Proteasome inhibitor induces nucleolar translocation of Epstein–Barr virus-encoded EBNA-5

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We have previously shown that Epstein–Barr virus (EBV)-encoded EBNA-5 is localized to PML bodies (PODs) in EBV-immortalized lymphoblastoid cell lines (LCLs). Here we have extended our study of the subnuclear localization of EBNA-5 and found a strict co-localization with PML in LCLs and in BL lines with an immunoblastic, LCL-like phenotype. Moreover, GFP–EBNA-5 accumulated in PML bodies upon transfection into LCLs. In contrast, transfection of cell lines of non-immunoblastic origin with an EBNA-5 expression construct showed preferential localization of the protein to the nucleoplasm. Since PML is involved in proteasome-dependent protein degradation, we investigated the total levels and sub-cellular localization of EBNA-5 upon inhibition of proteasome activity. We found that a proteasome inhibitor, MG132, induced the translocation of both endogenous and transfected EBNA-5 to the nucleoli in every cell line tested. The total EBNA-5 protein levels were not affected by the proteasomal block. EBNA-5 forms complexes with heat shock protein Hsp70. The proteasome inhibitor induced a rise in total levels of Hsp70 and dramatically changed its homogeneous nuclear and cytoplasmic distribution into nucleolar and cytoplasmic. This effect was EBNA-5-independent. The nucleolar localization of Hsp70 was enhanced by the presence of EBNA-5, however. EBNA-5 also enhanced the nucleolar translocation of a mutant p53 in a colon cancer line, SW480, treated with MG132. The coordinated changes in EBNA-5 and Hsp70 localization and the effect of EBNA-5 on mutant p53 distribution upon MG132 treatment might reflect the involvement of EBNA-5 in the regulation of intracellular protein trafficking associated with the proteasome-mediated degradation.

Introduction

Epstein–Barr virus (EBV) is the most potent transforming virus known. EBV infection of resting B cells in vitro induces blast transformation and leads to the establishment of immortal lymphoblastoid cell lines (LCLs). This process is associated with the expression of nine latent viral proteins: the nuclear antigens, EBNA1–6, and three membrane proteins, LMP-1, -2a and -2b. Six of these proteins are required for immortalization: EBNA-1, -2, -3 (3A), -5 (LP), -6 (3C) and LMP-1 (Rickinson & Kieff, 1996).

EBNA-5 and EBNA-2 are the first virally encoded proteins expressed after B cell infection (Alfiery et al., 1991). They can drive gp340-activated primary B cells into the G1 phase of the cell cycle (Sinclair et al., 1994). This suggests that EBNA-5 may play a role at the first steps of EBV transformation. EBNA-5 can co-operate with EBNA-2 in the activation of LMP-1 and Cp viral promoters (Harada & Kieff, 1997). The co-transfection of EBNA-3 and EBNA-5 into the DG75 line showed that EBNA-3 is tethered to the nuclear matrix fraction in the presence of EBNA-5 (Cludts & Farrell, 1998). This suggested that EBNA-5 may modify the intranuclear sorting of proteins.

After EBV infection of B cells, EBNA-5 is homogeneously distributed in the nucleus during the first 2 days. Later it accumulates in PML bodies or PODs (POD oncogenic domains; Szekely et al., 1995b, 1996). These are distinct nuclear domains associated with the nuclear matrix. PML, in complex with SUMO-1, is required to form the POD structure (Ishov et al., 1999; Zhong et al., 2000 a). PODs are specifically disrupted in human acute promyelocytic leukaemia cells where PML is fused to a retinoic acid receptor alpha gene. A number of cellular proteins are localized to the PODs: SP100, INT6, CBP/p300, Hsp70, a fraction of Rb, Daxx and SUMO-1 (for review see Zhong et al., 2000 b). Herpes simplex virus type 1 infection abrogates the modification of PML by SUMO-1 (Muller & Dejean, 1999), leading to rapid PML protein degradation (Chelbi-Alix & de The, 1999) and disruption of the
Fig. 1. Differential subnuclear localization of EBNA-5 in cell lines of different origin. (A) Double-staining of endogenously and exogenously expressed EBNA-5 and PML. LCL IARC171 expresses endogenous EBNA-5 that localized to both PML bodies and the nucleoplasm (upper row). BL line, DG75, and breast cancer line, MCF-7, were stably transfected with pBabe-EBNA-5. EBNA-5 was homogeneously distributed in the nucleoplasm and did not co-localize with PML (middle and lower row, respectively). (B) IARC171 (upper row), DG75 (middle row) and MCF-7 (lower row) were transfected with GFP–EBNA-5 and stained for PML 2 days later. GFP–EBNA-5 targeted PML bodies in IARC171, but not in DG75 or MCF-7. Red, PML; green, EBNA-5; blue, nuclei.
Fig. 2. Exogenously expressed EBNA-5 translocated to the nucleoli in MG132-treated MCF-7 cells. (A) MCF-7 derivative clone M1 stably transfected with the pBabe-EBNA-5 construct cultured in the presence of DMSO (a, b) or 20 µM MG132 (c, d) for 6 h. Panels b and d represent phase contrast fields of panels a and c, respectively. (B) High magnification image of the double-staining for B23 (a, green) and EBNA-5 (b, red) of clone M1 treated with 20 µM of MG132. Overlap of green and red fluorescence is shown in panel c. Panel d shows phase contrast.
PODs. The EBV growth and transformation-associated EBNA-5 localizes to the PODs without disrupting them (Szekely et al., 1996).

PML expression is induced by interferons. Cellular response to interferon requires normal PML function (Quignon et al., 1998; Wang et al., 1998). PML was recently shown to regulate MHC expression in untransformed fibroblasts and to induce the expression of the proteins involved in antigen processing and presentation (Zheng et al., 1998). The ubiquitin–proteasome system is involved in the processing of MHC class I antigens, providing a link between the cellular degradation machinery and PML. The ubiquitin–proteasome system is the major pathway of selective protein degradation in eukaryotic cells. Initially, the target proteins are conjugated to the polypeptide ubiquitin through a lysine residue on the proteins. In the second step, the ubiquitin-conjugated proteins are recognized by the 26S proteasome and degraded (for review see Ciechanover, 1998). It is likely that nuclear POD structures are involved in this process. A protein genetically modified for rapid degradation can accumulate in the ubiquitinated form in the PODs upon proteasome inhibitor treatment. This was accompanied by the attraction of the proteasomes to the PODs, suggesting that the PODs may represent an intermediate reservoir for the ubiquitinated proteins targeted for degradation (Anton et al., 1999).

In an attempt to understand the role of EBNA-5 targeting to the PML bodies and in proteasome-mediated protein degradation, we monitored the changes in subcellular localization and in the total EBNA-5 levels upon proteasome inhibitor treatment. We have also continued to analyse the subnuclear localization of EBNA-5 in different cell types and found that EBNA-5 targets preferentially the PODs in the LCLs and in the BLs with an LCL-like phenotype. Nucleoplasmic localization was found in other cell types. Inde-
Fig. 3. EBNA-5 dissociates from PML bodies and co-localizes with Hsp70 in the nucleoli after MG132 treatment of LCL IB-4. (A) DMSO (left panel) and 20 μM MG132-treated (right panel) IB-4 cells were double-stained for EBNA-5 (red) and PML (green). The overlap shows the co-localization between the two proteins (yellow). The image is reconstituted from a series of 17 optical sections. (B) Neither EBNA-2 nor LMP-1 change their localization after MG132 treatment. (C) MG132-treated IB-4 cells were double-stained for EBNA-5 (red) and Hsp70 (green). The overlap shows the co-localization between the two proteins (yellow) in both nucleoli and nuclear dots. Phase contrast visualizes nucleoli.
Fig. 3. For legend see previous page.
of the cells 2 days after GFP–EBNA-5 transfection with anti-PML MAb showed that GFP–EBNA-5 co-localized with PML in IARC171 (Fig. 1B, upper row).

Partial co-localization of EBNA-5 and PML in B and non-B cells

BL DG75 was transfected with the pBabe-EBNA-5 construct and a puromycin-resistant pool was tested for the pattern of EBNA-5 expression. The protein localized to the nucleoplasm (Fig. 1A, middle row). Transient transfection of BL lines DG75 and BL 28 with GFP–EBNA-5 showed nuclear localization and only seldom targeting of the PML bodies (Fig. 1B, middle row and not shown). The EBV-positive BL lines cultured in vitro change their original, BL tumour phenotype to an immunoblastic, LCL-like phenotype, and activate the expression of EBNAs and LMPs (Rowe et al., 1987). In one such line, Namalwa, EBNA-5 was localized to both nucleoplasm and PODs (not shown).

We have established clones of SW480 (clones S2, S8, S9) and MCF-7 (clones M1, M2, M3 and M5) that ectopically express EBNA-5. The clones were double-stained for PML and EBNA-5. EBNA-5 was homogeneously distributed in the nucleoplasm, avoiding the nucleioli in all lines mentioned, and did not co-localize with PML. This is shown for MCF-7 clone M5 (Fig. 1A, lower row) and MCF-7 transiently transfected with GFP–EBNA-5 (Fig. 1B, lower row). Transiently transfected GFP–EBNA-5 often accumulates in conglomerates of irregular shape, seen in one of the cells in Fig. 1(B), which do not co-localize with PML.

EBNA-5 translocates to the nucleoli after treatment with proteasome inhibitor MG132

Clones M1, M2 and M3 of MCF-7 expressed EBNA-5 at moderate levels and showed primarily homogeneous nuclear staining as shown in Fig. 2(A, panel a) for M1. Treatment with 20 µM of MG132 for 6 h led to a nearly complete translocation of EBNA-5 to the nucleoli (Fig. 2A, panel c). The phase contrast fields are shown in Fig. 2(A, panels b and d) to visualize the cells and the nucleoli. In order to prove the localization of EBNA-5 to the nucleolus, we double-stained M1 cells for EBNA-5 and nucleophosmin/B23 after MG132 treatment. Fig. 2(B) shows that EBNA-5 is localized deep inside the nucleoli and is surrounded by the B23 protein. The pattern of B23 staining was not changed by MG132 treatment (not shown).

LCL IB-4 expressed endogenous EBNA-5 that was co-localized with PML (Szekely et al., 1996) and Fig. 3(A, left panels). EBNA-5 and PML partially dissociated and the former accumulated in the nucleolus after treatment of IB-4 with 5 µM of MG132 for 6 h (Fig. 3A, right panels and C for EBNA-5 and phase contrast). We have also tested the localization of other EBV-encoded proteins and found that neither EBNA-2 (Fig. 3B) nor EBNA-6 (not shown) nor LMP-1 (Fig. 3B) changed their localization after MG132 treatment.

Hsp70 co-localizes with EBNA-5 and accumulates in the nucleoli of MG132-treated cells

We have shown previously that EBNA-5 co-localized with constitutively expressed Hsp70 in IB-4 cells under normal conditions and upon heat shock (Szekely et al., 1995a). EBNA-5 and Hsp70 co-localized in the nucleoli of IB-4 cells treated with MG132 for 6 h (Fig. 3C).

Exogenously expressed EBNA-5 co-localized with Hsp70 in clone M5 in the nucleoplasm (MCF-7 transfected with EBNA-5, Fig. 4A). This shows that localization to PML bodies is not a prerequisite for EBNA-5/Hsp70 co-localization. We noticed an increase in nuclear staining of Hsp70 in the clones of MCF-7 and SW480 expressing EBNA-5, as compared to the vector-transfected cells (Fig. 4B, panel b and not shown). This suggests that EBNA-5 tethers Hsp70 to the nucleus.

The antibody against Hsp70 recognizes both the inducible Hsp72 and the constitutive Hsc73 proteins. In untreated cells, Hsp70 showed homogeneous nuclear and cytoplasmic distribution (Fig. 4B, panel b). Upon MG132 treatment, Hsp70 completely changed its localization: it accumulated in the cytoplasm and nucleoli (Fig. 4B, panel f). EBNA-5 translocated to the nucleoli (Fig. 4B, compare panels a and e). Clone M5, in which only 10% of cells express high EBNA-5 levels, was chosen to demonstrate that the changes in the Hsp70 localization were independent of EBNA-5. We noticed, however, that cells expressing higher levels of EBNA-5 showed a stronger Hsp70 staining in the nucleoli (Fig. 4A, panels e, f, g). The same phenomenon was observed in the MG132-treated S2 clone of SW480 that expressed EBNA-5.

Total protein levels were monitored by Western blotting (Fig. 4C). EBNA-5 levels did not change after MG132 treatment in IB-4, the H6 clone of HeLa expressing EBNA-5 or clone M5 of MCF-7. Even 16 h treatment did not change the EBNA-5 levels in clone M1 (Fig. 4C, lower panel). Hsp70 levels were increased and a faster migrating band, representing the inducible Hsp72 protein, appeared in all MG132-treated cells including the EBNA-5-negative HeLa clone H4. This again demonstrates that Hsp70 is induced in response to MG132 and that this induction is independent of EBNA-5.

In order to test the effect of proteasome inhibition, we probed the same membrane with antibodies against p53, a short-lived protein degraded through the proteasome pathway. The presence of EBNA-5 did not change the levels of wild-type p53 in MCF-7 or in HeLa cells. MG132 treatment led to the accumulation of wild-type p53 in IB-4 and MCF-7 cells with the appearance of ubiquitinated forms of the protein in accordance with previously published data (Kubbutat et al., 1997). In HeLa cells, p53 is complexed with human papillomavirus-encoded E6 protein, which targets p53 for degradation. The inhibition of proteasome activity has led to the
Fig. 4. Concomitant changes in subnuclear distribution and total levels of EBNA-5 and Hsp70 in EBNA-5-expressing cell lines. (A) High magnification of EBNA-5 (red) and Hsp70 (green) double-staining of untreated MCF-7-derivative M5. (B) DMSO- and MG132-treated M5 cells that expressed low levels of EBNA-5 were double-stained for EBNA-5 (a and e, respectively) and Hsp70 (b and f, respectively). Overlap of green and red fluorescence is shown in panels c and g. Panel d, phase contrast of the
accumulation of p53 in both EBNA-5-expressing clone H6 and vector control H4 Hela cells.

### Presence of EBNA-5 enhances translocation of mutant p53 to the nucleoli of SW480 cells upon treatment with proteasome inhibitor

The colorectal cancer line SW480 expresses endogenous mutant p53 at high levels. p53 is localized to the nuclei mainly avoiding the nucleoli (Fig. 5A, panels c and e). P2 and S2 are pBabe vector- and pBabe-EBNA-5-transfected derivatives of SW480. The presence of EBNA-5 did not change the levels of mutant p53 (compare Fig. 5A, panels a and b). Treatment with 20 μM of MG132 for 6 h led to a nearly complete translocation of EBNA-5 to the nucleoli of S2 (Fig. 5A, panels a and b), to a partial accumulation of mutant p53 in the nucleoli in 10% of the P2 cells (Fig. 5A, panel f) and to a significant p53 accumulation in the nucleoli in 90% of S2 cells (Fig. 5A, panel d). Fig. 5(B) shows p53 inside the nucleoli in the MG132-treated S2 cells at high magnification. In order to substantiate this finding, we double-stained MG132-treated S2 cells for EBNA-5 and p53. Fig. 5(C) shows that p53 is preferentially accumulated in the nucleoli of cells when EBNA-5 is also in the nucleoli (arrows). In contrast, cells without EBNA-5 (square sign) or those where EBNA-5 has not changed its localization (asterisks) showed homogeneous p53 staining. These results show that EBNA-5 greatly enhances accumulation of mutant p53 in the nucleoli upon inhibition of proteasome activity.

The inhibition of wild-type p53 degradation by MG132 in MCF-7 cells led to the accumulation of the protein and its ubiquitinated forms (Fig. 4 C) in both nucleus and nucleolus (Fig. 6, panel d and phase contrast in panel f). Transient expression of GFP–EBNA-5 (Fig. 6, panels a and b) had no effect on the intranuclear distribution of wild-type p53 upon MG132 treatment (Fig. 6, panel d). Arrows point to the MG132-treated cell expressing GFP–EBNA-5 that has translocated to the nucleoli.

### Discussion

We have found that the treatment of different EBNA-5-expressing cell lines with a proteasome inhibitor, MG132, led to a dramatic change in the distribution of EBNA-5, namely its almost complete translocation to the nucleoli. MG132 increased the levels of the cellular chaperon Hsp70 in agreement with previously published data (Bush et al., 1997; Kim et al., 1999). We have found in addition that MG132 induced a nucleolar accumulation of Hsp70.

The Hsp70 proteins are involved in protein folding, protein translocation across membranes and thermal tolerance. In non-stressed cells, Hsp70 associates transiently with nascent polypeptide chains, polypeptides unfolded for translocation or other aberrantly folded proteins (for review see Hightower, 1991). Both major members of the Hsp70 family, the stress-inducible Hsp72 and the constitutive cognate Hsc73, were found to associate with the soluble form of EBNA-5 in co-immunoprecipitation experiments. The W9 region of EBNA-5 was required for binding (Mannick et al., 1995). Another study showed that the Y1 C-terminal exon region of EBNA-5 formed complexes with Hsp70 (Kitay & Rowe, 1996). We have found previously that EBNA-5 is co-localized with Hsp70 in PODs in the LCL IB-4 under normal conditions and in the nucleoli upon heat shock (Szekely et al., 1995a). Both Hsp72 and Hsc73 accumulate in the nucleoli in heat-shocked mammalian cells (for review see Hightower, 1996). We found that Hsp70 localized to the nucleoli upon MG132 treatment, independently of the presence or absence of EBNA-5. Considering the direct binding of EBNA-5 to Hsp70, it is possible that the latter transports EBNA-5 into the nucleoli. On the other hand, Hsp70 accumulated in the nucleoplasm of the EBNA-5- transfected clones of MCF-7 and SW480 (Fig. 4A and not shown). It also showed a high degree of co-localization with EBNA-5 in the nucleoplasm, suggesting that EBNA-5 expression recruits Hsp70 into complex formation. The nucleolar localization of Hsp70 in proteasome inhibitor-treated cells was enhanced by the presence of EBNA-5 (Fig. 4B), suggesting that there is an active component in the translocation of EBNA-5 to the nucleoli rather than passive transport by Hsp70.

EBNA-5 is strongly associated with the nuclear matrix (Szekely et al., 1995a). It can also tether another EBV-encoded nuclear protein, EBNA-3, to the nuclear matrix upon co-transfection (Cludts & Farrell, 1998). This suggests that EBNA-5 can influence the subnuclear localization of other proteins and therefore it may play a scaffolding role by forming bridges between the nuclear matrix and nucleoplasmic proteins.

We found in this study that EBNA-5 greatly enhanced the accumulation of a mutant, but not wild-type, p53 in the nucleoli upon MG132 treatment. Mutations in p53 lead to misfolding of the protein. Mutant p53 forms complexes with Hsp70 (Pinhasi-Kimhi et al., 1986). The high affinity binding sites for these proteins were mapped to the hydrophobic core of the central DNA binding domain, not accessible in a wild-type conformation (Fourie et al., 1997). In the absence of EBNA-5, mutant p53 was only rarely found in the nucleoli of MG132-treated cells. Our study suggests that EBNA-5 targets mutant p53 to the nucleoli upon proteasome inhibitor

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Fig. 5. For legend see facing page.
Fig. 5. EBNA-5 enhances the nucleolar accumulation of mutant p53 in SW480 cells treated for 6 h with the proteasome inhibitor MG132. (A) EBNA-5 (a, b) and mutant p53 (c, d, e, f) in the DMSO- (a, c, e) or MG132-treated (b, d, f) derivatives of the SW480 colon cancer cell line. Clone S2 (a–d) stably expressed EBNA-5. P2 (e, f) is a vector control-expressing clone. (B) High magnification of the MG132-treated S2 cells stained for p53 (a). Phase contrast is shown in panel (c). Overlap of panels a and c shows that p53 is localized to the nucleoli (b). (C) Double-staining of the mutant p53 (green) and EBNA-5 (red) in MG132-treated S2 cells. Note that a cell without EBNA-5 (square sign) or those cells where EBNA-5 has not changed its localization (asterisks) showed homogeneous p53 staining, while the cells with nucleoli-localized EBNA-5 have also nucleoli-accumulated p53 (arrows).

Alternatively, EBNA-5 binds and translocates mutant p53 independently of Hsp70.

The nucleoli are special nuclear domains involved in rRNA synthesis (fibrillar compartment) and ribosome formation (granular compartment). Nucleolar proteins are involved either in rRNA synthesis and processing or in ribosome assembly (for review see Scheer & Hock, 1999). The involvement of the
Fig. 6. Expression of GFP–EBNA-5 does not influence the proteasome inhibitor-induced nuclear and nucleolar accumulation of wild-type p53 in MCF-7 cells. MCF-7 cells, transfected with the GFP–EBNA-5 construct, were cultured 1 day after transfection in the presence of DMSO (a, c, e) or 10 µM of MG132 (b, d, f) for 16 h, and analysed for p53 expression by immunostaining. Panels a and b, GFP–EBNA-5 and DNA; panels c and d, wild-type p53; panel e, phase contrast of the field shown in panels a and c; panel f, phase contrast of panels b and d. Arrow indicates cell with GFP–EBNA-5 in the nucleoli.
nuclei in proteasome-mediated protein degradation has not been documented. Our data on proteasome inhibitor-induced nuclear accumulation of Hsp70, EBNA-5 and p53 may suggest that nuclei can be involved in proteasome-dependent protein degradation.

EBNA-5 accumulates in the PML bodies in cells with an immunoblastic phenotype but not in type I BLs or in non-B cells, suggesting the presence of EBNA-5-associated, B-blast-specific nuclear factors that target EBNA-5 into the PODs. Alternatively, it may require an additional EBV-encoded protein(s) or EBV genome in order to localize to the PODs.

It has been suggested that PML bodies are the nuclear analogues of the aggresomes that feed proteasomes with ubiquitinated substrates (Anton et al., 1999). PML bodies also contain Hsp70 even in unstressed cells (Szekely et al., 1995a), suggesting that misfolded nuclear proteins might accumulate in the PODs. It seems unlikely that EBNA-5 itself is degraded through the proteasome pathway since it does not contain lysines, which are the targets for ubiquitination. Also, our Western blot data showed no increase in EBNA-5 protein levels upon MG132 treatment in different cell lines even after 16 h of proteasomal block. It seems therefore unlikely that the PML bodies provide an intermediate reservoir for EBNA-5 on its way for degradation. On the other hand, our data suggest that POD-associated EBNA-5 might regulate the proteasome-mediated degradation of other proteins.

The PML bodies might be indirectly involved in transcription regulation by coordinating the degradation of transcription factors. Transcription co-activators such as CBP/p300, transcriptional repressors, such as Daxx and Tax, and the tumour suppressor protein Rb were detected in the PODs (for review see Zhong et al., 2000b). EBNA-5 was found to enhance EBNA-2-mediated transactivation of EBV promoters (Harada & Kieff, 1997). The mechanism of this co-operation is not known. Our data raise the possibility that EBNA-5 might cooperate with EBNA-2 through modifying the degradation rate of transcription regulators.

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