Cyclosporin A-sensitive induction of the Epstein–Barr virus lytic switch is mediated via a novel pathway involving a MEF2 family member

Shaofan Liu, Pingfan Liu, Ana Borrás¹,
Talat Chatila² and Samuel H. Speck³

Department of Pathology and Division of Molecular Oncology and
²Department of Pediatrics, Children's Hospital, Washington University
School of Medicine, St Louis, MO 63110 and ¹Division of Tumor
Virology, Dana-Farber Cancer Institute, Boston, MA 02115, USA

³Corresponding author

S.Lui and P.Lui contributed equally to this work

Induction of the Epstein–Barr virus (EBV) lytic cycle by crosslinking surface immunoglobulin is inhibited by the immunosuppressants cyclosporin A (CsA) and FK506. This correlates with the ability of CsA to inhibit Ca²⁺-dependent transcription of the lytic cycle switch gene BZLF1. It is shown here that CsA sensitivity maps to three sites (ZIA, ZIB and ZID) that bind the serum response factor-related protein MEF2D. A synthetic promoter containing multiple copies of a MEF2D site from Zp, in conjunction with a CREB/AP-1 site (ZII) from Zp, exhibits CsA-sensitive inducibility. Furthermore, the Zp MEF2D sites were functionally interchangeable with MEF2 sites derived from heterologous promoters. While no evidence of a NFAT family member binding to either the MEF2 or CREB/AP-1 sites was obtained, it could be demonstrated that CsA-sensitive induction of Zp was mediated by calcineurin and NFATc2 in synergy with either phorbol ester or especially with the EBV-induced Ca²⁺/calmodulin-dependent kinase type IV/Gr. These studies identify Zp as prototypic of a novel class of CsA-sensitive and NFAT-dependent promoters defined by the presence of MEF2 sites.

Keywords: BZLF1 promoter/cyclosporin A-sensitive induction/Epstein–Barr virus/MEF2D

Introduction

Epstein–Barr virus (EBV) is a lymphotropic human herpes-virus that latently infects B lymphocytes, resulting in a concomitant growth transformation of the infected cell. Propagation of EBV from host to host is dependent upon the activation of an estimated 100 or more viral genes, culminating in the production of infectious virions (Hummel and Kieff, 1982; Baer *et al.*, 1984; Cohen *et al.*, 1984). While these genes remain quiescent during latency, a switch in the genetic program leading to the expression of viral replication-associated genes can be accomplished *in vitro* by treatment of latently infected B lymphocytes with various reagents, including phorbol esters, butyrate, Ca²⁺ ionophores and anti-immunoglobulin (Tovey *et al.*, 1978; zur Hausen *et al.*, 1978; Bauer *et al.*, 1982; Faggioni *et al.*, 1986; Takada and Ono, 1989). Activation of the

lytic cascade by crosslinking surface immunoglobulin results initially in the expression of two viral genes, BZLF1 and BRLF1, which exhibit similar induction kinetics (maximal mRNA levels are reached between 2 and 4 h post-induction) (Takada and Ono, 1989; Flemington *et al.*, 1991). The protein products of both the BZLF1 gene (referred to here as Zta, but also called ZEBRA and EB1) and the BRLF1 gene (referred to as Rta) have been shown to be transcriptional activators (Countryman and Miller, 1985; Chevalier-Greco *et al.*, 1986; Takada *et al.*, 1986; Grogan *et al.*, 1987; Harwick *et al.*, 1988; Farrell *et al.*, 1989). Expression of Zta and Rta leads to the activation of early genes and ultimately viral replication. Of all the viral transactivators examined, Zta is unique in that its expression alone can initiate the entire lytic cascade (Miller, G. *et al.*, 1984; Countryman *et al.*, 1987; Grogan *et al.*, 1987) and regulation of Zta expression appears to be central to regulating entry into the lytic cycle.

The BZLF1 promoter (Zp) exhibits very low basal activity which is potently up-regulated by inducers of the viral lytic cycle (Flemington and Speck, 1990a; Shimizu and Takada, 1993; Daibata *et al.*, 1994). The region from –221 to +12 bp of Zp harbors the necessary *cis*-elements for maintaining low basal activity and activation by lytic cycle-inducing agents (Flemington and Speck, 1990a,b; Shimizu and Takada, 1993; Daibata *et al.*, 1994). Within this sequence, three distinct types of response elements have been defined (see Figure 1) (Flemington and Speck, 1990a,b). The first are A+T-rich sequences, termed ZI elements, four copies of which are interspersed in the promoter (ZIA–D). The second is represented by a unique element, ZII, which shares homology with consensus CREB/AP-1 binding sites. The third is composed of two sites, termed ZIIIA and ZIIIB, which bind the BZLF1 gene product Zta (Flemington and Speck, 1990b). ZIIIA, but not ZIIIB, is an AP-1 response element. Induction of the BZLF1 gene appears to involve two steps: (i) initial activation of the promoter by inducers of the lytic cycle, mediated through the ZI and ZII elements, which results in low level transcription of the BZLF1 gene; followed by (ii) auto-activation of the BZLF1 promoter, which is mediated through Zta binding to the ZIIIA and ZIIIB elements. It has previously been noted that Zta activation strongly synergizes with induction through the ZI and ZII elements (e.g. triggered by phorbol ester) (Flemington and Speck, 1990b). Thus, it has been proposed that the duration and magnitude of the initial signal may determine whether enough Zta is generated in an appropriate time interval to trigger the entire lytic cascade (Flemington and Speck, 1990b).

Recently, it was demonstrated that induction of the EBV lytic cycle by crosslinking surface immunoglobulin could be blocked by the immunosuppressants cyclosporin A (CsA) and FK506 (Goldfeld *et al.*, 1995), an effect

Fig. 1. (A) Organization of *cis*-elements in the BZLF1 promoter (Zp) as previously identified by DNase I footprinting in conjunction with functional analyses (Flemington and Speck, 1990a,b). The ZI domains were functionally grouped together on the basis of sequence similarity and binding competition studies (Flemington and Speck, 1990a). The ZII domain contains a consensus CREB/AP-1 binding site, while the ZIIIA and ZIIIB domains are binding sites for the BZLF1 gene product Zta, which serves to positively autoregulate Zp activity. (B) Sequence alignment of the ZI domains present in Zp. The core homology domain is highlighted in the shaded rectangle. Shown to the right of the ZI domain sequences are the complexes observed on EMSA with each domain employing crude nuclear extract from an EBV-negative B cell line, as previously described (Borras *et al.*, 1996). (C) Homology between individual ZI domains and the consensus binding site for the MEF2 family of transcription factors. Nucleotides which do not match the consensus MEF2 binding site are depicted in lower case letters.

which mapped to induction of the BZLF1 promoter. In this paper the known *cis*-elements in Zp were characterized for responsiveness to Ca^{2+} signals and sensitivity to CsA. These studies identify MEF2D as a cellular factor binding to the ZIA, ZIB and ZID elements and implicate this factor in the responsiveness of Zp to changes in the level of intracellular Ca^{2+} . This induction was mediated in part by a calcineurin pathway involving a member(s) of the NFAT family, although no evidence of NFAT binding to either the MEF2D or CREB/AP-1 sites was obtained. These studies outline a cellular signaling pathway that is likely to play an important role in triggering reactivation of latent EBV, in which MEF2D is downstream of CNM/NFAT.

Results

Identification of MEF2D binding to the ZIA, ZIB and ZID elements in Zp

Previous analyses of the regulatory sequences in Zp indicated that the ZI elements are not functionally identical (Borras *et al.*, 1996). Examination of the cellular factors binding to these elements demonstrated the formation of four specific complexes with the ZIA and ZID elements, three specific complexes with the ZIC element and only a single specific complex with the ZIB element. Competition analyses demonstrated that the ZIB and ZIC elements bind a subset of the cellular factors which bind to the ZIA and ZID elements (summarized in Figure 1B). When multiple copies of the ZIA, ZIC or ZID elements were

linked to a single copy of the CREB/AP-1 element (ZII element) from Zp, strong 12-*O*-tetradecanoylphorbol-13-acetate (TPA) inducibility was observed. However, an analogous reporter construct containing three copies of the ZIB element linked to a single copy of the CREB/AP-1 element ($[3 \times \text{ZIB}][\text{ZII}]\beta\text{GCAT}$) was only weakly responsive to TPA (Borras *et al.*, 1996). This suggested that the strong phorbol ester inducibility of ZIA, ZIC and ZID correlated with the cellular factors present in complexes 2–4, which have recently been demonstrated to be Sp1 and Sp3 (S.Liu, A.M.Borras, P.Liu, G.Suske and S.H.Speck, manuscript submitted). Synthetic promoters containing only three copies of the individual ZI elements or a single copy of the ZII element exhibited weak phorbol ester inducibility, indicating a requirement for both the ZI and ZII elements for efficient inducibility. This is consistent with our previous mutational analyses of Zp, which demonstrated that mutation of any ZI element or the ZII element greatly diminished inducibility of the promoter (Flemington and Speck, 1990a; Borras *et al.*, 1996).

Methylation interference analysis of complex 1 employing a probe containing the ZIA element demonstrated that cellular factor binding was centered over the region of homology between the ZI elements (summarized in Figure 1)(Borras *et al.*, 1996). Inspection of the sequences involved in binding complex 1 revealed a strong homology to the consensus recognition sequence for the myocyte enhancer factor 2 (MEF2) family of transcription factors (also known as related to serum response factor or RSRF) (see Figure 1C) (Pollock and Treisman, 1991; Yu *et al.*, 1992). To initially assess whether complex 1 might reflect binding of a MEF2 family member, binding competition was carried out employing a cold oligonucleotide probe containing either a consensus MEF2 site or a mutant MEF2 site (MEF2m1) (Figure 2A). This analysis clearly demonstrated that complex 1 was specifically competed by the consensus MEF2 site, but not by the mutant site. In addition, the formation of complexes 2–4 was unaffected by the oligonucleotide competitor containing the MEF2 site. As shown in Figure 2, both the ZIA and ZID elements appear to more avidly form complex 1 compared with the ZIB element.

Four different MEF2 genes have been identified (MEF2A, MEF2B, MEF2C and MEF2D) (Pollock and Treisman, 1991; Yu *et al.*, 1992; Breitbart *et al.*, 1993; Leifer *et al.*, 1993; McDermott *et al.*, 1993; Martin *et al.*, 1994). The expression of MEF2C is largely restricted to skeletal muscle and brain, while expression of the other family members has been observed in a wide variety of cell types. To assess whether a MEF2 family member might be the factor present in complex 1, antibodies specific for individual members of the MEF2 family (generously provided by Dr Ron Pruss, Columbia University) were assessed for their ability to supershift complex 1. As shown in Figure 2C, anti-MEF2D antibody was able to specifically supershift complex 1 formed with probes containing the ZIA, ZIB or ZID element. This antibody did not affect the formation or migration of complexes 2–4 (although a generalized enhancement of complex formation was observed in the presence of antibody, a phenomenon we have observed with other antibody reagents). Anti-MEF2B antibody did not exhibit any ability to interact with complex 1. However, anti-

Fig. 2. (A) Competition for cellular factor binding to the ZI domains by double-stranded oligonucleotide competitors containing either the consensus MEF2 binding site (mef2) or a mutated MEF2 binding site (mef2m1). The 32 P-labeled oligonucleotide probes employed are indicated at the top of each set of competitions. The specific complexes are numbered to the left of the gel and are as previously described (Borras *et al.*, 1996). The fastest migrating complex (labeled with a question mark) appears to be non-specific, since it is competed by irrelevant double-stranded oligonucleotides and mutational analyses across ZIA and ZIC failed to map this binding to a specific region of these domains (Borras *et al.*, 1996). (B) Longer exposure of the ZIB EMSA shown in (A), which better illustrates competition of complex 1 by the mef2 oligonucleotide but not by the mef2m1 oligonucleotide. (C) Analysis of MEF2 binding to the ZI domains employing antibodies against specific MEF2 family members. The 32 P-labeled oligonucleotide probes employed are indicated at the top of each set of antibody supershifts. The specific complexes are numbered to the left of the gel and are as previously described (Borras *et al.*, 1996). The asterisk indicates the supershifted complex observed when antibody specific for MEF2D was added to the EMSA binding reaction with the ZIA, ZIB or ZID probes. The specific conditions employed are described in Materials and methods.

MEF2A antibody appeared to weakly supershift a portion of complex 1 (as well as slightly alter the mobility of the remainder of complex 1) while not affecting complexes 2–4. It is not clear at this time whether the results with anti-MEF2A reflect the presence of low levels of MEF2A binding to the ZIA, ZIB and ZID elements or whether this represents weak cross-reactivity of this antibody with MEF2D. Overall, this data provides strong evidence that MEF2D is the predominant cellular factor present in complex 1 and most likely the only specific factor binding to the ZIB element.

The region of ZIB involved in binding MEF2D is required for ZIB function

Since the ZIB element does not form complexes 2–4, analysis of the functional properties of this element should

offer insights into the role of MEF2D in regulating Zp activity. To verify that the ZI homology region of the ZIB element is involved in the binding observed by EMSA, complex 1 was analyzed by methylation interference (Figure 3A). As anticipated, those bases whose methylation disrupted formation of complex 1 were localized to the region of homology between the ZI elements (summarized in Figure 3B). To determine whether the *in vitro* analysis of cellular factor binding correlated with the regions of ZIB required for activity, mutations were introduced across the ZIB element as illustrated in Figure 3C. These mutations were incorporated into each copy of ZIB present in the [3×ZIB][ZII]βGCAT heterologous promoter-driven reporter construct and their impact on activity assessed (Figure 3C). As discussed above, the [3×ZIB][1×ZII]βGCAT reporter construct was only weakly induced by phorbol ester. However, the combination of both phorbol ester and ionomycin resulted in strong induction (see wt; Figure 3C). The latter result demonstrated that the weak TPA inducibility of the heterologous promoter containing the ZIB element was not the result of a failure to clone the functional region of ZIB.

Functional analysis of the ZIB mutants revealed the anticipated pattern of activities (Figure 3C). Mutation m1, which lies upstream of the region implicated in binding MEF2D, did not significantly alter activity. However, the m2 mutation (which mutates bases shown by methylation interference to weakly affect binding) had a modest impact on activity. Mutations m3–m5, all of which lie completely within the region of homology to the MEF2 consensus site (as well as the region identified by methylation interference), completely abrogated inducibility of the reporter construct. Mutation m6, which mutates the G at the 3'-end of the MEF2D consensus site to a C, also significantly impaired activity (note that the entire m6 mutation lies within the region identified by methylation interference as involved in binding; see Figure 3B). Mutation m7, which lies downstream of the region involved in binding complex 1, exhibited enhanced activity compared with the unmutated ZIB site (wt).

The MEF2D sites in Zp, in conjunction with the ZII element, mediate Ca^{2+} signaling, which is CsA sensitive

The strong synergy between phorbol ester and Ca^{2+} ionophore in inducing the [3×ZIB][ZII]βGCAT reporter construct indicated that the ZIB element is responsive to changes in intracellular calcium. Heterologous promoter constructs containing only a single copy of the ZII element or three copies of the ZIB element exhibited little responsiveness to TPA, ionomycin or the combination of both inducers (data not shown). As discussed above (Introduction), we have previously observed that induction of the BZLF1 promoter by either crosslinking surface immunoglobulin or by a combination of phorbol ester and Ca^{2+} ionophore is CsA sensitive. Efforts to map the regions of Zp involved in responding to induction mediated by crosslinking surface immunoglobulin have implicated both the region containing the ZIA and ZIB elements as well as the ZII element (Shimizu and Takada, 1993; Daibata *et al.*, 1994). To further examine this phenomenon, we examined whether induction of the [3×ZIB][ZII]βGCAT reporter construct was CsA sensitive. Several

Fig. 3. (A) Methylation interference analysis of cellular factor binding to the ZIB element. The specific complex (complex 1) formed with partially methylated probe was excised from an EMSA gel and analyzed as described in Materials and methods. The sequences of the sense and antisense strands are indicated adjacent to the gel. Open circles indicate bases whose methylation strongly blocked formation of complex 1, while asterisks denote bases whose methylation had an intermediate effect on binding. The cleavage pattern observed with the unbound probe (P) and a G+A sequencing ladder (G+A) are also shown for both strands. (B) Summary of methylation interference analysis of complex 1 binding to the ZIB element. Bases whose methylation strongly affected binding are indicated with open circles, while bases whose methylation had an intermediate effect are indicated with asterisks. (C) Inducibility of ZIB mutants in the context of the $[3 \times \text{ZIB}][1 \times \text{ZII}]\beta\text{GCAT}$ reporter construct. The specific mutations introduced into the ZIB domain are indicated in the inset and were introduced into each copy of the ZIB domain present in the synthetic promoter. The mutant reporter constructs were transfected into the EBV-negative BL cell line DG75 as described in Materials and methods. Post-transfection the cells were divided into four aliquots which were either untreated, treated with TPA (20 ng/ml), treated with ionomycin (1 μM) or treated with a combination of TPA (20 ng/ml) and ionomycin (1 μM). Transfected cells were recovered 48–72 h post-transfection and CAT activity determined as described in Materials and methods. The activities shown are given relative to the activity observed with the unmutated $[3 \times \text{ZIB}][1 \times \text{ZII}]\beta\text{GCAT}$ reporter construct (wt) in the presence of TPA and ionomycin, which was defined as 1.0 ($n = 3$).

independent experiments demonstrated that synergistic induction of the -221ZpCAT or the $[3 \times \text{ZIB}][\text{ZII}]\beta\text{GCAT}$ reporter constructs by TPA and ionomycin could be blocked by CsA (Figure 4). However, induction of a heterologous promoter construct containing three copies of the ZIC element linked to a single copy of the ZII element ($[3 \times \text{ZIC}][\text{ZII}]\beta\text{GCAT}$) was not inhibited by CsA (Figure 4). Notably, as shown in Figure 2, the cellular factors that bind to the ZIB and ZIC elements are distinct. The ZIC element binds the ubiquitous cellular transcription factors Sp1 and Sp3 (S.Liu, A.M.Borras, P.Liu, G.Suske and S.H.Speck, manuscript submitted), while we have shown here that the ZIB element binds MEF2D. Since the ZII element is present in both heterologous promoter constructs, these results demonstrate that the CREB/AP-1 site is not sufficient to account for the CsA-sensitive

behavior of the $[3 \times \text{ZIB}][\text{ZII}]\beta\text{GCAT}$ reporter construct. This directly implicates MEF2D as a necessary component of the CsA-sensitive inducibility of Zp.

Since both the ZIA and ZID elements also bind MEF2D, the correlation between CsA-sensitive MEF2D binding inducibility could be extended by examining the functional properties of these elements. The activity of a heterologous promoter construct composed of three copies of the ZIA element cloned upstream of a single copy of the CREB/AP-1 site from Zp (ZII element) linked to the β -globin TATA box driving the luciferase reporter gene ($[3 \times \text{ZIA}][\text{ZII}]\beta\text{GLuc}$) was characterized (Figure 5A). Previous characterization of this heterologous promoter had demonstrated strong TPA inducibility compared with the analogous promoter construct containing three copies of the ZIB element (Borras *et al.*, 1996). This correlated

Fig. 4. CsA-sensitive induction of -221ZpCAT and [3xZIB][1xZII]βGCAT reporter constructs. The inducibility and CsA sensitivity of the indicated reporter constructs from several independent experiments are shown. The reporter constructs were transfected into DG75 cells and treated as described in the legend to Figure 3 and in Materials and methods. CsA (final concentration 1 μM) was added for 30 min post-transfection prior to the addition of TPA and ionomycin to the culture medium. CAT activity was determined as described in Materials and methods and is expressed as the percentage of chloramphenicol (CAM) acetylated.

with the ability of the ZIA element, but not the ZIB element, to form complexes 2–4 (binding of Sp1 and Sp3) (S.Liu, A.M.Borras, P.Liu, G.Suske and S.H.Speck, manuscript submitted). Consistent with the previous observation, the [3xZIA][ZII]βGLuc reporter construct exhibited pronounced TPA inducibility, which was augmented by the addition of ionomycin (Figure 5A). Addition of CsA was able to partially inhibit the observed synergy between phorbol ester and calcium ionophore. This is in contrast to the induction by phorbol ester and calcium ionophore of the heterologous promoter containing three copies of the ZIC element, which was not inhibited by CsA (see Figure 4). The inability to completely inhibit the synergy between TPA and ionomycin in induction of the [3xZIA][ZII]βGLuc reporter construct most likely reflects a component which involves Sp1/Sp3 binding, as observed with the heterologous promoter containing three copies of the ZIC element. Indeed, our previous functional analysis of the ZIA element demonstrated that regions involved in binding both Sp1/Sp3 and MEF2D were important for the observed activity of the [3xZIA][ZII]βGCAT reporter construct (Borras *et al.*, 1996). Thus, the activity of the [3xZIA][ZII]βGLuc reporter construct appears to be a hybrid of the activities observed with the

Fig. 5. (A) Inducibility and CsA sensitivity of synthetic promoter constructs containing multiple copies of the ZIA or ZIB elements. The indicated luciferase reporter constructs were transfected into DG75 cells as described in Materials and methods and treated as described in Figures 3 and 4. Transfected cells were harvested 48 h post-transfection and luciferase activity determined as described in Materials and methods. Activities of the reporter constructs are given as fold induction in luciferase activity compared with the activity of the respective reporter constructs in the absence of TPA and ionomycin (uninduced). (B) Mutations introduced into the ZIA and ZIB domains in the context of the -221ZpLuc reporter construct which either converted both domains to consensus MEF2 binding sites (ZIA/Bmef2) or introduced a mutation known to abrogate mef2 binding (ZIA/Bmef2m1). The sequences are shown relative to the unmutated ZIA and ZIB elements; dashes denote bases which were not changed. (C) Inducibility and CsA sensitivity of -221ZpLuc containing mutations in the ZIA and ZIB domains. The specific mutations introduced in the ZIA and ZIB domains are described in (B). Activities are expressed as fold induction in luciferase activity compared with the uninduced activity of the -221ZpLuc reporter construct (wt).

heterologous promoters containing three copies of either the ZIB or ZIC sites.

To further assess whether MEF2D binding to the ZI elements correlates with CsA-sensitive inducibility, mutations were introduced into the ZIA and ZIB elements (in the context of Zp sequences extending from -221 to +12 bp) which either altered the ZIA and ZIB elements to consensus MEF2 binding sites (ZIA/Bmef2) or introduced a minimal mutation known to abrogate MEF2D binding (ZIA/Bmef2m1) (see Figure 5B). As shown in Figure 5C, mutation of the ZIA and ZIB elements to consensus MEF2 sites (ZIA/Bmef2) did not significantly alter inducibility or CsA sensitivity. However, a 2 bp mutation in the consensus MEF2 binding site (ZIA/Bmef2m1) almost completely abrogated inducibility of

Fig. 6. CsA-sensitive induction of Zp is mediated through a calcineurin-regulated pathway and appears to involve NFATc2. **(A)** Activation of the -221ZpLuc and [3×ZIB][1×ZII]βGLuc reporter constructs in DG75 cells by constitutively active calcineurin (CNM) in conjunction with either TPA or a constitutively active form of the Ca²⁺/calmodulin-dependent kinase IV/Gr [ΔCaMKIV(c)]. DG75 cells were transfected with the indicated reporter constructs in the presence and absence of the indicated expression vectors, as described in Materials and methods. Where indicated, cells were treated post-transfection with the indicated reagents as described in the legends to Figures 3 and 4 and in Materials and methods. Activities are expressed as the fold induction in luciferase activity compared with the uninduced reporter construct. **(B)** Activation of the -221ZpLuc reporter construct by NFATc2 in conjunction with constitutively active CaMKIV/Gr. DG75 cells were co-transfected with both the -221ZpLuc and [NFAT]CAT reporter constructs and the indicated expression vectors as described in Materials and methods. Where indicated, the cells were treated post-transfection with the indicated reagents as described in the legends to Figures 3 and 4 and in Materials and methods. Cells were harvested 48 h post-transfection and divided into two aliquots for determining luciferase and CAT activities. Activities are expressed as the fold induction in either luciferase or CAT activity relative to the uninduced reporter construct. **(C)** Induction of -221ZpLuc and [NFAT]Luc reporter constructs by NFATc1 or NFATc2 in conjunction with constitutively active CaMKIV/Gr in HeLa cells. The indicated reporter constructs and expression vectors were transfected into HeLa cells as described in Materials and methods. Cells were harvested 48 h post-transfection and luciferase activity determined as described in Materials and methods. Activities are expressed as the fold induction in luciferase activity compared with the activity of the uninduced reporter construct.

Zp. It should be noted that it was previously demonstrated that mutation of any individual ZI element in the context of Zp significantly impaired inducibility (Borras *et al.*, 1996). Thus, this data provides strong support for MEF2D playing a critical functional role in the CsA-sensitive induction of Zp.

CsA-sensitive induction of Zp is mediated through a calcineurin-regulated pathway that appears to involve NFATc2

CsA and FK506 are known to mediate their immunosuppressive effects in T cells through inhibition of the calcium/calmodulin-regulated phosphatase calcineurin (see Crabtree and Clipstone, 1994). To determine whether calcineurin is involved in regulating the induction of Zp in B cells, a constitutively active form of calcineurin (CNM) was co-transfected along with either the -221Zp Luc or the [3×ZIB][ZII]βGLuc reporter construct (Figure 6A). In the absence of any other stimulation, constitutively active calcineurin was only able to weakly induce these promoters. However, in the presence of phorbol ester in the culture medium an ~60-fold induction of Zp activity

was observed (compared with only 10- to 15-fold induction with phorbol ester alone) and an ~30-fold induction of [3×ZIB][ZII]βG was observed (compared with <10-fold induction by phorbol ester alone). These results indicate that a calcineurin-regulated pathway is involved in the induction of Zp.

Notably, induction by phorbol ester and constitutively active calcineurin was not as strong as induction observed with the combination of phorbol ester and ionomycin (Figure 6A). This suggested that there may be other Ca^{2+} -dependent events involved in the induction of Zp. We have recently demonstrated that the calcium/calmodulin-dependent kinase IV/Gr (CaMKIV/Gr), whose expression is up-regulated by LMP1 in EBV-immortalized B cells (Mosialos *et al.*, 1994), can activate transcription from Zp (T.Chatila, H.Nga, P.Liu, S.Liu, G.Mosialos, E.Kieff and S.H.Speck, manuscript submitted). To determine whether CNM and CaMKIV/Gr can synergistically activate Zp, expression vectors encoding constitutively active forms of calcineurin and CaMKIV/Gr [$\Delta\text{CaMKIV(c)}$] were co-transfected with either the -221ZpLuc or the [3 $\times$ ZIB][ZII] β GLuc reporter construct into DG75 cells (Figure 6A).

As previously observed (T.Chatila, H.Nga, P.Liu, S.Liu, G.Mosialos, E.Kieff and S.H.Speck, manuscript submitted), expression of Δ CaMKIV(c) alone was able to induce Zp activity ~30-fold and $[3 \times \text{ZIB}][\text{ZII}]\beta\text{G}$ ~200-fold and this induction was not affected by the presence of CsA (Figure 6A). However, when Δ CaMKIV(c) was expressed in conjunction with constitutively active CNM both Zp and $[3 \times \text{ZIB}][\text{ZII}]\beta\text{G}$ promoter activities were dramatically up-regulated (>1000-fold induction) (Figure 6A). In addition, the synergy between Δ CaMKIV(c) and constitutively active calcineurin could be completely inhibited by CsA (Figure 6A). These results clearly demonstrate a role for a CNM-regulated pathway in the induction of Zp and dramatically illustrate the exquisite sensitivity of Zp to induction by both calcineurin and CaMKIV/Gr.

To further investigate calcineurin-regulated induction of Zp, the ability of individual NFAT family members to activate Zp was assessed. Expression vectors for NFATc1 (NFATc), NFATc2 (NFATp) or NFATc3 (generously provided by Dr Gerry Crabtree, Stanford University) were co-transfected into DG75 cells along with the -221ZpLuc reporter construct and a CAT reporter construct driven by a heterologous promoter containing three copies of the distal NFAT site from the IL2 promoter ([NFAT]CAT). The latter reporter construct was included as an internal control for activation by NFAT family members. It has been noted by others that ectopic expression of NFATs results in an apparent 'spill-over' of these proteins from the cytoplasm into the nucleus of the transfected cells, allowing NFAT-regulated transcription to be induced (Ho *et al.*, 1996). In DG75 cells, both NFATc1 and NFATc2 were able to weakly synergize with phorbol ester to induce the [NFAT]CAT reporter construct (Figure 6B). However, only NFATc2 exhibited any ability to synergize with TPA in inducing the -221ZpLuc reporter construct (Figure 6B). Indeed, the [NFAT]CAT reporter construct was also more strongly induced by NFATc2 than by NFATc1 (Figure 6B). The latter could reflect differences in the level of expression of these proteins in transfected DG75 cells or a functional difference. Although the levels of protein expression in the nucleus of the transfected cells were not examined, it should be noted that cDNAs encoding these gene products were cloned into the same expression vector. Expression of NFATc3 consistently failed to activate either the [NFAT]CAT or the ZpLuc reporter constructs (data not shown). The weak (or absent) synergy between NFAT proteins and TPA is in line with the relatively weak synergy observed with constitutively active CNM and TPA (see Figure 6A).

Since co-expression of constitutively active forms of calcineurin and CaMKIV/Gr was able to strongly induce Zp, we investigated co-expression of individual NFAT proteins with Δ CaMKIV(c). Co-expression of NFATc1 and Δ CaMKIV(c) resulted in a modest induction of -221ZpLuc and [NFAT]CAT reporter constructs in DG75 cells (Figure 6B). A similar level of induction of the [NFAT]CAT reporter construct was observed with co-expression of NFATc2 and Δ CaMKIV(c) (Figure 6B). However, co-expression of NFATc2 and Δ CaMKIV(c) strongly induced the -221ZpLuc reporter construct (>700-fold induction) (Figure 6B). The latter result appeared to recapitulate the hyperinduction of Zp observed with constitutively active calcineurin and Δ CaMKIV(c) and

suggests that Zp activation discriminates between the functions of NFATc1 and NFATc2. These results also indicate that induction through the IL2 distal NFAT site, although exhibiting many similar characteristics, is likely to be mechanistically distinct from the CsA-sensitive induction of Zp.

To assess whether the discrimination between NFATc1 and NFATc2 function in the induction of Zp was observed in a non-B cell system, the -221ZpLuc reporter construct was transfected along with the appropriate expression vectors into HeLa cells (Figure 6C). As a control, the [NFAT]Luc reporter construct (analogous to the [NFAT]-CAT reporter construct described above, in which the CAT gene has been replaced with the firefly luciferase gene) was also analyzed. As observed in the DG75 cell line, NFATc1 only weakly synergized with Δ CaMKIV(c) to induce Zp. However, NFATc1 was able to synergize with Δ CaMKIV(c) to induce [NFAT]Luc ~150-fold. In contrast to the results obtained with NFATc1, NFATc2 strongly synergized with Δ CaMKIV(c) to induce both -221ZpLuc and [NFAT]Luc reporter constructs. Notably, in HeLa cells the distal IL2 NFAT site was more potently induced by NFATc2 than by NFATc1 (Figure 6C), while in DG75 cells there was little difference between the activation through this site by NFATc1 and NFATc2 (Figure 6B). This may reflect differences in the AP-1 family members present in DG75 and HeLa cells and will require further investigation. Alternatively, this could reflect differences in the levels of NFAT protein present in the nucleus of the transfected cells.

Discussion

In this paper we have shown that CsA-sensitive induction of Zp requires: (i) multiple copies of a ZI element which binds MEF2D; (ii) the CREB/AP-1 site (ZII element) from Zp. In addition, this induction is mediated through a calcineurin-regulated pathway and appears to preferentially involve NFATc2 (NFATp). Analyses of cellular factor binding to the ZI and ZII elements, employing nuclear extracts prepared from uninduced and induced B cells, have consistently failed to identify any changes in factor binding upon induction (data not shown). Notably, extensive efforts to identify NFAT binding to the ZI or ZII elements have been unsuccessful. In addition to assessing cellular factor binding employing nuclear extracts prepared from uninduced and TPA + ionomycin-induced cells, we also assessed the ability of partially purified cytoplasmic NFATc2 and recombinant NFATc2 to bind to either the ZIB or ZII domain in the absence or presence of DG75 nuclear extract. While we consistently failed to demonstrate NFATc2 binding to either the ZII or ZIB *cis*-elements, binding of NFATc2 to a consensus NFAT site was readily detected in these assays (data not shown).

Inspection of the sequences in Zp does not reveal any sites which closely match the NFAT consensus binding sequence. Furthermore, the CsA-sensitive inducibility of the $[3 \times \text{ZIB}][\text{ZII}]\beta\text{G}$ heterologous promoter rules out the presence of an unidentified *cis*-element within Zp (i.e. NFAT binding site) which is required for NFAT-mediated induction of Zp. However, this does not rule out recruitment of an NFAT family member to the promoter by

MEF2D and/or a member of the CREB/AP-1 family of transcription factors. This could either solely involve protein-protein interactions between NFAT and MEF2D and/or CREB/AP-1 or might involve a very low affinity NFAT DNA binding site to which NFAT binding is stabilized by protein-protein interactions (although we have to date been unable to detect such an interaction by EMSA). Alternatively, Zp induction may lie downstream of an NFAT-regulated gene whose expression is required for induction of Zp. The latter is a formal possibility since we have previously demonstrated that efficient induction of transcription from Zp, triggered by crosslinking surface immunoglobulin, requires *de novo* protein synthesis (Flemington *et al.*, 1991). This was previously interpreted as a requirement for production of the BZLF1 gene product Zta, to allow autoactivation through the ZIIIA and ZIIIB elements in Zp. However, it could equally well reflect a requirement for the synthesis of an NFAT-regulated gene product.

Identification of the critical role of the ZI domains in the CsA-sensitive induction of Zp is consistent with previous studies which mapped *cis*-elements required for mediating induction of Zp by crosslinking surface immunoglobulin (Shimizu and Takada, 1993; Daibata *et al.*, 1994). The previous studies demonstrated that a deletion which removed the ZIA and ZIB domains or site-directed mutations in the ZIA and ZIB domains (Daibata *et al.*, 1994) dramatically diminished anti-Ig inducibility of Zp. Daibata *et al.* (1994) also showed that mutation of the ZII domain almost completely abolished anti-Ig inducibility of Zp. The requirement for the CREB/AP-1 site (ZII element) to generate the observed CsA-sensitive inducibility of Zp is consistent with the involvement of AP-1 or CREB family members observed in other CsA-sensitive promoters. In the case of the IL-2 gene promoter, in activated T cells NFAT proteins form a complex with AP-1 family members which then cooperatively bind to an NFAT response element (Jain *et al.*, 1992, 1993; Northrop *et al.*, 1993; McCaffrey *et al.*, 1994). For the tumor necrosis factor α gene promoter, Ca^{2+} induction in T cells is dependent on cooperation between NFATc2 and a heterodimer of ATF-2 and Jun (Tsai *et al.*, 1996).

The data presented here directly implicate Ca^{2+} signaling through MEF2D as CsA sensitive. Unlike NFAT, whose activation involves translocation from the cytoplasm to the nucleus, MEF2 family members are constitutively present in the nucleus of unactivated cells (Woronicz *et al.*, 1995). This suggests that activation through these factors requires a cofactor and/or a post-translational modification. Notably, CsA-sensitive induction of the NGFI-B gene (Nur77) also appears to involve a MEF2 family member (Woronicz *et al.*, 1995). In the latter studies, the role of calcineurin and individual NFAT family members was not examined. However, consistent with our results, these investigators failed to identify NFAT binding to the responsive region.

We have previously demonstrated that transcriptional activation of the BZLF1 promoter by CaMKIV/Gr could proceed independently of other signals and was dependent on the CREB/AP1 site (ZII element) in the BZLF1 promoter (T.Chatila, H.Nga, P.Liu, S.Liu, G.Mosialos, E.Kieff and S.H.Speck, manuscript submitted). CaMKIV/Gr directed transcription from a single heterologous ZII

element (weak induction) and mutations affecting the CREB/AP1 site in the ZII element abrogated activation of transcription from the BZLF1 promoter. The ZII element contains a CREB/AP1 response element and the finding that the CaMKIV/Gr activates transcription from this element are in line with the known effects of CaMKIV/Gr on both CREB and AP-1. CaMKIV/Gr phosphorylates CREB on Ser133, the same residue targeted for phosphorylation by cAMP-dependent protein kinase (Sheng *et al.*, 1991; Cruzalegui and Means, 1993; Enslen *et al.*, 1994; Mathews *et al.*, 1994; Sun *et al.*, 1994). Ser133 phosphorylation results in the activation of CREB and enables this factor to promote transcription from its response elements. CaMKIV/Gr also mediates Ca^{2+} -dependent activation of AP-1-dependent transcription. The capacity of CaMKIV/Gr to activate these bZIP complexes may underlie the synergy with NFAT in the induction of Zp. CaMKII, which fails to induce transcription from Zp (T.Chatila, H.Nga, P.Liu, S.Liu, G.Mosialos, E.Kieff and S.H.Speck, manuscript submitted), phosphorylates CREB on Ser133 and additionally on Ser143 (Sun *et al.*, 1994). The latter residue acts to inhibit the activation of CREB by phosphorylated Ser133.

The strong synergistic induction of Zp observed here by constitutively active forms of CaMKIV/Gr and calcineurin indicates that Ca^{2+} signaling may play an important role in reactivating latent EBV. It is of some interest to note that expression of CaMKIV/Gr is induced in EBV-immortalized B cells by the viral membrane protein LMP1 (Mosialos *et al.*, 1994). This may set the stage for later reactivation of the virus by appropriate extracellular signals. It should also be noted that in EBV-immortalized B cells signaling through the antigen receptor is blocked by another viral membrane protein LMP2a (Miller, C.L. *et al.*, 1995). The cytoplasmic domain of LMP2a contains the conserved (YXXL/I)₂ motif found in the cytoplasmic domains of the transducing subunits associated with B and T cell receptors which interacts with the SH2 elements of src family tyrosine kinases (Songyang *et al.*, 1993; Weiss and Littman, 1994). LMP2a is constitutively tyrosine phosphorylated and is associated with the tyrosine kinases Lyn and Syk (Miller, C.L. *et al.*, 1995). In LMP2a-expressing B cells, basal Lyn tyrosine kinase activity is low and there is a failure to up-regulate tyrosine kinase activity upon crosslinking surface immunoglobulin (Miller, C.L. *et al.*, 1995). Thus, in EBV-immortalized lymphoblasts LMP2a appears to act as a dominant negative receptor to block activation of Src family tyrosine kinases. However, replacement of the cytoplasmic domain of CD8- α with the LMP2a cytoplasmic tail results in a fusion protein which can signal when engaged by anti-CD8- α antibodies (Beaufils *et al.*, 1993). This raises the possibility that *in vivo* there may exist latently infected cells that express LMP2a in an 'inducible' form, perhaps under conditions where it does not aggregate in the membrane, which when appropriately stimulated can trigger viral reactivation. One mechanism which might lead to an inducible form of LMP2a could involve expression of LMP2b, a form of LMP2 which lacks the cytoplasmic domain containing the (YXXI/L)₂ motif. This form of LMP2 may serve to down-regulate the capacity of LMP2a to block activation of infected cells by dispersing the LMP2a aggregates in the membrane (Longnecker and

Fig. 7. Proposed scheme of second messenger pathways triggered by anti-Ig which leads to induction of the EBV BZLF1 promoter (Zp). Crosslinking of cell surface Ig causes activation of protein tyrosine kinases (which can be blocked by the EBV-encoded membrane protein LMP2a in EBV-immortalized lymphoblastoid cell lines; Miller, C.L. *et al.*, 1995), which subsequently activates phospholipase C (PLC). Hydrolysis of phosphatidylinositol biphosphate (PIP₂) through PLC activation yields diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃). Protein kinase C (PKC) is activated by DAG and IP₃ increases intracellular Ca²⁺, which induces activation of the calcium/calmodulin-dependent kinase type IV/Gr (CaMKIV) and the calcium/calmodulin-dependent phosphatase calcineurin. Expression of CaMKIV is up-regulated in EBV-immortalized B cells by the virally encoded membrane protein LMP1 (Mosialos *et al.*, 1994). Activation of calcineurin leads to translocation of cytoplasmic NFAT to the nucleus. The *cis*-elements in Zp targeted by these pathways are as indicated.

Miller, 1996). Whether this would lead to an inducible form of LMP2a or simply block the ability of LMP2a to inhibit signaling through antigen receptor is unclear.

The various pathways which have been shown to trigger transcription from Zp are illustrated in Figure 7. The ability of both a calcineurin-regulated pathway and CaMKIV/Gr to activate Zp strongly suggests that Ca²⁺ signaling is an important component of the reactivation stimulus. It remains to be determined how activation by NFATc2 is mediated; whether this reflects a direct recruitment to Zp by MEF2D, perhaps in conjunction with a CREB/AP-1 family member(s), or an indirect effect mediated through an NFAT-induced gene product. The independent identification of a MEF2 family member as being involved in CsA-sensitive induction of the NGFI-B gene (Woronicz *et al.*, 1995) substantiates a role for MEF2 proteins in mediating Ca²⁺ signaling in lymphocytes. Further characterization of the role of MEF2D in the induction of

Zp awaits identification of the cellular factor(s) interacting with the ZII element.

Materials and methods

Cell culture, transfections and reporter assays

The EBV-negative Burkitt lymphoma cell line DG75 was grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). Cell cultures were maintained in a 5% CO₂ atmosphere at 37°C.

DG75 cells were transfected using DEAE-dextran as described previously (Flemington *et al.*, 1992; Borrás *et al.*, 1996) with the following modifications. Samples of 10⁷ cells/transfection were spun down, washed and resuspended in 250 µl phosphate-buffered saline (PBS). Aliquots of 2 µg of the appropriate vector and 250 µl of a 1 mg/ml solution of DEAE-dextran were then added and the mixture was incubated at 23°C for 20 min. The cells were then subjected to dimethylsulfoxide shock for 2 min at a final concentration of 7%, washed in PBS and resuspended in RPMI 1640 containing 10% FCS and cultured at 37°C for 48–72 h. HeLa cells were transfected by calcium phosphate precipitation. TPA (final concentration 20 ng/ml) and ionomycin (final concentration 1 µM) were added immediately post-transfection. Where indicated, CsA (final concentration 1 µM) was added to transfected cells 30 min prior to addition of TPA and ionomycin.

For CAT assays cells were harvested 48–72 h post-transfection, resuspended in 100 µl 0.25 mM Tris-HCl, pH 7.5, and lysed by three consecutive rounds of freeze-thawing. Cellular debris was cleared by centrifugation at 12 000 g for 5 min and 80% of the supernatant was used to assess CAT activity as described elsewhere (Gorman *et al.*, 1982). The level of CAT activity was determined by quantitating the acetylated species of chloramphenicol using a PhosphorImager (Molecular Dynamics). For luciferase assays cells were harvested 48 h post-transfection, washed twice with PBS and resuspended in 300 µl luciferase lysis buffer (50 mM Tris-MES, pH 7.8, 1% v/v Triton X-100) containing 1 mM fresh dithiothreitol (DTT). The cells were incubated for 5 min at room temperature to allow cells to lyse and then cellular debris was removed by centrifugation at 12 000 g for 5 min at 4°C. The lysate was recovered and kept on ice until assayed for luciferase activity, as previously described (de Wet *et al.*, 1987).

Plasmids

The plasmid -221ZpCAT contains BZLF1 promoter sequences from -221 to +12 bp, relative to the BZLF1 gene transcription initiation site, cloned upstream of the CAT gene in a modified pGL2 vector (Promega) (Borrás *et al.*, 1996). The -221ZpLuc reporter construct contains the BZLF1 promoter sequences from -221 to +12 bp cloned upstream of the firefly luciferase gene in the pGL2 basic vector (Promega). The Zp (ZIA/Bmf2) and Zp(ZIA/Bmf2m1) mutants were derived by oligonucleotide site-directed mutagenesis of the -221ZpLuc reporter construct according to the manufacturer's protocol (Bio-Rad). The specific mutations introduced into the ZIA and ZIB elements are described in Figure 5B.

Heterologous promoter constructs containing specific *cis*-elements from the BZLF1 promoter were derived by cloning the indicated Zp element(s) into the βGAT vector which contains the β-globin TATA box upstream of either the CAT reporter gene in a modified pGL2 vector (Borrás *et al.*, 1996) or the firefly luciferase gene in the pGL2 basic vector (Promega). Three copies of the ZIA or ZIB element were cloned into the *Ava*I and *Xba*I restriction sites upstream of the β-globin TATA box and the 1×ZII element was cloned into the *Xba*I and *Bam*HI sites. The sequences of the sense strand oligonucleotides employed for the cloning were as follows: 3×ZIA(+), 5'-CCGGGGGCTGTCTATTTT-GACACCAGGCTGTCTATTTT-GACACCAGGCTGTCTATTTT-GACACCAT-3'; 3×ZIB(+), 5'-CCGGGCACCATGTTATTTAGACAC-TTCCACCAGCTTATTTAGACACTTCCACCAGCTTATTTAGACACTTCT-3'; 3×ZIC(+), 5'-CCGGGCTCCTCTCTTTAGAACT-CTCTCTCTCTTTAGAACTACTCTCTCTCTTTAGAACTAT-3'; 1×ZII(+), 5'-CATAGACGTCCCAACCATGCATCAGACAG-GAG-3'.

Expression vectors for constitutively active calcineurin and NFATc1, NFATc2 and NFATc3 were generously provided by Dr Gerry Crabtree (Stanford University) and have been previously described (Northrop *et al.*, 1994; Ho *et al.*, 1995). cDNA encoding FLAG epitope-tagged human CaMKIV/Gr was subcloned into pSG5 vector to generate pHGR-FLAG, as previously described (Mosialos *et al.*, 1994). The constitutively active CaMKIV/Gr mutant ΔCaMKIV/Gr(c) was prepared from pHGR-

FLAG by replacing the Gln318 codon of CaMK IV/Gr(wt) with a stop codon and the double mutant constitutive/inactive Δ CaMK IV/Gr(i) was prepared by mutating Lys75 of Δ CaMK IV/Gr(c) to Glu (Ho *et al.*, 1996).

Electrophoretic mobility shift assays

DG75 crude nuclear extracts were prepared as described previously (Borras *et al.*, 1996). A total reaction volume of 20 μ l containing 5 μ g extract, 5 μ l buffer D (20 mM HEPES, pH 7.9, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride and 0.5 mM DTT), 4.5 μ g bovine serum albumin, 0.5 μ l 0.1 M DTT and 2 μ g salmon sperm DNA was incubated at room temperature for 5 min. If competitor oligonucleotides were used, they were added at this point. The relevant 32 P-labeled double-stranded oligonucleotide (0.4 ng/reaction, corresponding to 200 000–300 000 c.p.m.) was added and incubated at room temperature for 30 min. Samples were loaded onto a running non-denaturing 4% acrylamide:0.1% bisacrylamide gel. The gel was run in 0.5 $\times$ TBE (1 $\times$ TBE is 90 mM Tris, 64.6 mM boric acid and 2.5 mM EDTA, pH 8.3). The 1 $\times$ oligonucleotides used were designed to include the entire region protected from DNase I digestion (Flemington *et al.*, 1990a) and their sequences were as previously described (Borras *et al.*, 1996).

Methylation interference

The double-stranded ZIB probe that was used for the EMSA was 32 P-labeled at only the 5'- or 3'-end as described (1,25), except 200 ng probe were used. Methylation interference was carried out essentially as previously described (Maniatis *et al.*, 1982; Ausubel *et al.*, 1992). A quarter of the sample was partially methylated with dimethylsulfate for 3 min. For the binding reaction, the EMSA protocol was scaled up 6-fold and 1–2 $\times$ 10⁶ c.p.m. of the probe was added. A 1-fold sample without extract was also run. After electrophoresis, the samples were transferred to a DEAE membrane using a semi-dry electrophoretic transfer unit, the membrane was exposed for autoradiography and the relevant bands were cut out and eluted (Singh *et al.*, 1986). The DNA was cleaved at the methylated bases using 1 M piperidine at 90°C for 30 min. The samples were loaded onto a 10% polyacrylamide/urea sequencing gel in 1 $\times$ TBE running buffer.

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