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Dissection of human papillomavirus type 33 L2 domains involved in nuclear domains (ND) 10 homing and reorganization

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Abstract

We have recently shown that the minor capsid protein L2 of human papillomavirus type 33 (HPV33) recruits the transcriptional repressor Daxx into nuclear domains (ND) 10 and causes the loss of the transcriptional activator Sp100 from these subnuclear structures (Florin et al., 2002b). In order to dissect L2 domains involved in nuclear translocation, ND10 homing, loss of Sp100, and recruitment of Daxx, a detailed deletion mutagenesis of L2 was performed. Using immunofluorescence and green fluorescent protein fusions, we have identified two nuclear localization signals (NLS) in the central and C-terminal part of L2, respectively, homologous to previously identified NLS in HPV6B L2 (Sun et al., 1995). We mapped the ND10 localization domain to within a 30 amino acid peptide in the C-terminal half of L2. L2-induced attraction of Daxx into ND10, coimmunoprecipitation of L2 and Daxx, as well as induction of the loss of Sp100 from ND10 require an intact ND10 localization domain. This domain contains conserved PXXP motives characteristic of some protein/protein interacting domains. Our data also suggest that the Daxx/L2 interaction may be the driving force for L2 accumulation in ND10.
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Keywords: Papillomavirus; L2; ND10; Sp100; Daxx

Introduction

Nuclear domains (ND) 10 are distinct subnuclear structures, which have been implicated in transcriptional regulation, growth suppression, and apoptosis (Doucas, 2000; Li and Chen, 2000; Li et al., 2000a; Gottifredi and Prives, 2001; Quignon et al., 1998; Torii et al., 1999; Wang et al., 1998; Zhong et al., 2000b). They have also been proposed to serve as transient deposition sites for nuclear factors. Promyelocytic leukemia (PML) protein is the main structural component of ND10 (Ishov et al., 1999; Zhong et al., 2000a), which was initially identified as fusion partner of the retinoic acid receptor α-oncogenic fusion protein in patients with acute promyelocytic leukemia (de The et al., 1990; Goddard et al., 1991; Kakizuka et al., 1991). Another major component of ND10 is Sp100, which has been shown to influence transcription and chromatin dynamics (Seeler et al., 1998, 2001). Additional proteins have been found transiently or permanently associated with these structures. These include SUMO-1, which can modify PML and Sp100 through covalent modification (Seeler et al., 2001; Zhong et al., 2000a), Daxx, which is involved in apoptosis and transcriptional repression (Li et al., 2000a, 2000b; Torii et al., 1999; Zhong et al., 2000b), and tumor suppressors like p53 (Gottifredi and Prives, 2001; Lain et al., 1999).

Specific regulatory early proteins of many large DNA viruses are targeted to ND10 inducing their destruction and/or complete reorganization. These include IE1 protein of cytomegalovirus (Korioth et al., 1996; Ahn et al., 1998), ICP0 protein of herpes simplex virus (Everett and Maul, 1994; Parkinson and Everett, 2000), and BZLF1 protein of Epstein-Barr virus (Adamson and Kenney, 2001). They interfere with the SUMO-1 modification of PML and/or Sp100, resulting in the complete disruption of ND10. Sometimes, PML is recruited into viral replication compartments later in the infection cycle (Burkham et al., 2001).

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In contrast to large DNA viruses, small DNA tumor viruses seem to utilize these nuclear sites for their replication without obvious reorganization. It was reported that SV40 DNA replicates in or near ND10 (Ishov and Maul, 1996; Tang et al., 2000). Using transient replication assays, it was also demonstrated that plasmids, carrying the replication origin of human papillomavirus type 11 (HPV11), mainly localized close to ND10, when the viral proteins E1 and E2 were expressed in trans (Swindle et al., 1999). Recent data suggest that ND10 are not only the sites of HPV-DNA replication, but may also serve as sites of virus morphogenesis. The minor capsid protein L2 of HPV-16 and -33 and of bovine papillomavirus type 1 accumulates in these nuclear bodies (Day et al., 1998; Florin et al., 2002b; Görnemann et al., 2002). The major capsid protein L1 is attracted into ND10 by L2 rather then being diffusely distributed in the nucleoplasm (Day et al., 1998; Florin et al., 2002b). Surprisingly, HPV-33 L2 accumulation in ND10 results in the reorganization of these domains (Florin et al., 2002b). Sp100 is lost and the transcriptional repressor Daxx is recruited into these sites. In contrast, an obvious change of PML was not observed in the presence of L2. Furthermore, L2 protein transiently colocalizes with Sp100-positive nuclear dots in HPV-infected lesions, suggesting that ND10 are indeed involved in virus morphogenesis. To further our understanding of the L2-induced reorganization of ND10 and in order to dissect L2 domains involved in nuclear translocation, ND10 homing, loss of Sp100, and recruitment of Daxx, we have now performed a detailed deletion mutagenesis of L2 encoded by HPV-33.

Results

A 30-amino acid peptide in addition to nuclear localization signals determines the subcellular localization of L2

N- and C-terminal deletion mutants of HPV33 L2 were constructed by PCR and expressed in the human osteosarcoma cell line HuTK^{-} using recombinant vaccinia viruses. The mutants are schematically depicted in Fig. 1A and their expression was demonstrated by Western blot (Fig. 1B). All mutant proteins displayed the expected migration in SDS-PAGE. For unknown reason, we reproducibly observe a lower steady state level of all mutant L2 proteins compared to wild-type (wt) L2. Subcellular localization of the mutant proteins was analyzed using immunofluorescence (Fig. 2A). All C-terminally deleted mutant proteins up to amino acids (aa) 330 (L2-1/330) still localized to the nucleus. In contrast, L2-1/300 was not actively translocated into the nucleus and was therefore mainly found in the cytoplasm. This is indicative of a nuclear localization signal (NLS) centered at amino acid position 300. The sequence is enriched for arginine without the characteristics of a classical NLS (Fig. 1A). The N-terminal deletion mutant L2-360/467, comprising only the C-terminal 108 amino acids, was also exclusively detected in the nucleus, suggesting the presence of a second NLS close to the C-terminus of L2 (Fig. 2A). A characteristic feature of this polypeptide is a stretch of six basic amino acids at position 450 to 455. This sequence is sufficient for nuclear translocation when fused to green fluorescent protein (GFP) (data not shown) suggesting that it functions indeed as NLS.

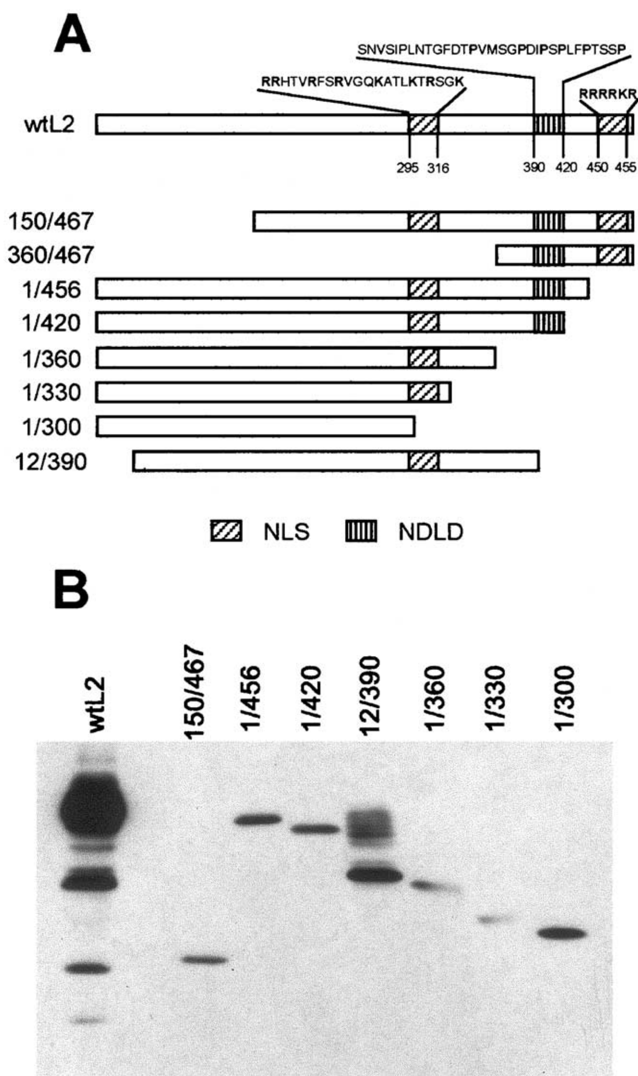


Fig. 1. (A) Schematical drawing of L2 mutants. Numbers on the left indicate the L2 amino acids still present in the construct. Amino acid sequences of the identified domains are given. NLS: nuclear localization signal; NDLD: ND10 localization domain. (B) Expression of the L2 mutants in HuTK^{-} cells using recombinant vaccinia viruses. L2 proteins were detected by Western blot using mAb 33L2-1, which binds to L2 amino acids 163 to 170 (Volpers et al., 1995).

Whereas the N-terminal deletion mutant L2-360/467 and the C-terminal mutant L2-1/420 were both found in the punctate nuclear pattern characteristic of wtL2 (Fig. 2A), removal of the C-terminal 77 amino acids (L2-12/390) or more (L2-1/360 and -1/330) resulted in a diffuse nucleoplasmic staining pattern (Fig. 2A). Confocal microscopy

demonstrated complete colocalization with PML of L2 mutants in the punctate pattern, indicative of ND10 localization (Fig. 2B). These data suggest that the sequence extending from aa 360 to aa 420 may be required for ND10 localization. The ND10 localization domain (NDLD) was further characterized using GFP-L2 fusion proteins. In agreement with the data obtained with the L2 deletion mutants, GFP-L2-360/467, -390/467, and -360/420, but not GFP-L2-390/420, accumulated in ND10. GFP-L2-360/420 and -390/420 do not comprise a NLS and reach the nucleus by passive diffusion. Therefore, cytoplasmic aggregates, which are commonly observed for over-expressed GFP fusion proteins, were found in addition (Fig. 2C). Our data indicate that L2 amino acids 390 to 420 form the core of the NDLD. As fusion with GFP, some adjacent L2 sequences at either end seem to be required for ND10 localization.

The NDLD of L2 is required for loss of Sp100

We have previously shown that wt L2 protein induces the dispersal of Sp100 from ND10 (Florin et al., 2002b). To test if this correlates with the presence of NDLD in L2, the effect of L2 mutant proteins on Sp100 was analyzed by immunofluorescence. L2 mutant proteins not localizing to ND10 (e.g. L2-1/360) had no detectable effect on Sp100 (Fig. 3). In contrast, L2 mutant proteins accumulating in ND10, like L2-1/456, -1/420, and -150/467 also affected Sp100 as indicated by our inability to detect this protein in ND10 (Fig. 3). So far, our data suggest that ND10 localization is essential for inducing the loss of Sp100.

NDLD of L2 is essential for recruitment of Daxx into ND10

We next analyzed L2 mutant proteins for their ability to interact with Daxx using immunofluorescence. As shown in Fig. 4A, wtL2, L2-150/467, -1/456, and -1/420 induced the redistribution of Daxx from the nucleoplasma into ND10. L2 mutants localizing to the nucleoplasm, as shown for L2-1/360 (Fig. 4A), or cytoplasm, like L2-1/300 (data not shown), had no detectable effect on Daxx. This also demonstrates that the use of recombinant vaccinia viruses alone does not induce the ND10 reorganization. We conclude that L2 mutant proteins, accumulating in ND10, also attract Daxx into these nuclear structures.

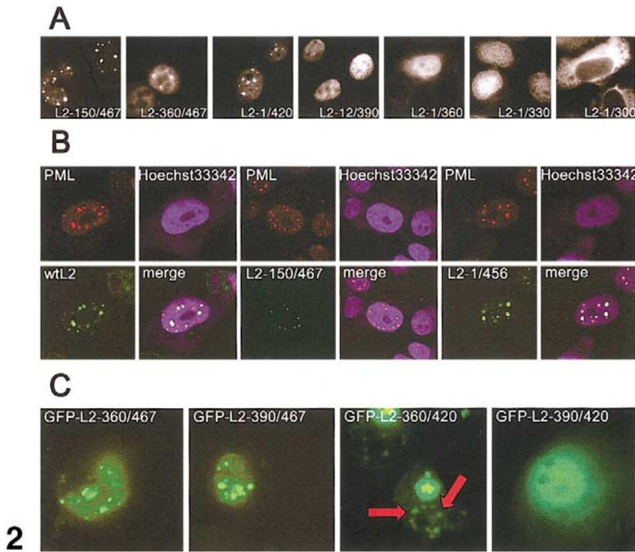
Immunoprecipitation analysis was used to confirm the interaction of Daxx with L2 mutant proteins. Whole cell extracts prepared from HuTK$^-$ cells expressing L2 mutants were subjected to immunoprecipitation using L2-specific monoclonal antibodies attached to magnetic beads. Lysates and bound proteins were analyzed by Western blotting. All extracts contained L2 proteins and similar amounts of Daxx (Fig. 4B). With the exception of L2-1/300, which was weakly expressed, all L2 mutant proteins were also detected in the precipitates. Whereas Daxx coimmunoprecipitated with wtL2, L2-1/420, and -150/467, neither L2-12/390 nor -1/330 precipitated Daxx (Fig. 4B). Taken together with the immunofluorescence analysis, we can conclude that L2 amino acids 390 to 420 are essential for Daxx interaction. Our data suggest that the capacity of L2 to accumulate in ND10 and to reorganize these sites, i.e., Sp100 loss and Daxx attraction, resides within this C-terminal L2 domain.

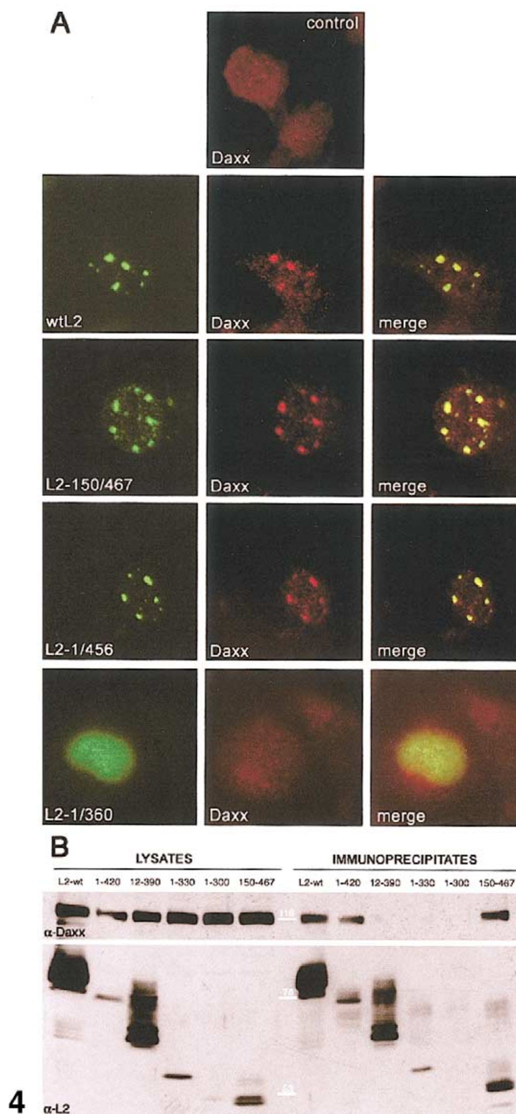
Discussion

Papillomavirus-encoded L2 proteins translocate into the nucleus independently of additional PV-encoded proteins (Florin et al., 2002a). The HPV6b L2 has been shown to contain three potential NLS, localized at the N-terminus, the C-terminus, and around amino acid position 286 to 306, respectively (Sun et al., 1995). Using deletion mutagenesis, we have identified two potential NLS within the HPV33 L2 protein. An N-terminal stretch of basic amino acids, homologous to HPV6B L2, does not function as NLS in the context of HPV33 L2 or when fused to GFP (data not shown). The characteristics of the L2 NLS are different from the classical, SV40 T-antigen type of NLS, as the C-terminal signal is rich in arginine and the central NLS seems to be bipartite (Fig. 1). It is likely that only one signal is utilized in vivo.

The HPV L2 protein was the first viral structural protein shown to accumulate in ND10 and, in addition, to reorganize these nuclear domains (Day et al., 1998; Florin et al., 2002b). We have now for the first time mapped a short continuous peptide responsible for directing a viral protein into ND10 and for the reorganization of this sub-nuclear structure. Previous analyses for viral proteins attracted into ND10, e.g. HCMV IE1, have revealed that ND10 localization cannot be confined to one distinct region, but rather requires several functional domains (Ahn et al., 1998; Wilkinson et al., 1998). The HPV33 L2 core sequence essential for ND10 homing comprises residues 390–420. Additional sequences at either side may be required in addition, since GFP-L2-390/420 did not accumulate in ND10, in contrast to GFP-L2-360/420 and -390/467. The adjacent amino acids may influence the folding and/or stability of the NDLD without directly contributing to the interaction interface. The core sequence of the HPV33 L2-NDLD is rich in proline and contains two PXXP-motives as characteristic feature. Such clusters form PPII-helices (Kay et al., 2000), which are involved in protein/protein interaction. Even though the amino acid sequence of the NDLD is rather diverse among papillomaviruses, prolines are conserved and PXXP-motives are present in most HPV types sequenced. The conservation of this protein interaction motif within the NDLD indeed suggests that it serves an important function.

The L2-induced Daxx attraction also resides within the NDLD. Since both proteins enrich concomitantly, the Daxx/L2 interaction may be the driving force for L2 accu-





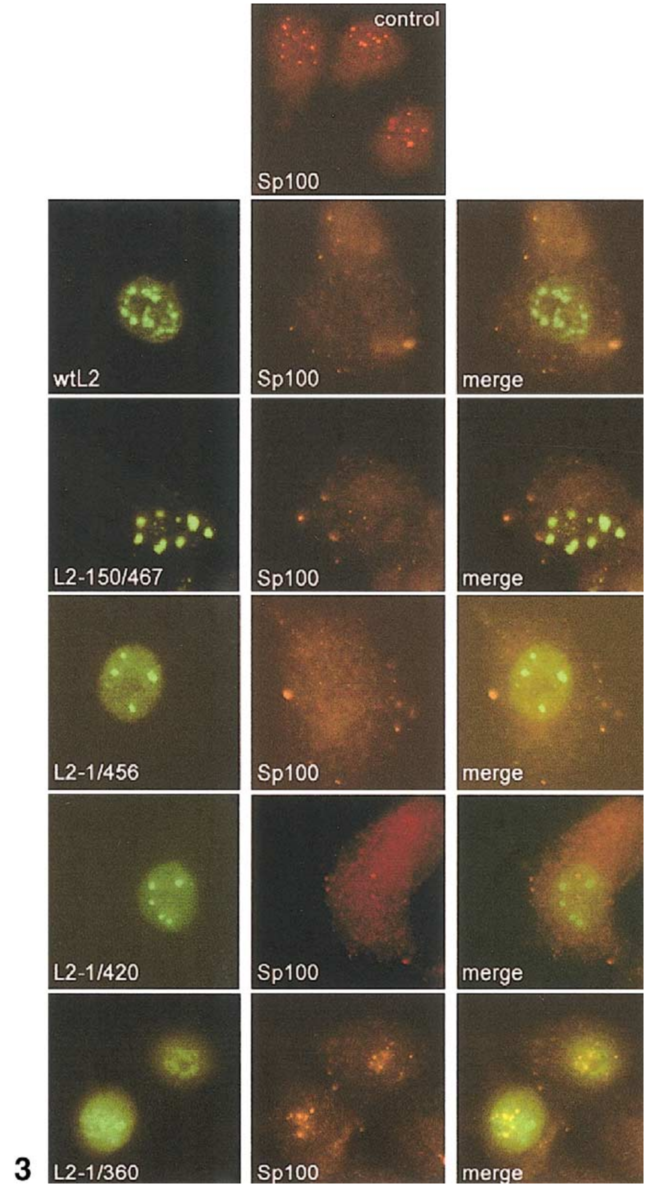


Fig. 2. Identification of domains determining L2 subcellular localization. (A and B) Indicated L2 mutants were expressed in HuTK$^-$ cells using recombinant vaccinia viruses. L2 protein was detected 10 h (wtL2, L2-150/467, -360/467, -1/456, -1/420, -12/390, -1/360, -130) and 6 h (L2-1/300) postinfection using mAb L2-1 and polyclonal antiserum K233 (L2-360/467). Costaining with PML is shown by confocal microscopy using a Zeiss 510 laserscan microscope (B). (C) Expression vectors carrying the indicated GFP-L2 fusions were transfected into COS-7 cells by electroporation. GFP fluorescence was monitored 48 h later. Arrows indicate GFP-L2 fusions localizing to nuclear domains 10.

Fig. 3. L2-induced loss of Sp100 requires ND10 localization. Indicated L2 variants were expressed in HuTK$^-$ cells using recombinant vaccinia viruses. 12 h postinfection, L2 and Sp100 were detected by immunofluorescence. Control: uninfected HuTK$^-$ cells stained for Sp100.

Fig. 4. L2-induced accumulation of Daxx requires an intact NDLD. Indicated L2 variants were expressed in HuTK cells using recombinant vaccinia viruses. (A) L2 and Daxx were detected by immunofluorescence 24 h postinfection. Control: uninfected HuTK$^-$ cells stained for Daxx. (B) Whole cell extracts were prepared and subjected to immunoprecipitation using mAb 33L2-1. Aliquots of the lysates and the immunoprecipitates were analyzed by Western blot for L2 and Daxx. Masses of molecular weight markers are given in kilodalton.

mulation in ND10. Daxx is enriched in ND10 in the absence of L2. It is therefore conceivable that the ND10-associated Daxx serves as nucleus for L2 accumulation in ND10. This can be achieved by direct interaction with Daxx but we cannot exclude the involvement of additional factors. Alternatively, L2 and Daxx may form a complex in the nucleoplasm, resulting in the accumulation of the complex in ND10. The latter seems to be more likely, since Daxx/L2 aggregates form independently of PML, e.g. in PML-negative cells (Becker et al., unpublished observations). In any case, the formation of these large aggregates requires multiple interaction domains within both proteins. Daxx has already been reported to contain at least two independent protein interaction domains (Hofmann et al., 2002; Ishov et al., 2002). The L2-NDLD also contains clusters of protein/protein interaction motives as mentioned above.

The L2-NDLD is also essential to induce the removal of Sp100 from ND10. As shown previously, the removal of Sp100 from ND10 is not controlled at the transcriptional level (Florin et al., 2002b). Our data suggest that L2 protein needs to be localized at ND10 in order to affect Sp100. Since the NDLD sequence overlaps with the Sp100 effector domain, ND10 homing of L2 itself may be sufficient for inducing the loss of Sp100. Additional functional L2 domains are probably not required. Sp100 is one of the main structural components of ND10 (Sternsdorf et al., 1997) and it is conceivable that L2 replaces Sp100 in this nuclear assembly. L2-induced recruitment of additional factors driving this process cannot, however, be excluded.

We have previously shown that L2-induced attraction of L1 into ND10 occurs only after ND10 reorganization, resulting in a delayed accumulation of L1 (Florin et al., 2002a). Using E. coli-expressed L1 and L2, Finnen and coworkers recently identified a L2 peptide interacting with L1, which largely overlaps with the herein described NDLD (Finnen et al., 2003). Highly likely, virions do not contain significant amounts of cellular proteins but histones. Therefore, the interaction of L2 with ND10 components has to be reversed for those L2 molecules, which incorporate into virions. It is tempting to speculate that the overlap of protein interaction domains within the L2-NDLD allows L1 to replace ND10 components, initiating the viral assembly, ensuring a timely coordinated morphogenesis, and finally the release of the virion from ND10.

Taken together, the HPV33 L2-NDLD seems to comprise multiple functions, including homing to ND10, binding of Daxx, dispersal of Sp100, and also attraction of L1 into these nuclear sub-structures (Becker et al., unpublished observations). These observations support the notion that L2 serves important nonstructural functions during virus morphogenesis. Since L2 is also essential for the infection process (Kawana et al., 2001; Roden et al., 2001; Unckell et al., 1997), one cannot imagine a complete papillomavirus life cycle in the absence of L2.

Materials and methods

Mutagenesis and generation of recombinant vaccinia viruses

C-terminal deletion mutants of HPV33 L2 were obtained by polymerase chain reaction (PCR) using pCMV33L2 as template and oligonucleotide 5′-GGT**GAATTC**CATGAGACACAAACGATCTAC-3′ (ON-33L2-1-5′) as forward primer. The following oligonucleotides were used as reverse primer: 5′-GCG**GGATCC**CTAACGTTTACGCCTGCGACG-3′(ON-33L2-456S-3′), 5′-AAA**GGATCC**CTATGGGCTAGATGTGGGAA-3′ (ON-33L2-420S-3′), 5′-AGA**GGATCC**CTAATCATTAATACTATAAGA-3′ (ON-33L2-360S-3′), 5′-CTA**GGATCC**CTAAGGACTTAAATCCTGATA-3′ (ON-33L2-330S-3′), and 5′-ACG**GGATCC**CTAACGCACAGTATGTCTACG-3′ (ON-33L2-300S-3′). N-terminal deletion mutants were generated by PCR using oligonucleotide 5′-GCG**GGATCC**CTAGGCCGCCACACGGACA-TC-3′ (ON-33L2-467S-3′) as reverse primer and oligonucleotides 5′-GGT**GAATTC**CATGGCATCTGCAACACAACT-3′ (ON-33L2-M12-5′), 5′-ATT**GAATTC**CATGTCATCTATTCAAACTAT-3′ (ON-33L2-M150-5′), and 5′-ATT**GAATTC**ATGGATGGTTTGTATGATGTTTATGC-3′ (ON-33L2-M360-5′) as forward primer. L2-12/390 was constructed by PCR using ON-33L2-M12-5′ as forward primer and 5′-GGT**GAATTC**CATGGCATCTGCAAC-ACAACT-3′ (ON-33L2-390S-3′) as reverse primer. Upstream of the ATG and downstream of the stop codon recognition sites for *EcoRI* and *BamHI*, respectively, had been added (highlighted in boldface). The resulting fragments were cloned into pCR2.1topo (Invitrogen). Fragments were cut out using *EcoRI* and *BamHI* and cloned into pTM1 cut correspondingly to obtain pTM33L2-1/456, -1/420, -1/360, -1/330, -1/300, -12/467, -91/467, -150/467, -360/467, and -12/390. The numbers indicate the 33L2 amino acids still present in the construct. Corresponding recombinant vaccinia viruses were obtained after cotransfection with wild-type vaccinia virus DNA following published procedures (Unckell et al., 1997). The construction of vac33L1 and vac33L2 has been described (Unckell et al., 1997). The helper virus VTF7-3, recombinant for the T7 RNA polymerase, was a generous gift by Bernhard Moss (Moss et al., 1990).

Generation of GFP-L2 fusions

Fragments of L2 were generated by PCR using ON-33L2-M360-5′ as forward primer and ON-33L2-467S-3′ and ON-33L2-420S-3′, as reverse primers, cut with *EcoR1* and *BamH1* and cloned into correspondingly cut pEGFP-C2 (Clontech) to obtain pGFP-33L2-360/467 and -360/420, respectively. Expression plasmids pGFP-33L2-390/467 and -390/420 were constructed similarly using ON-33L2-M390-5′ (5′-TCTGAATTCAATGAGCAATGTGTCTATACCT-3′) as forward primer, ON-33L2-467S-3′ and ON-33L2-420S-3′ as reverse primers, and pEGFP-C1 (Clontech).

Cell lines and antibodies

The osteosarcoma cell line HuTK^{-}143 B was obtained from Bernhard Moss (Moss et al., 1990). All cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum and antibiotics at 37°C. L1- and L2-specific mouse monoclonal and rabbit polyclonal antibodies have been described (Sapp et al., 1994; Volpers et al., 1993, 1995). Daxx-specific antibody was purchased from Santa Cruz. PML- and Sp100-specific rabbit polyclonal antiserum was obtained from Chemicon.

Infection of cells

Confluent cells were split 1:4 and grown for 24 h at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and antibiotics (Life Technologies). Cells were washed once with phosphate buffered saline (PBS), pH 7.3, and subsequently infected with recombinant vaccinia viruses diluted in serum-free DMEM at a multiplicity of infection of 2 for each virus. After incubation for 1 h at room temperature, virus-containing medium was replaced by supplemented DMEM. Cells were processed for Western blotting, immunoprecipitation, and immunofluorescence after the indicated periods of time at 37°C, respectively.

Immunofluorescence

Cells, grown on coverslips and infected with vaccinia viruses as described above, were fixated with methanol—0.02 M EGTA (−20°C) for at least 20 min, washed twice with PBS, and blocked in 5% goat serum dissolved in PBS. Coverslips were then incubated for 1 h at 37°C with the indicated antibodies. After extensive washing with PBS, coverslips were again blocked for 30 min with 5% goat serum and subsequently incubated at 37°C with Cy3-conjugated Affinipure goat anti-rabbit IgG and Cy2-conjugated Affinipure goat anti-mouse IgG (Jackson Immunoresearch Products) for 1 h. Coverslips were subsequently washed with PBS and mounted onto slides using Fluoprep mounting medium (bioMérieux). Pictures were taken using a ZEISS Axiovert 200M microscope and a ZEISS Axiocam digital camera. The Axiovision Software 3.0 was used for merging pictures.

Immunoprecipitation

5×10^6 cells, either uninfected or infected for 24 h with VTF7-3 and the indicated L2-recombinant vaccinia virus, were harvested, washed once with PBS, and suspended in 1 ml of PBS supplemented with 0.5% Nonidet P40 and a protease inhibitor cocktail. Cell extracts were prepared by sonication for 30 seconds at 20% output and 20% interval using a Branson Sonifier 250. This whole cell-lysate was added to goat anti-mouse IgG-coated dynabeads (Dynal), to which monoclonal antibodies (mAb) 33L2-1 and 33L2-2 had previously been coupled. After 4 h at 4°C at constant agitation the beads were collected and washed four times with 1 ml each of ice-cold PBS. Bound proteins were eluted with 1% sodium dodecylsulfate (SDS) at 100°C. Lysates and immunoprecipitates were subsequently analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) (Lämmli, 1970). Proteins were transferred to nitrocellulose membrane (Towbin et al., 1979). Daxx and L2 were stained using Daxx-specific polyclonal antibody and mAb 33L2-1, respectively, and horse radish peroxidase-coupled secondary antibody (Jackson Immunoresearch Products). The signal was visualized by enhanced chemiluminescence (Amersham Pharmacia).

Acknowledgments

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