Apoptin Nuclear Accumulation Is Modulated by a CRM1-Recognized Nuclear Export Signal that Is Active in Normal but not in Tumor Cells

Ivan K.H. Poon, Cristina Oro, Manisha M. Dias, Jingpu Zhang, and David A. Jans

Introduction

Chicken anemia virus viral protein 3 (VP3 or apoptin) is believed to be dependent on its ability to localize in the nucleus of transformed but not of primary or nontransformed cells. The present study characterizes the signals responsible for the novel nucleocytoplasmic trafficking properties of VP3 using two isogenic tumor/nontumor cell pairs. In addition to the tumor cell–specific nuclear targeting signal, comprising two stretches of basic amino acids in the VP3 COOH terminus which are highly efficient in tumor but not in normal cells, we define the CRM1-recognized nuclear export sequence (NES) within the VP3 tumor cell–specific nuclear targeting signal for the first time. Intriguingly, the NES (amino acids 97–105) is functional in normal but not in tumor cells through the action of the threonine 108 phosphorylation site adjacent to the NES which inhibits its action. In addition, we characterize a leucine-rich sequence (amino acids 33–46) that assists VP3 nuclear accumulation by functioning as a nuclear retention sequence, conferring association with promyelocytic leukemia nuclear bodies. This unique combination of signals is the basis of the tumor cell–specific nuclear targeting abilities of VP3. (Cancer Res 2005; 65(16): 7059-64)

Abstract

Tumor cell–specific activity of chicken anemia virus viral protein 3 (VP3 or apoptin) is believed to be dependent on its ability to localize in the nucleus of transformed but not of primary or nontransformed cells. The present study characterizes the signals responsible for the novel nucleocytoplasmic trafficking properties of VP3 using two isogenic tumor/nontumor cell pairs. In addition to the tumor cell–specific nuclear targeting signal, comprising two stretches of basic amino acids in the VP3 COOH terminus which are highly efficient in tumor but not in normal cells, we define the CRM1-recognized nuclear export sequence (NES) within the VP3 tumor cell–specific nuclear targeting signal for the first time. Intriguingly, the NES (amino acids 97–105) is functional in normal but not in tumor cells through the action of the threonine 108 phosphorylation site adjacent to the NES which inhibits its action. In addition, we characterize a leucine-rich sequence (amino acids 33–46) that assists VP3 nuclear accumulation by functioning as a nuclear retention sequence, conferring association with promyelocytic leukemia nuclear bodies. This unique combination of signals is the basis of the tumor cell–specific nuclear targeting abilities of VP3.

Materials and Methods

Expression constructs. Plasmid pEPI (7) was provided by H.J. Lipps and plasmids pEGFP-PM-C3 and pEGFP-HIPK2-C3 by A. Thorburn (8). Chicken anemia virus VP3 encoding plasmid constructs were prepared in the Gateway system (5), with VP3-encoding fragments generated by PCR using platinum Taq DNA polymerase (Invitrogen, Mt. Waverly, Victoria, Australia). Site-directed mutagenesis was done using the QuikChange site-directed mutagenesis system (Stratagene, La Jolla, CA). The fidelity of plasmid constructs was confirmed by DNA sequencing.

Mammalian cell culture and transfection. SAOS-2 and SR40 cells were cultured in McCoy’s media (JRH Bioscience, Brooklyn, Victoria, Australia), and COS-7 and CV-1 cells in DMEM, supplemented with 10% FCS in a 5% CO2 atmosphere at 37°C. Cells were transfected 24 hours after plating on coverslips using TransIT Transfection Reagent (Mirus, Madison, WI) according to the specifications of the manufacturer.

Immunofluorescence. Immunostaining for promyelocytic leukemia (PML) was carried out on 4% paraformaldehyde–fixed, 0.25% Triton X-100–permeabilized, bovine serum albumin–blocked cells (9, 10) using specific anti-PML primary (Santa Cruz, Los Angeles, CA) and Alexa 546–coupled secondary (Invitrogen) antibodies.

Confocal laser scanning microscopy and image analysis. Cells were imaged by confocal laser scanning microscopy (CLSM) using Bio-Rad MRC-600 CLSM (5, 9, 10) or Perkin-Elmer Ultra-view. For the former, a 488 × water immersion objective was used for live-cell imaging on a heated stage. In the case of the Ultra-view, a 100 × oil immersion objective was used for both live (heated stage) and fixed cell imaging. CLSM images were analyzed using the Image J 1.62 public domain software. The ratio (Fn/c) of nuclear fluorescence (Fn) to cytoplasmic fluorescence (Fc) was determined, subsequent to the subtraction of background fluorescence (5, 9, 10).
Results

VP3 localizes in the nucleus more efficiently in tumor than in normal isogenic cells. Two isogenic cell pairs identical in genotype, except for their transformed/nontransformed status, were selected to examine VP3 subcellular localization: the tumorigenic SAOS-2 line mutated in the retinoblastoma tumor suppressor gene product, together with its nontransformed SR40 counterpart derived by transfection of SAOS-2 with the full-length retinoblastoma cDNA (11, 12), and CV-1 African green monkey kidney cells (nontransformed) together with the SV40 virus–transformed derivative COS-7 line (13). Cells were transfected to express green fluorescent protein (GFP) or the various GFP-VP3 fusion constructs, and imaged live 16 hours later using CLSM. Full-length VP3 was found to confer nuclear localization on GFP in the transformed SAOS-2 and COS-7 lines (Fig. 1B and C), but in contrast to previous reports (2–4; see, however, refs. 5, 14), it also localized in the nucleus in both nontransformed cell types (SR40 and CV-1). Determination of the nuclear to cytoplasmic ratio (Fn/c) by image analysis revealed that the nontransformed lines accumulated GFP-VP3(1-121) to significantly (2-fold) lower levels than their transformed counterparts (P < 0.002; Fig. 1B and C). VP3 thus localizes to a greater extent in the nucleus of transformed compared with nontransformed cells.

Figure 1. Nuclear accumulation of VP3 is dependent on the COOH-terminal NLS1 and NLS2 sequences. A, schematic diagram of targeting sequences within VP3 (single-letter amino acid code), highlighting the putative NLSs (NLS1, amino acids 82-88; NLS2, amino acids 111-121), the two potential leucine-rich NESs (LRS, amino acids 33-46; NES, amino acids 97-105), and the phosphorylation site (T108). B, CLSM images of SAOS-2 cells (top rows) and SR40 cells (bottom rows), 16 hours posttreatment to express the indicated GFP-VP3 fusion proteins, and quantitative analysis (bottom) of the levels of nuclear accumulation [Fn/c, ratio of the nuclear fluorescence (Fn) to the cytoplasmic fluorescence (Fc) after the subtraction of background fluorescence], as determined using the Image J public domain image analysis software as previously described (10), from CLSM images such as those shown. Columns, mean Fn/c (n = 24); bars, SE. Significant differences (P values) for the Fn/c values between SAOS-2 and SR40 cells are indicated. C, CLSM images of COS-7 cells (topmost) and CV-1 cells (right) 16 hours posttreatment to express the indicated GFP-VP3 fusions and quantitative analysis of the levels of nuclear accumulation from CLSM images such as those shown (right). Columns, mean Fn/c (n = 15); bars, SE. Significant differences (P values) for the Fn/c values between COS-7 and CV-1 cells are indicated.
Definition of the VP3 tumor cell–specific nuclear targeting signal. To define the responsible sequences, GFP–VP3 truncated derivatives were initially examined. COOH-terminal truncations of even the last 10 amino acids of VP3, deleting NLS2 specifically, were found to confer predominantly cytoplasmic localization on GFP in the four cell lines (Fig. 1, and data not shown), resulting in an Fn/c < 1 (Fig. 1B and C). This was in contrast to the strong nuclear accumulation (Fn/c of ~20) in SAOS-2 and COS-7 cells of GFP–VP3(74-121) containing both NLS1 and NLS2: significantly higher (P < 0.0005) than in their nontransformed isogenic counterparts (Fn/c values of ~11 and 4 in SR40 and CV-1 cells, respectively). GFP–VP3(104-121), containing only NLS2, showed diffuse nuclear and cytoplasmic localization similar to that of GFP alone (Fig. 1C), indicating that NLS2 alone was not sufficient for nuclear accumulation. Finally, point mutations in either NLS (KK86/87 to NN in NLS1m, single-letter amino acid code, and RR117/118 to NN in NLS2m) abrogated nuclear accumulation in the context of full-length VP3 (Fig. 1B), demonstrating that both NLS1 and NLS2 are required for tumor cell–specific nuclear targeting. VP3 NLS1 and NLS2 thus constitute the tumor cell–specific nuclear targeting signal that is highly efficient in nuclear targeting in transformed but not in nontransformed cells. That cells expressing the NLS1- and NLS2-mutated GFP–VP3 derivatives, in contrast to the other proteins analyzed, showed discrete foci in the cytoplasm (see Fig. 1B) most likely relates to the reported ability of VP3 to aggregate/multimerize in the cytoplasm when not imported into the nucleus (e.g., in nontumor cells; ref. 4).

VP3 leucine-rich sequences differentially modulate VP3 nuclear targeting. To assess the contribution of the leucine-rich LRS and NES sequences (see Fig. 1A) to VP3 subcellular localization, localization of the GFP–VP3 constructs was examined in the absence and presence of a specific inhibitor of the nuclear export receptor CRM1, leptomycin B (Fig. 2B; ref. 15). In a similar fashion to its effect on the GFP-Rev control molecule containing the CRM1-recognized HIV-1 Rev protein NES, leptomycin B treatment significantly (P < 0.0001) increased nuclear accumulation of GFP–VP3(1-121) in SR40 cells, the Fn/c value of ~50 in its presence being equivalent to that in SAOS-2 cells in the absence of leptomycin B (Fig. 2B). In contrast, GFP–VP3(1-121) nuclear accumulation was not increased by leptomycin B in SAOS-2 cells, implying that CRM1-dependent nuclear export occurs differentially in the two isogenic lines; comparable differential effects of leptomycin B were also observed in COS-7/CV-1 cells (data not shown).

Point mutations within the two putative leucine-rich NESs had diverse effects; substitution of L1104/7 to AG (NESm) significantly increased (P < 0.0001) nuclear accumulation in SR40 but not in SAOS-2 cells, whereas mutations within the LRS at amino acids 33 to 46 (LRSm) resulted in significantly (P < 0.0001) reduced nuclear accumulation in both lines. The implication was that the COOH-terminal NES is functional and responsible in part for the reduced nuclear accumulation of VP3 in SR40 as opposed to SAOS-2 cells, whereas the NH2-terminal LRS is not a functional NES, but rather enhances nuclear import, possibly through facilitating nuclear retention (see below). That the LRS contributes strongly to VP3 nuclear localization is consistent with the fact that GFP–VP3(74-121), lacking the LRS, shows nuclear localization levels only about half of those of GFP–VP3(1-121) (see Fig. 1B and C). VP3 thus possesses a CRM1-recognized NES that is active in normal but not in tumor cells, in addition to an LRS that facilitates nuclear accumulation in normal and tumor cells.

Threonine 108 inhibits nuclear export of VP3 in normal but not in tumor cells. The possibility that phosphorylation of T108 in SAOS-2 cells (6) could regulate VP3 NES activity was addressed by examining the nuclear targeting properties and leptomycin B sensitivity of A108- and E108-substituted derivatives of GFP–VP3(1-121). The E108 derivative, with the negatively charged residue approximating T108-prephosphorylated VP3, was found to accumulate in the nucleus of SR40 cells to a level comparable to that of the wild-type protein in SAOS-2 cells (and to that of the NES mutant in both cell lines). No increase in nuclear localization of the derivative was effected by leptomycin B (Fig. 2B), consistent with the idea that phosphorylation of VP3 at T108 inhibits NES action, thereby contributing to the higher nuclear accumulation of VP3 in SAOS-2 compared with SR40 cells. Consistent with this, the nonphosphorylatable A108-substituted VP3 derivative showed significantly (P = 0.032) reduced nuclear accumulation in SAOS-2 cells compared with the wild-type protein (Fig. 2B), implying that the lack of phosphorylation allowed the NES to be active, resulting in reduced nuclear accumulation. That leptomycin B was able to increase nuclear accumulation of this derivative significantly (P < 0.0001) in SAOS-2 cells (Fig. 2B), in contrast to the wild-type protein, was further consistent with this idea. T108 thus seems to inhibit NES action specifically in tumor as opposed to normal cells, presumably through the fact that it is phosphorylated exclusively in tumor but not in normal cells (6).

VP3 localizes in promyelocytic leukemia nuclear bodies dependent on its NH2-terminal leucine-rich sequence. As evident in the CLSM images in Figs. 1 and 2, GFP–VP3(1-121) localizes in distinct substructures within the nucleus, which resemble PML nuclear bodies, whereas GFP–VP3(LRSm) shows nucleoplasmic localization (Fig. 2), implying that the LRS mutation impairs association with these substructures. To confirm this, immunostaining for PML protein, a predominantly PML nuclear body–localizing protein, was done on GFP–VP3(1-121)–expressing SAOS-2/SR40 cells (Fig. 3A), the yellow coloration in the merge images (rightmost) confirming colocalization of VP3 with endogenous PML. To further these observations, SAOS-2 cells were cotransfected with plasmids encoding GFP-PML and red fluorescent protein (DsRed)-VP3(1-121), DsRed-VP3(74-121), or DsRed-VP3(LRSm), results indicating not only colocalization of DsRed-VP3(1-121) with GFP–PML protein but also relocalization to the cytoplasm of coassociated VP3 and PML proteins in cells overexpressing both (Fig. 3B). Similar results were obtained for DsRed-VP3(1-121) and a GFP fusion of the PML nuclear body–localizing homedomain-interacting protein KIP2 (Fig. 3C; ref. 16). DsRed-VP3(LRSm), in contrast to DsRed-VP3(1-121), showed no colocalization with GFP-PML within the nucleus, and no redistribution of either to the cytoplasm (Fig. 3B), implying that the LRS mutation had eliminated VP3 association with PML-containing complexes, and therefore that the wild-type LRS sequence is responsible for this association. Consistent with this idea, cotransfection experiments using DsRed-VP3(74-121), which lacks the LRS, revealed no colocalization with or cytoplasmic redistribution of PML (Fig. 3B). Intriguingly, DsRed-VP3(LRSm) retained the ability to colocalize with cotransfected GFP–HIPK2 within the nucleus but not within PML nuclear bodies (Fig. 3C); thus, mutation of the LRS seemed to impair PML but not HIPK2 association, implying that LRS-conferring VP3 association with
PML nuclear bodies may be through direct binding to PML protein. These observations may relate integrally to the VP3 apoptotic mechanism in view of the fact that PML nuclear bodies, as well as PML and HIPK2 themselves, are linked to apoptosis (16).

**Discussion**

The present study uses quantitative approaches and isogenic tumor/nontumor cell pairs to show for the first time that tumor cell–specific nuclear targeting on the part of VP3 is determined by a set of unique targeting signals. These comprise a bipartite-type tumor cell–specific nuclear targeting signal (NLS1 and NLS2), a phosphorylation-inhibited CRM1-recognized NES within the tumor cell–specific nuclear targeting signal, and a LRS (amino acids 33-46) conferring interaction with PML nuclear body components (see schematic in Fig. 4). The most important finding is that inhibition of nuclear export in tumor cells contributes critically to the ability of VP3 to localize strongly in the nucleus of tumor but not of normal cells. We show for the first time here that the responsible NES lies within amino acids 97 to 105 and that it is recognized by CRM1. We also show that the NH2-terminal LRS, previously speculated to be a NES (1, 17), actually contributes to nuclear accumulation rather than to cytoplasmic retention or nuclear export, as clearly indicated by the fact that its absence [e.g., the GFP-VP3(74-121) construct] or inactivation by point mutation [the GFP-VP3(LRSm) construct] markedly reduces, rather than increases, tumor cell–specific nuclear targeting signal–dependent nuclear accumulation. The speculation that VP3 amino acids 33 to 46 represent a cytoplasmic retention sequence (18), based on qualitative analysis in primary cells of a small set of VP3 truncation mutants, is not consistent with our quantitative analysis here, particularly of LRS and NES point mutant derivatives in the context of full-length VP3. Our results show that the LRS facilitates nuclear accumulation of VP3 by conferring nuclear localizing function on the COOH-terminal CRM1-recognized NES. A, CLSM images of SAOS-2 cells (top) and SR40 cells (bottom) 16 hours posttreatment to express the indicated GFP fusion constructs in the absence (top) and presence of 2.8 µg/mL leptomycin B (LMB; added 5 hours before imaging) as indicated. B, quantitative analysis of the levels of nuclear accumulation from CLSM images such as those in (A) for SAOS-2 cells (left) and SR40 cells (right). Columns, mean Fn/c (n ≥ 30); bars, SE. Significant differences (P values) for the Fn/c values between cells treated with/without leptomycin B are indicated.
accumulation of VP3, with the activity, however, strongly dependent on the presence of NLS1 and NLS2.

Another important finding is that VP3 NES activity is inhibited by negative charge at T108. The A108 derivative VP3, lacking the ability to be phosphorylated at T108, shows nuclear export activity in tumor cells comparable to that observed in normal cells expressing wild-type VP3, whereas the E108 derivative shows resistance to nuclear export even in normal cells, comparable to the behavior of wild-type VP3 in tumor cells. Clearly, the action of T108 is specifically to inhibit nuclear export, in contrast to previous speculations (6) that the role of tumor cell–specific phosphorylation at T108 may be to enhance VP3 nuclear import. The data here clearly show that a critical contribution of T108 phosphorylation is to inhibit nuclear export, representing the mechanism of the tumor cell–specific activity of the NES activity (Fig. 4), whereas the basis of tumor cell–specific activity on the part of the tumor cell–specific nuclear targeting signal seems unclear. That specific phosphorylation can inhibit/modulate NLS/NES activity has been shown for a variety of proteins (see ref. 19); the unique aspect with respect to VP3 is that the key phosphorylation event seems to be tumor cell specific (see ref. 6). HIPK2, shown here to associate with VP3 (Fig. 3C), would seem to be of prime interest in this regard because preliminary bioinformatic analysis (20) indicates that it is likely to phosphorylate VP3 at T108, supporting the idea that it may be the

Figure 3. Localization of VP3 with PML nuclear bodies is dependent on an NH2-terminal leucine-rich sequence. A, immunostaining for endogenous PML in untransfected, fixed SAOS-2 and SR40 cells (left) or cells transfected to express GFP-VP3(1-121) (right). Staining was done using specific anti-PML primary (Santa Cruz) and Alexa 546–coupled secondary antibodies. Colocalization of endogenous PML transfected with GFP-VP3(1-121) is indicated by yellow coloration (Merge). B, CLSM images are shown of SAOS-2 cells transfected to express GFP-PML, DsRed-VP3(1-121), DsRed-VP3(74-121), and DsRed-VP3(LRSm) individually (top rows), or together (lower rows), as indicated. Cells were imaged live; colocalization of transfected GFP-PML and DsRed-VP3 is indicated by yellow coloration (Merge). C, CLSM images are shown of SAOS-2 cells transfected to express GFP-HIPK2, DsRed-VP3(1-121), and DsRed-VP3(LRSm) individually (top rows), or together (lower rows), as indicated. Cells were imaged live; colocalization of transfected GFP-HIPK2 and DsRed-VP3 is indicated by yellow coloration (Merge).
kinase responsible for the inhibition of VP3 nuclear export in tumor cells. As the focus of future studies in this laboratory, a critical first step in testing this possibility will be to determine relative levels of HIPK2 kinase activity in isogenic tumor/normal cell pairs, as well as in tumor tissues (6).

Acknowledgments


References


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