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The $(YXXL/I)_2$ signalling motif found in the cytoplasmic segments of the bovine leukaemia virus envelope protein and Epstein–Barr virus latent membrane protein 2A can elicit early and late lymphocyte activation events

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The cytoplasmic domains of the transducing subunits associated with B and T cell antigen receptors contain a common amino acid motif consisting of two precisely spaced Tyr-X-X-Leu/Ile sequences (where X corresponds to a variable residue). Expression of a single copy of this motif suffices to initiate B or T cell activation. The bovine leukaemia virus (BLV) is a B cell lymphotropic retrovirus which causes a non-neoplasic proliferation of B cells. The cytoplasmic domain of the BLV transmembrane envelope glycoprotein, gp30, possesses two overlapping copies of the Tyr-X-X-Leu/Ile-containing motif which could participate in the induction of B cell activation. Similarly, the N-terminal cytoplasmic domain of the latent membrane protein 2A (LMP2A) of the Epstein–Barr virus (EBV) contains a single copy of the Tyr-X-X-Leu/Ile-containing motif which could play a critical role in B cell transformation. To determine whether these two virus-encoded cytoplasmic domains are endowed with signalling functions, we constructed chimeric proteins by replacing the cytoplasmic tail of CD8-α with that of either BLV gp30 or EBV LMP2A. We show here that, once separately expressed in B or T cell lines, these chimeras are capable of triggering both calcium responses and cytokine production when cross-linked with an antibody to CD8-α. Furthermore, using site-directed mutagenesis, we demonstrated unequivocally that this signalling function may be accounted for by the Tyr-X-X-Leu/Ile motifs they contain. Since antibodies against the BLV envelope protein persist throughout infection, and LMP2A patches colocalize with the latent membrane protein 1 (LMP1), our data suggest that oligomerization of these viral proteins may trigger the activation and proliferation of infected B cells and contribute to virus persistence.

Key words: B cell activation/herpesvirus/retrovirus/signalling motifs

Introduction

The analysis of sequence elements responsible for the signalling properties of the transducing subunits of B and T cell antigen receptors has led to the identification of a recurrent functional domain made of ~20 amino acids (Irving and Weiss, 1991; Bonnerot *et al.*, 1992; Clark *et al.*, 1992; Letourneur and Klausner, 1992a; Romeo *et al.*, 1992; Wegener *et al.*, 1992; Kim *et al.*, 1993). Common to each copy of this domain is a pair of Tyr-X-X-Leu/Ile sequences (where X corresponds to a variable residue) separated by seven or eight variable amino acids. This consensus sequence is hereafter referred to as the $(YXXL/I)_2$ motif and expressed as a single copy in the cytoplasmic tail of the CD3-γ, CD3-δ, CD3-ε, Ig-α, Ig-β, FcεRIγ and FcεRIβ subunits (Reth, 1989). The CD3-ζ polypeptide appears to be unique among this set of signalling devices in the sense that it displays three copies of the $(YXXL/I)_2$ motif (Wegener *et al.*, 1992). Cross-linking of chimeric molecules composed of the extracellular and transmembrane parts of the CD4, CD8-α or CD25 molecules and of a single copy of the $(YXXL/I)_2$ motif suffices to elicit early or late activation events. The precise mechanisms by which the oligomerization of the $(YXXL/I)_2$ motifs generate intracellular signals remain unknown. However, as a result of ligand binding, the two tyrosines found in each motif are phosphorylated and likely to function as bidentate docking sites for the paired SH2 domains present in some lymphocyte-specific tyrosine kinases [e.g. syk (Taniguchi *et al.*, 1991; Hutchcroft *et al.*, 1992) and ZAP-70 (Chan *et al.*, 1992; Wange *et al.*, 1992)]. Upon recruitment to the inner face of the plasma membrane, these tyrosine kinases are believed to be responsible for the initiation of the signalling cascade (Songyang *et al.*, 1993).

Copies of the $(YXXL/I)_2$ motifs are also present in the cytoplasmic tail of two membrane proteins encoded by the bovine leukaemia virus (BLV) and Epstein–Barr virus (EBV). BLV is a B cell lymphotropic retrovirus which induces a chronic disease in cattle, often causing a non-neoplasic proliferation of B cells, and resulting in B cell leukaemia in a small number of infected animals (Burny *et al.*, 1987). The envelope protein of BLV is composed of a surface (gp51) and a transmembrane (gp30) subunit. gp51 determines the cellular tropism of the virus, whereas gp30 is responsible for anchoring the complex into the membrane and mediating virus–cell fusion (Burny *et al.*, 1987; Mamoun *et al.*, 1990). The 58 amino acid cytoplasmic tail of gp30 contains two overlapping copies of the $(YXXL/I)_2$ motif. EBV is a human herpesvirus which infects B lymphocytes and is the causative agent of infectious mononucleosis. EBV does not usually replicate in B lymphocytes but, instead, establishes latent infection (Kieff and Liebowitz, 1990). Latently infected B cells proliferate in immunocompromised individuals or in cell culture. The restricted set of EBV gene products which are expressed during latent infection constitutes the likely mediators of virus-induced B lymphocyte activation and proliferation. Two of them correspond to 'latent membrane proteins' denoted by LMP1 and LMP2A (Laux *et al.*, 1988; Sample *et al.*, 1989). LMP1 forms large, non-covalent membrane complexes and induces the expression of molecules associated with lymphocyte activation (e.g. CD23 and CD58). EBV LMP2A colocalizes with the LMP1 patches

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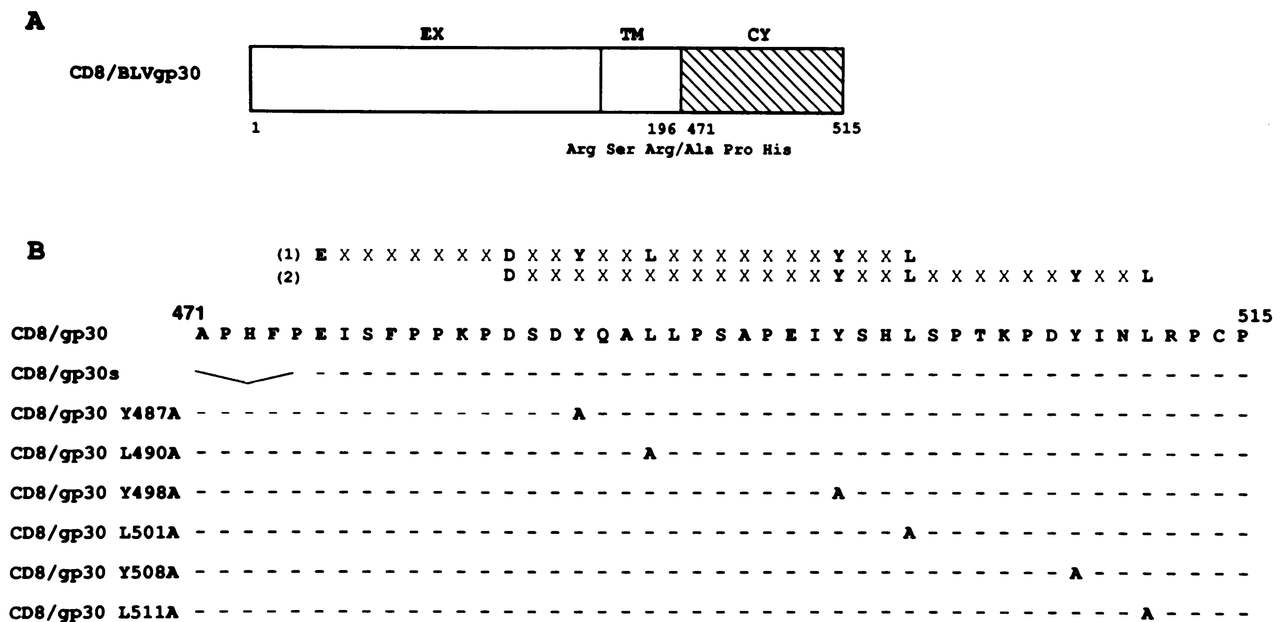


Fig. 1. Predicted structures of the CD8/BLV gp30 chimera. (**A**) A CD8/BLV gp30 chimera was assembled by linking the cDNA segment encoding the extracellular (EX) and transmembrane (TM) sequences of CD8-α to the sequence corresponding to the 45 C-terminal residues of the cytoplasmic segment (CY) of the BLV gp30 envelope protein. The amino acid sequence at the CD8/BLV gp30 junction is indicated under the chimera. The BLV gp30 residues are numbered according to Mamoun *et al.* (1990) (**B**) The sequence of the appended BLV gp30 cytoplasmic tail is shown in the single-letter amino acid code and the localization of the two $(YXXL/I)_2$ motifs is highlighted. The gp30 cytoplasmic segment contains three repeats of the sequence YXXL. These repeats may be arranged into two overlapping $(YXXL/I)_2$ motifs denoted as 1 and 2. All the BLV isolates sequenced to date show a strong conservation of both $(YXXL/I)_2$ motifs (Mamoun *et al.*, 1990). Note that the two YXXL sequences present in the gp30 C-terminal motif are spaced by six amino acids and thus differ from the seven or eight amino acid spaced YXXL/I sequences found in most of the functional $(YXXL/I)_2$ motifs analysed to date (see for instance Figure 9 in Wegener *et al.*, 1992). The sequences of the mutated BLV gp30 cytoplasmic tails used in this study are indicated under the wild-type sequence. Amino acid identities with the latter are indicated with dashes. In the CD8/gp30s molecule, residues 470–475 have been deleted.

in B lymphocyte plasma membrane and undergoes serine, threonine and tyrosine phosphorylations (Longnecker and Kieff, 1990). The N-terminal segment of LMP2A displays a cytoplasmic orientation, interacts with members of the src tyrosine kinase family (lyn, fyn; Burkhardt *et al.*, 1992) and contains a single copy of the $(YXXL/I)_2$ motif. To demonstrate that the BLV gp30 and the EBV LMP2A cytoplasmic tails share both structural and functional properties with the transducing subunits associated with antigen receptors, we expressed each of them individually in lymphoid cell lines and assessed their respective signalling capacity. Both of them were found able to elicit early and late activation events similar to those normally seen upon antigen–receptor triggering. Furthermore, using site-directed mutagenesis, we have formally ascribed these signalling properties to the $(YXXL/I)_2$ motif they contain.

Results

The BLV gp30 cytoplasmic tail induces IL-2 production in both T and B cells

In BLV-infected cells, the expression of the envelope gene products is under the control of the Rex regulatory protein (Felber *et al.*, 1989). To obviate this need, we have analysed the signalling capacity of the BLV gp30 cytoplasmic tail independently of other viral components by constructing a chimeric protein in which the cytoplasmic tail of the mouse CD8-α polypeptide has been replaced by that of the BLV gp30 protein (CD8/gp30, Figure 1A). In the absence of a bovine lymphoid cell line capable of signalling upon antigen–receptor triggering, and since the $(YXXL/I)_2$ motif is conserved between species (Wegener *et al.*, 1992), we stably expressed the CD8/gp30 chimera in the mouse cell lines IIA1.6 and BW5147 $\alpha^-\beta^-$. IIA1.6 is an FcγRII negative variant of the A20 B cell lymphoma (Bonnerot *et al.*, 1992). Cross-linking of the membrane IgG2a expressed on its surface triggers both an increase in intracellular calcium level and the activation of the interleukin-2 (IL-2) gene. BW5147 $\alpha^-\beta^-$ (hereafter referred to as BW^-) is a thymoma that fails to express most of the genes coding for the T cell antigen receptor/CD3 subunits (White *et al.* 1989; Wegener *et al.*, 1992). Expression of a single copy of the $(YXXL/I)_2$ motif suffices to confer to BW^- the capacity to produce IL-2 (Romeo *et al.*, 1992; Letourneur and Klausner, 1992a; A.M.K.Wegener and B.Malissen, unpublished data). IIA1.6 and BW^- transfectants expressing a CD8 wild-type molecule (CD8) were developed in parallel, and used to evaluate the possible contribution of the CD8-α segment present in the CD8/gp30 chimera. When analysed by flow cytofluorometry (Figure 2), the wild-type CD8 and CD8/gp30 molecules were readily detected at the surface of the corresponding BW^- and IIA1.6 transfectants. Examination of a number of independent transfectants showed that the levels of surface expression reached by the CD8/gp30 chimeras were on the average 5- to 10-fold lower than those observed with wild-type CD8 (Figure 2). This decrease in surface expression may result from possible retention/degradation signals present in the gp30 cytoplasmic tail (Letourneur and Klausner, 1992b). To establish whether the apparent molecular weight of the CD8/gp30 chimera corresponded to the one predicted by the sequence shown

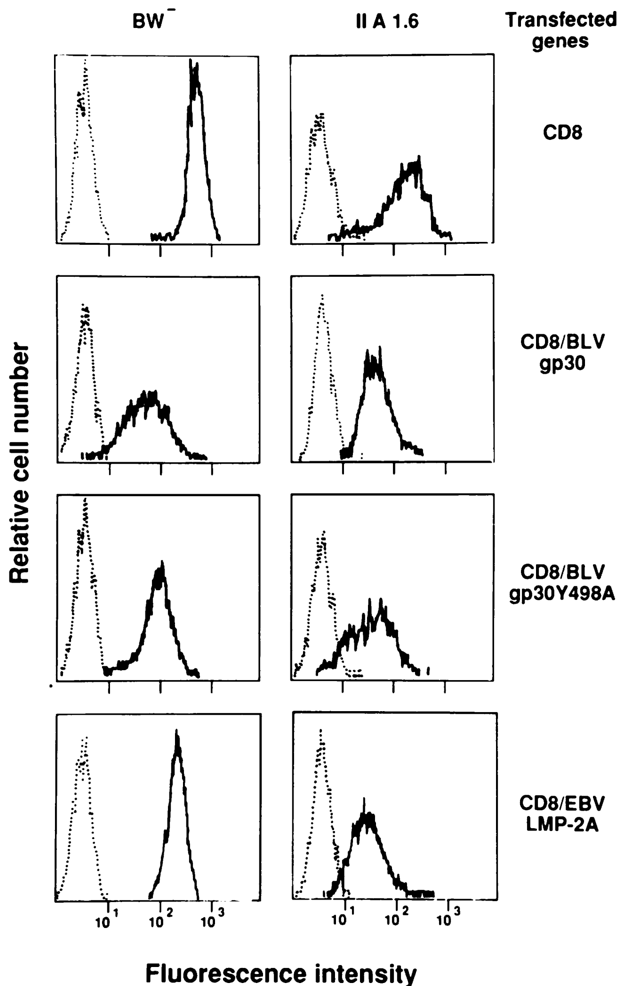


Fig. 2. Expression of the CD8 epitope at the surface of BW$^-$ and IIA1.6 cells following transfection of the CD8-α, CD8/BLVgp30, CD8/BLVgp30Y498A or CD8/EBV LMP2A gene constructs. The parental and transfected cell lines were analysed by flow cytometry after staining with the anti-CD8-α antibody H58-55.3 (solid lines). Each panel includes a negative control histogram obtained after staining with an anti-TCR Vβ10 antibody (dotted lines). The IIA1.6 samples showed similar levels of surface IgG after staining with FITC-conjugated rabbit anti-mouse IgG F(ab')$_2$ fragments (data not shown). Prior to transfection, the BW$^-$ and IIA1.6 recipient cells showed no staining above background with the anti-CD8-α antibody (data not shown).

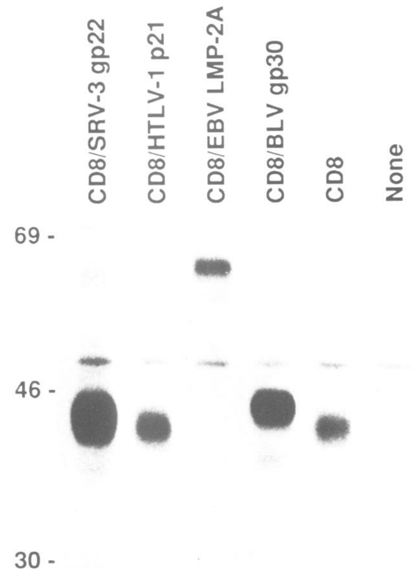


Fig. 3. Immunoprecipitation of the CD8 molecules and CD8 chimeras expressed by the various BW$^-$ transfectants. Lysates from cells biosynthetically labelled with [^{35}S]methionine (6 h) were incubated with the anti-CD8-α antibody 19/178. Immunoprecipitates were reduced and analysed on a SDS−10% polyacrylamide gel. The relative positions of the molecular mass markers (in kDa) are indicated.

in Figure 1, ^{35}S-labelled CD8/gp30 molecules were immunoprecipitated and analysed by SDS−PAGE under reducing conditions. As expected, immunoprecipitates from transfectants that received the CD8/gp30 gene displayed a single band, which migrated at a position slightly above the wild-type CD8-α polypeptide (Figure 3, compare lanes CD8 and CD8/gp30). The CD8/gp30 chimeras expressed on BW$^-$ and IIA1.6 cells were next tested for their ability to trigger IL-2 production in response to the anti-CD8 antibody 19/178. As shown in Figure 4, the CD8/gp30 chimera can transduce signals leading, in a dose-dependent manner, to IL-2 production in both BW$^-$ and IIA1.6 cells. In contrast, the wild-type CD8 molecule was unable to induce any detectable IL-2 production. None of the transfectants responded when cultured in microwells coated with an irrelevant anti-CD3-ϵ antibody, and all were capable of IL-2 production upon addition of phorbol 12-myristate 13-acetate and ionomycin (data not shown). Thus, the BLV gp30 cytoplasmic tail can function across species and induce cytokine production in both B and T cells.

Mutational analysis of the two (YXXL/I)$_2$ motifs present in the BLV gp30 cytoplasmic tail

Mutational analysis of the (YXXL/I)$_2$ motifs present in the CD3-ϵ and ζ subunits has shown that substitution of either of the two tyrosine or leucine/isoleucine residues abrogates their signalling capacity (Romeo *et al.*, 1992; Letourneur and Klausner, 1992a; A.M.K.Wegener and B.Malissen, unpublished results). The introduction of similar mutations in each of the two motifs identified in the BLV gp30 cytoplasmic segment should therefore allow analysis of their relative contribution to its signalling properties. Accordingly, the tyrosines 487, 498 and 508 were individually mutated to alanine to produce the mutants CD8/gp30Y487A, CD8/gp30Y498A and CD8/gp30Y508A (Figure 1B). Flow cytometry analysis showed that these mutated chimeras were properly expressed at the surface of BW$^-$ (Figure 2 and Table I) and IIA1.6 (Figure 2) cells. Despite levels of surface expression higher than those observed with CD8/gp30, the CD8/gp30Y498A chimera did not respond to anti-CD8 antibody (Table I). The results obtained with the other tyrosine and leucine mutants have been compiled in Table I. Substitution of residues Y487 or L490 resulted in the complete inactivation of the corresponding chimeras, whereas that of residues Y508 or L511 had no effect on IL-2 production. These data, together with those obtained with a CD8/gp30 shortened form (CD8/gp30s, Figure 1B and Table I), demonstrate that the functional domain of the BLV gp30 tail centres on the N-terminal (YXXL/I)$_2$ motif (denoted 1 in Figure 1B). However, in contrast to the observations made with the CD3-ϵ and ζ motifs (Romeo *et al.*, 1992; Letourneur and Klausner, 1992a), the L501A mutant was not drastically diminished in its signalling capacity. This differential requirement for a conserved C-

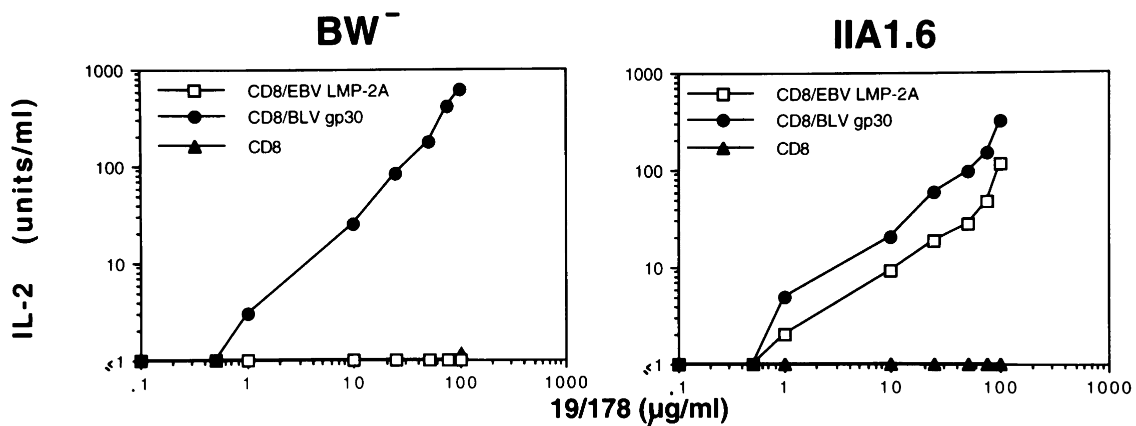
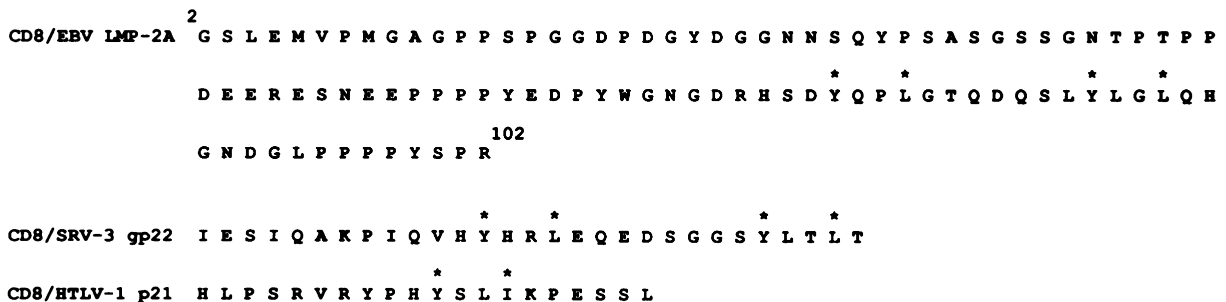


Fig. 4. IL-2 production of BW⁻ and IIA1.6 cells transfected with CD8-α, CD8/BLVgp30 or CD8/EBV LMP2A gene constructs in response to stimulation with anti-CD8-α antibodies. All the parental and transfected cells were capable of IL-2 production upon addition of phorbol 12-myristate 13-acetate and ionomycin (data not shown).

Table I. IL-2 secretion of BW⁻ cells transfected with CD8/HTLV-1 p21, CD8/SRV-3 gp22 or mutated CD8/BLV gp30 genes, in response to stimulation with antibodies directed to the CD8-α or CD3-ε molecules

	Mean fluorescence intensities after staining with antibodies specific for:			IL-2 secreted (U/ml) in response to stimulation with:		
Responder cells	CD8-α (H58-55.3)	Vβ 10 (B21.5)	CD45 (H129-326)	19/178 (CD8-α)	2C11 (CD3-ε)	PMA + ionomycin
BW⁻	3	3	199	<1	<1	512
BW⁻CD8	357	3	238	<1	<1	512
BW⁻CD8/gp30	43	3	181	256	<1	256
BW⁻CD8/gp30s	135	3	295	256	<1	256
BW⁻CD8/gp30Y487A	133	4	194	<1	<1	256
BW⁻CD8/gp30L490A	114	5	233	<1	<1	512
BW⁻CD8/gp30Y498A	56	3	194	<1	<1	128
BW⁻CD8/gp30L501A	23	6	157	32	<1	512
BW⁻CD8/gp30Y508A	47	3	319	256	<1	512
BW⁻CD8/gp30L511A	47	5	155	128	<1	256
BW⁻CD8/SRV-3 gp22	38	3	177	<1	<1	256
BW⁻CD8/HTLV-1 p21	171	5	203	<1	<1	128

Transfected cells (10^5) were cultured in microtitre wells precoated with 50 µl of a phosphate buffered saline solution containing 100 µg/ml of purified antibodies specific for either the CD8-α (19/178) or CD3-ε (2C11) molecules. After 24 h, the supernatants were assayed for IL-2 content. A concentration of 1 U/ml was the minimum detectable in our assay. All the transfected cells were capable of IL-2 production in response to the addition of PMA (phorbol 12-myristate 13-acetate, 10 ng/ml) and ionomycin (1 µM). The expression of the CD8 chimeric proteins at the surface of the various transfectants was monitored by flow cytometry using an antibody specific for the CD8-α polypeptide (H58-55.3). Background fluorescence levels are given after staining with an antibody specific for the product of the TCR Vβ10 gene segment. All cells showed similar bright staining with the anti-CD45 antibody H129-326.


CD8/EBV LMP-2A (2) G S L E M V P M G A G P P S P G G D P D G Y D G G N N S Q Y P S A S G S S G N T P T P P
D E E R E S N E E P P P P Y E D P Y W G N G D R H S D Y* Q P L* G T Q D Q S L Y* L G L* Q H
G N D G L P P P P Y S P R (102)

CD8/SRV-3 gp22 I E S I Q A K P I Q V H Y* H R L* E Q E D S G G S Y* L T L* T

CD8/HTLV-1 p21 H L P S R V R Y* P H Y S L I* K P E S S L

Fig. 5. Amino acid sequences of the cytoplasmic segments present in the CD8/EBV LMP2A, CD8/SRV-3 gp22 and CD8/HTLV-1 p21 chimeras. The YXXL/I sequences contained in each of these cytoplasmic segments are denoted by asterisks. Note that the $(YXXL/I)_2$ motif is found in only two out of the three SRV isolates analysed to date (Brody et al., 1992). The EBV LMP2A residues are numbered according to Sample et al. (1989).

Table II. IL-2 secretion of IIA1.6 cells transfected with mutated CD8/EBV LMP2A genes, in response to simulation with antibodies directed to the CD8-α or CD3-ϵ molecules

	Mean fluorescence intensities after staining with antibodies specific for:			IL-2 secreted (U/ml) in response to stimulation with:	
Responder cells	CD8-α (H58-5553)	Vβ10 (B21.5)	CD45 (H129-326)	19/178 (CD8-α)	2C11 (CD3-ϵ)
IIA1.6	3	3	144	<1	<1
IIA1.6 CD8	95	3	210	<1	<1
IIA1.6 CD8/EBV LMP2A	42	5	131	256	<1
IIA1.6 CD8/EBV LMP2A Y73A	74	3	153	<1	<1
IIA1.6 CD8/EBV LMP2A Y84A	23	3	175	<1	<1

Cells were analysed as described in the footnote to Table I.

terminal leucine residue may reflect some subtle differences in the functioning of the CD3 and BLV gp30 motifs.

The N-terminal cytoplasmic domain of the EBV LMP2A induces IL-2 production in B cells, but not in T cells

The structure of the EBV LMP2A has been predicted to consist of a 122 amino acid N-terminal domain, 12 putative transmembrane segments and a 27 amino acid C-terminal domain. The N- and C-termini are hydrophilic and both located on the cytoplasmic side of the plasma membrane (Laux *et al.*, 1988; Sample *et al.*, 1989; Longnecker and Kieff, 1990). The N-terminal domain contains a single copy of the $(YXXL/I)_2$ motif (Figure 5) and associates with intracellular tyrosine kinases (Burkhardt *et al.*, 1992). To determine whether the latter has a signalling function on its own, we constructed a cDNA which should direct the expression of a chimeric molecule composed of the extracellular and transmembrane segment of CD8-α and of most (101 out of 122 amino acids) of the N-terminal cytoplasmic sequence of LMP2A. To avoid assembling a synthetic gene of >300 nucleotides, we deliberately fused the N-terminal end of the LMP2A cytoplasmic sequence to the C-terminal end of the CD8-α transmembrane segment. Note that in this process, the 'floating' end of the LMP2A N-terminal cytoplasmic domain has been reversed. When analysed by flow cytofluorometry, the CD8/EBV LMP2A chimera was readily detected at the surface of the corresponding BW^- and IIA1.6 transfectants (Figure 2). As shown in Figure 3, under reducing condition, the CD8/EBV LMP2A chimeric protein was found to migrate as a single band with an apparent molecular weight consistent with its predicted sequence and the presence of glycosylation sites within the CD8 extracellular domain. Cross-linking of the CD8/EBV LMP2A chimera expressed in IIA1.6 cells elicited signals leading to IL-2 production (Figure 4). Despite the expression of a range of CD8 levels similar to or higher than those observed on the IIA1.6 transfectants (see Figure 2), none of the CD8/EBV LMP2A BW^- transfectants was able to respond to anti-CD8 cross-linking (Figure 4). Thus, in contrast to the CD8/gp30 chimera, the CD8/EBV LMP2A chimera was unable to elicit IL-2 production in T cells.

Mutational analysis of the $(YXXL/I)_2$ motif present in the N-terminal cytoplasmic domain of the EBV LMP2A

The seven Tyr residues contained in the LMP2A segment present in the CD8/EBV LMP2A chimera may allow it to interact with multiple SH2 domain-containing intracytoplasmic effectors (e.g. lyn, fyn or a 70 kDa protein tyrosine kinase with characteristics similar to those of syk; Burkhardt *et al.*, 1992; Miller *et al.*, 1993; Songyang *et al.*, 1993). As shown in Figure 5, two of these Tyr residues (Y73 and Y84) are part of a $(YXXL/I)_2$ motif. To determine whether this motif was responsible for the ability of the N-terminal cytoplasmic domain of LMP2A to trigger cytokine production, the tyrosine residues Y73 and Y84 were separately mutated to alanine to produce the mutants CD8/EBV LMP2AY73A and CD8/EBV LMP2AY84A. As summarized in Table II, IIA1.6 transfectants expressing either of the two mutated chimeras were completely unresponsive to cross-linking with anti-CD8 antibodies. Thus, these results suggest that the single $(YXXL/I)_2$ motif present in the N-terminal domain of LMP2A accounts for its ability to elicit a late B cell activation event.

Expression of CD8/EBV LMP2A did not block the increase in cytoplasmic free calcium induced by membrane Ig cross-linking

In most IIA1.6 transfected cells, cross-linking of the CD8/BLV gp30 or CD8/EBV LMP2A chimeric proteins with anti-CD8 antibodies induced a fast rise in intracellular calcium levels which then remained elevated for several minutes (Figure 6, top left and top middle panels). The calcium signals induced through the chimeras and those triggered by cross-linking membrane immunoglobulins (Ig) involved both calcium release from internal stores and transmembrane influx of extracellular calcium (data not shown). Note, however, that the traces observed with both chimeras returned to baseline levels with slower kinetics than those observed upon membrane Ig cross-linking. As expected, no significant increase in cytoplasmic free calcium was seen upon stimulation of IIA1.6 cells expressing wild-type CD8 molecules (Figure 6, top right panel). Therefore, chimeric transmembrane molecules carrying the cytoplasmic sequence of either BLV gp30 or EBV LMP2A can transduce early activation signals upon antibody-mediated cross-linking.

Expression of LMP2A in an EBV-negative Burkitt lymphoma cell line (BJAB) was recently shown to block the rise in intracellular free calcium which normally follows cross-linking of membrane Ig (Miller *et al.*, 1993). These results suggested that LMP2A diverted some essential components of the signal transduction pathway operated by the membrane Ig, and led to the view that LMP2A constitutes a negative regulator of signal transduction in B lymphocytes

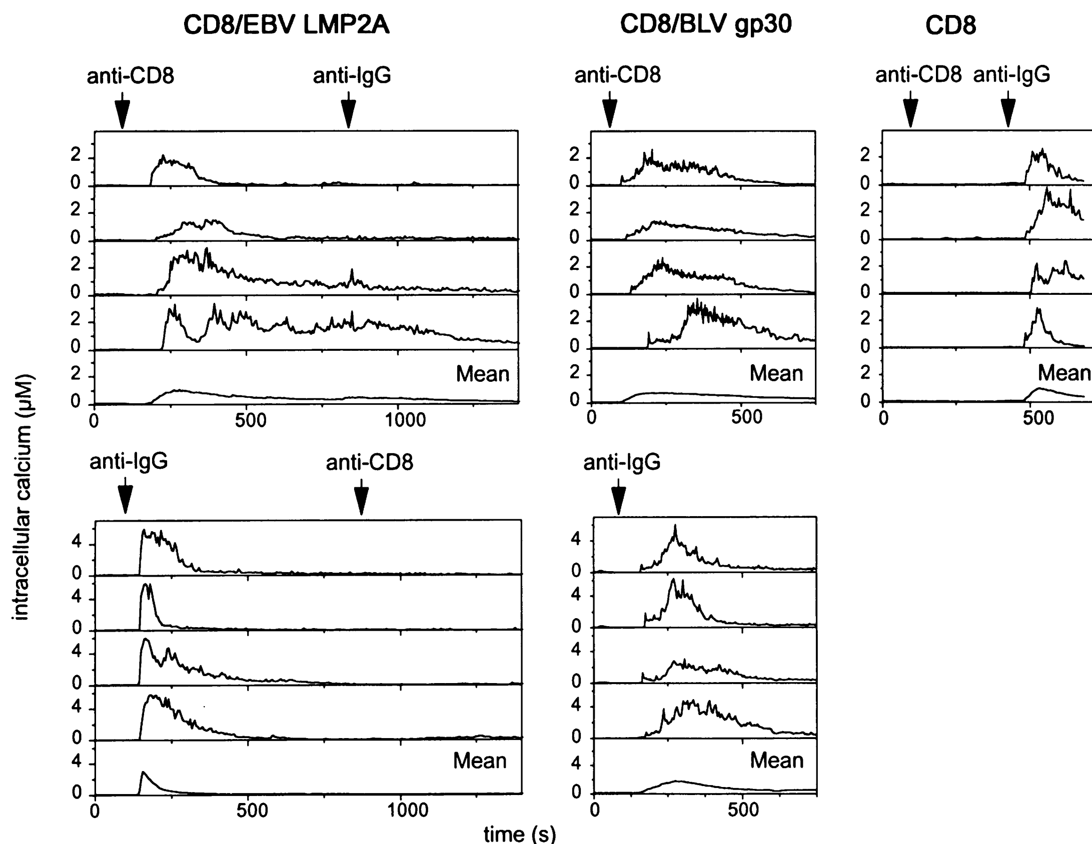


Fig. 6. Increase of intracellular free calcium ion in IIA1.6 CD8/EVB LMP2A, IIA1.6 CD8/BLV gp30 and IIA1.6 CD8 transfectants after cross-linking with anti-CD8 (H58-55.3) or anti-mouse IgG antibodies. Antibodies were added at the indicated times (arrows) and changes in the concentration of intracellular free calcium were recorded per single cell as described in Materials and methods. For each experiment, the traces corresponding to 30–100 cells have been averaged and represented in the bottom trace of each panel (mean).

(Miller *et al.*, 1993). Likewise, we determined whether the LMP2A segment present in the CD8/EBV LMP2A chimera was capable of shutting off membrane Ig signalling. As shown in Figure 6, bottom left panel, expression of the CD8/EBV LMP2A chimera by itself interfered neither with the increase in cytoplasmic free calcium induced by anti-Ig antibodies, nor with the percentage of cells responding to anti-Ig cross-linking. Similarly, transfectants expressing the CD8/BLV gp30 (Figure 6, bottom right panel) or CD8 wild-type (Figure 6, top right panel) molecules responded normally following cross-linking of membrane Ig. Thus, our results suggest that the N-terminal cytoplasmic segment present in the CD8/LMP2A chimera does not block the early signals induced by membrane Ig cross-linking. In contrast, responses elicited through membrane Ig and CD8/EBV LMP2A showed a full cross-desensitization (Figure 6, top and bottom left panels) as normally observed when receptors share common transducing pathways.

The cytoplasmic segment of the HTLV-1 and SRV-3 envelope proteins are unable to trigger IL-2 production

In view of the above data, we performed a similar analysis of the cytoplasmic segment of the human T cell leukaemia virus type I (HTLV-1) and simian retrovirus-3 (SRV-3 envelope proteins; Figure 5). SRV-3 (also called Mason–Pfizer monkey virus) is a retrovirus which causes an acquired immune deficiency in macaques and expresses a single $(YXXL/I)_2$ motif in the transmembrane subunit (gp22) of its envelope protein (Sonigo *et al.*, 1986; Brody *et al.*, 1992). HTLV-1 is a human retrovirus which shares a common life-style with BLV (Cann and Chen, 1990) and contains a single YXXI sequence in the transmembrane subunit (p21) of its envelope protein (Seiki *et al.*, 1983). Despite these similarities, both the CD8/SRV-3 gp22 and CD8/HTLV-1 p21 chimeras (Figure 3) were unable to induce IL-2 production when expressed in BW^- (Table I) or IIA1.6 (data not shown) cells. It should be noted that the two YXXL sequences present in the SRV-3 gp22 motif are spaced by eight amino acids and thus resemble one of the functional motifs (denoted as ζb in Wegener *et al.*, 1992) found in the CD3-ζ polypeptide. Therefore, based on the comparative analysis of the CD8/SRV-3 gp22 and CD8/ζb chimeras, we conclude that the presence of two properly spaced YXXL cytoplasmic sequences is not sufficient for IL-2 gene induction.

Discussion

We have constructed chimeric molecules by replacing the cytoplasmic tail of CD8-α with that of either BLV gp30 or EBV LMP2A. After expressing each of them in a B cell lymphoma, we have shown that they are capable of triggering both Ca^{2+} mobilization and IL-2 production when cross-linked with an antibody to CD8-α. When this manuscript was in preparation, a report appeared in which similar CD8/BLV gp30 and CD8/EBV LMP2A constructs were expressed in a mouse B lymphoma (K46) and found capable

of inducing both Ca^{2+} mobilization and tyrosine phosphorylation of various intracellular substrates (Alber *et al.*, 1993). Our experiments have extended these observations to a late activation event (e.g. cytokine production) and unequivocally demonstrated that its induction involved $(YXXL/I)_2$ motifs similar to those found in the transducing subunits of the B and T cell antigen receptors. In contrast to the CD8/BLV gp30 motif, the one present in CD8/EBV LMP2A was unable to induce IL-2 production in T cells. This difference may reflect the fact the functional $(YXXL/I)_2$ motif present in the BLV gp30 cytoplasmic segment is more promiscuous and capable of recruiting both B and T cell specific intracytoplasmic effectors.

At present we can only speculate as to the *in vivo* importance of the signalling properties of $(YXXL/I)_2$ motifs found in the BLV gp30 and EBV LMP2A cytoplasmic segments. For instance, upon interaction with its cellular receptor or the anti-envelope antibodies which persist throughout infection (Burny *et al.*, 1987), the BLV envelope proteins expressed at the host cell surface may aggregate and trigger the activation and proliferation of infected B cells. The resulting increase in the pool of infected B cells may augment the spread of viral infection to a new host. Equally, upon attachment and fusion to a B cell, the BLV virion releases close to 100 envelope proteins into the B cell membrane (Ozel *et al.*, 1988). After aggregation, these envelope proteins may promote B cell activation and proliferation, and consequently permit proviral integration (Demotz *et al.*, 1992; Coffin, 1990). Similarly, after associating with LMP1 in the plasma membrane, LMP2A may trigger the production of cytokines and synergize with LMP1 to constitutively activate human B lymphocytes (Henderson *et al.*, 1991). In this context, it should be noted that the N-terminal cytoplasmic domain of LMP2A associates with members of the src tyrosine kinase family (Burkhardt *et al.*, 1992), and is likely to intersect the same intracellular signalling pathways as the ones operated by the Ig-α and Ig-β subunits of the B cell antigen receptor (Taniguchi *et al.*, 1991; Clark *et al.*, 1992; Hutchcroft *et al.*, 1992; Kim *et al.*, 1993). Using second site homologous recombination, EBV mutants were constructed with a deletion of most of the LMP2A N-terminal cytoplasmic domain (Longnecker *et al.*, 1992). Surprisingly, this N-terminal domain was found to be non-essential to initiate and maintain primary B lymphocyte growth transformation *in vitro*. However, LMP2A is expressed in all infected B lymphocytes *in vivo* (Qu and Rowe, 1992) and thus likely to be important during EBV infection. For instance, human B lymphocytes have recently been shown to secrete large amounts of human interleukin-10 (IL-10) after EBV infection (Burdin *et al.*, 1993). This property, which is yet to be formally assigned to the $(YXXL/I)_2$ motif present in the N-terminal domain of LMP2A, may suppress the antiviral arms of the immune system and contribute towards initiating and maintaining latent infection *in vivo*. Therefore, the $(YXXL/I)_2$ signalling motifs found in the BLV gp30 and EBV LMP2A cytoplasmic segments may represent additional examples of the many strategies that viruses have evolved to manipulate the host immune system (Ben-David and Bernstein, 1991; Gooding, 1992). Finally, our data should prompt the search for signalling motifs in the unusually long intracytoplasmic domains found in the envelope proteins of lentiviruses (Cohen *et al.*, 1992).

Materials and methods

Cells

BW5147 $\alpha^-\beta^-$ is a variant of the BW5147 thymoma lacking functional TCRα and TCRβ chain genes (White *et al.*, 1989). IIA1.6 is an FcγRII negative variant of the A20 B cell lymphoma (Bonnerot *et al.*, 1992).

Plasmid construction

Polymerase chain reaction (PCR) was used to amplify a segment of a mouse CD8-α cDNA clone corresponding to the extracellular and transmembrane domains of the CD8-α polypeptide and cDNA segments corresponding to the cytoplasmic tail of the BLV gp30, EBV LMP2A, SRV-3 gp22 or HTLV-1 p21 proteins. Primers corresponding to the 3′-most sequences of the CD8 fragment and the 5′-most sequences of the various viral polypeptides were designed to overlap such that annealing of each pair of PCR products yielded a hybrid template (Wegener *et al.*, 1992). From these templates, chimeric cDNA were amplified using external primers containing restriction sites compatible with the ones present in the vector pSRα-neo. The expression vector pSRα-neo was designed both to select stable transfectants in the presence of G418-sulfate and to place the various cDNA constructs under the control of the SV40 early promoter and part of the long terminal repeat of HTLV-1 (Takebe *et al.*, 1988). The sequence of the various PCR products was confirmed using the dideoxy-chain termination method. The tyrosine and/or leucine residues present in the EBV LMP2A $(YXXL/I)_2$ motif and the two BLV gp30 $(YXXL/I)_2$ motifs were changed to alanine via PCR. Each of the mutated cDNAs was sequenced and cloned into pSRα-neo.

Transfection by protoplast fusion

Transfection of BW 5147 $\alpha^-\beta^-$ and IIA1.6 cells with protoplasts and selection in the presence of G418-sulfate were performed as described (Wegener *et al.*, 1992).

Cytofluorometric analysis

Transfected cells were stained with saturating concentrations of rat antibodies, washed twice and incubated with FITC-conjugated mouse anti-rat IgG $F(ab')_2$ fragments (Jackson). After washing, samples were analysed with a FACScan flow cytometer.

19/178 and H58-55.3 are two anti-CD8-α antibodies (Blanc *et al.*, 1988). 145-2C11 (2C11) is a hamster anti-mouse CD3-ε antibody (Leo *et al.*, 1987). H129-326 is an anti-CD45 antibody (Pierres *et al.*, 1984). B21.5 is an antibody specific for the product of the TCR Vβ10 gene segment (Necker *et al.*, 1992).

Stimulation of transfectants with antibodies

Cells (10^5) were cultured in microtitre wells coated with the indicated concentrations of a purified anti-CD8-α antibody (19/178). After 24 h, supernatants were assayed for interleukin-2 (IL-2) content (Wegener *et al.*, 1992).

Metabolic labelling, immunoprecipitation and SDS–PAGE

These were all performed essentially as described (Wegener *et al.*, 1992).

Recording of intracellular Ca^{2+} levels

Digital calcium imaging experiments were performed on fura-2 loaded cells. Fura-2 was added to IIA1.6 cells (10^6/ml) at a final concentration of 0.25 μM for 15 min at 37°C. Cells were then stuck on poly-lysine coated glass coverslips and placed on a 300 μl recording chamber containing Ringer medium (140 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, 10 mM glucose and pH adjusted to 7.2). An epifluorescence inverted microscope (Nikon Diaphot, les Ullis, France) equipped with 20× and 40× objectives was used for recording. Images were collected through a 510 nm interference filter with an intensified CCD camera (Hamamatsu, Paris, France), during alternate excitation at 340 and 380 nm produced by a rotating filter wheel (Sutter instruments, Novato, CA) placed in the light path of a xenon lamp. An image processor (ETM systems, Irvine, CA) averaged and stored ratio 340/380 images on line until analysis by the Vprobe software. Usually eight consecutive images (33 ms per image) were averaged at each wavelength, and one ratio stored every 2 or 5 s. Calibrations were performed as described (Choquet *et al.*, 1993). The ratio R varied from ±0.4 (low Ca^{2+} levels) to ±10 (high Ca^{2+} levels) with an apparent dissociation constant for Ca^{2+} binding to fura-2 of 2.5 μM. Calcium mobilization by the CD8/BLV gp30 and CD8/EBV LMP2A did not require additional cross-linking of the primary anti-CD8 antibodies.

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