Avian Sarcoma and Leukosis Virus Cytopathic Effect in the Absence of TVB Death Domain Signaling

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The cytopathic effect (CPE) seen with some subgroups of avian sarcoma and leukosis virus (ASLV) is associated with viral Env activation of the death-promoting activity of TVB (a tumor necrosis factor receptor-related receptor that is most closely related to mammalian TNF-related apoptosis-inducing ligand [TRAIL] receptors) and with viral superinfection leading to unintegrated viral DNA (UVD) accumulation, which is presumed to activate a cellular DNA damage response. In this study, we employed cells that express signaling-deficient ASLV receptors to demonstrate that an ASLV CPE can be uncoupled from the death-promoting functions of the TVB receptor. However, these cell-killing events were associated with much higher levels of viral superinfection and DNA accumulation than those seen when the virus used signaling-competent TVB receptors. These findings suggest that a putative cellular DNA damage response that is activated by UVD accumulation might act in concert with the death-signaling pathways activated by Env-TV B interactions to trigger cell death. Such a model is consistent with the well-established synergy that exists between TRAIL-signaling pathways and DNA damage responses which is currently being exploited in cancer therapy regimens.

A number of retroviruses can cause a cytopathic effect (CPE) that is attributed, at least in part, to a function of the viral envelope glycoprotein (Env) (14, 29, 30, 33). For many retroviruses, the CPE is also associated with massive levels of viral superinfection and/or viral DNA accumulation (13, 17, 27, 36, 37, 40). Increased levels of viral DNA have been postulated to directly trigger a DNA damage response either before or after the formation of the gapped retroviral DNA integration intermediate in cells that are deficient in the nonhomologous DNA end-joining pathway (10, 11, 21). However, if viral DNA accumulation is important for stimulating cell death, then its role cannot be universal because human immunodeficiency virus type 1 can cause a CPE in the absence of viral DNA synthesis (5, 20).

We are studying avian sarcoma and leukosis viruses (ASLVs) to better understand how retroviruses can kill their target cells. Certain subgroups of ASLV are cytopathic (e.g., subgroups B, D, and F). Infection by the cytopathic viral subgroups can lead to the death of up to 30 to 40% of target cells in culture during the acute phase of infection (36, 37). This CPE is associated with viral superinfection and increased levels of viral DNA that are not seen during infection by noncytopathic subgroups of ASLV. The determinants of the ASLV-B surface (SU) Env subunit that are important for triggering the viral CPE are also the same as those required for receptor usage (14), leading to the suggestion that Env-receptor interactions might contribute to the viral CPE. This notion was strengthened when the TVB receptor for cytopathic viral sub-

groups B and D was identified as a tumor necrosis factor receptor (TNFR)-related death receptor that is most closely related to the mammalian DR4 (TRAIL-R1) and DR5 (TRAIL-R2) receptors for the TNF-related apoptosis-inducing ligand (TRAIL) (8, 23, 26).

Three highly related TVB proteins have been described, TVB^{S1} and TVB^{S3} from the chicken and TVB^T from the turkey. TVB^{S3} is a receptor for subgroups B and D viruses (8), TVB^{S1} is a receptor for subgroups B, D, and E viruses (2), and TVB^T is a receptor for subgroup E virus (1). These proteins contain three extracellular TNFR-related cysteine-rich domains (CRD1 to -3) and a cytoplasmic death domain that can activate cellular apoptosis signaling pathways via the formation of a death-inducing signaling complex (7–9).

The identification of TVB as a death receptor suggested a model in which the Env proteins of cytopathic ASLV subgroups B and D play a direct role in virus-induced cell killing by activating receptor-associated death-promoting signaling pathways. Indeed, it has been proposed that the viral Env-TV B interaction is sufficient to cause the ASLV CPE and that viral superinfection does not play a role (12). According to that model, Env proteins expressed at the surfaces of infected cells elicit bystander killing by triggering the death-promoting activity of the TVB receptor on neighboring uninfected cells.

In this study, we have directly tested the requirement for death receptor signaling in the ASLV CPE and show that ASLV can elicit a CPE in cells that express signaling-deficient viral receptors. Intriguingly, the CPE that is induced under these conditions is associated with higher than normal levels of viral superinfection and DNA accumulation. These observations lead to a model in which TVB death domain signaling and viral superinfection might act in concert to promote the ASLV CPE.

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MATERIALS AND METHODS

Cells and viruses. Human embryonic kidney HEK 293 cells were obtained from the American Type Culture Collection. DF-1 cells were described previously (31). The RCASH-A, RCASH-B, RCASE-Hgr, RCASBP(A)-EGFP, and RCASBP-EGFP viral vectors used to generate viral stocks were described previously (8, 34, 41).

TVB constructs. The synthetic TVB^{S1} gene (TVB^{S1}ΔDD-EGFP) was described elsewhere (18). The protein encoded by this gene contains amino acid residues 1 to 186 of TVB^{S1} with enhanced green fluorescent protein (EGFP) fused in frame after the ninth amino acid residue of the cytoplasmic tail. The TVB^{S3}ΔDD-EGFP and TVB²⁰⁸ΔDD-EGFP proteins are identical except that residue 62 is a serine in the TVB^{S3} protein (2) and residues L36/Q37/L41/Y42 in the TVB²⁰⁸ protein were changed to alanines to specifically abolish ASLV-B interaction (19). The TVBΔDD-EGFP genes were subcloned between the EcoRI and NotI restriction enzyme sites of the pLIB MLV (murine leukemia virus) vector, and their authenticity was validated by DNA sequencing. The MLV vector encoding TVA800 was described previously (25). The MLV vectors encoding the different ASLV receptors were packaged into recombinant MLV virions containing the vesicular stomatitis virus G protein from transiently transfected 293 cells (6, 25). Populations of DF-1 cells expressing the various recombinant TVB receptors were sorted based upon EGFP expression using a FACS-Vantage-SE/DiVa cell sorter (Becton Dickinson). These cell populations were designated DF-1:TVB^{S3}ΔDD-EGFP, DF-1:TVB^{S1}ΔDD-EGFP, DF-1:TVB²⁰⁸ΔDD-EGFP^{lo}, DF-1:TVB²⁰⁸ΔDD-EGFP^{hi}, and DF-1:TVA800.

Flow cytometric analysis of altered TVB receptor expression. The flow cytometric method used to monitor cell surface expression levels of the ASLV receptors using subgroup A-specific (SUA-IgG), subgroup B-specific (SUB-IgG), and subgroup E-specific (SUE-IgG) SU-immunoglobulin fusion proteins has been described previously (2). The secondary antibody used was an allophycocyanin (APC)-conjugated goat anti-rabbit antibody (Molecular Probes) used at a 1:400 dilution. Samples of 10,000 cells were analyzed on a Becton Dickinson flow cytometer.

Adherent cell numbers. Cells were plated in six-well tissue culture plates at 10⁵ cells per well, and 24 h later they were challenged with the ASLV vectors. The cells were passed as described in the figure legends. The adherent cell number was determined by washing the cells with phosphate-buffered saline, harvesting them with trypsin-EDTA, and counting them using a hemocytometer. Routinely, greater than 98% of adherent cells excluded trypan blue, indicating that they were mostly viable.

Quantitative PCR amplification. Cells were harvested in trypsin-EDTA, counted, pelleted, and incubated for 20 min at room temperature in 0.3 ml of a buffer containing 20 mM HEPES, 5 mM EDTA, 150 mM salt, 1% sodium dodecyl sulfate, and 100 μg/ml proteinase K (Boehringer-Mannheim). All samples were phenol-chloroform-isoamyl alcohol extracted once, and the DNA was precipitated and resuspended in water at a concentration equivalent to 5,000 cells/μl. PCR amplifications were performed in a final volume of 50 μl of 1× Universal PCR Master Mix (Applied Biosystems) using the DNA template from 25,000 cell equivalents and 200 nM each of the following oligonucleotides derived from the long terminal repeat of the RCAS vector: 5'-ACTGAATTC CG CATTGCAGAG-3' (sense), 5'-CCATCAACCCAGGTGCACA-3' (antisense), and 5'-TGCCTAGCTCGATAACAATAAACGCCATTG (FAM-labeled probe; Applied Biosystems). The PCR samples were incubated at 50°C for 2 min and 95°C for 10 min and then for 45 cycles of 95°C for 15 s and then 60°C for 1 min, and viral DNA measurements were obtained using an ABI 7700 instrument (Applied Biosystems). A serial dilution of RCASH-A proviral DNA was used to generate a standard curve for absolute quantitation of the amounts of viral DNA present in each sample. In the case of the DF-1:TVA800 experiments, the cell number was also corrected for by quantitative PCR analysis using primers that amplify an intron of the chicken *tvb* gene compared to a standard curve generated from serially diluted samples of the cloned *tvb* gene in plasmid pBK9 (8). The *tvb* primers used were 5'TCCTTGCATGGCTGAGCTCT 3' (sense), 5'TTCAGCTCTGGAAGCCCTTG 3' (antisense), and 5'TGCTTTCCTCTGCC TTGAACCGATGAA 3' (VIC-labeled probe; Applied Biosystems).

RESULTS

Subgroup B virus-induced cell death in DF-1 cells engineered to overexpress signaling-deficient TVB proteins lacking the cytoplasmic death domain. To test the role of the TVB receptor in the ASLV-B-induced CPE in DF-1 cells, an immortalized chicken embryo fibroblast line, we took advantage

of the well-established paradigm that signaling-incompetent forms of TNFR-related death receptors, lacking a functional cytoplasmic death domain, act as dominant-negative inhibitors of apoptosis pathways activated by the wild-type receptors through mixed-trimer formation (3, 22). Death domain deletion-containing forms of the TVB^{S3} and TVB^{S1} receptors were generated (designated TVB^{S3}ΔDD-EGFP and TVB^{S1}ΔDD-EGFP). These proteins consist of amino acid residues 1 to 186 of TVB^{S3} or TVB^{S1} with EGFP added after the ninth amino acid of their cytoplasmic tails to facilitate protein detection (18).

DF-1 cells which are homozygous for the subgroup B- and D-specific *tvb*^{S3} allele were transduced with an MLV-based vector encoding the different modified TVB proteins. Flow cytometric analysis, performed with ASLV SU-immunoglobulin fusion proteins, confirmed that the modified DF-1 cells expressed a higher level of cell surface subgroup B viral receptors than the parental DF-1 cells (Fig. 1A to C). As expected, these cells expressed endogenous levels of the TVA receptor for subgroup A ASLV (4) (Fig. 1A to C).

The different DF-1 cell populations were challenged with either subgroup A viral vector RCASH(A) or subgroup B viral vector RCASH(B) (41). The numbers of adherent cells remaining in each population were then monitored over time, and the level of viral superinfection was also measured at different time points using a quantitative PCR amplification-based assay. These studies revealed that at early time points postinfection (days 3 and 6), RCASH(B) induced a more pronounced CPE in cells expressing the signaling-incompetent TVB proteins compared to the parental DF-1 cells (Fig. 1D). These time points reflect the peak of the acute viral CPE over the 21 days that the cultures were monitored. These effects were correlated with increased viral superinfection as judged by determining the number of viral DNA genomes that had accumulated per cell. At day 3 postinfection, cells expressing TVB^{S3}ΔDD-EGFP or TVB^{S1}ΔDD-EGFP that were infected by RCASH(B) contained 3.2-fold and 4.7-fold more viral DNA than did the corresponding subgroup B virus-infected DF-1 cell population (Fig. 1E). Taken together, these data show that overexpression of signaling-deficient TVB receptors does not inhibit, but rather enhances, the ASLV CPE in DF-1 cells by a mechanism that is associated with increased viral superinfection compared with parental DF-1 cells.

Subgroup E virus-induced CPE in DF-1 cells engineered to express a death domain deletion-containing subgroup E-specific TVB receptor. To more thoroughly probe the role of the TVB death domain in the viral CPE, DF-1 cells which do not express any subgroup E receptors were engineered to express a death domain deletion-containing subgroup E-specific TVB receptor. DF-1 cells were transduced with a retroviral vector encoding an altered version of TVB^{S1}ΔDD-EGFP (designated TVB²⁰⁸ΔDD-EGFP) with the major ASLV-B interaction determinants (Leu-36, Gln-37, Leu-41, and Tyr-42) all changed to alanines so that it supports subgroup E, but not subgroup B, virus entry (19). Modified DF-1 cells expressing either low or high levels of the subgroup E-specific viral receptor were isolated by flow cytometric sorting on the basis of cell-associated EGFP fluorescence intensity. Flow cytometric analysis performed using ASLV SU-immunoglobulin fusion proteins con-

FIG. 1. Subgroup B virus CPE in DF-1 cells engineered to express signaling-deficient TVB receptors. (A to C) DF-1 cells and DF-1 cells overexpressing either TVB^{S3}ΔDD-EGFP or TVB^{S1}ΔDD-EGFP were analyzed by flow cytometry for cell surface expression of TVA and TVB receptors using the subgroup A-specific SUA-rlgG (open), the subgroup B-specific SUB-rlgG (grey), or no SU-immunoglobulin fusion protein (dark shaded) and an APC-conjugated secondary antibody. The cells were infected with RCASH(A) (open bars) or RCASH(B) (grey bars) at an MOI of 1.0 hygromycin B-resistant CFU, and the cells were passaged 1:5 every 3 days. The resultant adherent cell number at different time points was monitored (compared to the numbers of uninfected cells). Data taken from days 3 and 6 postinfection are shown in panel D. The number of viral DNA molecules accumulated per cell was measured using a quantitative real-time PCR-based assay (panel E). These experiments were performed at least twice with samples in triplicate. The average mean values of a representative experiment are shown with the standard deviation of the data indicated with error bars.

firmed that these cell populations expressed different levels of the subgroup E-specific viral receptor (Fig. 2A and B).

These cell populations were challenged with either the RCASH(A), RCASH(B), or RCAS(E)-Hgr viral vector, and viral superinfection and CPEs were measured as before. These studies revealed that the subgroup E viral vector induced a CPE that was greater in magnitude than that seen with the ASLV-B vector and that was correlated with the level of subgroup E viral receptor expression (Fig. 2C). These effects were also correlated with increased viral superinfection in the subgroup E virus-infected populations compared to the subgroup B virus-infected populations; at day 3 postinfection, the TVB²⁰⁸ΔDD-EGFP^{lo} population contained 7.2-fold more viral DNA and the DF-1:TVB²⁰⁸ΔDD-EGFP^{hi} population contained 13.3-fold more viral DNA (Fig. 2D). In this case, the increase in viral DNA that was detected preceded the onset of the CPE (Fig. 2C and D). These data demonstrate that expression of a death domain deletion-containing subgroup E viral receptor can render DF-1 cells susceptible to a CPE induced

by an ASLV-E vector. The CPE that was induced under these conditions was again correlated with higher levels of viral superinfection and DNA accumulation than was seen with subgroup B viruses and the parental DF-1 cells.

Subgroup A virus-induced CPE in DF-1 cells engineered to overexpress TVA. The results described so far indicate that viral superinfection and DNA accumulation might play a role in the ASLV CPE but that the level of viral DNA that accumulates normally during a cytopathic subgroup B virus infection may not be sufficient to trigger cell death in the absence of TVB death domain signaling. This might explain why the CPE that is observed in cells expressing signaling-deficient TVB receptors is associated with higher than normal levels of viral superinfection. To test this idea further, TVA, a low-density lipoprotein receptor-related protein with no known death-promoting activity (4), was overexpressed in DF-1 cells to determine if its overexpression would lead to superinfection and a CPE caused by a subgroup A virus. DF-1 cells were transduced with an MLV vector encoding TVA800, a glycosylphosphati-

FIG. 2. Subgroup E virus CPE in DF-1 cells that express a death domain deletion-containing ASLV-E receptor. Transduced DF-1 cells expressing low (A) or high (B) levels of the TVB²⁰⁸ΔDD-EGFP receptor were analyzed by flow cytometry for cell surface TVA and TVB expression as described in the legend to Fig. 1, with the exception that the subgroup E-specific SUE-rIgG protein (dark-shaded histogram) was also included in this analysis. Both cell types were challenged with RCASH(A), RCASH(B), or RCAS(E)-Hgr at an MOI of 1.0 hygromycin B-resistant CFU. Adherent cell numbers (C) and the average mean number of viral DNA molecules accumulated per cell (D) were then monitored using the approaches described in the legend to Fig. 1. Results obtained with RCASH(A)-, RCASH(B)-, and RCAS(E)-Hgr-infected cells are shown with open, grey, and black bars, respectively (C and D). Cells were passaged 1:5 every 3 days. These experiments were performed at least twice, each time in triplicate, and a representative example is shown with the standard deviation of the data indicated with error bars.

dylinositol-linked form of the TVA receptor (4). Flow cytometric analysis performed with a subgroup A-specific SU-immunoglobulin protein confirmed that the TVA800 receptor was overexpressed at the surface of the transduced DF-1: TVA800 cells (Fig. 3A).

FIG. 3. TVA overexpression renders DF-1 cells susceptible to an ASLV-A-induced CPE. (A) Flow cytometric analysis was performed with DF-1 cells and DF-1:TVA800 cells using the SUA-rIgG protein and the APC-conjugated secondary antibody (2°) as in Fig. 1. The cells were infected with RCASBP(A)-EGFP and RCASBP(B)-EGFP at an initial MOI of 2.0 GFP-transducing units, and the resultant adherent cell numbers were determined at different time points after infection (B). As in the previous figures, the adherent cell numbers are reported relative to a control population of uninfected cells. During the time course, the cells were plated and reseeded on days 2, 4, 8, 10, 12, and 14 at 100,000 cells per well of a six-well tissue culture plate. DNA samples were also harvested for quantitative real-time PCR-based measurements of viral DNA (C), normalized to the levels of an intron of the *tvb* gene. These experiments were performed with samples in triplicate, and the average mean values are shown with the standard deviation of the data indicated with error bars.

DF-1:TVA800 cells with the highest levels of the glycosylphosphatidylinositol-linked TVA receptor were sorted by flow cytometry and challenged with the subgroup A-specific RCASBP(A)-EGFP viral vector (34) or, for control purposes, with the subgroup B-specific RCASBP(B)-EGFP viral vector. As predicted, the subgroup A virus-infected DF-1:TVA800

cells exhibited a CPE although it was manifested mainly at a later time point in the infection (day 14) compared to subgroup B virus-infected cells, where the CPE was most pronounced at day 8 (Fig. 3B). Strikingly, the subgroup A virus-infected DF-1:TVA800 cells did not display any significant CPE during the first 8 days postinfection, even though these cells contained levels of viral DNA the same as or higher than those seen in the subgroup B virus-infected cell population (Fig. 3B and C). Instead, the CPE observed in these cells was again associated with a marked increase in the viral DNA levels relative to subgroup B virus-infected cells (Fig. 3B and C). These data confirm that DF-1 cells which overexpress TVA become susceptible to an ASLV-A-induced CPE and extend the notion that an ASLV CPE which is induced in the absence of TVB death domain signaling is associated with higher levels of viral superinfection. Moreover, the data also suggest that if viral superinfection does play a role in cell killing, then the level of viral DNA that normally accompanies cytopathic subgroup B virus infection is unlikely by itself able to trigger cell death; otherwise, the subgroup A virus-infected cells would have been expected to be killed with more rapid kinetics.

DISCUSSION

The results described in this report demonstrate that ASLV is capable of inducing a CPE independently of the death-promoting activity of the TVB receptor. An ASLV-E-induced CPE was observed in DF-1 cells that were engineered to express a death domain deletion-containing form of a subgroup E-specific viral receptor. An ASLV-A-induced CPE was seen in DF-1 cells that were engineered to overexpress the TVA receptor (which has no known death-promoting function). Moreover, an ASLV-B-induced CPE occurred with DF-1 cells that were engineered to overexpress signaling-deficient forms of the TVB receptor. In each of these instances, the CPE induced by these viruses was associated with higher levels of viral superinfection than were seen with parental DF-1 cells. This increase in superinfection is most likely due to elevated cell surface receptor expression levels in the modified DF-1 cells.

These findings seem to contradict the conclusions drawn by Diaz-Griffero et al., who have proposed that the CPE is due solely to Env-TVB signaling and that viral superinfection plays no role in cell killing (12). Evidence supporting this model includes the finding that DF-1 cells challenged with subgroup B virus at high multiplicities of infection (~10 infectious units) prevents the CPEs even though the cells seemed to contain significant amounts of unintegrated viral DNA (UVD). However, the actual levels of viral DNA present in each cell were not precisely measured in that study. Moreover, in cultures of DF-1 cells that were undergoing viral CPEs it was found that the DF-1 cells which were dying by apoptosis did not appear to express the EGFP marker protein encoded by a subgroup B viral vector. One interpretation of this finding is that the infected cells induce the bystander killing of neighboring uninfected cells via the EnvB-TVB interaction (12). However, that conclusion seems inconsistent with the fact that the cells were infected at a multiplicity of infection (MOI) of ~1 EGFP transducing unit and then analyzed 5 days postinfection (12). Under these conditions, it would be expected that every cell in

the culture expressing TVB would have become infected with the replication-competent subgroup B viral vector. Thus, we propose an alternative explanation for this result, namely, that the dying cells were infected with virus but lost EGFP expression either prior or during the time that they underwent apoptosis. Clearly, additional experiments are required to test the bystander killing model more thoroughly. These authors also observed a CPE in quail QT6 cells that were engineered to express wild-type, but not signaling-deficient, TVB receptors. While differences in the cell type used might also account for the opposite results obtained in that earlier study, neither the level of viral superinfection nor receptor expression in the modified QT6 cells was assessed. Taken together, it is our opinion that the data of Diaz-Griffero et al. are consistent with a role for the death domain of TVB in promoting the viral CPE but they do not exclude the possibility that viral superinfection also plays a role.

Based upon the results described in this report, we suggest that the ASLV CPE might result from death signaling pathways that result from Env-TVB interactions acting in concert with those of putative DNA damage responses which result from multiple rounds of viral superinfection and UVD accumulation. If so, we would predict that a certain threshold level of UVD must be achieved in order to trigger cell death, presumably by activating a cellular DNA damage response. This putative threshold level of UVD would presumably be lower under conditions in which ASLV Env also activates the death-promoting functions of the TVB receptor. This putative model for viral cell killing is similar to the well-established paradigm that a TNFR-related death receptor-signaling pathway can collaborate with DNA damage responses activated by chemotherapeutic agents to trigger tumor cell death (15, 16, 24, 28, 32, 35, 38, 39). In these studies, it has been shown that treatment of cells with agonists against TRAIL receptors (mammalian structural homologs of TVB) renders tumor cells susceptible to killing by lower doses of DNA-damaging chemotherapeutic agents. We are in the process of rigorously testing this provocative new model for the ASLV CPE.

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