Equine Herpesvirus Protein E10 Induces Membrane Recruitment and Phosphorylation of its Cellular Homologue, Bcl-10

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Abstract. v-E10, a caspase recruitment domain (CARD)-containing gene product of equine herpesvirus 2, is the viral homologue of the bcl-10 protein whose gene was found to be translocated in mucosa-associated lymphoid tissue (MALT) lymphomas. v-E10 efficiently activates the c-jun NH_2-terminal kinase (JNK), p38 stress kinase, and the nuclear factor (NF)-κB transcriptional pathway and interacts with its cellular homologue, bcl-10, via a CARD-mediated interaction. Here we demonstrate that v-E10 contains a COOH-terminal geranylgeranylation consensus site which is responsible for its plasma membrane localization. Expression of v-E10 induces hyperphosphorylation and redistribution of bcl-10 from the cytoplasm to the plasma membrane, a process which is dependent on the intactness of the v-E10 CARD motif. Both membrane localization and a functional CARD motif are important for v-E10–mediated NF-κB induction, but not for JNK activation, which instead requires a functional v-E10 binding site for tumor necrosis factor receptor–associated factor (TRAF)6. Moreover, v-E10–induced NF-κB activation is inhibited by a dominant negative version of the bcl-10 binding protein TRAF1, suggesting that v-E10–induced membrane recruitment of cellular bcl-10 induces constitutive TRAF-mediated NF-κB activation.

Key words: herpesvirus • bcl-10 • CARD • NF-κB • TRAF

Introduction

Members of the γ-herpesvirus family are characterized by the ability to establish latent infections in lymphoid cells and some of the primate γ-herpesviruses, such as Epstein-Barr virus (EBV)[1] and Kaposi's sarcoma–associated human herpesvirus-8, are closely associated with abnormal cell proliferation and cancer (for review see Damania et al., 2000). Equine herpesvirus (EHV)-2 is a slow growing lymphotropic γ-herpesvirus widely distributed in the equine population (Telford et al., 1995), but its precise role as a pathogen remains largely uncharacterized. The virus has been reported to induce chronic pharingitis associated with lymphoid proliferation in infected horses (Blakeslee et al., 1975). The persistence of the virus in infected lymphocytes suggests that it has evolved strategies to counteract the proapoptotic and growth-arresting processes naturally occurring in the infected host cell.

Many γ-herpesviruses encode proteins (called viral FLIPs) which inhibit induction of apoptosis through caspase-8–dependent death receptors (for review see Tschopp et al., 1998). Some γ-herpesviruses also encode membrane proteins which actively induce proliferation of the infected cells by activation of the nuclear factor (NF)-κB transcriptional pathway (for review see Damania et al., 2000). The tumor necrosis factor receptor–associated factor (TRAF) family of adapter proteins, which are important mediators of cellular stress responses triggering the NF-κB and c-jun NH_2-terminal kinase (JNK) transcriptional pathways (for review see Arch et al., 1998), play an essential role in this activation. Indeed, the transforming activity of the EBV latent membrane protein (LMP)1 is dependent, at least in part, on the TRAF-mediated activation of the NF-κB pathway, which induces the expression of antiapoptotic genes such as bcl-2 and A20 (Henderson et al., 1991; Laherty et al., 1992; Opipari et al., 1992). The herpesvirus Saimiri transforming protein was also shown to activate NF-κB through interaction with TRAFs (Lee et al., 1999).

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[1]*Abbreviations used in this paper:* CARD, caspase recruitment domain; EBV, Epstein-Barr virus; EHV, equine herpesvirus; JNK, c-jun NH_2-terminal kinase; LMP, latent membrane protein; MALT, mucosa-associated lymphoid tissue; NF, nuclear factor; TRAF, tumor necrosis factor receptor–associated factor; VSV, vesicular stomatitis virus.

We have shown previously that the EHV-2 protein E10 activates the NF-κB transcriptional pathway as well as JNK and p38 stress kinases (Thome et al., 1999). v-E10 binds to TRAF3 and TRAF6, whereas its cellular homologue bcl-10 binds TRAF1, 2, and 5 (Thome et al., 1999; Yoneda et al., 2000). v-E10 and bcl-10 contain a highly homologous NH$_2$-terminal caspase recruitment domain (CARD) motif which allows for both homo- and heterodimerization of the two proteins (Koseki et al., 1999; Srinivasula et al., 1999; Thome et al., 1999). However, the two proteins differ in their COOH-terminal extensions. v-E10 contains a glycine-rich extension of ∼200 amino acids, which shares very little sequence homology with the shorter, serine/threonine-rich COOH terminus of bcl-10 (Srinivasula et al., 1999; Thome et al., 1999), suggesting that the two related proteins may target the same biological pathway but with a different physiological outcome.

The in vivo function of bcl-10 is still poorly defined. Various reports have suggested a role for bcl-10 in induction of apoptosis and NF-κB activation (Costanzo et al., 1999; Koseki et al., 1999; Srinivasula et al., 1999; Thome et al., 1999; Yan et al., 1999; Yoneda et al., 2000). A potential tumor-suppressing function of bcl-10 has been postulated based on genetic studies in which bcl-10 cDNA mutation and/or abnormal expression of bcl-10 RNA has been correlated with the formation of mucosa-associated lymphoid tissue (MALT) B cell lymphomas and various other types of tumors (Willis et al., 1999; Zhang et al., 1999). However, others have reported a naturally occurring high degree of *BCL-10* gene polymorphism which may be unrelated to tumor formation (Apostolou et al., 1999; Fakruddin et al., 1999).

In this study, we have investigated the subcellular distribution of v-E10 and of its cellular homologue, bcl-10, in the context of the induction of the JNK and NF-κB transcriptional pathways. We find that v-E10 constitutively associates with the plasma membrane and induces hyperphosphorylation and partial redistribution of cytoplasmic bcl-10 to the membrane. Moreover, both membrane localization and an intact CARD motif of v-E10 are important for the activation of the NF-κB, but not for activation of the JNK pathway, which instead requires v-E10 binding to TRAF6. These results provide new insights into the molecular mechanism underlying v-E10 and bcl-10 function.

Materials and Methods

Expression Vectors

Expression vectors for v-E10 and bcl-10 with an NH$_2$-terminal FLAG, or vesicular stomatitis virus (VSV), tag and for dominant negative TRAF1 have been described previously (Thome et al., 1999; Irmler et al., 2000). A deletion construct of v-E10 lacking the two COOH-terminal cysteine residues, cysteine 310 and 311 (v-E10ΔCC), point mutants of v-E10 where either one of both cysteine residues was replaced by an alanine residue (v-E10-CA and v-E10-AC); the CARD-mutant (L49R); and the TRAF6-binding mutant (P$_{278}$QE→GQA) were obtained by amplification on cloned full length wild-type v-E10 cDNA using standard PCR conditions and Pwo polymerase (Boehringer). Amplified products were sequenced in both directions and subcloned into expression vectors derived from pCR-3 to yield expression constructs with an NH$_2$-terminal FLAG or VSV tag. The TRAF6 expression vector was a gift of V. Dixit (Genentech, San Francisco, CA) and reporter plasmids NF-κBLuc and FLAG-JNK were gifts of V. Jongeneel and C. Widmann (University of Lausanne), respectively.

Cell Lines and Culture Conditions

293T, HeLa, and NIH3T3 cells were grown in DME and Jurkat cells were grown in RPMI 1640 medium, both supplemented with 10% heat-inactivated FCS and penicillin/streptomycin (100 μg/ml of each).

Transient Cell Transfection, Immunoprecipitation, JNK, and NF-κB Activation Assays

These techniques were performed essentially as described previously (Bodmer et al., 1997; Thome et al., 1999). Antibodies used for Western blotting include anti-FLAG M2 and anti-VSV P5D4 monoclonal antibodies (Sigma-Aldrich), rabbit anti-TRAF6 (H-274; Santa Cruz Biotechnology, Inc.), and an antibody specifically detecting the activated (phosphorylated) form of JNK (Promega).

Cellular (endogenous) bcl-10 was detected using an affinity-purified polyclonal rabbit antibody (AL114) directed against a peptide encompassing 24 NH$_2$-terminal amino acids (SLTEEDLTEVKKDALENYRVL-CEK) of murine bcl-10, synthesized using the multiple antigen technology (Francis et al., 1991).

Generation of v-E10–expressing Jurkat and NIH3T3 Clones

For generation of stable v-E10–expressing Jurkat clones, an NH$_2$-terminally FLAG-tagged v-E10 construct was subloned into an expression vector with IPTG-inducible promoter (LacSwitch inducible mammalian expression system; Stratagene). 8 × 10^6 exponentially grown Jurkat cells were transfected with 20 μg of v-E10 vector or empty vector by electroporation at 250 kV and 960 μF and stable clones were selected in RPMI complete medium with 300 μg/ml of hygromycin B (Calbiochem). For analysis of v-E10 isoprenylation, Jurkat cells were treated for 48 h with 10 mM IPTG and the indicated concentrations of lovastatin (Calbiochem) previously converted to the open acid form as described (Liu et al., 1999).

For generation of stable v-E10–expressing NIH3T3 clones, an NH$_2$-terminally FLAG-tagged v-E10 construct in pCR-3 or an empty vector was transfected into NIH3T3 cells using Effectene transfection reagent (QIAGEN) according to the manufacturer's instructions, and stable clones were selected in DME complete medium with 1 μg/ml of G418 (GIBCO BRL).

Protein Dephosphorylation

Transfected 293T cells from a 5.5-cm dish were lysed in 1% NP-40 buffer containing 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 0.25 μg/ml Pefabloc® SC (Serva) on ice. Aliquots (20 μg) of postnuclear lysates were incubated with lambda protein phosphatase (New England Biolabs, Inc.) in buffer supplied by the manufacturer for 1 h at 30°C.

Cellular Fractionation

293T cells (∼5 × 10^5) from a 5.5-cm dish were harvested and washed twice with cold PBS, and cell pellets were resuspended in 100 μl of ice-cold lysis buffer (20 mM Tris-HCl, pH 7.4, 10 mM EDTA, 5 mM EGTA, 0.1% β-mercaptoethanol, 1 mM Na$_3$VO$_4$, and protease inhibitors [100 ng/ml benzamidine, Sigma-Aldrich; 10 ng/ml antipain and 2.5 ng/ml leupeptin, Boehringer]) for 30 min on ice. Lysates were homogenized by 50 strokes through a syringe with a 25G5/8 needle (0.5 × 16 mm) and centrifuged at 1,500 g for 5 min to remove nuclei and cellular debris. Postnuclear lysates were centrifuged at 100,000 g for 1 h to yield the cytosolic supernatant and the membrane pellet which were boiled in reducing SDS-PAGE sample buffer for subsequent SDS-PAGE and Western blot analysis.

Triton X-114 Extraction

Cells (∼10^6) were harvested and submitted twice to Triton X-114 extraction (Bordier, 1981). Proteins of the aqueous and diluted detergent phases were precipitated by the addition of MeOH/CHCl$_3$ (1:4, vol/vol) and the resulting protein pellet was dried, resuspended in reduced SDS-PAGE sample buffer, sonicated, and boiled before loading for SDS-PAGE and Western blotting.

Immunostaining and Confocal Laser Scanning Microscopy

HeLa cells of 10–20% confluency were transfected on 20-mm glass coverslips by the calcium phosphate/BES method (Ausubel et al., 1999). 30 h
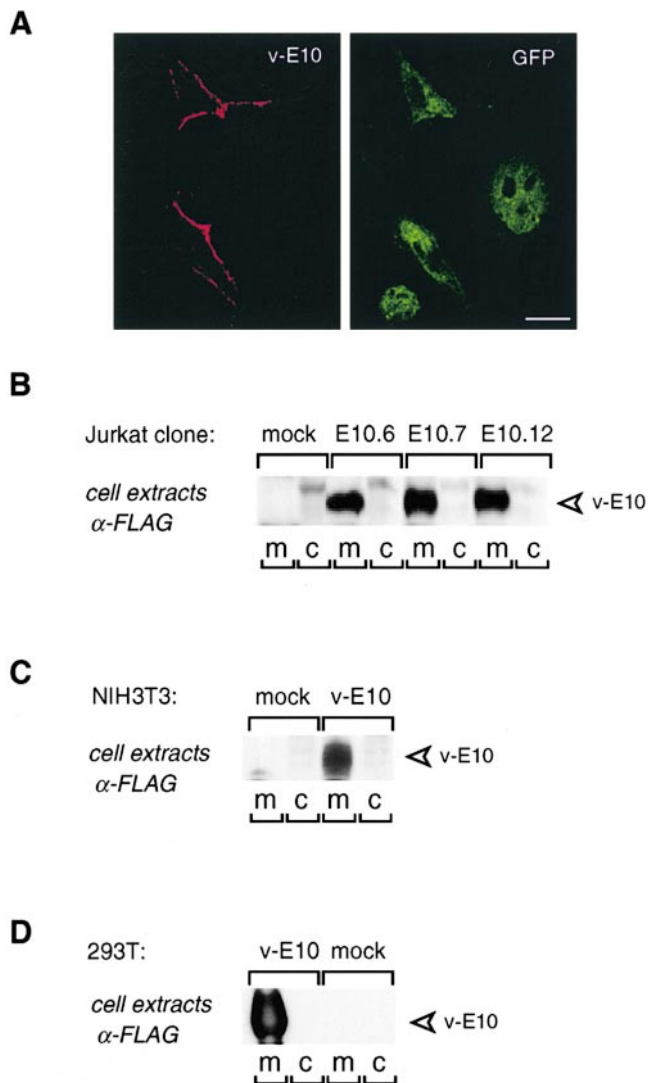
A

B

Jurkat clone: mock   E10.6   E10.7   E10.12

cell extracts
α-FLAG   ◁ v-E10

m c   m c   m c   m c

C

NIH3T3:   mock   v-E10

cell extracts
α-FLAG   ◁ v-E10

m c   m c

D

293T: v-E10   mock

cell extracts
α-FLAG   ◁ v-E10

m c   m c

Figure 1. v-E10 is localized at the plasma membrane. (A) HeLa cells were cotransfected with expression plasmids for GFP and VSV-tagged v-E10 and cells were stained using anti-VSV and Cy5-labeled secondary antibody. Protein expression was analyzed by confocal laser scanning microscopy at 488 nm (green staining of GFP) and 633 nm (red staining of VSV-tagged v-E10). (B) Clones of Jurkat cells expressing FLAG-tagged v-E10 were analyzed by Triton X-114 extraction, SDS-PAGE, and anti-FLAG Western blot for repartition of v-E10 protein into cytoplasmic (c) and membrane (m) fractions. (C) Mock-transfected and v-E10–expressing stable NIH clones were analyzed as in B. (D) 293T cells were transfected with mock or v-E10 expression vectors and the subcellular localization of v-E10 was analyzed as in B. Bar, 10 μm.

after onset of transfection, cells on coverslips were washed twice with cold PBS, fixed for 12 min in 4% paraformaldehyde at room temperature, washed again, and permeabilized in PBS with 0.1% saponine (PBSS) overnight at 4°C. Coverslips were blocked for 30 min at room temperature with 5% milk in PBSS (PBSSM), stained with anti-VSV (1:50,000; Sigma-Aldrich) or anti-FLAG (0.2 μg/ml; Zymed Laboratories) in PBSSM for 1 h at room temperature, washed three times with PBSS, and stained with Cy5-labeled (Jackson ImmunoResearch Laboratories), Alexa-488–labeled (Molecular Probes) secondary anti–mouse, or anti–rabbit antibody (1:100 in PBSSM) for 1 h in the dark. Finally, coverslips were washed three times with PBS and mounted with FluorSave reagent (Calbiochem). Confocal microscopy was performed on an Axiovert 100 micro-

scope (laser scanning microscope model 510; ZEISS) with a 63× oil objective. To detect the Cy5 fluorochrome, a helium laser was filtered at 633 nm; an argon laser at 488 nm was used to detect the Alexa fluorochrome. Standardized conditions for the pinhole size, gain, and offset (brightness and contrast) were used for image capture. Each image was the average of 16 scans. Digital images were prepared using Adobe Photoshop®.

Results

HeLa cells were transfected with an expression vector encoding FLAG-tagged v-E10 and the cellular distribution of the transfected protein was analyzed by confocal laser scanning microscopy. v-E10 was found exclusively localized at the plasma membrane (Fig. 1 A). This subcellular localization of v-E10 was corroborated biochemically by Triton X-114 extraction (Fig. 1, B–D). v-E10 from transiently transfected 293T and HeLa cells was found to partition exclusively to the detergent-soluble membrane fraction (Figs. 1 D and 2 A). Similar results were obtained using stable clones of Jurkat and NIH3T3 cells expressing v-E10 (Fig. 1, B and C).

v-E10 does not contain a putative transmembrane segment and must therefore be targeted to the plasma membrane in some other manner. Covalent lipid modifications, such as myristilation, palmitoylation, farnesylation, or geranylgeranylation, can mediate membrane targeting of proteins that lack a transmembrane domain (Casey and Seabra, 1996; Resh, 1999). Indeed, the amino acid sequence of v-E10 terminates in two cysteine residues and thus contains a cognate consensus site for geranylgeranylation by geranylgeranyltransferase II (rab geranylgeranyltransferase), which transfers geranylgeranyl to proteins terminating in CC, CXC, or CCXX (Seabra et al., 1992; Glomset and Farnsworth, 1994). To investigate whether the two COOH-terminal cysteine residues of v-E10 are implicated in its membrane localization, we generated mutants of v-E10 lacking both cysteine residues (v-E10ΔCC) or in which either one of the two cysteine residues was replaced by an alanine residue (v-E10-CA and v-E10-AC, respectively). Biochemical fractionation and confocal laser scanning showed that v-E10 lacking both COOH-terminal cysteine residues was localized in the cytoplasm and no longer detectable in the membrane fraction (Fig. 2, A and B). The subcellular distribution of the mutants v-E10-CA and v-E10-AC was similar to the one observed for the deletion mutant, indicating that both Cys residues are required for plasma membrane targeting of v-E10 (Fig. 2 B). The 3-hydroxymethylglutaryl-coenzyme A reductase inhibitor lovastatin inhibits isoprenylation, e.g., farnesylation and geranylgeranylation, by decreasing cellular pools of farnesylpyrophosphate and geranylgeranylpyrophosphate, respectively. Pretreatment of stably v-E10–expressing Jurkat cells with lovastatin significantly reduced the membrane localization of v-E10, but not of the unrelated transmembrane protein Fas, whereas it did not affect the overall expression levels of v-E10 (Fig. 2, C and D).

v-E10 associates with bcl-10 both in two-hybrid assays and in coimmunoprecipitation experiments (Costanzo et al., 1999; Koseki et al., 1999; Srinivasula et al., 1999; Thome et al., 1999). This association requires an intact CARD region of v-E10 since a v-E10 CARD point mutation (L49R) homologous to a mutation previously demonstrated to be important for CARD–CARD interaction in
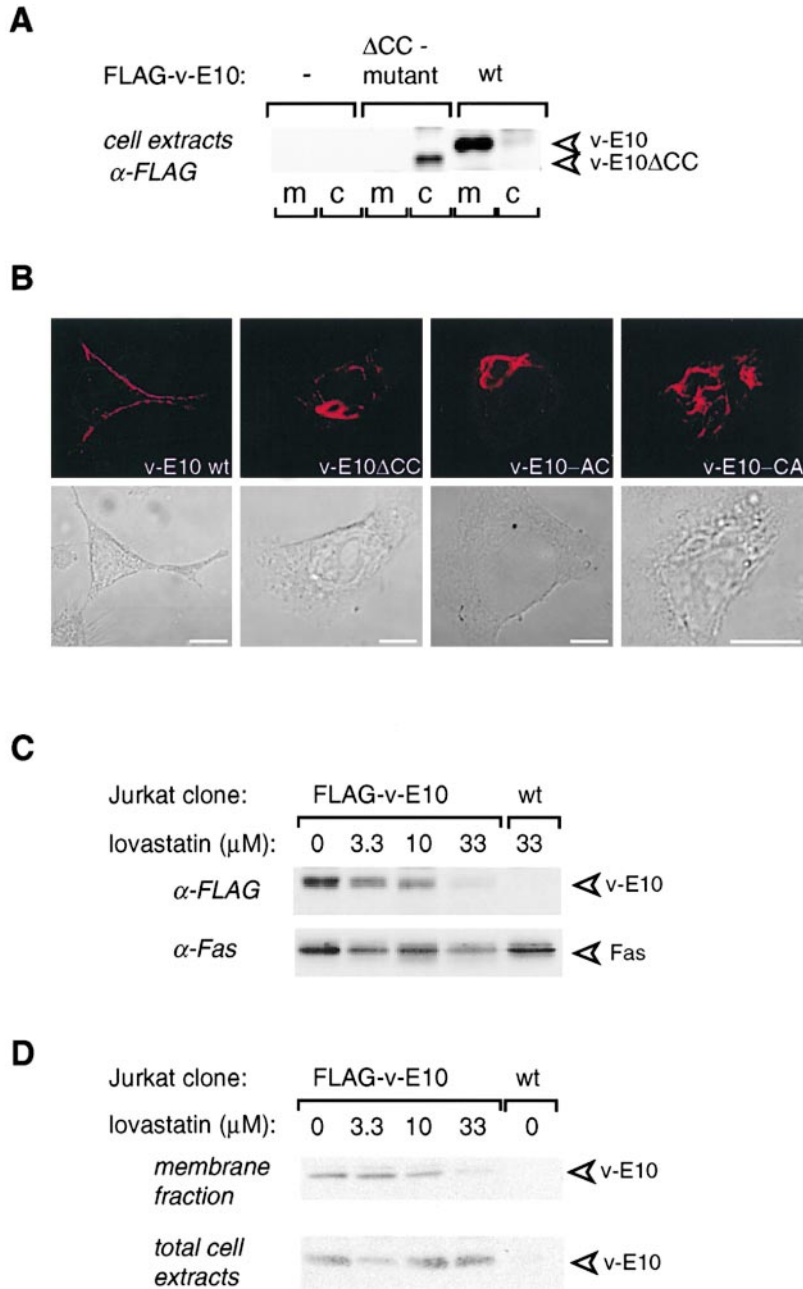
Figure 2. v-E10 is targeted to the plasma membrane via an isoprenylated COOH-terminal Cys–Cys motif. (A) HeLa cells were transfected with expression vectors for wild-type v-E10 (wt) or v-E10 lacking the two COOH-terminal cysteine residues (v-E10ΔCC), and the subcellular localization of both proteins was analyzed by Triton X-114 extraction as described in the legend to Fig. 1 B. (B) HeLa cells were transfected as in A and the subcellular distribution of wild-type v-E10 and its mutants v-E10ΔCC, v-E10-CA, and v-E10-AC was analyzed by confocal staining using anti-VSV– and Cy5-labeled secondary antibody. (C) Jurkat cells expressing FLAG-tagged v-E10 or the parental clone (wt) were treated for 60 h with the indicated concentrations of lovastatin, and membrane proteins were analyzed by Triton X-114 extraction, SDS-PAGE, and Western blotting with the indicated primary antibodies. (D) Jurkat cells expressing FLAG-tagged v-E10 or the parental clone (wt) were treated for 60 h with the indicated concentrations of lovastatin, and total cellular extracts or Triton X-114–extracted membrane proteins were analyzed by SDS-PAGE and anti-FLAG Western blotting. Bars, 10 μm.

bcl-10 (Koseki et al., 1999; Srinivasula et al., 1999; Yan et al., 1999) abolished the interaction (Fig. 3 A). Moreover, v-E10 also binds to TRAF6 (Thome et al., 1999) and mutation of a PQE consensus binding site ($P_{278}QE \rightarrow GQA$) located within the COOH-terminal part of v-E10 abolishes the interaction in 293T cells (Fig. 3 B).

We took advantage of the mutants described above and next analyzed whether membrane targeting of v-E10 or an intact CARD motif or TRAF6 binding site were required for previously described v-E10 effector functions, namely activation of the JNK and NF-κB transcriptional pathways. To this purpose, 293T cells were transfected with expression constructs for wild-type or mutant v-E10, and cells were assayed for JNK and NF-κB activation by cotransfection with suitable reporter plasmids (Fig. 3, C and D). Mutation of the COOH-terminal membrane-targeting motif of v-E10

did not appear to interfere with JNK activation, whereas the TRAF6-binding mutant was greatly impaired in its capacity to activate JNK (Fig. 3 C). Mutation of the CARD motif completely abolished NF-κB induction by v-E10, whereas mutation of the COOH-terminal Cys-residues had a partial effect on NF-κB activation (Fig. 3 D and data not shown). Indeed, in three independent experiments done in two different cell lines (293T and HeLa) and using variable plasmid concentrations, the mean values of NF-κB induction by the v-E10 geranylgeranylation mutants were reduced compared with the wild-type v-E10 construct: to 47% of wild-type levels ($P < 0.005$) for the v-E10ΔCC mutant, to 52% ($P < 0.0004$) for the v-E10-CA mutant, and to 42% ($P < 0.03$) for the v-E10-AC mutant. Therefore, we conclude that the geranylgeranylation mutants show a statistically significant (approximately twofold) reduction in NF-κB ac-
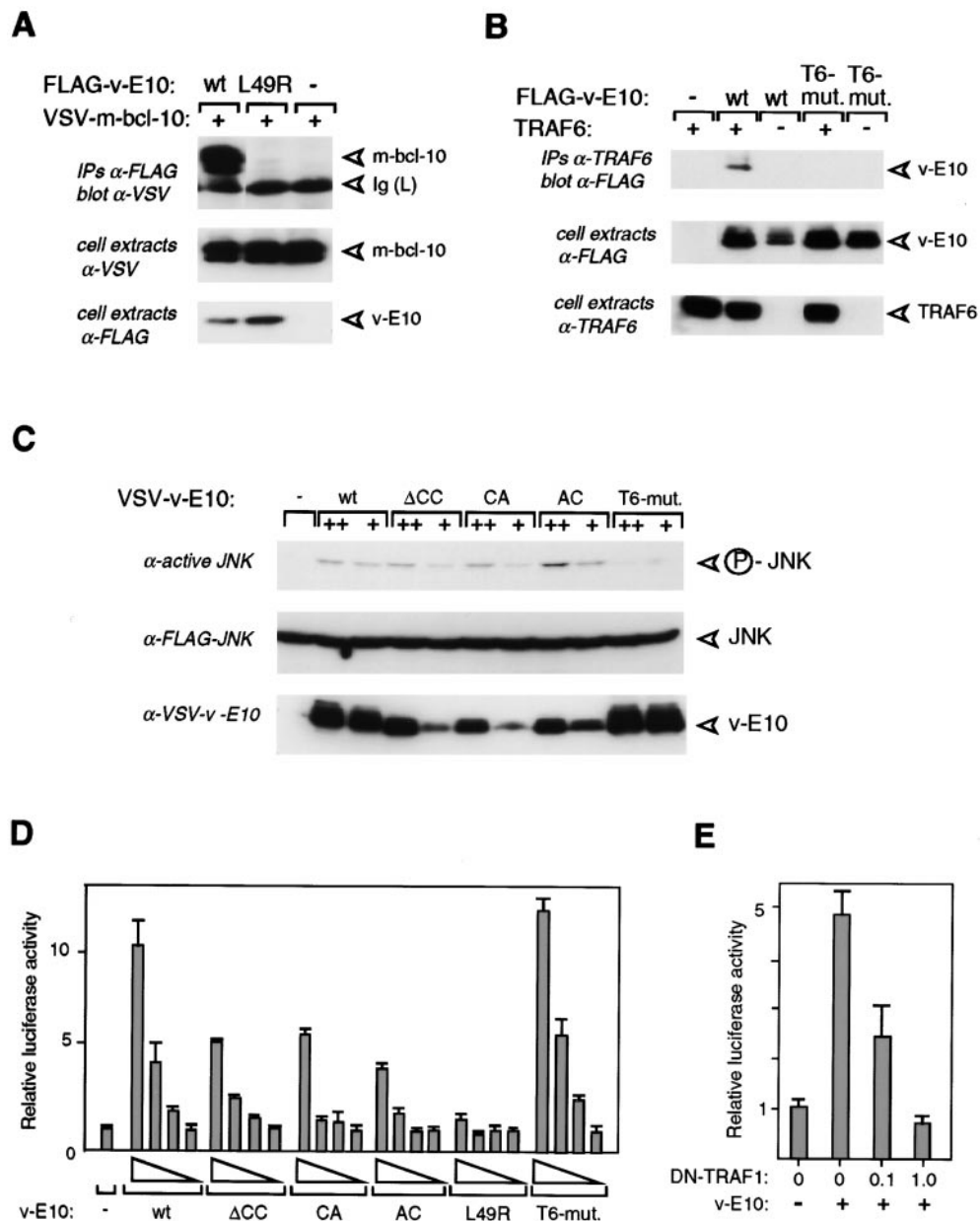
Figure 3. Membrane targeting and an intact CARD motif of v-E10 are required for the activation of NF-κB, whereas TRAF6-binding mediates JNK activation. (A) Association of v-E10 and bcl-10 requires an intact CARD domain. 293T cells were cotransfected with expression vectors for FLAG-tagged wild-type v-E10 or its CARD mutant, v-E10 L49R, and VSV-tagged wild-type bcl-10 and anti-FLAG immunoprecipitates were analyzed for the presence of bcl-10 by anti-VSV Western blotting. The lower panels show the expression levels of the FLAG- and VSV-tagged proteins in the cellular extracts. (B) v-E10 binds TRAF6 via a PQE binding motif. 293T cells were cotransfected with expression vectors for FLAG-tagged wild-type v-E10 or its TRAF6 binding mutant ($P_{278}QE \rightarrow GQA$) and human full length TRAF6, and anti-TRAF6 immunoprecipitates were analyzed for the presence of v-E10 by anti-FLAG Western blotting. The lower panels show the expression levels of FLAG–v-E10 and TRAF6 in the cellular extracts. (C) 293T cells (2×10^5) were transfected with 2 μg of a JNK expression vector together with 5 μg (++) or 1 μg (+) of expression vectors for v-E10, its COOH-terminal mutants v-E10ΔCC, v-E10-CA, and v-E10-AC, and its TRAF6-binding mutant (T6-mut). 24 h after transfection, the presence of FLAG-tagged JNK and of its activated form was assessed by Western blotting using anti-FLAG M2 and an antibody directed against the activated phosphorylated form of JNK (P-JNK), respectively. Expression levels of the various v-E10 mutants were assessed by Western blotting of total cell extracts using anti-VSV. (D) HeLa cells (2×10^5) were cotransfected with 2 μg of an NF-κB luciferase reporter plasmid, 0.5 μg of β-galactosidase expression vector, and 10, 1, 0.1, or 0.01 μg of the indicated v-E10 constructs. The total amount of plasmid was normalized with empty vector. Luciferase activities were determined 24 h after transfection and normalized on the basis of β-galactosidase values. Data shown are mean values ± standard deviations from one representative out of three independent experiments, each done in triplicate. (E) v-E10–induced NF-κB activation is inhibited by dominant negative (DN) TRAF1. HeLa cells (2×10^5) were transfected with 100 ng (+) of a v-E10 expression vector, together with the indicated amounts (in μg) of a dominant negative TRAF1 construct, and luciferase activities were determined as in B.

tivation. Mutation of the TRAF6 binding site, on the other hand, did not affect NF-κB activation (Fig. 3 D).

In summary, these results show that membrane recruitment and an intact CARD motif of v-E10 are important for induction of the NF-κB pathway, whereas JNK activation appears to be mediated by the interaction of v-E10 with TRAF6.

In addition, we found that a dominant negative form of the adaptor molecule TRAF1, which has been shown previously to bind to bcl-10, but not to v-E10 (Thome et al., 1999), was able to inhibit v-E10–induced NF-κB activation (Fig. 3 E), suggesting that NF-κB activation by v-E10 is mediated by its interaction with bcl-10.

This led us to investigate whether the subcellular distribution of bcl-10 was altered in the presence of v-E10. Bcl-10 has been reported to be localized in the cytoplasm in filamentous stress fiber–like structures (Yan et al., 1999; Guiet and Vito, 2000). However, in our hands
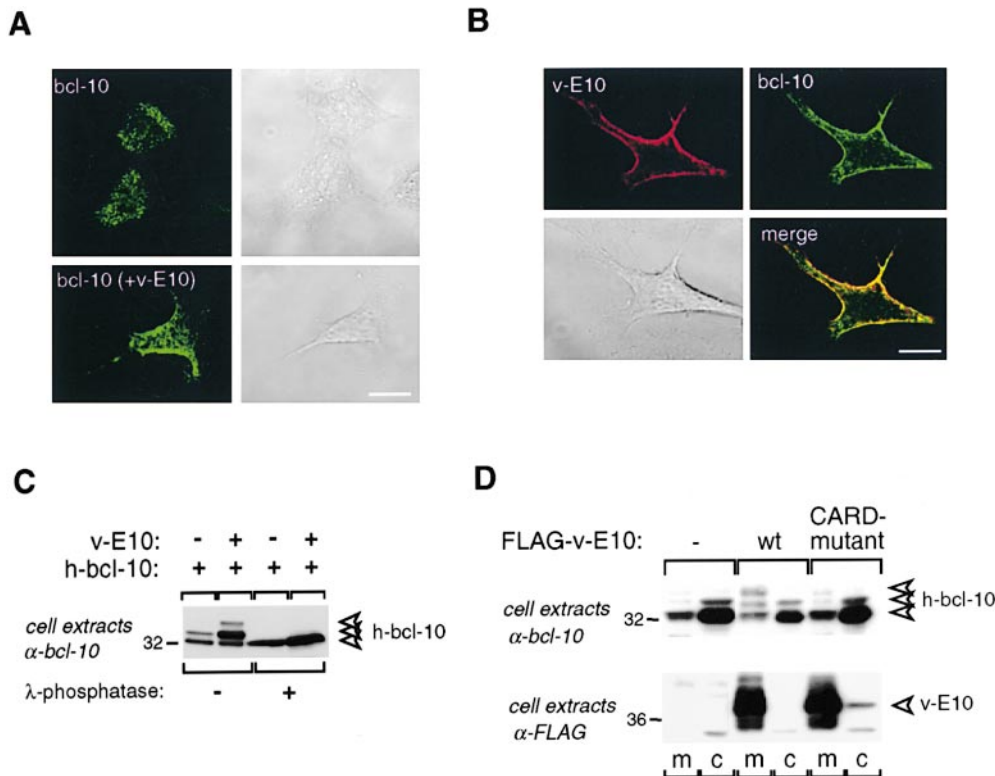
Figure 4. v-E10 induces hyperphosphorylation and plasma membrane recruitment of bcl-10. (A) Bcl-10 is localized in the cytoplasm of HeLa cells. HeLa cells were transfected with 0.5 μg of an expression vector for FLAG-tagged bcl-10 together with 4.5 μg of mock vector; the subcellular distribution of the transfected bcl-10 protein was analyzed by confocal staining using rabbit anti-FLAG antibody and Alexa-488–labeled anti–rabbit secondary antibody. (B) v-E10 and bcl-10 colocalize at the plasma membrane. HeLa cells were cotransfected with expression vectors for FLAG-tagged bcl-10 (0.5 μg), VSV-tagged v-E10 (2 μg), and mock vector (2.5 μg); the subcellular distribution of the transfected proteins was analyzed by confocal staining using rabbit anti-FLAG and mouse anti-VSV antibody followed by Alexa-488–labeled anti–rabbit and Cy5-labeled anti–mouse secondary antibody. (C) v-E10 induces hyperphosphorylation of bcl-10. HeLa cells were cotransfected with expression vectors for FLAG-tagged bcl-10 (1 μg) and VSV-tagged v-E10 (3 μg), and cell extracts of transfected cells were incubated for 1 h in the presence or absence of λ-phosphatase, as indicated. Cell lysates were analyzed by Western blotting using affinity-purified anti–bcl-10 antibody (AL114). Arrowheads indicate the migration position of the nonphosphorylated and the hyperphosphorylated forms of bcl-10. (D) v-E10 recruits hyperphosphorylated bcl-10 to the plasma membrane. HeLa cells were transfected with 3 μg of an expression vector for FLAG-tagged v-E10 and the subcellular distribution of the transfected v-E10 and the endogenous bcl-10 proteins was analyzed by cellular fractionation and Western blotting using anti-FLAG and anti–bcl-10 (AL114) antibodies, respectively. Bars, 10 μm.

bcl-10 shows a granular staining pattern in the cytoplasm of transfected HeLa cells (Fig. 4 A). Filamentous structures were observed only when considerably higher concentrations of the bcl-10 expression vector were used (data not shown). In cells cotransfected with v-E10, a significant portion of bcl-10 was now redistributed to the plasma membrane (Fig. 4 A). Coimmunostaining of HeLa cells transfected with both v-E10 and bcl-10 expression constructs and analysis by confocal laser scanning microscopy revealed a clear colocalization of the two proteins at the plasma membrane in cotransfected cells (Fig. 4 B). Consistent with this observation, cellular (endogenous) bcl-10 was found to partially redistribute to the membrane fraction in v-E10–transfected HeLa cells (Fig. 4 D).

Bcl-10 has been suggested to be posttranslationally modified by phosphorylation (Koseki et al., 1999; Srinivasula et al., 1999; Thome et al., 1999; Yoneda et al., 2000). Coexpression of v-E10 induces the formation of hyperphosphorylated forms of bcl-10, as shown by treatment of the lysates with λ-phosphatase (Fig. 4 C). Interestingly, the membrane-targeted population of cellular bcl-10 was clearly enriched in these hyperphosphorylated forms (Fig. 4 D). This suggests that v-E10–induced recruitment of bcl-10 to the membrane is required for its phosphorylation.

Discussion

In this report, we have studied the molecular function of v-E10 and of its cellular homologue, bcl-10, in the context of their subcellular distribution. v-E10 was found to localize to the plasma membrane via a COOH-terminal Cys–Cys motif, which represents a consensus site for geranylgeranylation by geranylgeranyltransferase II (Farnsworth et al., 1994). Mutation of the Cys–Cys consensus site or pretreatment of the cells with lovastatin significantly and specifically reduced the membrane localization of v-E10. It is reasonable to assume that both cysteine residues of v-E10 are geranylgeranylated, since in Rab proteins terminating in Cys–Cys, both cysteines were found to be geranylgeranylated (Peter et al., 1992; Wei et al., 1992; Farnsworth et al., 1994).

Ectopic expression of v-E10 induced the membrane recruitment and concurrent phosphorylation of endogenous bcl-10. Currently, very little is known about the in vivo function of bcl-10. The *BCL-10* gene was originally identified based on its chromosomal location close to the t(1; 14)(p22; q32) translocation breakpoint associated with some aggressive cases of MALT B cell lymphomas (Willis et al., 1999; Zhang et al., 1999). (Over)expression of mutated and/or truncated forms of bcl-10 mRNA has been suggested to be causally related to the development of

MALT lymphomas and various other tumors lacking the t(1;14)(p22; q32) translocation (Willis et al., 1999; Zhang et al., 1999). However, other studies have subsequently identified similar sequence variations with comparable frequency in normal tissues, suggesting that the observed bcl-10 mutations represent a naturally occurring genetic polymorphism rather than tumor-specific mutations (Apostolou et al., 1999; Fakruddin et al., 1999).

Interestingly, a proapoptotic and antiproliferative (thus potentially NF-κB inhibitory) role of bcl-10 was suggested by a recent study of bcl-10 transgenic mice (Yoneda et al., 2000). These mice showed lymphoid depletion and atrophy of the thymus and spleen, which could be correlated with accelerated apoptosis of B and T cells, suggesting that an increase in the cellular bcl-10 expression levels could perturb the regulation of apoptosis and proliferation in lymphoid cells. It is interesting to note that the phenotype of bcl-10 transgenic mice is similar to that of TRAF2-deficient mice reported previously (Yeh et al., 1997) and TRAF2 has been reported to interact with bcl-10 (Yoneda et al., 2000). This suggests that bcl-10 may interfere with TRAF2-dependent signaling pathways necessary for lymphocyte proliferation.

Bcl-10 has also been identified based on the presence within its NH$_2$ terminus of a predicted CARD motif (Hofmann et al., 1997; Koseki et al., 1999; Srinivasula et al., 1999; Thome et al., 1999; Yan et al., 1999), which suggests an involvement of the bcl-10 protein in CARD-dependent signaling pathways commonly associated with the regulation of apoptotic or inflammatory processes. In overexpression studies, bcl-10 has been reported to induce apoptosis and activation of NF-κB (Costanzo et al., 1999; Koseki et al., 1999; Srinivasula et al., 1999; Willis et al., 1999; Yan et al., 1999; Zhang et al., 1999). However, relative to its viral counterpart, activation of NF-κB by bcl-10 is very weak (Srinivasula et al., 1999; Thome et al., 1999), suggesting distinct roles of v-E10 and bcl-10 in the regulation of NF-κB. Consistently, whereas wild-type bcl-10 was found to suppress transformation of rat embryonic fibroblasts by ras and cooperating oncogenes, v-E10 was found to enhance this process (Willis et al., 1999), further supporting the idea that v-E10 and bcl-10 may exert antagonistic functions in vivo.

Expression of v-E10 has been shown to activate both the JNK and the NF-κB transcriptional pathways (Koseki et al., 1999; Srinivasula et al., 1999; Thome et al., 1999; Willis et al., 1999), most likely through interaction with specific members of the TRAF family (Thome et al., 1999). We found that JNK activation by the viral protein was independent of its membrane localization or intactness of its CARD (data not shown), but required an intact TRAF6-binding site. In contrast, mutation of the COOH-terminal membrane-targeting Cys–Cys motif of v-E10 partially impaired the activation of NF-κB and mutation of the CARD motif completely abolished NF-κB activation. Therefore, bcl-10 may represent a natural inhibitory regulator of the NF-κB activation pathway and its inhibitory function would be overcome by its phosphorylation and interaction with v-E10 at the plasma membrane (or potentially by its aggregation in overexpression studies). This hypothesis is supported by (a) the observation that NF-κB activation by v-E10 involves both membrane localization and a functional v-E10–CARD, (b) v-E10–induced NF-κB activation is inhibited by a dominant negative version of the bcl-10 binding protein TRAF1, and (c) the phenotype of the bcl-10 transgenic mouse (see above).

It is interesting to compare v-E10 with the EBV protein, LMP1. LMP1 is a protein with six transmembrane-spanning domains that contributes to EBV-induced transformation of B lymphocytes (Damania et al., 2000). LMP1 mimics the function of the B lymphocyte activation antigen CD40 by recruiting TRAFs to the membrane, thereby activating the NF-κB pathway. Similarly, v-E10 may mimic the function of an unknown receptor or membrane-associated protein that recruits bcl-10 and hence TRAFs to the membrane. Like LMP1 that contributes to EBV-induced transformation of B lymphocytes, v-E10 may thus contribute to the cellular proliferation occurring in lymphocytes of EHV-2–infected horses.

Finally, our analysis of the v-E10 function also extends our understanding of the reported phosphorylation of bcl-10 (Koseki et al., 1999; Srinivasula et al., 1999; Thome et al., 1999; Yoneda et al., 2000). Phosphorylation of bcl-10 at the plasma membrane may be important for the regulation of its physiological function. Most likely, relocalization of bcl-10 to the membrane facilitates its phosphorylation by an unknown membrane-proximal kinase in vivo. Alternatively, phosphorylated bcl-10 may be continuously generated but rapidly turned over, and the interaction of bcl-10 with v-E10 (or its postulated functional homologue in uninfected cells) at the plasma membrane may stabilize the otherwise short-lived phosphorylated forms of bcl-10. Further studies aimed at the identification and subsequent mutation of the bcl-10 phosphorylation sites should help to clarify this issue.

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