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Signals That Dictate Nuclear, Nucleolar, and Cytoplasmic Shuttling of the $\gamma_1$34.5 Protein of Herpes Simplex Virus Type 1

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The $\gamma_1$34.5 protein of herpes simplex virus type 1 (HSV-1) is required for viral neurovirulence in vivo. In infected cells, this viral protein prevents the shutoff of protein synthesis mediated by double-stranded-RNA-dependent protein kinase PKR. This is accomplished by recruiting protein phosphatase 1 to dephosphorylate the α subunit of translation initiation factor eIF-2 (eIF-2α). Moreover, the $\gamma_1$34.5 protein is implicated in viral egress and interacts with proliferating cell nuclear antigen. In this report, we show that the $\gamma_1$34.5 protein encoded by HSV-1(F) is distributed in the nucleus, nucleolus, and cytoplasm in transfected or superinfected cells. Deletion analysis revealed that the Arg-rich cluster from amino acids 1 to 16 in the $\gamma_1$34.5 protein functions as a nucleolar localization signal. The region from amino acids 208 to 236, containing a bipartite basic amino acid cluster, is able to mediate nuclear localization. $R^{215}A$ and $R^{216}A$ substitutions in the bipartite motif disrupt this activity. Intriguingly, leptomycin B, an inhibitor of nuclear export, blocks the cytoplasmic accumulation of the $\gamma_1$34.5 protein. $L^{134}A$ and $L^{136}A$ substitutions in the leucine-rich motif completely excluded the $\gamma_1$34.5 protein from the cytoplasm. These results suggest that the $\gamma_1$34.5 protein continuously shuttles between the nucleus, nucleolus, and cytoplasm, which may be a requirement for the different activities of the $\gamma_1$34.5 protein in virus-infected cells.

The $\gamma_1$34.5 gene of herpes simplex viruses (HSVs) is located in the inverted repeats of the viral genome flanking the unique long sequence and is present in two copies per genome (1, 13, 14). In HSV type 1 (HSV-1) strain F, the $\gamma_1$34.5 gene encodes a protein of 263 amino acids consisting of an amino-terminal domain, a linker region of three amino acid repeats (Ala-Thr-Pro), and a carboxyl-terminal domain (13). The triplet repeats are a constant feature of the $\gamma_1$34.5 protein in HSV-1, but the number of repeats varies from strain to strain (3, 13, 16). In HSV-2, these triplet repeats are not present in the $\gamma_1$34.5 protein, as determined by nucleotide sequence analysis (32). Interestingly, the carboxyl-terminal domain of the $\gamma_1$34.5 protein is partially homologous to the corresponding domains of the murine myeloid differentiation primary-response protein MyD116 (28; D. J. McGeoch and B. C. Barnett, Letter, Nature **353:**609, 1991), the human and hamster growth arrest and DNA damage response protein GADD34 (46), and virulence factor NL/I14L of African swine fever virus (18, 47).

The $\gamma_1$34.5 protein is essential for HSV to display neurovirulence in experimental animal models (10, 29, 44). Deletion or nonsense mutation in the $\gamma_1$34.5 gene abates the ability of HSV to replicate in the central nervous system neurons, and therefore, the mutant is incapable of causing encephalitis (10, 37). In human neuroblastoma cell lines infected with HSV-1, the $\gamma_1$34.5 protein is expressed to prevent the shutoff of protein synthesis mediated by the double-stranded-RNA-dependent protein kinase (PKR) (9, 11). This function requires the carboxyl terminus of the $\gamma_1$34.5 protein to recruit cellular protein phosphatase 1 (PP1), forming a high-molecular-weight complex that dephosphorylates the α subunit of the translation initiation factor 2 (eIF-2α) (8, 12, 21, 22). In virus-infected cells, the $\gamma_1$34.5 protein-mediated eIF-2α dephosphorylation contributes to HSV resistance to the antiviral effect of alpha/beta interferon (7).

The carboxyl-terminal domains of the $\gamma_1$34.5 protein and GADD34/MyD116 are functionally interchangeable in the context of the HSV genome (20). GADD34/MyD116 belongs to a family of proteins induced under conditions of genotoxic stress, growth arrest, differentiation, and apoptosis (23, 28, 46). GADD34 promotes apoptosis induced by ionizing radiation or methyl methanesulfate, and this activity is negatively regulated by Src kinase Lyn (19, 23). It is also involved in the negative regulation of a stress-inducible gene, CHOP (36). Like the $\gamma_1$34.5 protein, GADD34 complexes with proliferating cell nuclear antigen (PCNA) through its carboxyl-terminal domain (6). It has been proposed that the interaction of GADD34 or the $\gamma_1$34.5 protein with PCNA may release cells from growth arrest and facilitate viral replication in HSV-infected cells (6). Studies show that the $\gamma_1$34.5 protein is required for viral glycoprotein processing and maturation from infected cells (3, 5). In mouse 3T6 cells, the $\gamma_1$34.5 deletion mutant is defective in viral egress and the growth of the mutant is severely affected in resting but not in actively dividing cells (4, 5).

Early studies indicate that the $\gamma_1$34.5 protein of HSV-1(F) is a soluble protein that accumulates in both the nucleus and cytoplasm late in infection (1). However, studies with the $\gamma_1$34.5 protein of HSV-1(17+) suggest that it is a cytoplasmic protein (33, 34). In analyzing the domain function of the $\gamma_1$34.5 protein, we found that although it is predominantly found in the cytoplasmic fraction, the $\gamma_1$34.5 protein is also present in the nuclear fraction (20). Interestingly, deletion of the carboxyl

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terminus or substitution of it with the corresponding region of MyD116 leads to an increased accumulation of the $\gamma_1$34.5 protein in the nuclear fraction (20). These observations raise the possibility that the $\gamma_1$34.5 protein may contain *cis* elements that determine its subcellular localization.

In the present study, we further examined the cellular localization of the $\gamma_1$34.5 protein derived from strain HSV-1(F). We show that in transfected cells the $\gamma_1$34.5 protein is distributed in the nucleus, nucleolus, and cytoplasm. We demonstrate, by the use of deletions and site-specific mutations, that the $\gamma_1$34.5 protein contains nuclear import and export signals that control its nuclear, nucleolar, and cytoplasmic accumulation. We also find that leptomycin B inhibits the cytoplasmic localization of the $\gamma_1$34.5 protein. These findings suggest that the $\gamma_1$34.5 protein continuously shuttles between the nucleus, nucleolus, and cytoplasm. While this work was in progress, it was reported that the cellular localization of the $\gamma_1$34.5 protein is affected by the number of ATP repeats and the arginine-rich cluster in the amino terminus (31).

MATERIALS AND METHODS

Cells and reagents. HeLa, Vero, and human neuroblastoma SK-N-SH cell lines were obtained from the American Type Culture Collection and propagated in Dulbecco's modified Eagle's medium supplemented with 5% (HeLa and Vero cells) or 10% (SK-N-SH cells) fetal bovine serum. Recombinant virus R3616, which has a 1-kb deletion in the $\gamma_1$34.5 gene, was generously provided by Bernard Roizman (10). Leptomycin B and 4′,6′-diamidino-2-phenylindole (DAPI) were purchased from Sigma and Vector Laboratories, respectively.

Plasmids. Mammalian expression vectors pEYFP-C1 and pEYFP-N1 were purchased from Clontech. pRB143 has the *Bam*HI S fragment of HSV-1(F) encoding the $\gamma_1$34.5 protein (21). pGF9907 contains a *Bam*HI-*Stu*I fragment that encodes the entire coding region of the $\gamma_1$34.5 protein (8). To construct pGF9912, a *Bam*HI-BspEI fragment from pRB143 was blunt-ended with Klenow fragment and cloned into the *Eco*RV site of pTet-Splice (GIBCO BRL). In this plasmid, the expression of the $\gamma_1$34.5 gene is driven by a cytomegalovirus promoter. To construct pGF2101, a *Nco*I/Klenow-*Hin*dIII fragment from plasmid pGF9907 was cloned into the BspEI/Klenow and *Hin*dIII sites of pEYFP-C1. In this plasmid, the full-length $\gamma_1$34.5 protein was fused in frame to the carboxyl terminus of the green fluorescent protein (GFP). Deletions in the $\gamma_1$34.5 gene were generated by PCR with Platinum high-fidelity *Taq* polymerase (GIBCO BRL) and pRB143 as a template (8). To construct plasmid pGF2110(ΔN16), oligo17-N (ATGCGCTAGCCATGGGGCCCACGGGCGCCGTCCCAACCG CACAG) and oligoR34.5-Hind (ATCGAAGCTTTATATGCGCGGCTCCTG CCATCGTCTCTCC) were used. The PCR fragment was digested with *Nco*I/Klenow and *Hin*dIII and then ligated into the BspEI/Klenow and *Hin*dIII sites of pEYEP-C1. In this plasmid, the DNA fragment encoding amino acids 17 to 263 of the $\gamma_1$34.5 protein was fused in frame to the carboxyl terminus of GFP. In a similar way, the following deletion mutants were generated: pGF2109, with a deletion of amino acids 1 to 28 (ΔN28); pGF2104, with a deletion of amino acids 1 to 52 (ΔN52); pGF2105, with a deletion of amino acids 1 to 83(ΔN83); pGF2106, with a deletion of amino acids 1 to 116 (ΔN116); pGF2107, with a deletion of amino acids 1 to 146 (ΔN146); pGF2117, with a deletion of amino acids 1 to 187 (ΔN187); and pGF2118, with a deletion of amino acids 1 to 216 (ΔN216). In the PCR amplification, oligoR34.5-Hind was used as a downstream primer. The upstream primers were oligo29-N (ATGCGCTAGCCATGGTAA CCTCCACGCCCAACTCGGAACCCGCG) for pGF2109(Δ28), oligo53-N (ATGCGCTAGCCATGGCCAGTGGGCCCCCGCCTTCTTGTTCGC) for pGF2104(Δ52), oligo84-N (ATGCGCTAGCCATGGACAGCCCCCCGCCCG AGCCGGCGCCAG) for pGF2105(Δ83), oligo117-N (ATGCGCTAGCCATG GCTAACCCCTCCCACCCCCCCTCACGCCCCTTCCG) for pGF2106(Δ116), oligo147-N (ATGCGCTAGCCATGGTGCGCCTGCGACGCGCGGGCGGGG AGGGGGCG) for pGF2107(Δ146), oligo188-N (ATGCGCTAGCCATGGCGAC CCCCGCGCGGGTGCGCTTCTCGC) for pGF2117(Δ187), and oligo217-N (AT GCGCTAGCCATGGGCTCGTGGGCCCGCGAGCGGGCCGACC) for pGF2118(Δ216). To construct pGF2121(N16), oligoN16-Nhe (CTAGCCAT GGCCCGCCGCCGCCGCCATCGCGGCCCCCGCCGCCCCCGGCCGCC CC) and its complement, oligoN16-Xho (TCGAGGGGCGGCCGGGGGCG GCGGGGGCCGCGATGGCGGCGGCGGCGGGCCATGG), were cloned into the *Nhe*I and *Xho*I sites of pEYFP-N1. In this plasmid, amino acids 1 to 16 of the $\gamma_1$34.5 protein were fused to the amino terminus of GFP. To construct pGF2142(188-236), PCR amplification was carried out with oligo188-N and oligo236-C (ATGCCTCGAGGGCCTCCGCCACCCGGCG CCGGAACCGAGC) and the PCR fragment digested with *Nhe*I and *Xho*I was ligated into the *Nhe*I and *Xho*I sites of pEYFP-N1. In this plasmid, amino acids 188 to 236 of the $\gamma_1$34.5 protein were fused to the amino terminus of GFP. Similarly, to construct pGF2143(208-236), oligoN208-Nhe (CTAG CCATGGCCTCGGCCGCCCGCCTGGCGCGCCGCGGCTCGTGGGCCC GCGAGCGGGCCGACCGGGCTCGGTTCCGGCGCCGGGTGGCGGAG GCCC) and its complement, oligoN236-Xho (TCGAGGGCCTCCGCCACC CGGCGCCGGAACCGAGCCCGGTCGGCCCGCTCGCGGGCCCACGA GCCGCGGCGCGCCAGGCGGGCGGCCGAGGCCATGG), were cloned into the *Nhe*I and *Xho*I sites of pEYFP-N1. In this plasmid, amino acids 208 to 236 of the $\gamma_1$34.5 protein were fused to the amino terminus of GFP. To construct pGF2231, oligoN208/2R-Nhe (CTAGCCATGGCCTCGGCCGCC CGCCTGGCGGCCGCCGGCTCGTGGGCCCGCGAGCGGGCCGACCG GGCTCGGTTCCGGCGCCGGGTGGCGGAGGCCC) and its complement, oligoN236/2R-Xho (TCGAGGGCCTCCGCCACCCGGCGCCGGAA CCGAGCCCGGTCGGCCCGCTCGCGGGCCCACGAGCCGGCGGCCG CCAGGCGGGCGGCCGAGGCCATGG), were cloned into the *Nhe*I and *Xho*I sites of pEYFP-N1. In this plasmid, amino acids 208 to 236 of the $\gamma_1$34.5 protein were fused to the amino terminus of GFP, with Arg^{215} and Arg^{216} replaced by Ala. To construct plasmid pGF2144, a two-step PCR was performed by using plasmid pRB143 as the template. In the first round of PCR, two fragments were amplified. One fragment was amplified with oligoR34.5-Nco (ATGCGCTAGCCATGGCCCGCCGCCGCCGCCATCGCGGC) and oligoNES-C (GCTCTGCGGTGACGCGCGCGCGGGCGGCGAGGCGCG GCG), and the other was amplified with oligoNES-N (CGCCGCGCCTCG CCGCCCGCGCGCGCGTCACCGCAGAGC) and oligoR34.5-Hind. Using a mixture of two PCR fragments as a template, a second round of PCR was carried out with oligoR34.5-Nco and oligoR34.5-Hind, and the PCR fragment digested with *Nco*I/Klenow and *Hin*dIII was cloned into the BspEI/Klenow and *Hin*dIII sites of pEYFP-C1. In this plasmid, the full-length $\gamma_1$34.5 protein, with both Leu^{134} and Leu^{136} mutated to Ala, was fused in frame to the carboxyl terminus of GFP.

Antibodies. Plasmid pRB4893 encoding a glutathione *S*-transferase–$\gamma_1$34.5 fusion protein (amino acids 146 to 263) was transformed into *Escherichia coli* BL21. Expression of the fusion protein was induced by addition of isopropyl-β-D-thiogalactopyranoside to the bacterial culture, followed by affinity purification of the fusion protein from lysates on agarose beads conjugated with glutathionine (22). The fusion protein was used for immunization of rabbits for production of polyclonal anti-$\gamma_1$34.5 antibody. Mouse monoclonal anti-C23 antibody was purchased from Santa Cruz Biotechnology.

Transfection and leptomycin B treatment. Cells were grown on glass coverslips to 60% confluency. An aliquot of plasmid DNA (0.5 to 1.0 μg) was mixed with 3 μl of Lipofectamine reagent (GIBCO BRL) and added to cells as suggested by the manufacturer. Five hours after incubation, the cells were placed in serum-containing medium and further incubated for 36 h at 37°C. Leptomycin B (Sigma) was added to the medium at a final concentration of 5 nM at 36 h after transfection. The incubation was continued with 5% CO_2 at 37°C for 3 h.

Fluorescence confocal microscopy. After transfection, the medium was aspirated and cells were washed twice with phosphate-buffered saline, fixed with 4% paraformaldehyde for 20 min, and examined directly by a fluorescence microscope. For indirect-immunofluorescence analysis, cells plated on glass coverslips were fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.2% Triton X-100 for 30 min, blocked with 1% bovine serum albumin, and then incubated with the rabbit polyclonal anti-$\gamma_1$34.5 antibody (1:250 dilution) or mouse monoclonal anti-C23 antibody (1:100 dilution) overnight at 4°C. After extensive washing with phosphate-buffered saline, cells were incubated with goat anti-rabbit or mouse secondary antibody conjugated with Rhodamine (Santa Cruz Biotechnology) at a 1:100 dilution. Cells were then visualized with a fluorescence microscope. The procedure for DAPI staining was carried out as described previously (27). Briefly, after direct- or indirect-fluorescence analysis, cells were counterstained with DAPI (1.5 μg/ml) in the VECTASHIELD mounting medium (Vector Laboratories). The slides were then examined by an Olympus IX70 inverted digital confocal microscope. For each sample, approximately 100 cells from different fields were examined. All images, representing a middle focal plane of cells, were captured with a Cooke Sensican camera, analyzed with Slidebook3 software (Intelligent Imaging Innovation, Inc.), and presented by use of Adobe Photoshop 5.0.

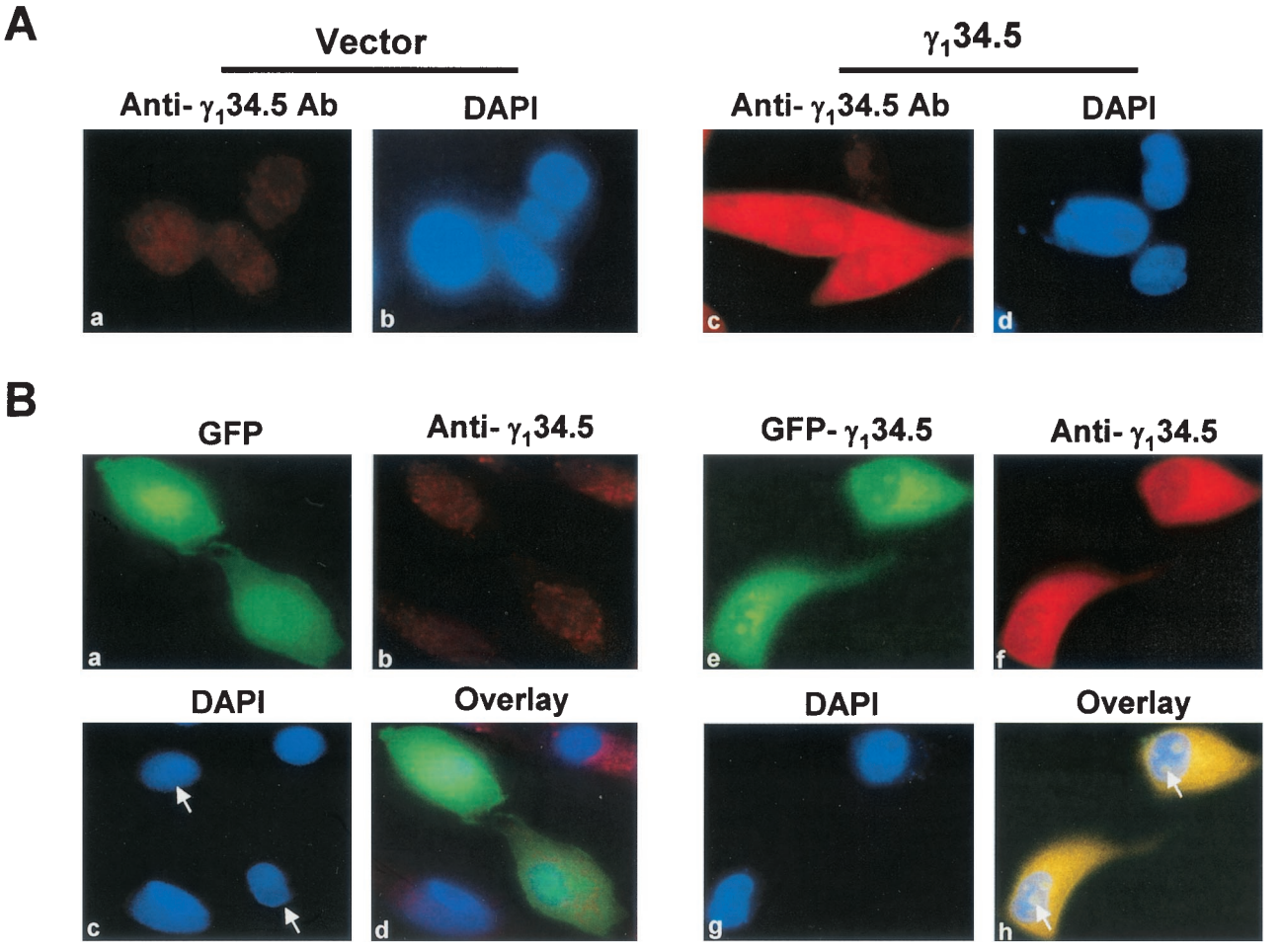

FIG. 1. (A) Cellular localization of the $\gamma_1$34.5 protein. HeLa cells grown on glass coverslips were transfected with either vector (panels a and b) or pGF9912 expressing the $\gamma_1$34.5 protein of HSV-1(F) (panels c and d). Thirty-six hours after transfection, cells were fixed with 4% paraformaldehyde and the samples were processed as described in Materials and Methods. Cells were visualized by indirect immunostaining with anti-$\gamma_1$34.5 serum, followed by incubation with Rhodamine-conjugated goat anti-rabbit secondary antibody (Santa Cruz) (panels a and c). DNA in the nucleus was stained with DAPI (1.5 μg/ml) (panels b and d). (B) Localization of GFP-$\gamma_1$34.5 fusion protein. HeLa cells were transfected with a GFP vector (panels a to d) or GFP-$\gamma_1$34.5 construct (panels e to h), and samples were processed as described in Materials and Methods. Cells were visualized by direct-fluorescence microscopy analysis (panels a and e) or indirect immunostaining with anti-$\gamma_1$34.5 serum, followed by incubation with Rhodamine-conjugated goat anti-rabbit secondary antibody (panels b and f). DNA in the nucleus was stained with DAPI (1.5 μg/ml) (panels c and g). Overlaid images indicate the superimposed signals of GFP and anti-$\gamma_1$34.5 antibody, which are shown in yellow (panels d and h). Arrows denote the corresponding cells transfected with vector or GFP-$\gamma_1$34.5 construct.

RESULTS

The $\gamma_1$34.5 protein encoded by HSV-1(F) is localized in the cytoplasm and a distinct region in the nucleus. In order to examine the distribution pattern of the $\gamma_1$34.5 protein in intact cells, a DNA fragment encoding the full-length $\gamma_1$34.5 protein of HSV-1(F) was cloned into the mammalian expression vector pTet-Splice. This plasmid construct or a vector alone was transfected into HeLa cells. Thirty-six hours after transfection, the cells were probed with the anti-$\gamma_1$34.5 antibody and visualized by indirect immunostaining by using a Rhodamine-conjugated secondary antibody. As shown in Fig. 1A, a very weak fluorescent signal (panel a), which may have resulted from background staining, was observed in cells transfected with a vector. In cells transfected with plasmid expressing the $\gamma_1$34.5 protein, an intense fluorescent signal was seen both in the cytoplasm and nucleus (Fig. 1A, panel c). It is obvious that besides being concentrated in the cytoplasm, the $\gamma_1$34.5 protein was localized to distinct regions of the nucleus, most likely the nucleoli (Fig. 1A, panels c and d), which are recognized by their exclusion from counterstaining by DAPI, a dye that specifically reacts with double-stranded DNA. Moreover, the $\gamma_1$34.5 protein was also present in a much lower level in the region between the cytoplasm and discrete areas in the nucleus.

To facilitate the assay, the full-length $\gamma_1$34.5 protein was fused to the GFP in vector pEYFP. When expressed in HeLa cells, this fusion protein, like the wild-type $\gamma_1$34.5 protein, is able to mediate dephosphorylation of eIF-2α (data not shown). After transfection of HeLa cells with a vector or plasmid expressing the GFP-$\gamma_1$34.5 fusion protein, the intracellular localization of these proteins was examined by fluorescence confocal microscopy. The results shown in Fig. 1B indicate that the

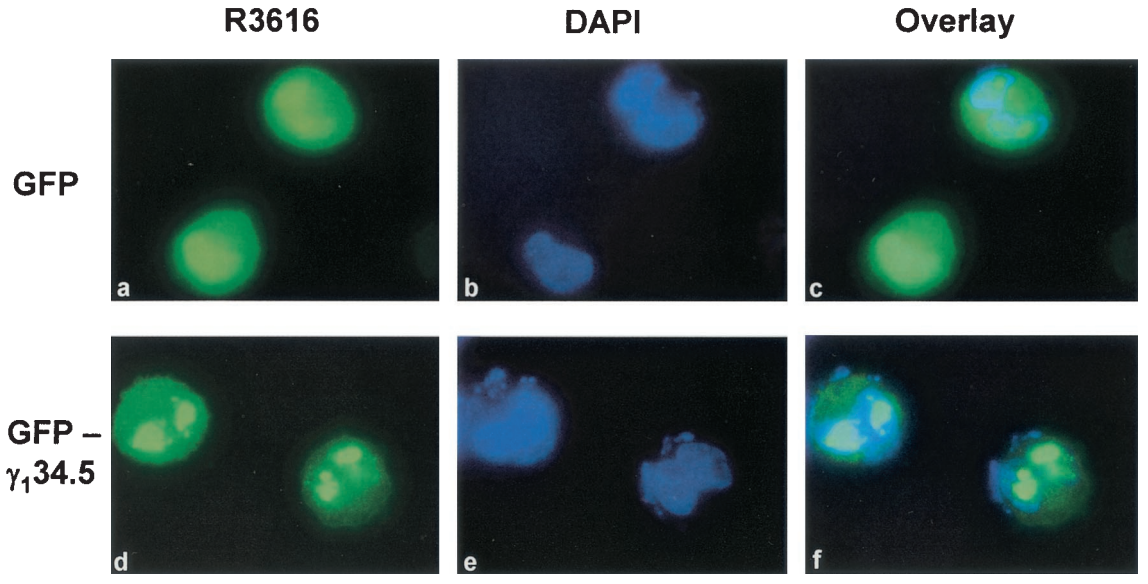

FIG. 2. Cellular localization of GFP-$\gamma_1$34.5 protein in HSV-1-infected cells. HeLa cells were transfected with a GFP vector (panels a to c) or GFP-$\gamma_1$34.5 construct (panels d to f) as described in Materials and Methods. Sixteen hours after transfection, cells were infected with recombinant virus R3616, from which the $\gamma_1$34.5 gene has been deleted (5 PFU/cell). Nine hours after infection, cells were fixed with 4% paraformaldehyde and processed for direct-fluorescence analysis. DNA in the nucleus was stained with DAPI (1.5 μg/ml) (panels b and e). Overlaid images indicate the superimposed signals of GFP and DAPI staining (panels c and f).

GFP control diffused throughout the cells (panels a and c). Although the GFP-$\gamma_1$34.5 fusion protein was also seen in both the cytoplasm and nucleus, its distribution within the nucleus was quite different, with the fusion protein mainly targeted to the subnuclear structures (Fig. 1B, panels e and g). Among the 100 cells from different fields that were examined, over 90% displayed this localization pattern. To confirm that the observed green fluorescence represents the intracellular localization of the $\gamma_1$34.5 protein, the same cells were probed with the anti-$\gamma_1$34.5 antibody and visualized by indirect immunostaining using a Rhodamine-conjugated secondary antibody. As shown in Fig. 1B, panels b and f, the anti-$\gamma_1$34.5 antibody reacted strongly with the GFP-$\gamma_1$34.5 fusion protein, with the $\gamma_1$34.5 protein distributed similarly in the cytoplasm and distinct regions of the nucleus. The faint fluorescence in the GFP-transfected cells is likely due to the background staining. The overlaid images (Fig. 1B, panels d and h) clearly indicate that the bulk of the protein is present in the cytoplasm and the distinct regions of the nucleus. These results indicate that localization of the GFP-$\gamma_1$34.5 fusion protein does not differ from that of the wild-type $\gamma_1$34.5 protein. When Vero cells or human neuroblastoma SK-N-SH cell lines were transfected with the GFP-$\gamma_1$34.5 construct, a similar distribution pattern was observed (data not shown).

To examine whether virus infection affects the localization of the $\gamma_1$34.5 protein, HeLa cells were transfected with either the GFP-$\gamma_1$34.5 construct or vector plasmid. Sixteen hours after transfection, cells were infected with the HSV-1 mutant R3616 (5 PFU/cell), from which the $\gamma_1$34.5 gene is deleted (10). At 9 h postinfection, cells were examined for the cellular localization of the $\gamma_1$34.5 protein by fluorescence microscopy. The confocal microscopy images shown in Fig. 2 indicate that while GFP alone spread throughout cells (panels a, b, and c), the GFP-$\gamma_1$34.5 fusion protein was localized primarily to the cytoplasm and the discrete areas of the nucleus (panels d, e, and f). The distorted nucleus structures in the round cells were the result of virus infection. Essentially, the localization of the GFP-$\gamma_1$34.5 fusion protein is similar to that seen in cells transfected but not infected with virus. We conclude from these collective experiments that the $\gamma_1$34.5 protein of HSV-1(F) is a cytoplasmic and nuclear protein.

Deletions in the $\gamma_1$34.5 protein affect intracellular localization. The $\gamma_1$34.5 protein of HSV-1(F) has 159 amino acids in the amino-terminal domain, 30 amino acids in the central domain arranged as triplet repeats (ATP), and 74 amino acids in the carboxyl-terminal domain (Fig. 3). Given that it is distributed in the cytoplasm and nucleus, we examined whether deletions in the $\gamma_1$34.5 protein had any effect on its intracellular distribution. For this purpose, we constructed a series of $\gamma_1$34.5 deletion mutants that were fused to the carboxyl terminus of GFP (Fig. 3). These mutants, with deletions spanning amino acids 1 to 216, include ΔN28 (a deletion of amino acids 1 to 28), ΔN52 (a deletion of amino acids 1 to 52), ΔN83 (a deletion of amino acids 1 to 83), ΔN116 (a deletion of amino acids 1 to 116), ΔN146 (a deletion of amino acids 1 to 146), ΔN187 (a deletion of amino acids 1 to 187), and ΔN216 (a deletion of amino acids 1 to 216). When transfected into HeLa cells, these mutants were expressed, as detected by Western blot analysis, with anti-GFP and anti-$\gamma_1$34.5 antibodies (data not shown). HeLa cells transfected with these mutants were then examined for intracellular distribution by confocal microscopy. The fluorescence confocal microscopy images shown in Fig. 4, panels a to p, represent a middle focal plane of cells transfected with the various GFP-$\gamma_1$34.5 constructs and stained with DAPI. Unlike the full-length GFP-$\gamma_1$34.5 fusion protein, ΔN28 and ΔN52 were primarily found in the cytoplasm, and no

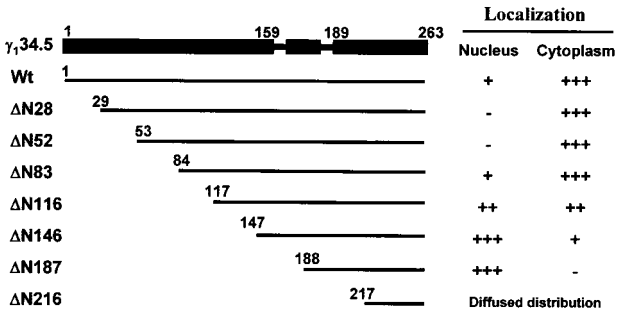

FIG. 3. Schematic diagrams of the full-length $\gamma_1$34.5 protein of HSV-1(F) and deletion mutants. The designations for the wild-type (Wt) $\gamma_1$34.5 protein and each mutant are indicated on the left. The filled bar at the top represents the domain structure, and the numbers indicate amino acid positions. The amino-terminal domain contains amino acids 1 to 159, the ATP repeat region contains amino acids 160 to 188, and the carboxyl-terminal domain contains amino acids 189 to 263. Thin lines indicate the coding regions retained in the wild-type $\gamma_1$34.5 protein or deletion mutants. The number at the left of each line denotes the starting amino acid in each construct. All constructs were fused in frame to the carboxyl terminus of GFP. A summary of the cellular localization of wild-type $\gamma_1$34.5 or mutants is presented on the right. −, negative signal; +, positive signal, with each additional + indicating a stronger signal.

obvious nuclear accumulation was observed in over 90% of transfected cells. This suggests that the region containing amino acids 1 to 28 is required for the $\gamma_1$34.5 protein to be localized in the distinct region within the nucleus.

Further analysis indicated that mutants ΔN83 and ΔN116 exhibited different patterns (Fig. 4, panels g to j). These mutants were found in both the cytoplasm and nucleus. The mutant ΔN83 appeared to be localized to regions resembling the plasma membrane. Moreover, this mutant was distributed either in the cytoplasm or throughout the cells. The frequency of cytoplasmic localization was approximately 80% in cells expressing the ΔN83 mutant. While present throughout the cells, the mutant ΔN116 displayed a more nuclear localization pattern. Intriguingly, the next-shorter truncation mutant, ΔN146, was almost completely restricted to the nucleus, displaying a uniform distribution pattern (Fig. 4, panels k and l). These results may be interpreted as indicating that the region containing amino acids 83 to 146 is likely involved in either nuclear export or cytoplasmic anchoring. As shown in Fig. 4, panels m and n, mutant ΔN187 was also present only in the nucleus. Thus, the lack of the amino-terminal domain and the central domain containing the triplet repeats had no effect on the nuclear localization. However, further deletion of amino acids to amino acid 216, as represented by ΔN216, resulted in a diffused distribution pattern throughout the cells (Fig. 4, panels o and p). Notably, an additional deletion of amino acids 188 to 216 from the mutant ΔN187 completely abolished its exclusive nuclear distribution. The diffused distribution pattern is similar to that seen for GFP alone (Fig. 1B, panels a and d), which is likely due to passive diffusion. These results indicate that the region spanning amino acids 187 to 263 is required for

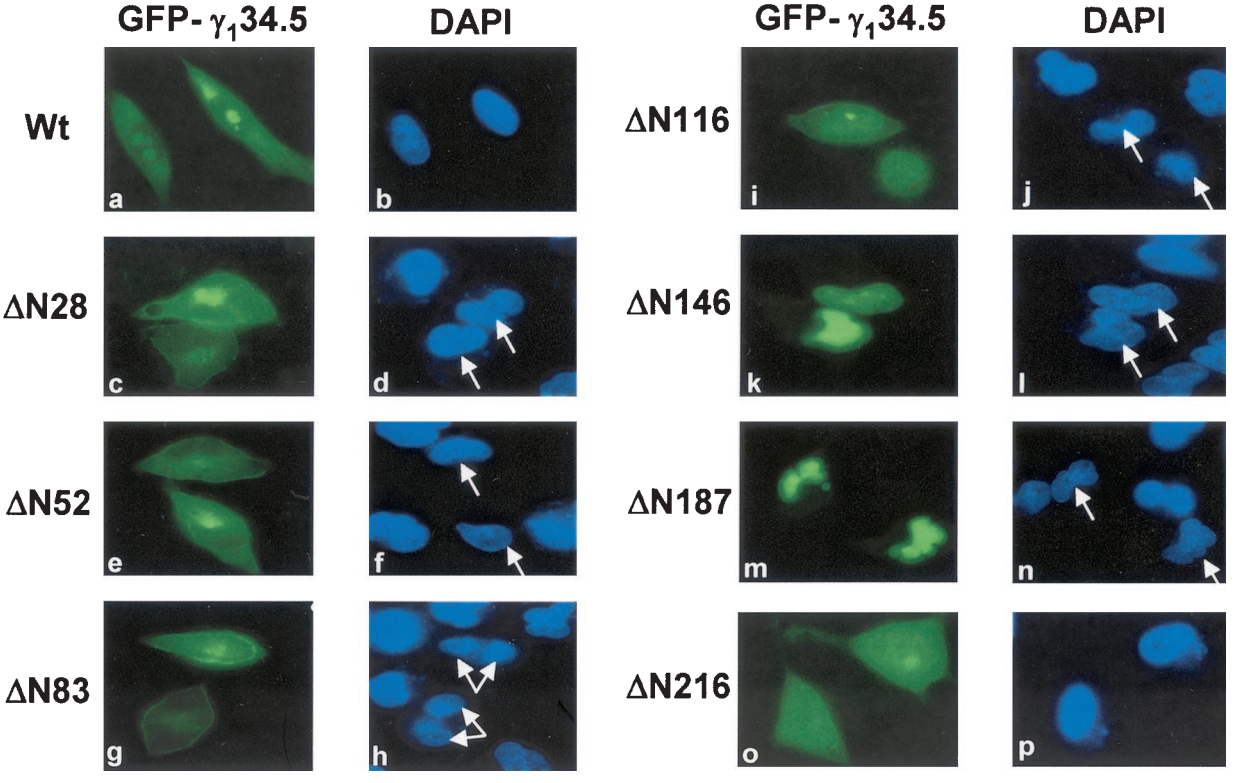

FIG. 4. Fluorescence microscopy images of the full-length $\gamma_1$34.5 protein and deletion mutants. HeLa cells, transfected with plasmid constructs as described in the legend to Fig. 3, were fixed with 4% paraformaldehyde at 36 h after transfection and visualized by direct-fluorescence microscopy as described in the legend to Fig. 1. The intracellular distribution of each mutant is shown along with DAPI staining for nuclear DNA. Arrows denote the corresponding cells transfected with various GFP-$\gamma_1$34.5 constructs. Wt, wild type.

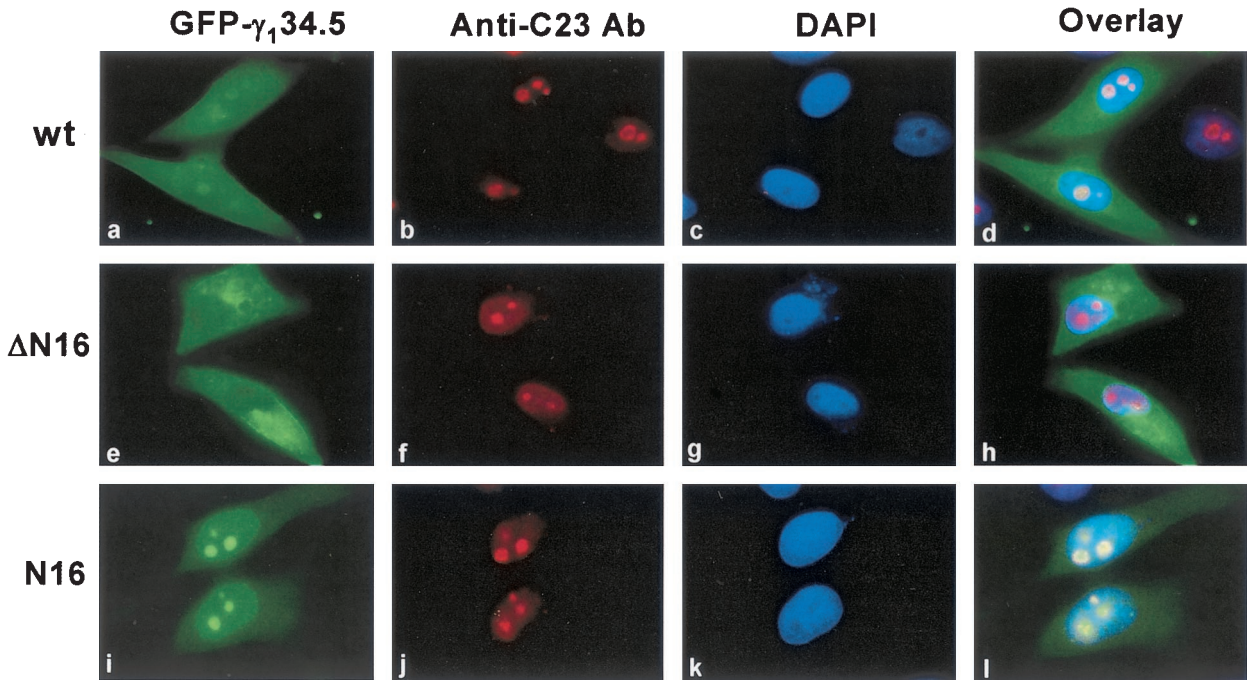

FIG. 5. Characterization of nucleolar localization signal in the $\gamma_1$34.5 protein. HeLa cells transiently expressing the full-length GFP-$\gamma_1$34.5 fusion protein (wt), mutant ΔN16, or mutant N16 were fixed with 4% paraformaldehyde and visualized by direct-fluorescence microscopy. Mutant ΔN16 has a deletion of amino acids 1 to 16 (MARRRRHRGPRRPRPP) from the $\gamma_1$34.5 protein which is fused to the carboxyl terminus of GFP. Mutant N16 expresses only amino acids 1 to 16 from the $\gamma_1$34.5 protein which is fused to the amino terminus of GFP. The C23 protein in the nucleolus was detected by indirect-immunofluorescence staining (panels b, f, and j). Cells were incubated with mouse anti-C23 monoclonal antibody, followed by reaction with Rhodamine-conjugated goat anti-mouse immunoglobulin G. DNA in the nucleus was stained with DAPI (panels c, g, and k). Overlaid images represent the superimposed signals of GFP, anti-C23 antibody, and DAPI (panels d, h, and l).

nuclear localization. Taken together, these data strongly suggest that both the amino-terminal and the carboxyl-terminal domains have *cis* elements that determine the intracellular localization of the $\gamma_1$34.5 protein.

A stretch of basic amino acids from amino acids 1 to 16 in the $\gamma_1$34.5 protein directs nucleolar localization. The results of the deletion analysis shown in Fig. 4, panels a to f, indicate that the first 28 amino acids are involved in the translocation of the $\gamma_1$34.5 protein into discrete regions within the nucleus. This region, from amino acids 1 to 16, consists of a cluster of arginine residues (MARRRRHRGPRRPRPP). This cluster is rich in basic amino acids that are reminiscent of nucleolar localization signals found in the Rev protein of human immunodeficiency virus (HIV) (30), the Rex protein of human T-cell leukemia virus type 1 (HTLV-1) (38), the MEQ protein of Marek's disease virus (27), and the NL/I14L protein of African swine fever virus (18). To precisely define the function of this motif, two mutants were constructed. In the mutant ΔN16, the $\gamma_1$34.5 protein with a deletion of amino acids 1 to 16 was fused to the carboxyl terminus of GFP. In the mutant N16, only amino acids 1 to 16 from the $\gamma_1$34.5 protein were fused to the amino terminus of GFP. When examined for cellular localization, the two mutants displayed different staining patterns. As shown in Fig. 5, panels a to l, a deletion of amino acids 1 to 16 from the $\gamma_1$34.5 protein resulted in a cytoplasmic localization. Essentially, the mutant ΔN16 was excluded from the nucleus. In sharp contrast, the mutant N16 was accumulated in the discrete regions within the nucleus resembling the nucleoli or nucleolus-organizing regions. To verify that these distinct regions or structures within the nucleus represent the nucleoli, the same cells were stained for the major nucleolar protein C23 or nucleolin with the anti-C23 antibody (26). The punctate structures within the nucleus, as shown in Fig. 5, panels b, f, and j, were recognized specifically by anti-C23 antibody. Remarkably, localization of the full-length GFP-$\gamma_1$34.5 protein and the mutant N16 coincided well with these intensely stained spots within the nucleus (Fig. 5, panels a, b, i, and j). This is clearly indicated in the superimposed images (Fig. 5, panels d and l). These data demonstrate that the motif containing amino acids 1 to 16 functions independently as a nucleolar localization signal.

The region from amino acids 208 to 236 in the carboxyl terminus of the $\gamma_1$34.5 protein is able to mediate nuclear localization. Figure 4, panels m to p, shows that the mutant ΔN187, which expresses amino acids 188 to 263, was capable of localizing to the nucleus, whereas the mutant ΔN216, which expresses amino acids 217 to 263, diffused throughout the cells. One interpretation of this phenotype is that amino acids 188 to 217 in the carboxyl terminus of the $\gamma_1$34.5 protein are crucial for nuclear localization. To examine the role of this domain, two additional deletion mutants were constructed. In the mutant 188-236, the region from amino acids 188 to 236 in the $\gamma_1$34.5 protein was fused to GFP. Similarly, in the mutant 208-236, the region from amino acids 208 to 236 was fused to GFP. Fluorescence confocal microscopy analysis showed that both mutants were predominantly localized to the nucleus in

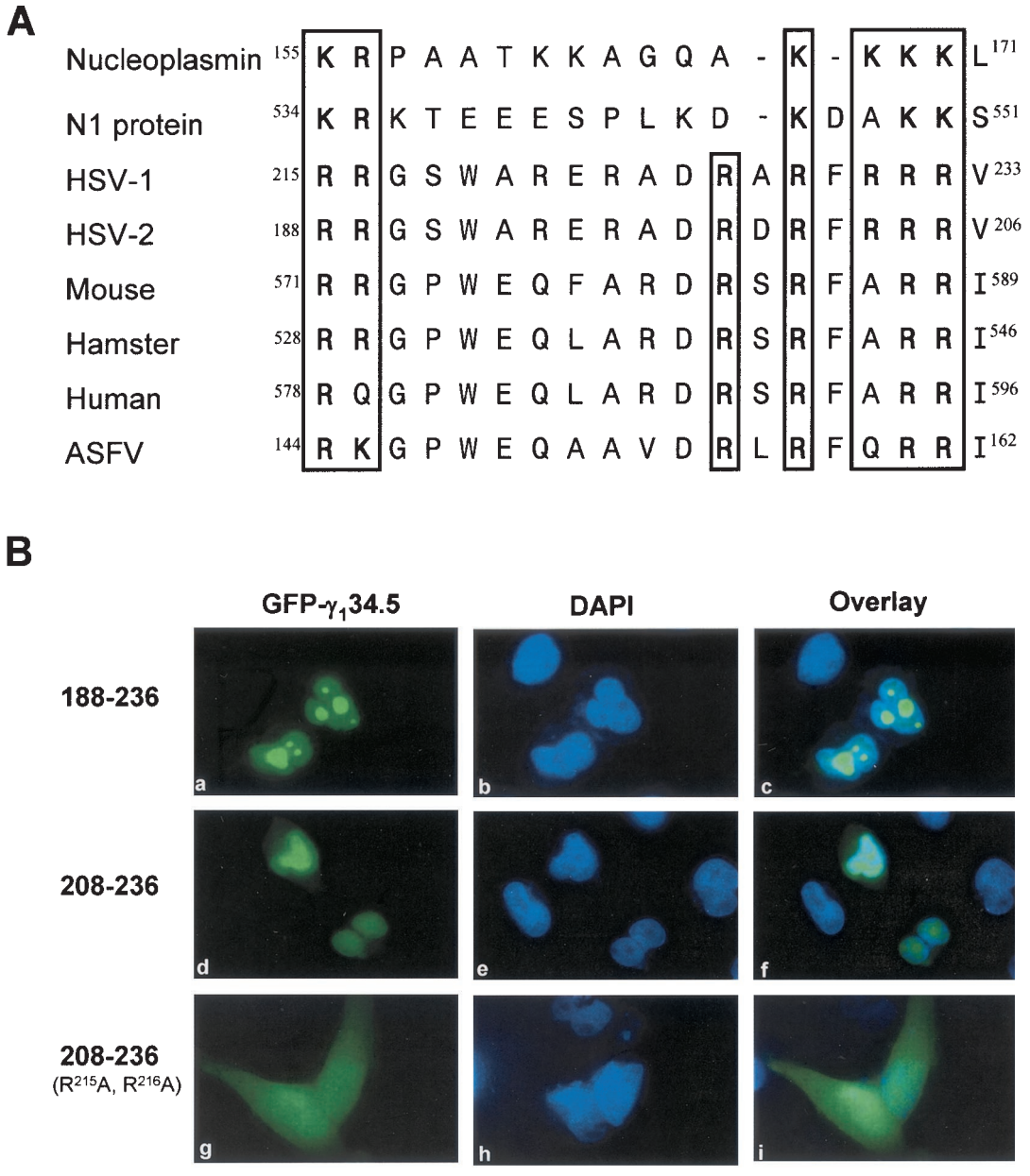

FIG. 6. Analysis of the bipartite nuclear localization signal in the $\gamma_1$34.5 protein. (A) Amino acid sequence alignment of bipartite nuclear localization signals of nucleoplasmin and N1 from *Xenopus laevis* (15); the $\gamma_1$34.5 protein from HSV-1 and HSV-2 (13, 32); GADD34 from human (23), mouse (28), and hamster (46); and NL/I14L from African swine fever virus (47). (B) Immunofluorescence confocal microscopy images of the $\gamma_1$34.5 deletion mutants. Mutant 188-236 contains amino acids 188 to 236 of the $\gamma_1$34.5 protein fused to GFP. Mutant 208-236 contains amino acids 208 to 236 of the $\gamma_1$34.5 protein fused to GFP. Mutant 208-236sb was derived from mutant 208-236 by $R^{215}A$ and $R^{216}A$ substitutions. These mutants were transfected into HeLa cells, and samples were processed as described in Materials and Methods. Cells were visualized by direct-fluorescence microscopy (panels a, d, and g). DAPI was used to stain DNA in the nucleus (panels b, e, and h). The superimposed signals of GFP and DAPI are indicated by overlaid images (panels c, f, and i).

over 90% of transfected cells (Fig. 6B, panels a to f). Interestingly, the mutant 188-236 also concentrated to the regions resembling nucleoli (Fig. 6B, panels a to c). These results demonstrate that the region containing amino acids 208 to 236 is sufficient to direct nuclear localization. Inspection of the amino acid sequence of this region revealed two clusters of basic amino acids separated by 9 to 11 spacer amino acids (Fig. 6A), indicating that it bears the signature motif of bipartite nuclear localization signals identified in other proteins, such as nucleoplasmin and N1 from *Xenopus laevis* (15).

To further analyze the bipartite motif in the $\gamma_1$34.5 protein, site-specific mutations were introduced into the mutant 208-236. In the 18-amino-acid sequences of the $\gamma_1$34.5 protein shown in Fig. 6A, two conserved basic amino acids (R^{215} and R^{216}) in the first cluster were replaced by alanine. The images shown in Fig. 6B, panels g to i, indicate that the mutant 208-

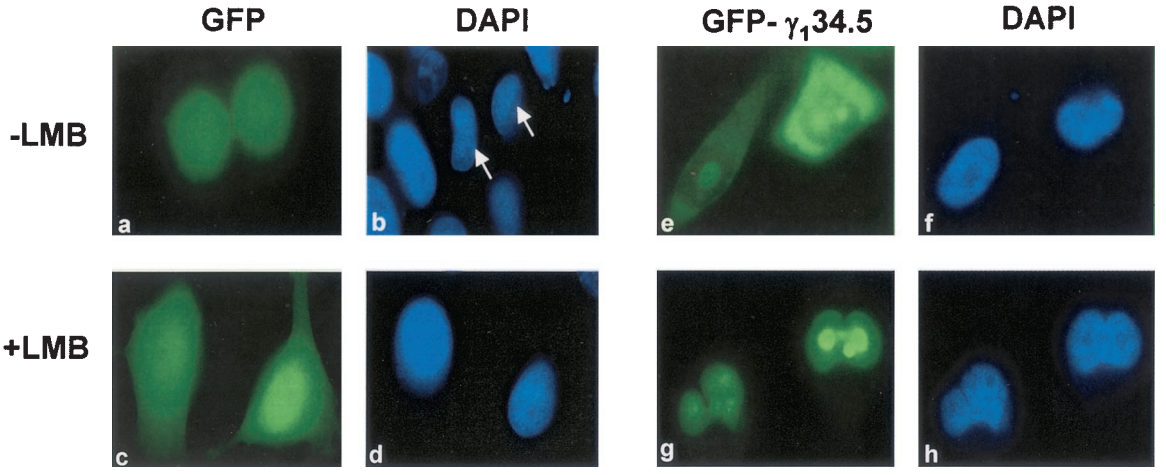

FIG. 7. Effect of leptomycin B (LMB) on cellular localization of the $\gamma_1$34.5 protein. HeLa cells transfected with a GFP vector (panels a to d) or GFP-$\gamma_1$34.5 construct (panels e to h) were either left untreated or treated with leptomycin B (5 nM) for 3 h, fixed with 4% paraformaldehyde, and visualized by direct-fluorescence microscopy. DAPI was used to stain DNA in the nucleus (panels b, d, f, and h). Arrows denote the corresponding cells transfected with GFP vector.

236sb, with the $R^{215}A$ and $R^{216}A$ substitutions, diffused throughout the cells. Compared to mutant 208-236, there is a significant increase in the cytoplasmic accumulation and a concomitant decrease in nuclear localization for mutant 208-236sb (Fig. 6B, panels f and i). This result indicates that the two conserved arginines are required for efficient nuclear localization, which is consistent with the feature of bipartite nuclear localization signals (15). Combined with the diffused distribution of the mutant ΔN216 (Fig. 4, panels o and p), in which the amino-terminal deletion extended to both R^{215} and R^{216} in the first cluster, these data strongly suggest that the bipartite motif in the $\gamma_1$34.5 protein is critical for nuclear localization. It is interesting that this motif is present not only in the $\gamma_1$34.5 protein from HSV-1 and HSV-2 but also in the NL/I14L protein from African swine fever virus and GADD34 from mouse, hamster, and human (Fig. 6A).

Leptomycin B inhibits the cytoplasmic accumulation of the $\gamma_1$34.5 protein. Since the $\gamma_1$34.5 protein is found in the cytoplasm, we examined whether nuclear export or cytoplasmic anchoring is required for its cellular localization. Specifically, HeLa cells were transfected with the GFP or GFP-$\gamma_1$34.5 construct. Thirty-six hours after transfection, cells were left untreated or treated with leptomycin B, a specific inhibitor of CRM1-mediated active nuclear export (17, 45). Subcellular localization of GFP or GFP-$\gamma_1$34.5 was determined by fluorescence microscopy. As shown in Fig. 7, panels a to h, prior to treatment with leptomycin B, the GFP control alone diffused throughout the cells. GFP-$\gamma_1$34.5 predominantly localized in the cytoplasm and in the nucleolus. After leptomycin B treatment was carried out as reported previously (17), the GFP control still showed a diffused staining pattern whereas all GFP-$\gamma_1$34.5 became localized in the nucleus and nucleolus. The strong staining in the nucleolus is attributed to the nucleolar signal present in the amino terminus of the $\gamma_1$34.5 protein. These results suggest that active nuclear export via the CRM1 pathway contributes to the cytoplasmic localization of the $\gamma_1$34.5 protein.

$L^{134}A$ and $L^{136}A$ substitutions in the leucine-rich motif result in nuclear accumulation of the $\gamma_1$34.5 protein. To map the *cis* element required for nuclear export, HeLa cells were transfected with the deletion mutants ΔN28, ΔN52, ΔN83, and ΔN116, and the effect of leptomycin B on intracellular distribution of these mutants was examined. As shown in Fig. 8A, all of these mutants were primarily localized in the nucleus, which is different from the phenotype seen in the cells not treated with leptomycin B (Fig. 4). The data indicate that all of these mutants are still capable of moving in and out of the nucleus. Comparison of the cellular distribution of all of these mutants with that of ΔN146 (Fig. 4, panels k and l), which is restricted to the nucleus, suggests that the sequence consisting of amino acids 116 to 146 is required for nuclear export.

Based on the results described above, we analyzed the amino acid sequence between amino acids 116 to 146 and identified a leucine-rich motif containing LPPRLALRLR from amino acids 128 to 137. This cluster bears a signature sequence of nuclear export signals present in protein kinase A inhibitor (43), REV from HIV (42), NS1 from influenza A virus (25), and ICP27 from HSV-1 (39) (Fig. 8B). To further demonstrate the function of this leucine-rich motif in nuclear export, we introduced the $L^{134}A$ and $L^{136}A$ substitutions in the full-length $\gamma_1$34.5 protein. This mutant was examined for intracellular localization in transfected HeLa cells. As shown in Fig. 8C, while the wild-type $\gamma_1$34.5 protein was found in the nucleus, nucleolus, and cytoplasm, the mutant was exclusively present in the nucleus and nucleolus (panels a to f). This localization pattern was observed in 90% of the cells examined. These results strongly suggest that the leucine-rich motif is required for the cytoplasmic localization of the $\gamma_1$34.5 protein through active nuclear export.

DISCUSSION

We have demonstrated that the $\gamma_1$34.5 protein of HSV-1(F) is present in the nucleus, nucleolus, and cytoplasm in transfected or superinfected cells. These results are consistent with previous findings that the $\gamma_1$34.5 protein of HSV-1 accumu-

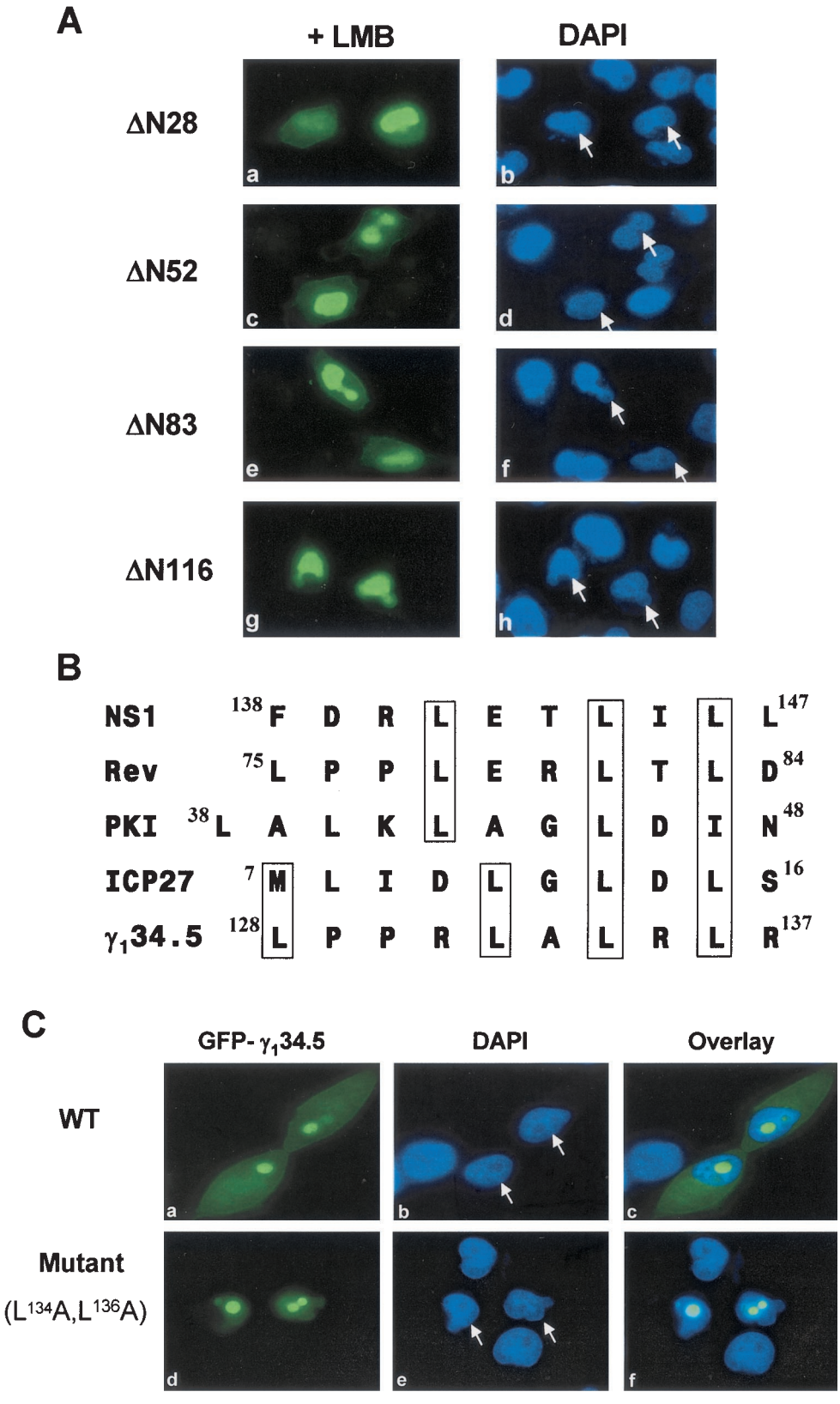

FIG. 8. Characterization of the nuclear export signal of the $\gamma_1$34.5 protein. (A) HeLa cells transfected with the indicated GFP-$\gamma_1$34.5 deletion mutants were treated with 5 nM leptomycin B (LMB) for 3 h, fixed with 4% paraformaldehyde, and visualized by direct-fluorescence microscopy (panels a, c, e, and g). DAPI was used to stain DNA in the nucleus (panels b, d, f, and h). (B) Alignment of the nuclear export signals of protein kinase A inhibitor (PKI) (43), NS1 from influenza virus (25), REV from HIV (42), ICP27 (39) and $\gamma_1$34.5 (this work) from HSV-1. (C) Cellular localization of the $L^{134}A$ $L^{136}A$ mutant. HeLa cells expressing wild-type (WT) $\gamma_1$34.5 (panels a, b, and c) or the mutant, in which both L^{134} and L^{136} have been replaced by A (panels d, e, and f), were analyzed by direct-fluorescence microscopy. The superimposed signals of GFP and DAPI are indicated by overlaid images (panels c and f). Arrows denote the corresponding cells transfected with the GFP-$\gamma_1$34.5 constructs.

lates in both the nucleus and cytoplasm late in infection (1). Nuclear and cytoplasmic localization of the $\gamma_1$34.5 protein was also demonstrated by a recent study of different HSV-1 isolates (31). Furthermore, the data presented here indicate that the amino-terminal domain of the $\gamma_1$34.5 protein contains a nucleolar localization signal and nuclear export signals, whereas the carboxyl-terminal domain contains a nuclear localization signal. An important observation emerging from these studies is that the $\gamma_1$34.5 protein of HSV-1(F) continuously shuttles between the nucleus, nucleolus, and cytoplasm.

Our studies indicate that the region containing amino acids 1 to 16 in the $\gamma_1$34.5 protein is a nucleolar localization signal. This region is rich in arginine residues and resembles the nucleolar localization signals found in other viral proteins such as Rev of HIV type 1 (30), Rex of HTLV-1 (38), MEQ of Marek's disease virus (27), and NL/I14L of African swine fever virus (18). A common feature of the nucleolar localization signals of these virus proteins is an array of a various number of basic amino acids (R/K), which are required to direct nucleolar localization. As shown in Fig. 5, deletion of this region led to the complete cytoplasmic localization of the $\gamma_1$34.5 protein. Further, addition of this sequence to GFP is sufficient to mediate nucleolar localization. It was recently reported that the Arg-rich motif of the $\gamma_1$34.5 protein derived from different HSV-1 strains directs proteins to both the cytoplasm and discrete regions of the nucleus (31). However, as shown in Fig. 5, when amino acids 1 to 16 were fused to GFP, the protein was highly concentrated in the nucleolus and very little of it was seen in the cytoplasm. These different phenotypes could result from differences in the strains or in the numbers of amino acids in the $\gamma_1$34.5 protein fused to GFP. Nevertheless, the data presented in this study strongly suggest that amino acids 1 to 16 of the $\gamma_1$34.5 protein of HSV-1(F) primarily target the protein to the nucleolus.

Deletion mutagenesis suggests that a bipartite nuclear localization signal is located in the carboxyl terminus of the $\gamma_1$34.5 protein (Fig. 6A and B). While both the mutant 188-236 and the mutant 208-236 are capable of directing nuclear accumulation of GFP, the mutant 188-236 tends to concentrate in areas resembling the nucleolus. It is likely that the presence of a region containing amino acids 188 to 208 may affect the activity of the bipartite motif. Importantly, these mutants contain a sequence with two clusters of basic amino acids that is similar to the pattern of bipartite nuclear localization signals defined in nucleoplasmin and N1 from *Xenopus laevis* (15). These signals typically consist of two clusters of basic amino acids separated by a short spacer of 10 to 12 amino acids. Notably, substitution of alanine for arginine^{215} and arginine^{216} in the first cluster of the bipartite motif significantly increased the cytoplasmic localization (Fig. 6B, panels g to i). In agreement with this observation, ΔN216, in which the amino-terminal deletion extended into arginine^{215} and arginine^{216}, lost this exclusive nuclear localization pattern (Fig. 4, panels m to p). These results suggest that the first two arginines in the bipartite motif are critical for nuclear import. The nuclear localization sequence in the $\gamma_1$34.5 protein of HSV-1 seems to fit the bipartite pattern with 9 to 11 spacer amino acids. This sequence is also conserved in the $\gamma_1$34.5 protein from HSV-2, the NL/I14L protein from African swine fever virus, and GADD34 from human, hamster, and mouse (Fig. 6A). It is likely that this element may play a role that is common to these proteins.

Although harboring a bipartite basic motif, the full-length $\gamma_1$34.5 protein is predominantly found in the nucleolus and the cytoplasm (Fig. 1A and B). One possibility is that the bipartite motif normally functions to direct nuclear import, but it works less efficiently than the nucleolar localization signal or nuclear export signal in the $\gamma_1$34.5 protein. The net outcome is the predominantly nucleolar and cytoplasmic distribution. The notion that the bipartite basic motif of the $\gamma_1$34.5 protein is a functional element is supported by the fact that the mutant ΔN28, which normally localizes to the cytoplasm, accumulates in the nucleus after leptomycin B treatment (Fig. 4, panel c, and 8A, panel a). Since the nucleolar localization signal from amino acids l to 16 was deleted in ΔN28, this phenotype was due to a block in nuclear export and continued nuclear import by the bipartite motif. Another possibility is that the bipartite nuclear localization signal is an element that functions only under certain conditions such as posttranslational modifications or protein-protein interactions. Interpreted within the framework of this model, it is notable that the $\gamma_1$34.5 protein is found in a high-molecular-weight complex containing PP1 in virus-infected cells (8, 21). Interestingly, the $\gamma_1$34.5 protein encoded by the KOS strain of HSV-1 colocalizes with PP1 in the nucleus (31). In addition, replacement of the carboxyl terminus of the $\gamma_1$34.5 protein with the corresponding region from MyD116/GADD34 leads to an increased nuclear accumulation of the $\gamma_1$34.5 protein (20).

The amino-terminal domain of the $\gamma_1$34.5 protein has a nuclear export signal. This signal sequence contains a leucine-rich cluster found in the nuclear export signals of many proteins such as the NS1 protein of influenza A virus (25), the Rev protein of HIV (42), protein kinase A inhibitor (43), and p53 (40). Two lines of evidence support this conclusion. First, leptomycin B, an inhibitor of the nuclear export receptor CMR1 (17), blocked the cytoplasmic accumulation of the $\gamma_1$34.5 protein (Fig. 7). Second, as shown in Fig. 8C, amino acid substitutions ($L^{134}A$ and $L^{136}A$) in the leucine-rich cluster completely abolished the cytoplasmic localization of the $\gamma_1$34.5 protein. This result indicates that the leucine-rich-cluster-mediated nuclear export is critical for maintaining the cytoplasmic pool of the $\gamma_1$34.5 protein. Because the leucine-rich cluster is present in the $\gamma_1$34.5 protein of HSV-1 and HSV-2 but not in the NL/I14L protein from African swine fever virus or GADD34/MyD116 (Fig. 8B), we speculate that this nuclear export signal might be related to the functions that are unique to the $\gamma_1$34.5 protein.

Our analysis is consistent with the model that the $\gamma_1$34.5 protein shuttles continuously between different cellular compartments. Both the amino-terminal and carboxyl-terminal domains are involved in this process. Presently, it is not clear whether the cellular localization of the $\gamma_1$34.5 protein derived from HSV-1(F) is a regulated process. However, it should be noted that the amino-terminal and the carboxyl-terminal domains of the $\gamma_1$34.5 protein are relatively constant, but the number of triplet repeats (ATP) in the central region varies among different HSV-1 strains (3, 13, 16). The results of recent experiments have indicated that variations in the number of triplet repeats in the $\gamma_1$34.5 protein affect its intracellular distribution (31). The $\gamma_1$34.5 protein with a long ATP repeat

tends to be present in the cytoplasm (31). In light of these observations, it is conceivable that the triplet repeat may indirectly affect the cellular localization of the $\gamma_1$34.5 protein by masking or unmasking the nuclear import or export signals within the $\gamma_1$34.5 protein.

Finally, the question arises as to why the $\gamma_1$34.5 protein shuttles dynamically between the nucleus and the cytoplasm. A number of studies have indicated that the $\gamma_1$34.5 protein of HSV is critical for promoting virulence in experimental animal models (2, 10, 24, 37, 41). More than one function associated with this viral factor has been suggested to contribute to the observed phenotype in vivo (4–6, 10, 20, 35). In cell culture, this viral protein is required to prevent the shutoff of protein synthesis mediated by the double-stranded-RNA-dependent protein kinase PKR (9, 11). In doing so, the $\gamma_1$34.5 protein binds to and redirects PP1 to dephosphorylate the α subunit of eIF-2α (8, 21, 22). Consistent with previous findings, a recent study demonstrated that the $\gamma_1$34.5 protein and PP1 colocalize in the cytosol (31). Therefore, the cytoplasmic $\gamma_1$34.5 protein is more likely involved in mediating eIF-2α dephosphorylation and therefore blocks the cellular antiviral response mediated by interferon. This activity does not exclude the additional functions it performs in the cytoplasm. The $\gamma_1$34.5 protein is also implicated in viral egress and glycoprotein processing (3, 5). An additional function of the $\gamma_1$34.5 protein is suggested by the observation that it associates with PCNA involved in DNA replication and cell cycle regulation (6). Thus, in principle, the nuclear $\gamma_1$34.5 protein may be required to perform these last two functions. These processes require the $\gamma_1$34.5 protein to be rapidly shuttled between the different cellular sites. It remains an open question whether the nuclear and cytoplasmic shuttling of the $\gamma_1$34.5 protein has as-yet-unidentified functions. Further experiments are needed to explore these possibilities.

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