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The Transmembrane Domains of the EBV-Encoded Latent Membrane Protein 1 (LMP1) Variant CAO Regulate Enhanced Signalling Activity

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Sequence variants of the Epstein–Barr virus (EBV)-encoded latent membrane protein-1 (LMP1) have been reported in association with EBV-linked malignancies but little is known about their effects on signalling pathways and phenotype. We have examined the ability of the nasopharyngeal carcinoma (NPC)-derived variant, CAO-LMP1 to activate the transcription factors NF-κB and AP-1 in epithelial cells. In this study, transient expression of CAO-LMP1 was found to activate higher levels of NF-κB and AP-1 than the prototype B95.8-LMP1 in human embryonic kidney (HEK) 293 cells and SV40-transformed keratinocytes (SVK). In addition, pulse–chase analysis revealed that CAO-LMP1 has a longer half-life than B95.8-LMP1. Chimera studies localised these phenomena to the transmembrane domains of CAO-LMP1, suggesting that this enhanced signalling capacity may be a consequence of its prolonged half-life. The ability of CAO-LMP1 to activate higher levels of NF-κB and AP-1 may contribute to its potent transforming properties.

Key Words: LMP1; CAO; NF-κB; AP-1.

INTRODUCTION

Epstein–Barr virus (EBV), a gamma herpes virus, is associated with a number of malignancies, including Burkitt's lymphoma, nasopharyngeal carcinoma (NPC), and Hodgkin's disease (Rickinson and Kieff, 1996). A subset of viral genes is expressed when EBV transforms resting B lymphocytes into lymphoblastoid cell lines (LCLs), including latent membrane protein-1 (LMP1). LMP1 is a 63-kDa integral membrane protein, consisting of a short highly charged cytoplasmic amino terminus of 23 aa, 6 hydrophobic transmembrane domains required for protein aggregation, and a long acidic cytoplasmic carboxyl-terminus of 200 aa (Fennewald *et al.,* 1984). The carboxyl-terminus comprises two functional domains: the membrane proximal CTAR1 (residues 194–232 also referred to as TES1) and the membrane distal CTAR2 (residues 351–386 also referred to as TES2) (Huen *et al.,* 1995) through which LMP1 activates several signalling pathways.

Compelling evidence for the importance of LMP1 in B cell transformation was provided by EBV recombinant genetic analysis in which recombinant EBV expressing mutated LMP1 was non-transforming (Kaye *et al.,* 1993). The amino terminus, transmembrane domains and CTAR1 are critical for this process (Izumi *et al.,* 1997) whilst CTAR2 is required for long-term outgrowth of the transformed B cells (Izumi and Kieff, 1997; Kaye *et al.,* 1995). The residues between CTAR1 and CTAR2 (aa 232–351) are dispensable for initial B cell transformation and maintenance of long-term outgrowth (Izumi *et al.,* 1999). In addition, LMP1 has the ability to growth transform rodent fibroblasts in culture and is thus considered an oncogene (Wang *et al.,* 1985).

In B cells, in the absence of other EBV gene products, LMP1 expression results in upregulation of the cell surface markers, CD21, CD23, CD39, CD40, CD54, LFA-1, and LFA-3 (Wang *et al.,* 1988, 1990), and also protects these cells from apoptosis by the induction of bcl-2 (Henderson *et al.,* 1991), Mcl-1 (Wang *et al.,* 1996) and A20 (Laherty *et al.,* 1992). In epithelial cells, LMP1 upregulates CD40, CD54, and A20 expression (Huen *et al.,* 1995; Miller *et al.,* 1997) and inhibits differentiation (Dawson *et al.,* 1990) and cell growth (Eliopoulos *et al.,* 1996; Hammerschmidt et al., 1989; Kaykas and Sugden, 2000).

The membrane-proximal CTAR1 domain contains a PxQxT motif (residues 204–208) similar to that found in CD40 and TNF-R2, and it is through this motif that CTAR1 binds TRAFs 1, 2, 3, and 5 (Brodeur *et al.,* 1997; Mosialos *et al.,* 1995; Devergne *et al.,* 1996; Sandberg *et al.,* 1997). CTAR2 was identified as the major NF-κB activation domain (Huen *et al.,* 1995; Mitchell and Sudgen, 1995) and this NF-κB activation mapped to the far C terminus, residues 379–384 (Floettmann and Rowe, 1997). Although CTAR2 is unable to bind TRAFs directly, a dominant negative TRAF2 molecule partially inhibits CTAR2-mediated NF-κB activation, suggesting it may interact indirectly (Eliopoulos *et al.,* 1997; Kaye *et al.,* 1996). CTAR2 is able to bind the TNF receptor-associated death domain protein (TRADD), which augments NF-κB activation by LMP1 (Eliopoulos *et al.,* 1999a; Izumi and Kieff, 1997). Downstream of TRAF2, NF-κB activation from both CTAR domains is mediated through NF-κB-inducing ki-

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nase (NIK) (Eliopoulos *et al.,* 1999a; Sylla *et al.,* 1998). Other signalling pathways activated by LMP1 include the c-Jun amino terminal kinase (JNK) pathway. LMP1-induced JNK activation is mediated entirely by CTAR2 (Eliopoulos and Young, 1998; Kieser *et al.,* 1997) and involves both TRADD and TRAF2 (Eliopoulos *et al.,* 1999a; Eliopoulos and Young, 1998). LMP1 also activates the extracellular signal regulated kinase (ERK) pathway in a ras-dependent manner (Roberts and Cooper, 1998), the p38 MAPK cascade via both CTAR1 and CTAR2 (Eliopoulos *et al.,* 1999b) and JAK3 resulting in the activation of the STAT1 transcription factor, which was maps to the repeat region of LMP1 (Gires *et al.,* 1999).

LMP1 is similar to growth factor receptors in that it is turned over rapidly. Pulse–chase analysis has shown that it has a half-life of between 2 and 5 h, depending on the cell type (Baichwal and Sugden, 1987; Mann and Thorley-Lawson, 1987; Moorthy and Thorley-Lawson, 1990). Recent work has identified amino terminal ubiquitination followed by proteasomal degradation as the mechanism by which LMP1 is turned over (Aviel *et al.,* 2000).

LMP1 sequence variants have been reported in association with EBV-linked malignancies including NPC. The CAO-LMP1 variant was cloned from the genomic library of a nude mouse-propagated Chinese NPC and, compared to B95.8-LMP1, has nucleotide substitutions located mostly in the amino terminus and transmembrane domains, three additional 33-bp repeats in the cytoplasmic carboxyl-terminus, and a 30-bp deletion proximal to the start of CTAR2 (Hu *et al.,* 1991). This LMP1 variant is more tumourigenic than prototype B95.8-LMP1 in keratinocytes (Hu *et al.,* 1993) and less immunogenic (Trivedi *et al.,* 1994). Analysis of LMP1-activated signalling pathways in lymphoid cell lines found that CAO-LMP1 activated higher levels of NF-κB than B95.8-LMP1 but paradoxically was impaired in its ability to upregulate phenotypic markers (Johnson *et al.,* 1998). This study demonstrates that CAO-LMP1 confers enhanced NF-κB and AP-1 signalling in epithelial cells and implicates increased protein stability in these phenomena.

RESULTS

Analysis of B95.8 and CAO-LMP1 sequence

DNA sequencing of pSG5 B95.8-LMP1 (Huen *et al.,* 1995) and pSG5 CAO-LMP1 (Johnson *et al.,* 1998) confirmed the published sequences for B95.8-LMP1 (Baer *et al.,* 1984) but not for CAO-LMP1 (Hu *et al.,* 1991). In contrast to the published sequence (Hu *et al.,* 1991), changes were not found at amino acids 7, 8, 11, 12, in agreement with a recent study (Johnson *et al.,* 1998), and 120, which were identical to those of the B95.8 sequence. Two additional changes were found, one in the transmembrane domains at amino acid 122, a leucine instead of isoleucine, and the other in the carboxyl-terminus at amino acid 337, an asparagine instead of serine. A schematic representation of these molecules is shown in Fig. 1.

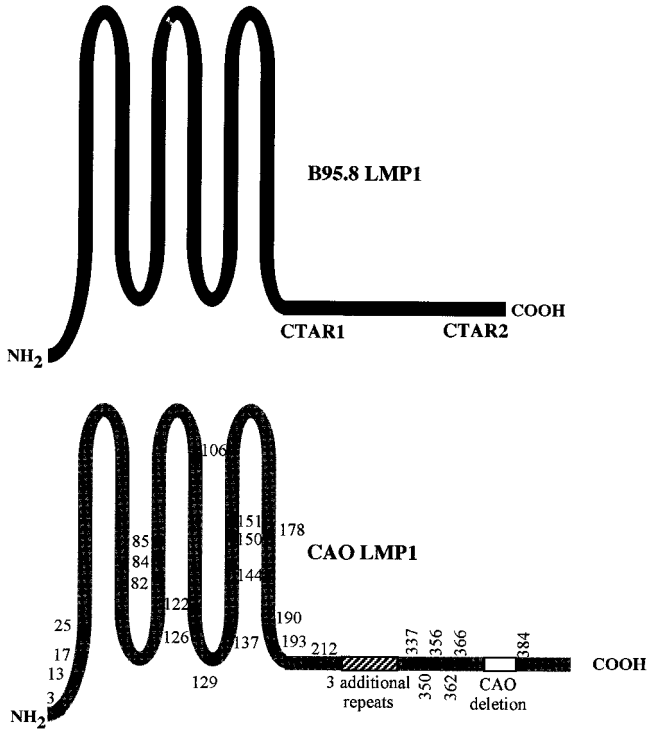

FIG. 1. Schematic representation of prototype B95.8 and NPC-derived CAO-LMP1. Schematic representation of B95.8 and CAO-LMP1. The cytoplasmic carboxyl-terminus comprises two functional domains, known as C-terminal activating region 1 (CTAR1, aa 194–232) and CTAR2 (aa 351–386). The region linking CTAR1 and CTAR2 contains several repeating motifs. The amino acids in CAO-LMP1 that were found to differ from B95.8-LMP1 are denoted by black circles and their position shown. CAO-LMP1 also contains three additional repeats and a 10 aa deletion in the cytoplasmic carboxyl-terminus compared to B95.8-LMP1.

CAO-LMP1 activates higher levels of NF-κB and AP-1 than B95.8-LMP1

The effect of CAO-LMP1 on cellular signalling pathways has been studied transiently in lymphoid cells and in stably expressing epithelial cells with interesting results. In transiently transfected lymphoid cells, CAO-LMP1 activates higher levels of NF-κB but is impaired in its ability to upregulate cell surface markers (Johnson *et al.,* 1998). In stably expressing epithelial cells, CAO-LMP1 activates comparable levels of NF-κB to B95.8 but is impaired in its ability to activate AP-1 and cell surface markers (Dawson *et al.,* 2000). To examine the transient effects of B95.8 and CAO-LMP1 on NF-κB in epithelial cells, HEK 293 and SVK cells were transiently transfected with 5 μg pSG5 or pSG5-based LMP1 constructs in the presence of a NF-κB luciferase reporter plasmid. Thirty-six hours posttransfection, cells were harvested and lysates analysed for LMP1 expression by immunoblotting with the CS.1–4 monoclonal antibodies (Rowe *et al.,* 1987), and NF-κB activity by reporter and electrophoretic

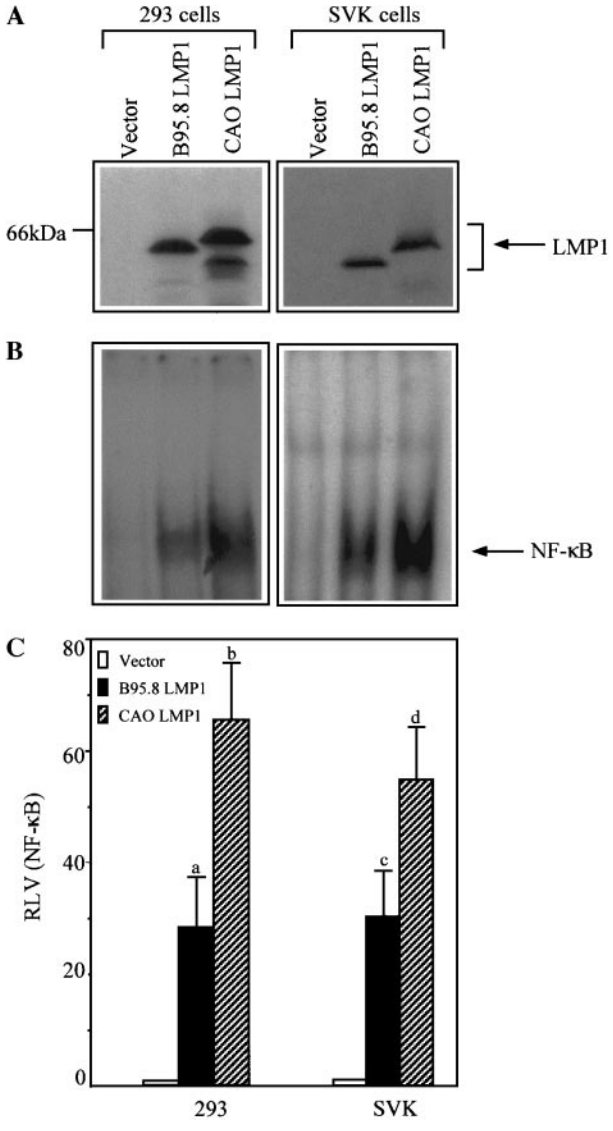

FIG. 2. NF-κB activation by the LMP1 variants B95.8 and CAO. HEK 293 and SVK cells were transfected with 5 μg pSG5 or pSG5-based LMP1 constructs in the presence of a NF-κB luciferase reporter plasmid (κBconA-Luc) and equivalent amounts of a β-galactosidase expression vector (CMV-β-gal). Thirty-six hours posttransfection, cells were harvested and samples were analysed for LMP1 expression by immunoblotting and NF-κB activity by reporter assay and EMSA. (A) Immunoblot of a representative experiment showing LMP1 expression in transiently transfected HEK 293 cells (left) and SVK cells (right) using the CS.1–4 monoclonal antibodies. (B) NF-κB binding assay in 293 cells (left) and SVK cells (right) transiently transfected with 5 μg pSG5 or pSG5-based LMP1 constructs. Thirty-six hours posttransfection, nuclear extracts were isolated and examined for NF-κB binding activity using a ^{32}P-labeled HIV-κB probe in EMSAs. (C) NF-κB reporter assay in 293 and SVK cells transfected with 5 μg pSG5 or pSG5-based LMP1 constructs in the presence of κBconA-Luc and CMV-β-gal. Relative luciferase values [RLV (NF-κB)] were calculated as the ratio of luciferase versus β-galactosidase activity and results are depicted as means ± standard deviation of four independent experiments. RLV were compared using the Student's *t* test: a/b $P < 0.01$, c/d $P < 0.05$.

mobility shift assays (EMSAs). Figure 2A shows representative immunoblots, which verify that CAO-LMP1 has a higher molecular weight than B95.8-LMP1, and shows CAO-LMP1 is expressed at marginally higher levels in both cell lines. Interestingly, CAO-LMP1 was found to induce higher levels of NF-κB binding by EMSA (Fig. 2B) and significantly higher levels of NF-κB activation by luciferase reporter assay (Fig. 2C) in both HEK 293 and SVK cells. To compare the effects of B95.8 and CAO-LMP1 on AP-1 in epithelial cells, HEK 293 cultures were transiently transfected with 5 μg pSG5 or pSG5-based LMP1 constructs in the presence of an AP-1 luciferase reporter plasmid. Thirty-six hours posttransfection, cells were harvested and lysates analysed for AP-1 activity by EMSA and reporter assay. Compared with B95.8-LMP1, CAO-LMP1 was consistently found to induce higher levels of AP-1 binding by EMSA (Fig. 3A) and higher levels of AP-1 activation by luciferase reporter assays (Fig. 3B)

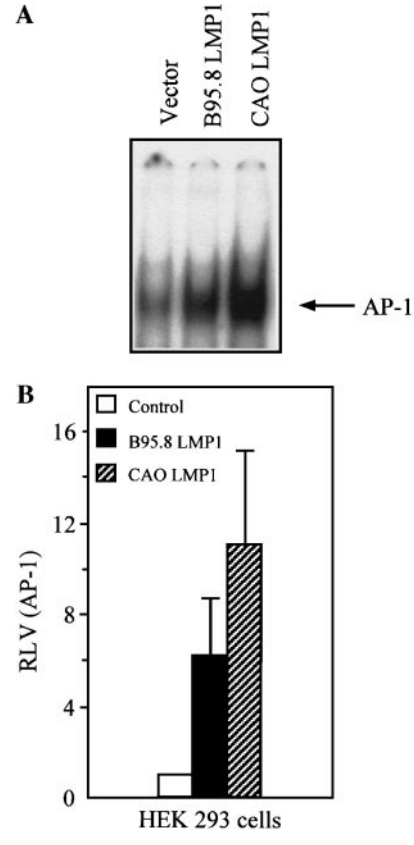

FIG. 3. AP-1 activation by the LMP1 variants B95.8 and CAO. HEK 293 cells were transfected with 5μg pSG5 or pSG5-based LMP1 constructs in the presence of an AP-1 luciferase reporter plasmid and equivalent amounts of CMV-β-gal. Thirty-six hours posttransfection, cells were harvested and samples were analysed for AP-1 activity by reporter assay and EMSA. (A) AP-1 reporter assay in HEK 293 cells transfected with 5 μg pSG5 or pSG5-based LMP1 constructs in the presence of AP-1 reporter and CMV-β-gal. Relative luciferase values [RLV (AP-1)] were calculated as the ratio of luciferase *vs* β-galactosidase activity, and results are depicted as mean ± standard deviation of three independent experiments. (B) AP-1 binding assay in 293 cells transiently transfected with 5 μg pSG5 or pSG5-based LMP1 constructs. Thirty-six hours posttransfection, nuclear extracts were isolated and examined for AP-1 binding activity using a ^{32}P-labeled collagenase TRE probe in EMSAs.

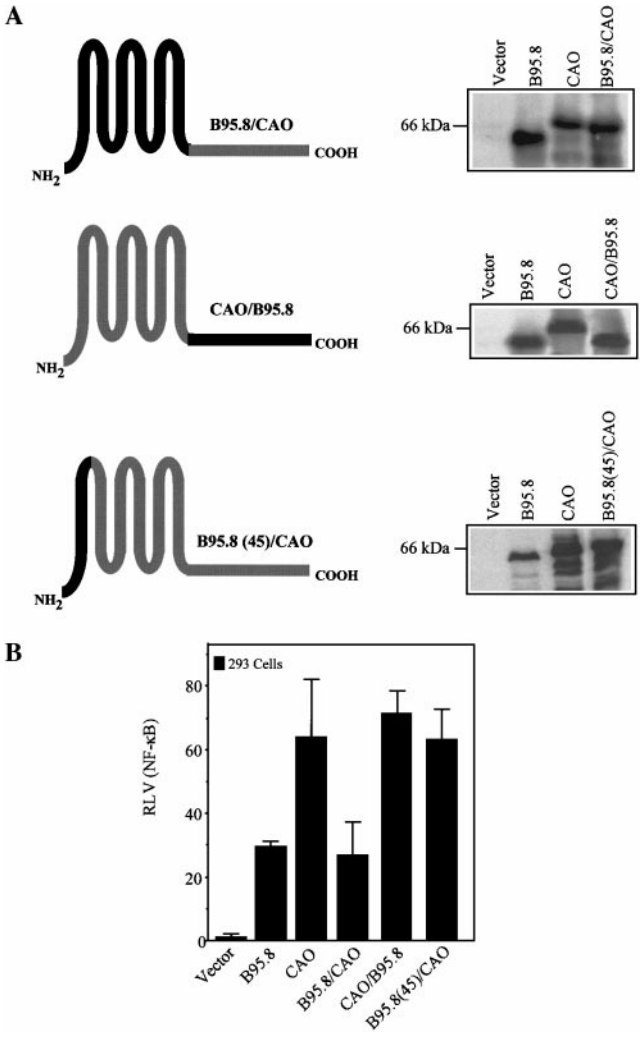

FIG. 4. NF-κB activation by B95.8 and CAO-LMP1 chimeras. (A) Schematic representation of B95.8 and CAO-LMP1 chimeras generated in this study. Solid black lines represent B95.8-LMP1 sequence and dotted lines denote CAO-LMP1 sequence. HEK 293 cells were transfected with 5 μg pSG5 or pSG5-based LMP1 wild-type and chimera constructs in the presence of a NF-κB luciferase reporter plasmid (κBconA-Luc) and equivalent amounts of a β-galactosidase expression vector (CMV-β-gal). Thirty-six hours posttransfection, cells were harvested, and samples were analysed for LMP1 expression by immunoblotting (A) and NF-κB activity by reporter assays (B). Relative luciferase values [RLV (NF-κB)] were calculated as the ratio of luciferase *vs* β-galactosidase activity and results are depicted as means ± standard deviation of three independent experiments.

in HEK 293 cells although the difference was not statistically significant.

Enhanced NF-κB activation by CAO-LMP1 localises to the transmembrane domains

To map the domain of CAO-LMP1 responsible for enhanced signalling activity, a series of chimeras were constructed that are schematically represented in Fig. 4A. Generation of chimeric molecules rather than deletion mutants was chosen since large deletions may alter the conformation of the protein and thus the association of adapter proteins and downstream signalling pathways. HEK 293 cells were transiently transfected with 5 μg pSG5 or pSG5-based LMP1 wild-type and chimera constructs in the presence of 50 ng κBconA-Luc and 50 ng CMV-β-gal. Cells were harvested 36 h posttransfection and the lysates subjected to immunoblotting and reporter assays. Immunoblot analysis demonstrated that, as expected, B95.8/CAO-LMP1 and B95.8(45)/CAO-LMP1 had molecular weights identical to CAO-LMP1 and CAO/B95.8-LMP1 had a molecular weight identical to B95.8-LMP1 (Fig. 4A). Luciferase reporter assays showed that B95.8/CAO behaved in a similar manner to B95.8-LMP1, whilst the CAO/B95.8 and B95.8(45)/CAO behaved in a similar manner to CAO-LMP1 with respect to NF-κB activation (Fig. 4B), thus implicating sequences within the transmembrane domains as responsible for increased NF-κB activation by CAO-LMP1.

CAO-LMP1 has a prolonged half-life

LMP1 is similar to growth factor receptors in that it is turned over rapidly, and pulse–chase analysis has shown that it has a half-life of between 2 and 8 h, depending on the cell type (Baichwal and Sugden, 1987; Mann and Thorley-Lawson, 1987; Martin and Sugden, 1991a,b; Moorthy and Thorley-Lawson, 1990). Deletion analysis identified the amino terminus and transmembrane domains as important in regulating the turnover of B95.8-LMP1 (Martin and Sugden, 1991b). To determine whether CAO-LMP1's ability to activate higher levels of NF-κB than B95.8-LMP1 could be explained by a difference in half-life, pulse–chase analysis was performed in HEK 293 cells transiently transfected with 5 μg pSG5-based LMP1 constructs. Figure 5 (top) shows a representative experiment of HEK 293 cells transiently transfected with 5 μg pSG5 B95.8-LMP1 or pSG5 CAO-LMP1 with pulse–chase analysis at 0, 2, 4, 6, and 10 h. Following autoradiography, half-life was quantified by laser densitometry and plotted as a percentage of the original signal (bottom). In three independent experiments, B95.8-LMP1 was found to have a half-life of 2.9 ± 0.6 h, consistent with the half-life of B95.8-LMP1 reported in other cell types (Baichwal and Sugden, 1987; Mann and Thorley-Lawson, 1987; Martin and Sugden, 1991a,b). However CAO-LMP1 was found to have an extended half-life of 7.25 ± 1.78 h. To determine the region of CAO-LMP1 responsible for this difference in half-life, HEK 293 cells were transiently transfected with 5 μg pSG5 B95.8/CAO (Fig. 6A), CAO/B95.8 (Fig. 6B), or B95.8(45)/CAO (Fig. 6C), and pulse–chase analysis was performed. In three independent experiments, B95.8/CAO had a half-life of 3.9 ± 0.45 4, similar to that of B95.8-LMP1, whilst CAO/B95.8 had a half-life of 7.17 ± 1.26 h and B95.8(45)/CAO had a half-life of 6.95 ± 1.34 h, both similar to that of CAO-LMP1. These results suggest that the transmembrane domains are responsible for increased half-life of CAO-LMP1.

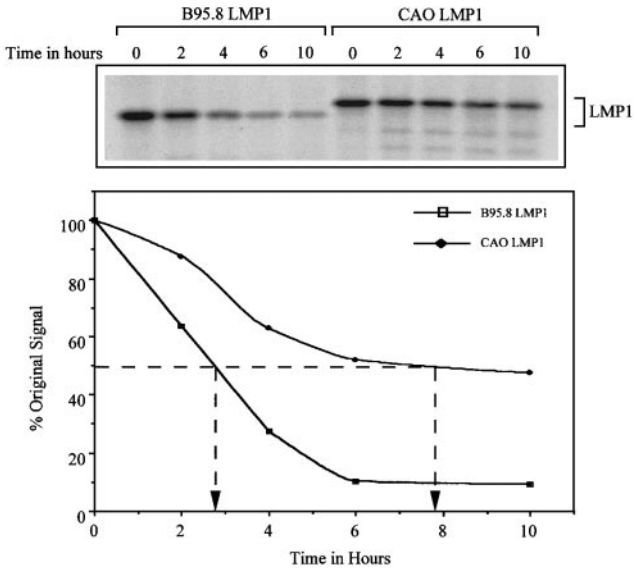

FIG. 5. CAO LMP1 has a prolonged half-life. HEK 293 cells were transiently transfected with 5μg pSG5 B95.8 or CAO-LMP1. Pulse chase analysis was performed 24h post-transfection and cells harvested at time points of 0, 2, 4, 6 and 10 h. The upper panel shows a representative experiment and the lower panel shows half-life quantified by densitometry and plotted as a percentage of the original signal. In three independent experiments B95.8-LMP1 was found to have a half-life of 2.9 h $\pm$ 0.6, whilst CAO-LMP1 has a half-life of 7.25 h $\pm$ 1.78.

DISCUSSION

Whether particular LMP1 variants contribute to EBV-associated oncogenesis remains unclear, and the functional significance of mutations and deletions in LMP1 is largely unknown. This study demonstrates that the transmembrane domains of the NPC-derived LMP1 variant CAO confer enhanced signalling activity in transiently transfected epithelial cells. The region of CAO-LMP1 responsible for this enhanced signalling also maps to that which prolongs the half-life of the protein, suggesting that the effects of CAO-LMP1 on NF-κB and AP-1 may be a direct result of increased protein stability. These results are consistent with a recent study in lymphoid cell lines (Johnson *et al.,* 1998) but contrast with observations in stably expressing epithelial cells where, although able to activate equivalent levels of NF-κB, CAO-LMP1 is unable to substantially upregulate AP-1 (Dawson *et al.,* 2000). It is an interesting paradox that, although able to signal NF-κB and AP-1, CAO-LMP1 appears to be unable to block differentiation, induce IL-6 or IL-8 secretion, or upregulate CD54 or CD40 (Dawson *et al.,* 2000; Johnson *et al.,* 1998). The chimeras generated in this study may enable mapping of these phenomena.

Given that B95.8 and CAO-LMP1 are virtually identical over the CTAR1 and CTAR2 domains, it was perhaps surprising that there should be a difference in their ability to activate cellular signalling pathways. The major difference in the carboxyl-termini of these two variants lies in the repeat region between CTAR1 and CTAR2 in which there are three additional 33-bp repeats and a 30-bp deletion. The effect of these changes and whether they affect LMP1's ability to engage signalling adapter molecules such as TRAFs and TRADD are unknown. This region has recently been shown to interact with JAK3 and activate STAT proteins (Gires *et al.,* 1999), and the ability of CAO-LMP1 to activate this pathway will be an interesting area of future investigation. However, using chimeric molecules, this study has implicated the transmembrane domains of LMP1 in regulating its signalling capacity. The role of the transmembrane domains is considered predominantly structural, serving to localise the protein in the plasma membrane and enable oligomerisation. However, the transmembrane domains have also been found to contribute to toxicity in both rodent and lymphoid cell lines (Hammerschmidt *et al.,* 1989), and more recent work demonstrates that these regions can inhibit cell proliferation (Kaykas and Sugden, 2000) and activate the Rho-like GTPase Cdc42, leading to actin polymerisation and filopodia formation in fibroblasts (Puls *et al.,* 1999). Further studies are in progress to identify mechanisms by which the transmembrane domain of LMP1 influences these functional properties.

There has been an abundance of data in recent years describing deletions and mutations within the carboxyl-terminus of LMP1 in EBV-associated malignancies (Berger *et al.,* 1999; Chen *et al.,* 1992; Cheung *et al.,* 1996, 1998; Chiang *et al.,* 1999; Hu *et al.,* 1991; Kingma *et al.,* 1996; Knecht *et al.,* 1993, 1995; Palefsky *et al.,* 1996; Sandvej *et al.,* 1994; Santon *et al.,* 1995), but few studies have considered sequence variation in the transmembrane domains. The enhanced NF-κB activation from CAO-LMP1 is consistent with the Mediterranean NPC-derived C15 LMP1 (Miller *et al.,* 1998). Comparative sequence analysis of the transmembrane domains of B95.8-LMP1 and the NPC-derived variants CAO and C15 identified four loci at which CAO and C15 LMP1 differed from B95.8 but were identical to each other (residues 85, 106, 122, and 129). However, mutagenesis at these four loci failed to identify a single residue as responsible for CAO-LMP1's enhanced NF-κB activation (data not shown). Further analysis with various combinations of these point mutants may identify the critical residues involved in regulating signalling capacity. Similar amino acid changes to those in the transmembrane domain of CAO-LMP1 have also been observed in the NPC-derived LMP1 variant 1510 (Chen *et al.,* 1992), four NPC biopsies (Cheung *et al.,* 1998), and three cases of hairy leukoplakia (Palefsky *et al.,* 1996), and further analysis is required to determine the significance of these changes.

The regions of LMP1 required for correct protein turnover include the amino acids 322–364 (Moorthy and Thorley-Lawson, 1993), and thus it has been postulated that the 30-bp deletion observed in many LMP1 variants may lead to a prolonged half-life (Sandvej *et al.,* 1994). This study has shown that CAO-LMP1 has a prolonged half-life, but this property was localised to the transmembrane domains. These domains of CAO-LMP1 contain

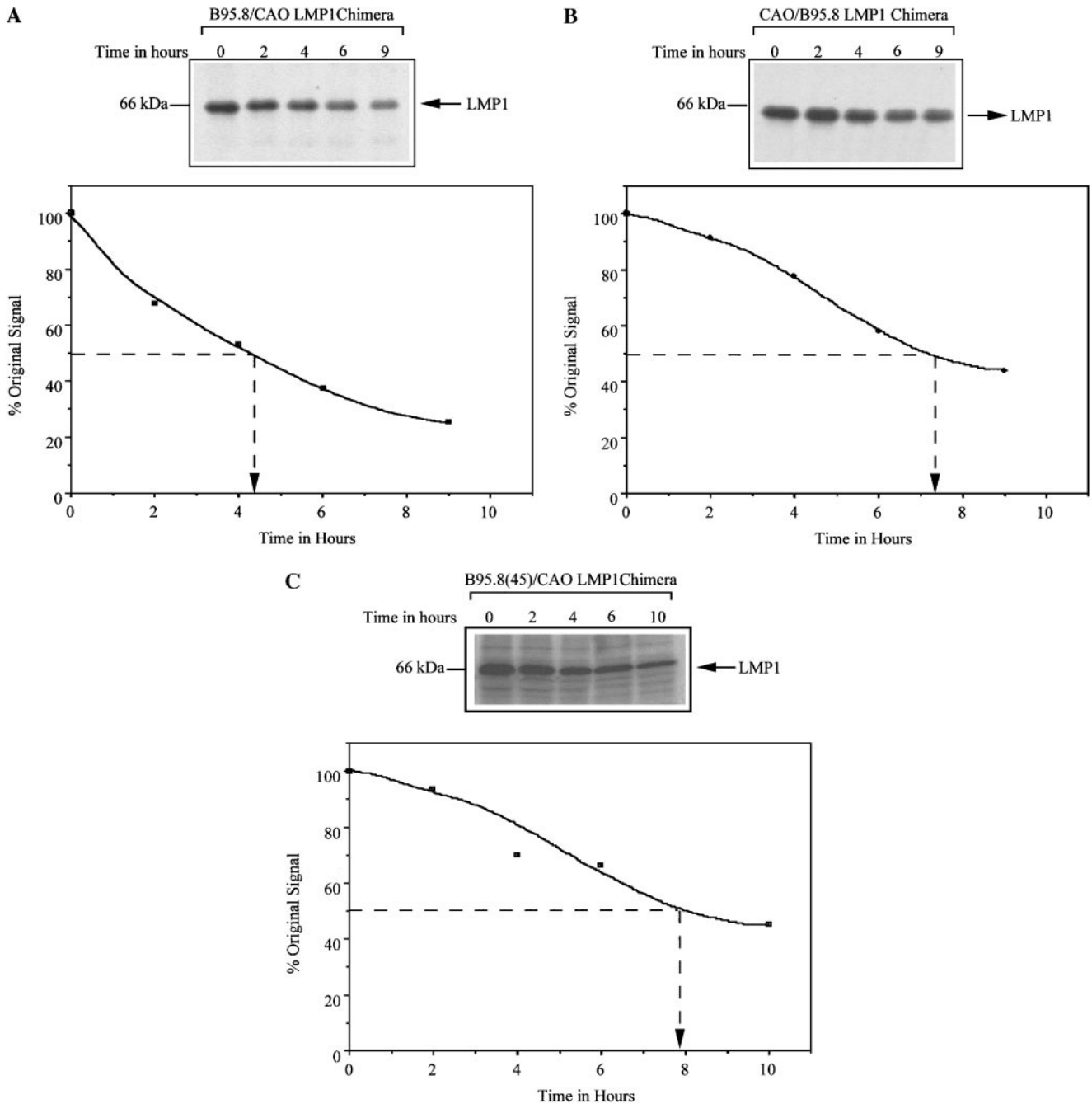

FIG. 6. The prolonged half-life of CAO-LMP1 is mediated through its transmembrane domains. HEK 293 cells were transiently transfected with 5 μg pSG5-based LMP1 constructs, and LMP1 half-life was determined by pulse–chase analysis. Pulse–chase analysis was performed 24 h posttransfection and cells harvested at various time points. The top panels show representative experiments and the bottom panels show half-life quantified by densitometry and plotted as a percentage of the original signal. (A) HEK 293 cells were transiently transfected with 5 μg pSG5 B95.8/CAO-LMP1. In three independent experiments, B95.8/CAO-LMP1 was found to have a half-life of 3.9 $\pm$ 0.45 h. (B) HEK 293 cells were transiently transfected with 5 μg pSG5 CAO/B95.8-LMP1. In three independent experiments, CAO/B95.8-LMP1 was found to have a half-life of 7.17 $\pm$ 1.26 h. (C) HEK 293 cells were transiently transfected with 5 μg pSG5 B95.8(45)/CAO LMP1. In three independent experiments, B95.8(45)/CAO-LMP1 was found to have a half-life of 6.95 $\pm$ 1.34 h.

the majority of the mutations, and one possibility is that these mutations enable an interaction with another membrane protein thus stabilising CAO-LMP1 in the membrane. An alternative possibility is that these mutations in CAO-LMP1 block its degradation. Recent findings have implicated the ubiquitin-proteasome pathway and phosphorylation in the degradation of LMP1, with its short amino terminus shown to be essential for subsequent degradation (Aviel *et al.,* 2000). Given the mutations in the amino terminus of CAO-LMP1, it would be interesting to investigate its ubiquitination and phosphorylation and determine the mechanism by which it is degraded. Further detailed analysis of the amino terminus of LMP1 may identify the residues essential for

ubiquitination and phosphorylation and thereby help to reconcile the relative contributions of the extreme amino terminus and the transmembrane domains to the prolonged half-life of CAO-LMP1.

In summary, this study has implicated increased protein stability in the ability of the NPC-derived LMP1 variant CAO to activate enhanced levels of NF-κB and AP-1. Taking into account the role of these transcription factors in oncogenesis (Ip and Davis, 1998; Rayet and Gelinas, 1999), this data may have important implications for our understanding of the enhanced transforming capacity of CAO-LMP1 (Hu *et al.,* 1993).

MATERIALS AND METHODS

DNA constructs

pSG5 B95.8-LMP1 and pSG5 CAO-LMP1 have been previously described (Huen *et al.,* 1995; Johnson *et al.,* 1998). To generate a B95.8/CAO-LMP1 chimera comprising the amino terminus and transmembrane domains of B95.8-LMP1 and the carboxyl-terminus of CAO-LMP1, an artificial *Kpn*I site was first introduced at amino acid (aa) 192 of pSG5 B95.8-LMP1 using site-directed mutagenesis as previously described (Eliopoulos and Young, 1998) (mutated oligonucleotide primers used were 5′-GGACAACGACACGGTACCGAACACCACCACG-3′ and its complimentary oligo; mutated positions are underlined). The carboxyl-terminus of B95.8-LMP1 was then removed by digestion with *Bam*HI and *Kpn*I. The carboxyl-terminus of CAO-LMP1 (aa 192–404) was PCR amplified using a 5′-primer with an artificial *Kpn*I site (5′-ACGACACGGTACCGAACACCA-3′; mutated positions are underlined) and a 3′-primer with an artificial *Bam*HI site (5′-CGTGCGGATCCTTAGTCATAGTAG-3′; mutated positions are underlined). The PCR product was then cloned in frame into the amino terminal/transmembrane sequences of pSG5 B95.8-LMP1 to generate B95.8/CAO. To generate the chimera comprising the amino terminus and transmembrane domains of CAO-LMP1 and the carboxyl-terminus of B95.8-LMP1, site-directed mutagenesis was used to introduce an artificial *Kpn*I site at aa 192 of pSG5 CAO-LMP1 (mutated oligonucleotide primers used were 5′-GGACCACGACACGGTACCGAACACCACCACG-3′ and its complimentary oligo; mutated positions are underlined). The carboxyl-terminus of CAO-LMP1 was then removed by digestion with *Bam*HI and *Kpn*I. The carboxyl-terminus of B95.8-LMP1 (aa 192–386) was isolated from pSG5 B95.8-LMP1 containing a *Kpn*I site at aa 192, following digestion with *Kpn*I and *Bam*HI. The isolated product was then cloned in frame into the amino terminal/transmembrane sequences of pSG5 CAO-LMP1 to generate CAO/B95.8. To generate a chimera comprising the amino terminus and first transmembrane loop of B95.8-LMP1 (aa 1–45) and the remainder of CAO-LMP1 (45–404), site-directed mutagenesis was used to introduce an artificial *Kpn*I site at aa 45 of both pSG5 B95.8-LMP1 and pSG5 CAO-LMP1 (mutated oligonucleotide primers used were 5′-ATCGTTATGGGTACCTGGACTGGAGGAGC-3′ and its complimentary oligo; mutated positions are underlined). Amino acids 45–386 of B95.8-LMP1 were then removed by digestion with *Bam*HI and *Kpn*I, and a *Bam*HI/*Kpn*I fragment corresponding to aa 45–404 of CAO-LMP1 was then cloned in frame into pSG5 B95.8-LMP1 (1–44) to generate B95.8(45)/CAO. The sequences of these hybrid constructs were verified by DNA sequencing, which was performed on an ABI Prism377 DNA Sequencer using the Big Dye DNA Sequencing Kit (Perkin–Elmer).

Cell culture, transfections, and reporter assays

Human embryonic kidney (HEK) 293 cells were grown in DMEM supplemented with 10% foetal calf serum, 2 mM glutamine, 1000 U/ml penicillin, and 1 mg/ml streptomycin. SV40-transformed human keratinocytes (SVK) were grown in Joclics supplemented with 8% foetal calf serum, 2 mM glutamine, 1000 U/ml penicillin, 1 mg/ml streptomycin, and 0.4 μg/ml hydrocortisone. For transient transfection of HEK 293 cells, 1×10^6 cells were plated out in a 25-cm^2 flask and transfected the following day using a standard calcium phosphate technique. For transient transfection of SVK cells, 1.5×10^6 cells were plated out in a 25-cm^2 flask and transfected the following day using a modified DEAE-dextran method (Gonzalez and Joly, 1995).

SDS–PAGE and immunoblotting

Immunoblotting was performed using the and the proteins transferred to a nitrocellulose membrane. Following incubation in blocking buffer (5% skimmed milk in 0.1% PBS-Tween) for 1 h at room temperature, the membrane was incubated overnight at 4°C with agitation in CS.1–4 monoclonal antibody. The membrane washed for 30 min in 0.1% PBS-Tween and incubated for 1 h at room temperature with agitation in HRP-conjugated secondary antibody. The membrane was washed in PBS-Tween 20 for ≥1 h with several changes of buffer and the proteins detected by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech).

Electrophoretic mobility shift assays

EMSA probes were prepared by first annealing complementary synthetic oligonucleotides at 95°C for 5 min in 1× buffer B (Boehringer Mannheim). The probes were end-labeled in a reaction containing 3 μCi [γ-^{32}P]ATP, 1 pmol annealed probe, 5 μl 10× polynucleotide kinase (PNK) buffer and 2 μl PNK (Boehringer Mannheim) for 1 h at 37°C. The probe was then isolated using the Qiaquick-Nucleotide Removal Kit (Qiagen) according to the manufacturer's instructions. To prepare nuclear extracts, cells were washed twice in PBS and resuspended in 100 μl buffer A (10 mM HEPES pH 7.9, 2% v/v N-P40, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 100 μg/ml leupeptin, 0.5 mM PMSF, and 1 mM benzamidine), which lyses the cell

membrane. Cell nuclei were pelleted by centrifugation at 13,000 rpm for 10 s and the nuclei lysed in 100 μl buffer C (20 mM HEPES pH 7.9, 25% v/v glycerol, 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 0.5 mM DTT, 100 μg/ml leupeptin, 0.5 mM PMSF, and 1 mM benzamidine) by incubation on ice for 30 min. Nuclear debris was pelleted by centrifugation at 13,000 rpm at 4°C for 5 min, and nuclear protein concentration was determined using the Biorad protein assay. The reaction containing 4 μg nuclear extract, 1 μg poly dIdC (Pharmacia), 5 mM DTT, and 0.1 ng probe was incubated on ice for 30 min. The reaction was resolved by electrophoresis on a 5% polyacrylamide gel in 0.5× TBE buffer. The gel was dried and exposed to X-ray film. The probes used in this study to assess NF-κB and AP-1 binding were described previously (Eliopoulos *et al.,* 1997; Eliopoulos and Young, 1998).

Pulse–chase analysis and immunoprecipitation

Transiently transfected cells were incubated in methionine/cysteine-free DMEM for 30 min. This media was then replaced with met/cys-free DMEM containing 0.2 mCi/ml [^{35}S]methionine/cysteine protein labelling mix (NEN) and the cells incubated at 37°C for 1 h (pulse). Following the incubation, the cells were washed three times with methionine/cysteine-free DMEM, complete media was added and the cells were returned to 37°C. The cells were harvested at various time points (chase). Cells were harvested and lysed in RIPA buffer (50 mM Tris pH 8, 150 mM NaCl, 1% v/v N-P40, 0.5% w/v deoxycholate, 0.1% w/v SDS, 5 μg/ml leupeptin, 2 μg/ml aprotinin, 0.5 mM sodium orthovanadate, and 1 mM PMSF) on ice for 30 min, and the cell debris pelleted by centrifugation at 13,000 rpm for 5 min at 4°C. Protein concentration was determined using the Biorad protein assay. Two hundred microgams protein was precleared with 10 μl of 50% v/v sepharose–protein G at 4°C with rotation for 1 h. The CS.1 monoclonal antibody (Rowe *et al.,* 1987) was added to the pre-cleared supernatants and the samples incubated at 4°C with rotation for 2 h. Twenty-five microliters of 50% v/v protein G–sepharose beads were added and the samples rotated at 4°C for 2 h. The beads were pelleted by centrifugation and washed four times with RIPA buffer. Following the final wash, the beads were drained with a fine-gauge Hamilton syringe, 50 μl GSB added, and the samples boiled for 4 min. The samples were then electrophoresed and the gel stained with 0.1% w/v Coomassie Blue stain for 30 min and destained in Destain solution I (45% v/v methanol, 10% v/v glacial acetic acid) for 1 h. The gel was rehydrated in Destain solution II (5% v/v methanol, 5% v/v glacial acetic acid) for 1 h, incubated in Amplify (Amersham Pharmacia Biotech) for 15 min to enhance the signal and dried. Autoradiography and densitometry were used to quantitate the assays.

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