Communication

G₀/G₁ Growth Arrest Mediated by a Region Encompassing the Basic Leucine Zipper (bZIP) Domain of the Epstein-Barr Virus Transactivator Zta*

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The Epstein-Barr virus (EBV) immediate early transactivator Zta is a basic leucine zipper (bZIP) transcription factor that causes G₀/G₁ cell cycle arrest through induction of the tumor suppressor protein, p53, and the cyclin-dependent kinase inhibitors, p21 and p27 (Cayrol, C., and Flemington, E. K. (1996) EMBO J. 15, 2745–2759). Here, we report a genetic analysis of Zta-mediated G₀/G₁ growth arrest and p21 induction. The majority of the Zta transactivation domain can be deleted (Z₁–128) without significantly affecting the ability of Zta to elicit growth arrest. A larger amino-terminal deletion (Z₁–167) abrogates the ability of Zta to inhibit proliferation, mapping the growth-inhibitory domain to a carboxy-terminal region encompassing the bZIP domain (amino acids 128–245). The integrity of the bZIP domain is required for growth suppression since a two-amino acid mutant which is defective for homodimerization, fails to induce cell cycle arrest. Western blot analysis of p21 expression in cells expressing Zta mutants reveals that the ability of Zta mutants to cause G₀/G₁ growth arrest is intimately related to their capacity to induce p21 expression. Together, these data demonstrate that a carboxy-terminal region of Zta that includes the bZIP domain is sufficient to mediate G₀/G₁ growth arrest and p21 induction.

The Epstein-Barr virus (EBV) lytic switch transactivator Zta (also referred to as BZLF1, EB1, and Zebra) is a sequence-specific DNA-binding protein related to the bZIP family of transcription factors, which plays a key role in the EBV replicative cycle (1). By transactivating several early lytic cycle viral promoters, Zta initiates the ordered cascade of EBV gene expression that results in the induction of an estimated 100 or more viral replication-associated genes and culminates in virus production (2). In addition to its role in EBV lytic gene expression (3) and replication (4, 5), Zta can also regulate the expression of cellular factors such as the growth suppressive cytokine transforming growth factor-β (6).

Zta is a member of the bZIP family of transcription factors and binds as a homodimer to multiple AP1 or ZRE (Zta-responsive elements) sites in the promoters of target genes (7). The carboxy-terminal bZIP domain of Zta has significant amino acid homology with the basic DNA-binding and dimerization domains of c-Fos (7) and C/EBP (8). The bZIP domain of Zta mediates homodimerization through a coiled-coil interaction, although Zta lacks the heptad repeat of leucine residues found in the leucine zipper proteins (8–10). The amino-terminal region of Zta does not appear to influence dimerization or DNA binding, but plays a role in activation of transcription (11–13). The Zta activation domain has been shown to mediate association with the general transcription factor TFIIA (14, 15) and the TATA box-binding protein TBP (12). Amino acids between 25 and 86 were shown to be critical for this latter interaction (12).

We have recently demonstrated that Zta inhibits proliferation by causing cell cycle arrest in G₀/G₁ in several epithelial tumour cell lines (16). Zta-mediated G₀/G₁ arrest was found to result from induction of the tumor suppressor protein, p53, and the cyclin-dependent kinase (CDK) inhibitors, p21/WAF-1/CIP-1 and p27/KIP-1, two pleiotropic mediators of cell cycle arrest, that inhibit kinase activity of various cyclin-CDK complexes (17–21). Inactivation of the retinoblastoma tumor suppressor protein (pRb), a known target of cyclin-dependent kinases (22), was shown to overcome Zta-mediated G₀/G₁ arrest, indicating that pRb or pRb-related proteins are involved in the pathway of growth suppression induced by Zta (16).

Here we report on the ability of Zta mutants to cause G₀/G₁ growth arrest and demonstrate that growth arrest is independent of the transactivation function of Zta. These results suggest a key role for the bZIP domain and flanking sequences in Zta-mediated growth arrest and induction of p21.

EXPERIMENTAL PROCEDURES

Construction of Plasmids—The pMARK vector encoding the cell surface marker, CD7, was a generous gift from Seth Alper. pMARK-Zta expression plasmids used in transient transfection assays were obtained by subcloning the indicated Zta gene sequences into pMARK, downstream from the SV40 promoter (9, 13, 16, 23).

Transfections and Western Blot—Transient transfection experiments using the human cervical carcinoma cell line, HeLa, were performed employing the calcium phosphate precipitation procedure (24). Cells were grown in high-glucose Dulbecco’s modified Eagle’s medium (Cellgro), supplemented with 10% fetal bovine serum (Life Technologies, Inc.), in a 5% CO₂ environment. To analyze the effects of Zta mutants on p21 expression, 10⁶ cells were transfected with 5 µg of pMARK control plasmid, 5 µg of pMARK-Zta plasmid, or 5 µg of pMARK-Zta mutant expression vectors (pMARK-Z31–128, pMARK-Z1–167, pMARK-Zdbm1, pMARK-Zdim). Cells were harvested 72 h following transfection, and extracts were assayed for p21 expression by Western blot analysis with a mouse monoclonal antibody against p21 (sc-187, Santa Cruz Biotechnology), as described previously (16).

Cell Cycle Analysis by Flow Cytometry—The effects of Zta on cell cycle distribution were determined as described previously (16). Briefly,
The amino terminus of Zta is not required for Zta-induced G₀/G₁ growth arrest. Zta amino-terminal deletion mutants were analyzed for their ability to inhibit the growth of HeLa cells. Cells were transiently transfected with 5 µg of pMARK (control encoding only cell surface CD7 marker) pMARK-Zta (encoding both CD7 marker and Zta), or pMARK-Zta deletion mutants vectors, pMARK-Z1–128 and pMARK-Z1–167 (encoding both CD7 marker and Zta mutants). Cell cycle distribution was determined on transfected cells (CD7⁺) by FACS. The experiment shown here is representative of three experiments.

1 × 10⁶ HeLa cells were cotransfected with 5 µg of pMARK-Zta (or Zta mutants) plasmid encoding both the CD7 cell surface marker and Zta together with 25 µg of pGL2 carrier plasmid, using a standard calcium phosphate precipitation method (24). Control experiments were performed using 5 µg of pMARK plasmid encoding only CD7 marker and 25 µg of pGL2 carrier plasmid. Three days after transfection, cells were collected, washed in PBS, and incubated with a fluorescein isothiocyanate-conjugated CD7 monoclonal antibody (3A1, diluted 1/10; Sigma) for 1 h on ice. Cells were then washed in PBS, fixed with 70% cold ethanol for at least 30 min, washed with PBS, treated for 30 min at 37 °C with RNase A (0.1 mg/ml), and stained with propidium iodide (69 µM) in 38 mm sodium citrate. Cells were then analyzed by fluorescence-activated cell sorting (FACSScan; Becton Dickinson) for both DNA content and CD7 staining. Analyses were performed three times on 40,000 cells with similar results.

RESULTS

The Amino-terminal Region of Zta Is Not Required for Induction of G₀/G₁ Growth Arrest—To identify regions of Zta that are important for its growth-inhibitory activity, a transient transfection assay was employed (16). pMARK-Zta plasmids were constructed that allow co-expression of Zta (or Zta mutants) and a signal transduction-defective CD7 cell surface marker (25). HeLa cells were transfected with pMARK-Zta, pMARK-Zta amino-terminal deletion mutants, or the parental plasmid, pMARK. Three days after transfection, the CD7 positive cell population was separated employing fluorescence-activated cell sorting (FACS) and subjected to DNA content analysis to assess the cell cycle distribution (Fig. 1). A G₀/G₁ cell cycle arrest is observed in the CD7⁺ population from pMARK-Zta transfected HeLa cells (G₀/G₁: 88%; S: 5.7%) but not in the CD7⁺ population of pMARK transfected HeLa cells (G₀/G₁: 52.4%; S: 31%). Deletion of the majority of the Zta activation domain (ZΔ1–128) does not significantly affect the ability of Zta to elicit a G₀/G₁ growth arrest since only 3.9% of cells expressing ZΔ1–128 were in S phase compared with 31% in control cells transfected with pMARK. This amino-terminal truncation severely impairs the ability of Zta to induce transcriptional activation (13) and data not shown) and deletes the region shown previously to mediate association with the TATA box-binding protein TBP (12). Therefore, Zta-mediated growth arrest is likely independent of its ability to activate transcription or to interact with TBP. To further refine the analysis of Zta sequences involved in mediating growth arrest, a mutant with a larger amino-terminal deletion was tested (Fig. 1). Deletion of the first 167 amino acids (ZΔ1–167) abrogated the ability of Zta to cause growth arrest, indicating that key Zta growth-inhibitory sequences are located in a carboxyl-terminal region of Zta (amino acids 128–245). Essentially identical results were obtained when these constructs (Zwt, ZΔ1–128, ZΔ1–167) were expressed in the EBV-negative nasopharyngeal cell line, AdAH, and the EBV-positive nasopharyngeal cell line, NPC-KT, two other epithelial cell lines sensitive to the growth-inhibitory effects of Zta (16) and data not shown).

The Integrity of the bZIP Domain Is Required for Zta-mediated G₀/G₁ Growth Arrest—To determine whether the bZIP domain plays a role in growth inhibition, we tested two Zta mutants with alterations in this domain, a Zta DNA-binding mutant (Zdbm1), which has lost the ability to recognize ZRE (Zta-responsive elements) sites (23) and a mutant with a two-amino acid substitution in the Zta dimerization domain (Zdim), which is defective for dimerization (9). The Zta DNA-binding mutant, Zdbm1, retains the capacity to block cell cycle progression with an efficiency similar to that of wild type Zta since 86% of cells expressing Zdbm1 were found in the G₀/G₁ population compared with 83% of cells expressing wild type Zta (Fig. 2). This further suggests that Zta-mediated G₀/G₁ growth arrest is independent of its ability to transactivate AP1 or ZRE containing cellular promoters. In contrast to Zdbm1, the dimerization mutant, Zdim, has lost the ability to induce cell cycle arrest (only 55% of cells in G₀/G₁), indicating that dimerization and/or
the integrity of the Zta coiled-coil dimerization structure is required for growth suppression (Fig. 2). Western blot analysis revealed that the expression level of the mutants which fail to induce growth arrest (Zdbm1, ZΔ1–167) is similar to that of mutants that block cell cycle progression (data not shown). In addition, all mutants are localized to the nucleus as judged by immunofluorescence analysis (data not shown). Together, these data demonstrate that Zta-induced growth arrest is mediated by a carboxyl-terminal region and requires an intact bZIP domain.

The Capacity of Zta Mutants to Cause G0/G1 Arrest and Induction of p21 Are Intimately Related—Our previous studies demonstrated that Zta causes a p53-dependent induction of p21 levels suggesting that this may be a key response leading to cellular growth arrest (16). Based on this, the genetics of Zta-mediated growth arrest might be expected to parallel the genetics of p21 induction. As shown in Fig. 3, p21 is induced by Zta, ZΔ1–128, and Zdbm1 but not ZΔ1–167 or Zdim. Therefore, a tight correlation exists between the ability of Zta mutants to elicit a G0/G1 growth arrest and to induce p21 expression.

**DISCUSSION**

Several conclusions can be derived from this limited genetic analysis. First, a tight correlation exists between growth arrest and induction of p21. We showed previously that induction of p21 by Zta is mediated through p53 (16), and, indeed, induction of p53 follows the same genetics as that of p21 (data not shown). Therefore, this pathway is likely to play an important role in transmitting Zta growth arrest signals. These studies also demonstrate that a carboxyl-terminal region of Zta encompassing the bZIP domain is sufficient to cause a G0/G1 growth arrest. Deletion of the amino-terminal half of Zta does not significantly affect its capacity to block G1/S progression since the mutant, ZΔ1–128, inhibited the percentage of cells in S phase, as efficiently as wild type Zta (respectively 3.9% and 5.7% versus 31% in untransfected cells). We note, however, that the G1/S population is not reduced in ZΔ1–128 expressing cells indicating an inability of cells to traverse through G2/M. This suggests that sequences between amino acids 1 and 128 are required for progression through the G1/S checkpoint in the context of other Zta-mediated alterations in cellular growth control pathways. This apparent G1/M arrest is not evident in Zdbm1-transfected cells indicating that the inability of ZΔ1–128 to transactivate ZRE containing cellular promoters is not the sole defect leading to a block in G1/M progression. Instead, it is possible that these sequences contribute to some functional interactions with factors involved in controlling the G2/M checkpoint control.

The ability of the Zta DNA-binding mutant, Zdbm1, to efficiently induce cellular growth arrest provides additional support for the idea that the transactivation function of Zta is not required for inducing growth arrest. We have previously reported that Zta DNA binding mutants, including Zdbm1, can activate certain promoters in transient reporter assays probably through protein-protein interactions (23). However, in these studies, we found that Zdbm1 and wild type Zta elicited transcriptional activation through distinct promoter elements and they may therefore have distinct promoter specificities. Although we cannot rule out the possibility that transcriptional activation plays a role in eliciting cellular growth arrest, we favor a model whereby Zta induces growth arrest through protein-protein interactions with key cell cycle control proteins. Previous studies demonstrated an interaction between p53 and the dimerization domain of Zta (26). Moreover, in this study, it was suggested that the Zta-mediated post-transcriptional induction of p53 might arise through masking of p53 sequences involved in targeting it for ubiquitin-dependent degradation (26). Since the results presented here point to a critical role for Zta’s bZIP domain in eliciting G0/G1 growth arrest and p21 induction, this is a reasonable possibility.

Results presented here suggest that sequences upstream from the bZIP domain, amino acids 128 to 167, are also required for p21 induction and for cellular growth arrest. At this time we don’t know whether this sequence might contribute to or stabilize interactions with key cellular proteins or whether this region provides conformational contributions to the bZIP structure.

The finding that the carboxyl-terminal region of Zta encompassing the bZIP domain is sufficient to block cell proliferation further emphasizes the critical role of bZIP domains and bZIP transcription factors in the control of cell proliferation. Other bZIP factors have previously been implicated in affecting cell growth control pathways. For example, the AP1 family bZIP factors, c-Fos and c-Jun, promote cell proliferation in some settings and are associated with differentiation in several cellular differentiation model systems (27–31). The Caenorhabditis elegans bZIP protein Ces-2 has been shown to induce programmed cell death (32). Further investigations into mechanisms driving Zta-mediated G0/G1 growth arrest should reveal additional insights into the role of bZIP transcription factors in cell proliferation.

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