely to be due to lower surface IgM expression, since IgM was still abundant on these clones. Furthermore, a vector control clone with surface IgM levels similar to those of LMP2A-converted cell lines still had responses which were indistinguishable from other controls. Of the cells in this vector population, 80% responded to anti-IgM treatment, and with a 140% increase in the mean intracellular calcium level (data

TABLE 2. Calcium mobilization in BJAB stable transfectants following stimulation through the MHC class II, CD19, or IgM receptors[a]

Receptor and transfectant	% of cells responding	% Change in intracellular calcium
Class II		
BJAB	71	105
VECTOR.1	39	47
VECTOR.2	18	17
LMP2A.1	1	9
LMP2A.2	6	5
LMP2A.3	9	14
2A/1.1	31	20
2A/1.2	10	6
2A/1.3	19	28
LMP1.1	46	65
LMP1.2	38	57
LMP1.3	41	59
CD19		
BJAB	18	20
VECTOR.1	30	48
VECTOR.2	14	21
LMP2A.1	3	8
LMP2A.2	2	5
LMP2A.3	2	6
2A/1.1	7	14
2A/1.2	2	6
2A/1.3	3	11
LMP1.1	28	38
LMP1.2	14	19
LMP1.3	30	43
IgM		
BJAB	74	180
VECTOR.1	93	251
VECTOR.2	82	198
LMP2A.1	23	43
LMP2A.2	36	57
LMP2A.3	11	19
2A/1.1	41	116
2A/1.2	38	90
2A/1.3	38	88
LMP1.1	57	110
LMP1.2	67	161
LMP1.3	78	237

[a] Changes in intracellular calcium levels as determined by flow cytometry of indo-1-loaded cells. A baseline was established prior to the addition of antibodies against IgM, MHC class II, or CD19 as outlined in Materials and Methods. Class II and CD19 experiments involved the addition of anti-IgG after the primary antibody as described in Materials and Methods. The percentage of cells responding to the indicated antibody treatment was calculated by integration and subtraction of the background events. The percentage of change in intracellular calcium was determined by comparing the mean fluorescence of the cells before and after antibody treatment.

not shown). Additionally, calcium mobilization experiments in which anti-IgG as a secondary antibody was added after the anti-IgM antibody to increase the surface IgM cross-linking and potentiate the responses were done. This amplification of cross-linking is an accepted strategy for inducing calcium mobilization through less abundant receptors such as MHC class II and CD19 (29, 31). After cross-linking anti-IgM antibodies, LMP2A-expressing cell lines did not increase calcium mobilization over the anti-IgM treatment alone (data not shown). Thus, the effect of LMP2A in blocking anti-IgM-induced calcium mobilization is not due to less abundant IgM expression.

DISCUSSION

These experiments indicate that expression of the EBV latency-associated membrane protein LMP2A in B lymphocytes disrupts normal signal transduction following receptor stimulation, as measured by calcium mobilization. LMP2A-converted BJAB cells displayed diminished calcium mobilization in response to anti-IgM, -class II, or -CD19 treatments. LMP2A-superconverted LMP1 clones also displayed diminished responses relative to those of vector controls, but to a lesser degree than LMP2A cell lines. In contrast, LMP1-converted cells mobilized calcium to degrees similar to those of vector controls. However, LMP1-converted clones returned to baseline calcium levels with slower kinetics than vector controls. LMP2A-superconverted LMP1 cells which responded to receptor cross-linking also displayed slower returns to baseline calcium levels. In summary, LMP2A expression in B cells results in a diminution of calcium mobilization following IgM, CD19, and class II cross-linking. LMP1 partially reverses this block in calcium mobilization and may cause a slower return to baseline calcium level.

The B-lymphocyte antigen receptor is a complex structure consisting of surface immunoglobulin and transducer/transporter substructures. Surface immunoglobulin is noncovalently associated with heterodimers of α, β, and γ chain products of the *mb-1* and B29 genes (10, 14, 24, 25, 43). These molecules associate with members of the *src* tyrosine kinase family as well as other SH2-containing molecules such as phosphatidylinositol 3-kinase (5, 55, 56). Surface immunoglobulin cross-linking triggers the activation of receptor-associated tyrosine kinases, resulting in phosphorylation and activation of cellular proteins including PLCγ1 and PLCγ2 (3, 8, 9, 11, 12, 15, 16, 20, 26). Subsequent phosphoinositide hydrolysis and calcium mobilization lead to transcriptional activation of immediate-early genes and ultimately to B-cell proliferation (7, 17, 19, 42, 44).

LMP2A interacts with *lyn* and *fyn src* tyrosine kinases and has been shown to be a major tyrosine kinase substrate in both transfected B-lymphoma cells and EBV-transformed lymphoblastoid cell lines (4, 33). LMP2A also induces the phosphorylation of an associated cellular 70-kDa tyrosine kinase substrate in vivo and in in vitro kinase reactions. This protein may be related to the 70-kDa phosphotyrosine protein found in B lymphocytes immediately after immunoglobulin cross-linking (5, 11, 20). The association of LMP2A with LMP1 and cellular components of the immunoglobulin receptor complex suggests an involvement of LMP2A in normal cell signaling. The results presented here are consistent with this suggestion and clearly demonstrate LMP2A functions in B cells to downmodulate calcium mobilization in response to receptor cross-linking. On the basis of known interactions between LMP2A and IgM-associated tyrosine kinases, it is likely that LMP2A blocks calcium mobilization by preventing transduction of signal at an early point in the pathway. LMP2A may compete directly with cellular *lyn* and *fyn* kinase substrates for interaction with these kinases.

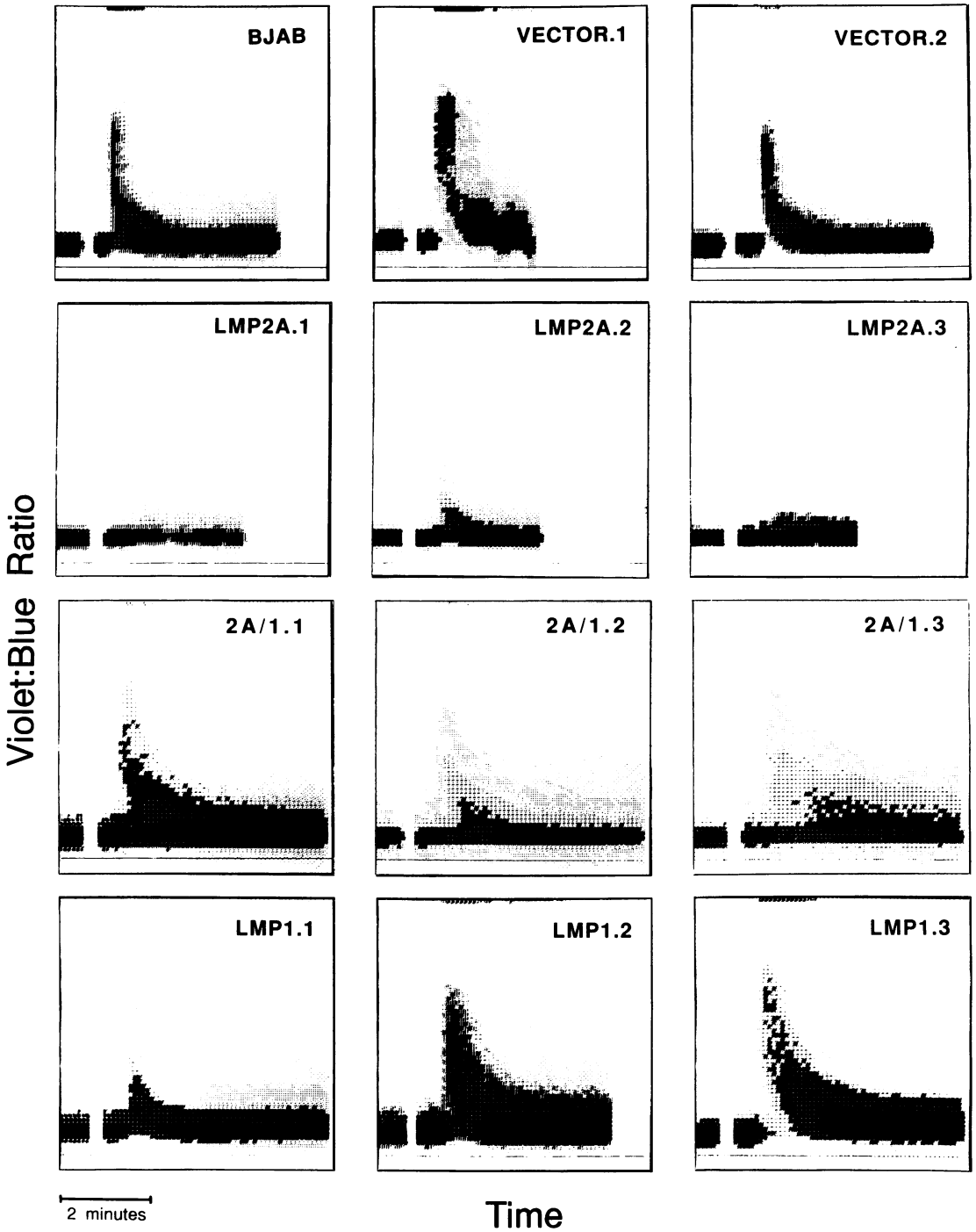

FIG. 3. Effects of LMP2A and LMP1 on intracellular free calcium after IgM cross-linking. Calcium mobilization was monitored over time by changes in the ratio of violet to blue (405 nm to 485 nm) fluorescence of cells loaded with the calcium-sensitive dye indo-1 and analyzed by flow cytometry. Flow rate was maintained at 900 cells per s. Data are presented as a histogram of the number of cells (indicated by the intensity of dots) with a particular ratio of violet to blue fluorescence (indicated by position of the dot along the *y* axis) over the time after IgM cross-linking (indicated by position along the *x* axis). At each point in time, the violet-to-blue ratio distribution among the population of approximately 6,000 cells is indicated by the vertical dot distribution. The break in the graphs on the left side indicates the interval during which the cells were removed from the flow cytometer for addition of antibody.

Alternatively, LMP2A could be a *fyn* or *lyn* substrate through interaction with a *fyn*- or *lyn*-associated molecule such as pp70 or a putative membrane protein. The observed IgM reduction on LMP2A-converted BJAB cells could be explained by LMP2A interfering with normal interactions between receptor-associated proteins, ultimately resulting in destabilization and turnover of the receptor complex. Determination of the effect of LMP2A on protein tyrosine phos-

phorylation following IgM cross-linking may clarify the point at which LMP2A interacts with this pathway.

LMP1 is an important component of the EBV latent life cycle, demonstrated by its essentiality in the transformation process (27). LMP1 has broad activation effects on B lymphocytes, which include induction of activation and adhesion molecules, as well as vimentin (32, 53, 54). It is therefore interesting that LMP1 partially reverses the block on receptor-mediated calcium mobilization caused by LMP2A. LMP1 and LMP2A may compete for interaction with cellular components of the IgM signaling pathway. Alternatively, LMP1 could antagonize LMP2A effects by acting to increase intracellular free calcium levels independently of interaction with LMP2A-associated proteins. Previous data have indicated that LMP1 may cause an increase in steady-state intracellular calcium in human B lymphocytes (53).

The exact role of LMP2A in EBV latent infection is unknown and may be difficult to discern in vitro. In in vitro transformation assays of B lymphocytes, LMP2A and LMP2B are dispensable (35, 36). However, LMP2A is expressed in all latently infected B lymphocytes in vitro and in vivo, is detected in nasopharyngeal carcinoma biopsies, and nasopharyngeal carcinoma patients express antibodies to LMP2A (2, 6, 34, 46). There is also selection against LMP2A, since LMP2A has T-cell reactive epitopes that can mediate a cytoxic T-cell response (41). The association of LMP2A with key components of signaling pathways in B cells and the expression of LMP2A in spite of selective pressure against its expression suggest an important role of LMP2A in EBV infection and growth transformation in vivo.

The results reported here implicate LMP2A as a negative modulator of normal signal transduction in B lymphocytes. LMP2A may function to downregulate the EBV-induced activation state by repressing activation properties of LMP1, allowing EBV-infected cells to less actively proliferate in certain tissues and evade destruction by natural killer or host cytotoxic T cells in vivo. Alternatively, the block on receptor-mediated calcium mobilization by LMP2A could provide a mechanism by which reactivation of EBV is downregulated in certain tissues in vivo. Reactivation can be induced by cross-linking surface immunoglobulin on latently infected lymphocytes or by treatment with calcium ionophore and protein kinase C agonist (16a, 50). Therefore, LMP2A may function to limit reactivation in response to calcium mobilization induced through cross-linking of surface receptors on latently infected lymphocytes. Such hypotheses will require testing in experimental models of human EBV infection such as SCID mice, cotton top tamarins, or Old World primates.

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