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The Epstein-Barr Virus Glycoprotein 110 Carboxy-Terminal Tail Domain Is Essential for Lytic Virus Replication

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To investigate the importance of the Epstein-Barr virus (EBV) glycoprotein 110 (gp110) tail domain in the intracellular localization of gp110 and virus lytic replication, three carboxy-terminal truncation mutants of gp110 were constructed. Deletion of 16 amino acids from the carboxyl-terminal tail resulted in gp110 intracellular localization which was indistinguishable from that of wild-type gp110, whereas deletion of either 41 or 56 amino acids from the carboxyl-terminal tail of gp110 resulted in loss of retention of gp110 in the endoplasmic reticulum and nuclear membrane. None of the gp110 truncation mutants was able to complement EBV(gp110−)$^{+}$ lymphoblastoid cell lines in transformation assays, indicating the importance of the gp110 tail domain in virus lytic replication. In electron microscopy analysis, no nucleocapsids or enveloped viruses were detected in EBV(gp110−)$^{+}$ lymphoblastoid cell lines induced for lytic replication.

Epstein-Barr virus (EBV) is one of eight human herpesviruses which latently infect humans (34). Following primary infection, EBV establishes latency in B lymphocytes (19). Periodically throughout the lifetime of the host, lytic replication is induced in latently infected B lymphocytes, leading to production and shedding of virus progeny (19). Many homologous proteins have been identified in the various herpesvirus families that infect human and animal hosts (19, 34). Membrane glycoproteins are one class of viral proteins conserved among the herpesvirus family members. They have been shown to be important in a variety of important processes, including specific binding to the cell surface, fusion of viral membranes during entry, virion assembly, and egress (17, 34–36).

Compared to those of their homologs among the alphaherpesviruses, the nucleotide and amino acid sequences of the nine glycoproteins known to be encoded by the human gammaherpesvirus range from substantially conserved to virtually unrelated (17). For instance, a gH-gL complex homolog (gp85-gp25) is present in EBV-infected cells, but it contains a third glycoprotein, gp42, which has no known relatives in the other human herpesviruses (12, 14, 17, 21, 26, 27). This additional component is important for infection of B lymphocytes but not of epithelial cells (21). Reflecting its specialized cellular niche in B lymphocytes, EBV binding to the cell surface utilizes a specific interaction between the type 2 complement receptor, CR2 (CD21), and gp350/220, which has no known herpesvirus homolog (21, 28, 29, 38, 39). Despite its amino acid homology to gB, gp110 is not a major component of the virion, unlike gB, and it does not occur in appreciable amounts in the plasma membrane of infected cells; rather, gp110 localizes predominantly to the inner and outer membranes of the nucleus and the endoplasmic reticulum (ER) (7, 10, 11, 32). Antibodies currently available and directed against gp110 fail to neutralize EBV infectivity (10). Although gp110 and gB have been shown to be essential for viral replication (3, 15), the unique features of gp110 may indicate that it has a different role from gB in viral infection.

Both gp110 and gB consist of three domains: a large amino-terminal ectodomain of either 685 (gp110) or 726 (gB) amino acids, three hydrophobic regions spanning amino acids 686 to 753 (gp110) and 727 to 794 (gB), and a carboxyl-terminal tail domain of either 104 (gp110) or 109 (gB) amino acids (30). Extensive data on the functions of the three herpes simplex virus (HSV) gB domains has been generated both by site-specific mutations and by analysis of virus variants containing mutations in the gB gene (31). The carboxyl-terminal tail domain of gB is one of the most extensively studied domains of HSV gB (1–4, 6, 8, 33). Deletion mutagenesis has shown that the last 41 amino acids are not required for production of infectious virus (16). To begin to explore important domains for function of gp110, three truncation mutations within the tail domain of gp110 were constructed by removal of 16, 41, or 56 amino acids from the carboxyl-terminal tail domain of gp110. The mutations were then assayed for function in EBV lytic replication by using a novel complementation assay with EBV(gp110−)$^{+}$ lymphoblastoid cell lines (LCLs). The distinct intracellular localization of the gp110 deletion mutations was also tested by immunofluorescence, and virus assembly and maturation in EBV(gp110−)$^{+}$ LCLs were tested by electron microscopy.

Construction of gp110 tail domain deletion mutants. To investigate the role of the gp110 tail domain in lytic viral replication and intracellular localization, three deletion mutants with truncations of the gp110 carboxyl-terminal tail after 801, 816, or 841 amino acids were constructed (Fig. 1). Two of the constructs, DM(1-816) and DM(1-801), were constructed by insertion of an *Xba*I linker containing stop codons in all three reading frames into unique restriction sites within the region encoding the gp110 tail contained in a previously described gp110 expression vector, pSVgp110 (15). For DM(1-816), the *Xba*I linker was inserted into a unique *Eag*I site, which results in termination of gp110 after 816 amino acids; this removed 41 amino acids from the 104-amino-acid gp110 carboxyl-terminal tail. Two additional amino acids, leucine and valine, were added to the carboxyl-terminal tail of gp110 as a result of the cloning manipulations. For DM(1-801), the *Xba*I linker was inserted into a unique *Eco*47III site, which results in termination of gp110 after 801 amino acids without any additional amino acids. For DM(1-841), a *Bbs*I-to-*Eco*RV fragment was removed from pSVgp110, resulting in termination of gp110 after 841 amino acids. An additional arginine and an

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gp110 Tail Mutants | **Complementation**

Mutant	Sequence	Complementation
1-841	RRTRQMSQQPVQMLYPGI-DELAQQH---ASGEGPGINPISKTELQ-AIMLALHEQNQEQKRAAQRAAGPSVASRALQAARDRFPGLRRRRYH	−
1-816	RRTRQMSQQPVQMLYPGI-DELAQQH---ASGEGPGINPISKTELQ-AIMLALHEQNQEQKRAAQRAA	−
1-801	RRTRQMSQQPVQMLYPGI-DELAQQH---ASGEGPGINPISKTELQ-AIMLAL	−

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                                                                                    ****
gp110 Tail  754 RRTRQMSQQPVQMLYPGI-DELAQQH---ASGEGPGINPISKTELQ-AIMLALHEQNQEQKRAAQRAAGPSVASRALQAARDRFPGLRRRRYHDPETAAALLGEA-ETEF 857
                :     :  :::   ::    :::::     :  :                   :   : ::  :    : ::                  : : :
gB Tail     795 RYVMRLQSNPMKALYPLTTKELKNPTNPDASGEGEEGGDFDEAKLAEAREMIRYMALVSAMERTEHKAKKKGTS-ALLSAKVTDMVMRKRRNTNYTQVPNKDGDADEDDL 903
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gB Tail Mutants

Mutant	Sequence	Complementation
186	RYVMRLQSNPMKALY	−
025	RYVMRLQSNPMKALYPLTTKELKNPTNPDASGEGEEGGDFDEA	−
1513	RYVMRLQSNPMKALYPLTTKELKNPTNPDASGEGEEGGDFDEAKL	−
176	RYVMRLQSNPMKALYPLTTKELKNPTNPDASGEGEEGGDFDEAKLAEAREMIRY	−
162	RYVMRLQSNPMKALYPLTTKELKNPTNPDASGEGEEGGDFDEAKLAEAREMIRYMA	−
1528	RYVMRLQSNPMKALYPLTTKELKNPTNPDASGEGEEGGDFDEAKLAEAREMIRYMALVSA	−
ambB1	RYVMRLQSNPMKALYPLTTKELKNPTNPDASGEGEEGGDFDEAKLAEAREMIRYMALVSAMERTEHKA	+
1511	RYVMRLQSNPMKALYPLTTKELKNPTNPDASGEGEEGGDFDEAKLAEAREMIRYMALVSAMERTEHKAKKKGTS-ALLSAKV	+

FIG. 1. Alignment of the amino acid sequences for the EBV gp110 and HSV-1(F) gB tail domains (30). Gaps (−) inserted to account for the difference in sequence length and residues which are identical in the aligned sequences (:) are shown. The gp110 truncation mutants whose sequences are shown at the top were described in this study. The amino acid sequences of the gB tail domain truncation mutants were published previously (186, 025, 176, and 162, reference 3; 1513, 1520, and 1511, reference 1; ambB1, reference 16). The ability of each of the truncation mutations to function in complementation assays using the respective viral systems is also indicated.

additional leucine were added to the gp110 cytoplasmic tail as a result of the plasmid collapse. The reading frames was confirmed by DNA sequencing (data not shown). Both the DM(1-816) and the DM(1-801) gp110 tail deletion mutations remove the basic four arginine residues at amino acids 836 to 839. Similar basic oligopeptides characterize nuclear localization (13, 18) or ER retention signals (9, 20).

Expression of the gp110 tail deletion mutants in EBV (gp110−)$^+$ LCLs. Expression of the gp110 truncation mutants was verified by transfection followed by immunoprecipitation in a previously described EBV(gp110−)$^+$ LCL designated M.2, in which the gp110 coding domain has been interrupted by a cassette expressing the hygromycin phosphotransferase gene driven by the simian virus 40 early promoter (SVHYG) (15). The insertion results in the complete absence of expression of gp110 in EBV(gp110−)$^+$ LCLs (15). M.2 cells were transfected with the DM(1-841), DM(1-816), or DM(1-801) expression construct as previously described (15). The parental expression vector pSG5 (Stratagene, La Jolla, Calif.) and the wild-type gp110 expression vector pSVgp110 were used as negative and positive controls, respectively. After transfection, cells were radiolabeled with [^{35}S]methionine-cysteine, lysed in 1% Nonidet P-40 lysis buffer, and immunoprecipitated with the mouse monoclonal antibody L2 (Chemicon, Temecula, Calif.) against gp110 as previously described (15). Immunoprecipitates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Gels were fixed, dried, and subjected to autoradiography. As expected, wild-type gp110 was readily detected in M.2 cells transfected with pSVgp110 (Fig. 2, lane 1). Each of the deletion mutants had a reduction in size of gp110 as expected (Fig. 2, lanes 2 to 4). In addition, the level of expression of each of the gp110 deletion mutants was similar to the level of expression of wild-type gp110 (Fig. 2; compare lane 1 with lanes 2 to 4). No proteins reactive with L2 were detected in pSG5-transfected cells, except for background bands evident in all of the immunoprecipitations (Fig. 2, lane 5, below the 85-kDa marker).

Intracellular localization of gp110 tail deletion mutants. The intracellular localizations of gp110 and gp350 were investigated in the EBV-infected partially permissive marmoset cell

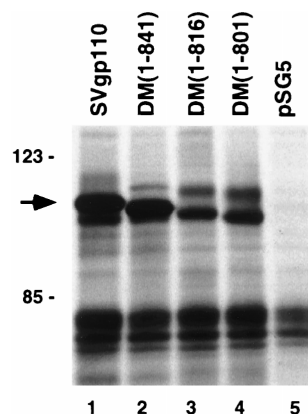

FIG. 2. Expression of gp110 and the gp110 tail domain deletion mutants in EBV(gp110−)$^+$ LCLs. M.2 cells (10^7) were transfected with 50 μg of each expression vector or control vector (pSG5). Transfected cells were labeled with [^{35}S]methionine-cysteine. Twenty-four hours after transfection, cells were washed with phosphate-buffered saline and lysed in 1% Nonidet P-40 lysis buffer. Following centrifugation, the cleared lysates were immunoprecipitated with monoclonal antibody L2, which is reactive with gp110. Immunoprecipitated proteins from equivalent cell numbers were loaded in each lane and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Gels were dried and subjected to autoradiography. Molecular weight standards are on the left, in thousands. The location of the wild-type gp110 is indicated by an arrow. Lane 1 demonstrates the wild-type gp110, lane 2 shows the 17-amino-acid-smaller gp110 of the DM(1-841) mutant, lane 3 shows the 41-amino-acid-smaller gp110 of the DM(1-816) mutant, and lane 4 shows the 57-amino-acid-smaller gp110 of the DM(1-801) mutant.

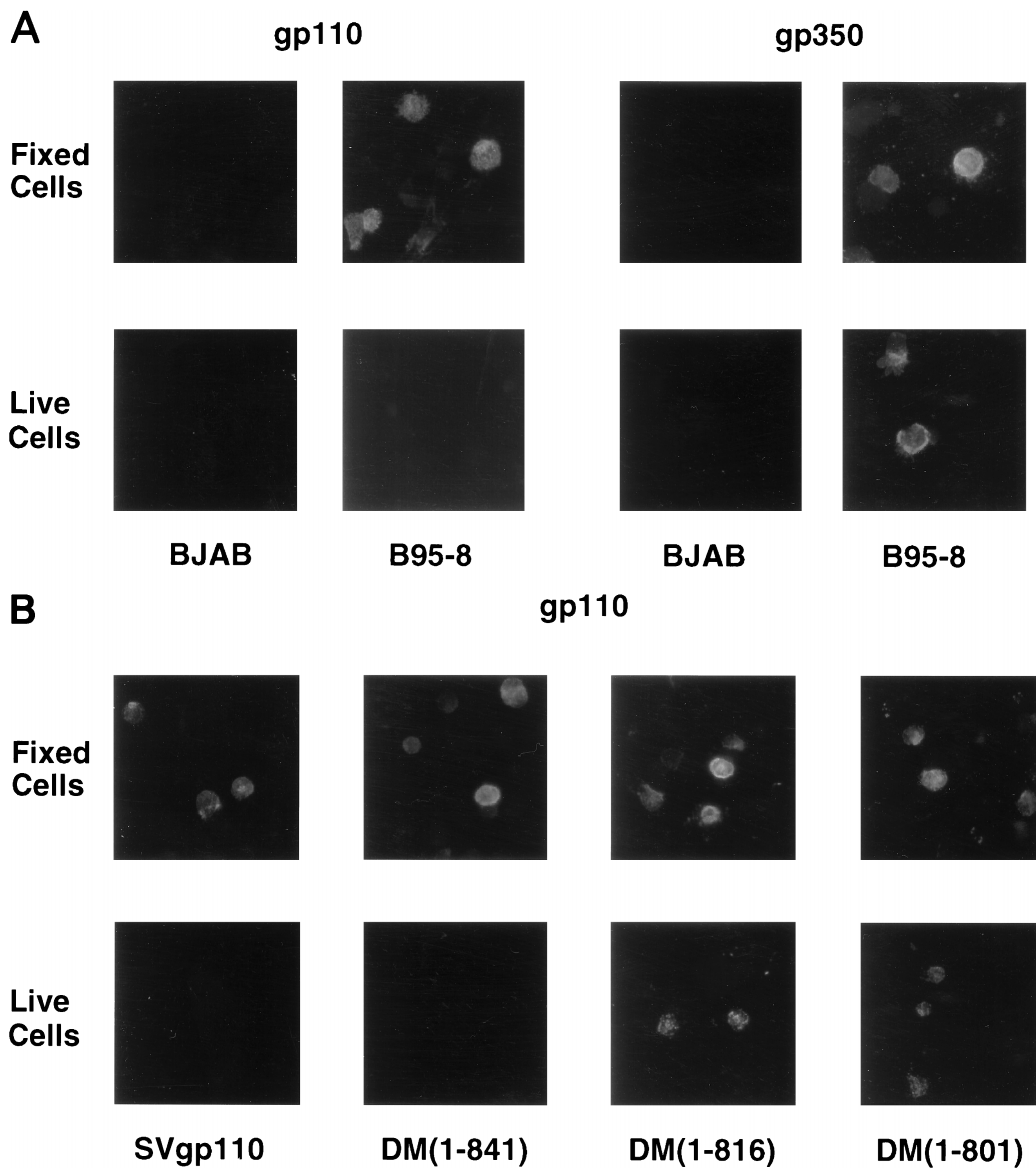

FIG. 3. Immunofluorescence microscopy of TPA-treated B95-8 cells and BJAB cells transfected with gp110 expression vectors. For fixed-cell immunofluorescence, cells were fixed to glass slides and incubated with either L2 (mouse monoclonal anti-gp110 antibody) or 2L10 (mouse monoclonal anti-gp350 antibody). For live-cell immunofluorescence, 1 ml of cell suspension was pelleted in a tube and resuspended in 100 μl of 20% normal goat serum containing L2 or 2L10 antibodies. Cells were incubated for 1 h on ice with gentle agitation every 15 min. After two washes with phosphate-buffered saline, cells were dried on glass slides. Following the primary antibody treatment and washing, slides were incubated with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G and viewed with a Zeiss fluorescence axioscope. (A) B95-8 cells were treated with 20 ng of TPA per ml for 3 days to induce lytic replication. The EBV-negative B lymphoma BJAB was used as a negative control. (B) BJAB cells were transfected with 50 μg of the wild-type or deletion mutants of gp110. Twenty-four hours later, fixed- and live-cell immunofluorescence microscopy was performed as described for panel A. L2 was used to detect the wild-type and gp110 deletion mutants.

line B95-8 (24, 25). Lytic replication was induced by treatment with 20 ng of tetradecanoyl phorbol acetate (TPA) per ml for 3 days. Fixed- or live-cell preparations were then incubated with monoclonal antibodies directed against gp110 or gp350, stained with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G, and viewed by fluorescent microscopy. Anti-gp350 monoclonal antibody 2L10 (Chemicon) readily detected gp350, which is expressed on the plasma membrane of EBV-infected cells in both fixed- and live-cell preparations (Fig. 3A). In contrast, gp110, which is expressed on the nuclear membrane and the ER but not on the plasma membrane of infected cells (7, 10, 11, 32), was detected only in fixed-cell preparations with the anti-gp110 monoclonal antibody L2 (Chemicon). In live cells, the plasma membrane remains intact during antibody treatment procedure, preventing antibody access to intracellular compartments. No reactivity was observed in EBV-negative BJAB Burkitt's lymphoma cells with L2 or 2L10 antibodies (Fig. 3A).

To assess the effect of the tail deletion mutations on the intracellular localization of gp110, BJAB cells (23) were transfected with DM(1-841), DM(1-816), and DM(1-801) expression constructs as previously described (15). The wild-type expression vector pSVgp110 was included as a control. As observed in B95-8 cells, wild-type gp110 was readily detected in fixed-cell preparations but not in live-cell preparations (Fig. 3B). Like wild-type gp110, the deletion mutant DM(1-841), which has 16 amino acids deleted from the carboxyl end of the cytoplasmic tail, was detected in the fixed-cell preparations but not in the live-cell preparations (Fig. 3B). However, deletion mutants DM(1-816) and DM(1-801), lacking 41 and 56 amino acids, respectively, of the gp110 tail, had positive staining in both fixed- and live-cell preparations (Fig. 3B), indicating that an important intracellular localization domain of gp110 had been deleted.

Lymphocyte transformation by gp110 tail deletion mutants. To investigate if the tail domain of gp110 is important in lytic virus replication, complementation experiments were performed as described previously (15). Wild-type EBV(gp110+)$^+$ LCLs (LCL1, LCL2, and LCL10) and EBV(gp110−)$^+$ LCLs (M.2, 6.16, and KC.1) were transfected with pSG5, pSVgp110, DM(1-841), DM(1-816), or DM(1-801), as indicated in Table 1. To induce lytic replication, the LCLs were also transfected with pSVNaeZ (37) and cultured for 3 to 5 days following transfection in the media containing 20 ng of TPA per ml. Virus production was monitored by assessing transforming activity by culturing freshly isolated primary B lymphocytes with lethal gamma-irradiated transfected cells or cell-free virus preparations. Six to eight weeks after infection, transformed cells were identified and expanded. LCL1, LCL2, and LCL10 produced infectious virus which readily transformed primary B cells and were unaffected by transfection with any of the gp110 expression vectors or the vector control, as expected (Table 1). Of all of the EBV(gp110+)$^+$ LCLs, LCL2 routinely produced the greatest amount of transforming virus, and in general all wells were positive for all transfections (data not shown). In contrast, transforming activity was observed with EBV (gp110−)$^+$ LCLs only when wild-type gp110 was transfected (Table 1). No transforming activity was observed when any of the gp110 tail deletion mutants or the vector alone was transfected (Table 1). This lack of activity was observed in three different EBV(gp110−)$^+$ LCLs (Table 1), indicating the importance of the gp110 tail in EBV lytic replication.

Virus assembly and maturation in EBV(gp110−)$^+$ LCLs. To investigate the assembly and maturation of viral particles in EBV(gp110−)$^+$ LCLs, lytic replication was induced in M.2 and KC.1 LCLs by transfection with pSVNaeZ and treatment with TPA. B95-8, LCL1, and LCL10 were included as wild-type controls. Induction of lytic replication was quantified by immunofluorescence for gp350. B95-8 cells were routinely 25% positive for expression of gp350. All the LCLs were routinely found to be 5 to 7% positive for gp350 expression. Following

TABLE 1. Transformants obtained by passage of EBV(gp110+)$^+$ and EBV(gp110−)$^+$ LCLs[a]

Experimental group	Cell line	No. of clones obtained for:				
		pSG5	pSVgp110	DM (1-841)	DM (1-816)	DM (1-801)
EBV(gp110+)$^+$ LCLs (cocultivation)	LCL1	1	4	1	1	1
		1	0	1	0	0
		0	1	0	0	0
		2	4	2	0	1
		0	6	0	0	0
		2	0	0	0	0
		0	0	0	1	1
		4	9	11	5	10
		0	0	0	1	0
	LCL10	0	0	1	1	1
		0	0	3	0	1
	Total	10	24	19	9	15
EBV(gp110+)$^+$ LCLs (cell free)	LCL1	1	2	2	5	2
		2	0	1	0	0
		3	5	4	3	4
		0	1	0	0	0
		0	1	0	4	0
	LCL10	0	0	1	0	0
		0	2	0	0	0
	Total	6	11	8	12	6
EBV(gp110−)$^+$ LCLs (cocultivation)	M.2	0	1	0	0	0
		0	3	0	0	0
		0	1	0	0	0
		0	3	0	0	0
		0	3	0	0	0
		0	1	0	0	0
		0	5	0	0	0
	6.16	0	1	0	0	0
		0	1	0	0	0
		0	1	0	0	0
		0	1	0	0	0
	KC.1	0	5	0	0	0
		0	2	0	0	0
	Total	0	28	0	0	0
EBV(gp110−)$^+$ LCLs (cell free)	M.2	0	1	0	0	0
		0	1	0	0	0
	Total	0	2	0	0	0

[a] Cells were transfected with the indicated plasmids. Three to five days post-transfection, primary human B lymphocytes were cocultivated with gamma-irradiated transfected cells or infected with cell-free virus. Wells positive for EBV transformation were counted 6 to 8 weeks after the infection. One 96-well plate was used for each transfection. Each row of data represents a different experiment. Totals represent the sum of the number of clones that emerged for each experimental group. The presence of the SVHYG insertion in the newly obtained LCLs from EBV(gp110−)$^+$ LCLs was verified by PCR (data not shown) as previously described (15). Control amplifications for the EBV genome using primers specific for the BHRF1 gene (22) were positive for all EBV$^+$ LCLs, as expected (data not shown).

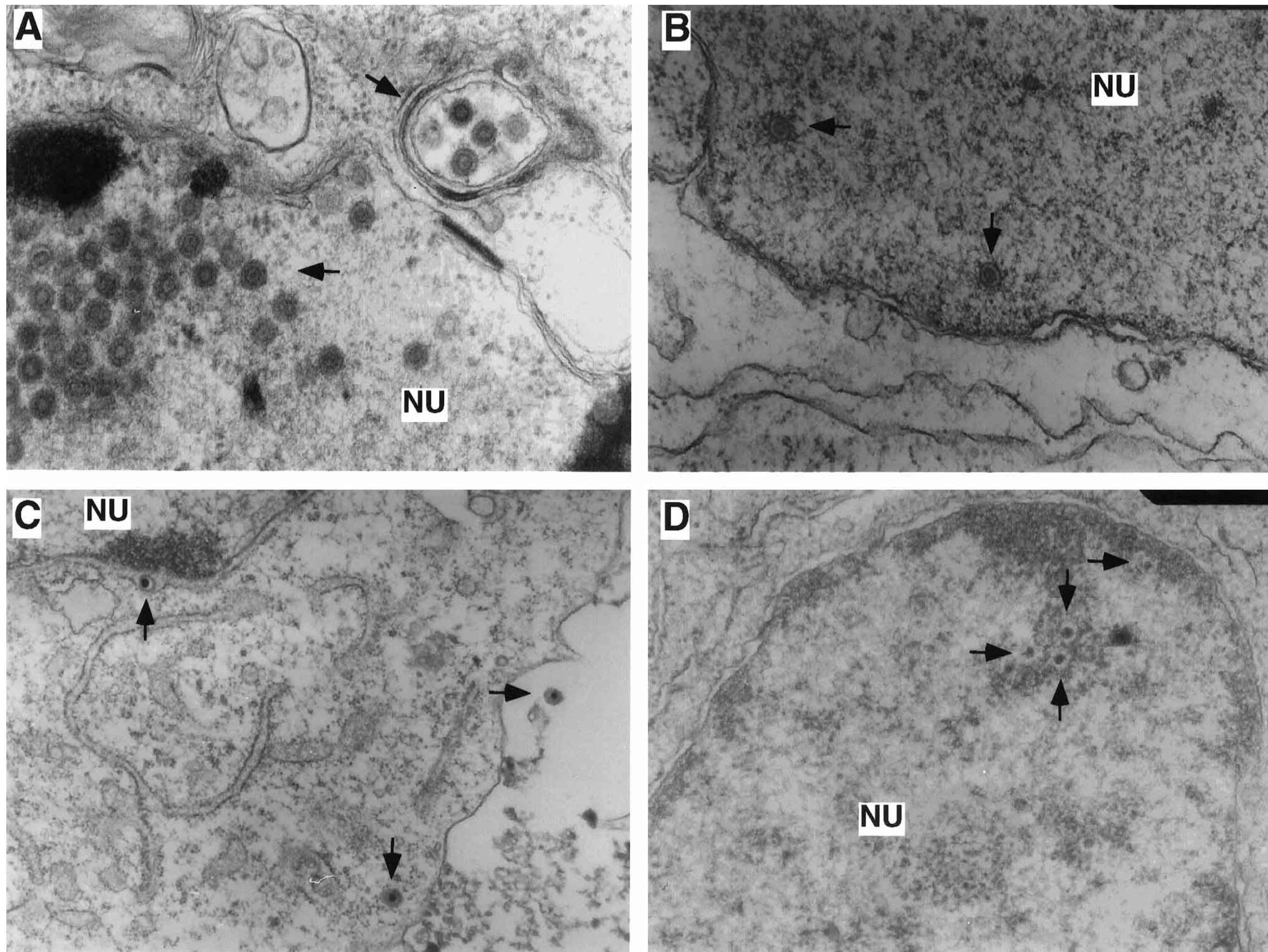

FIG. 4. Electron micrographs of B95-8 cells, EBV(gp110+)$^+$ LCLs, and EBV(gp110−)$^+$ LCLs in which lytic replication was induced by transfection with pSVNaeZ and treatment with TPA. NU, nucleus. (A) B95-8 cells exhibiting numerous viral particles (arrows). Magnification, ×25,200. (B) EBV(gp110+)$^+$ LCL1 in which viral particles are readily observed in the nucleus (arrows). Magnification, ×25,200. (C) EBV(gp110+)$^+$ LCL10 demonstrating budding through the nuclear membrane, a cytoplasmic virion, and an extracellular virion (arrows). Magnification, ×12,600. (D) EBV(gp110−)$^+$ KC.1 in which morphologically abnormal structures which may represent partially assembled nucleocapsids are observed in the nucleus (arrows). Magnification, ×12,600. No morphologically normal virions or nucleocapsids were observed in EBV(gp110−)$^+$ LCLs.

induction of lytic replication, cells were prepared and viewed by electron transmission microscopy (5). B95-8 cells reproducibly yielded the highest number of cells producing virus. Virus was readily observed in the nuclear, cytoplasmic, and extracellular compartments. A representative electron micrograph is shown in Fig. 4A. In EBV(gp110+)$^+$ LCLs, virus was observed in approximately 1 of 100 cells for LCL1 and in 1 of 200 cells for LCL10. Morphologically normal virus was readily observed in both the nuclear and the cytoplasmic intracellular compartments as well as in extracellular spaces. Representative electron micrographs are shown in Fig. 4B and C. In contrast, no morphologically normal virus was observed in EBV(gp110−)$^+$ LCLs, despite screening over 1,500 M.2 cells and 1,500 KC.1 cells. In approximately 5% of both the M.2 and the KC.1 cells, morphologically unusual structures were observed in the nuclei of the infected cells, correlating with the 5 to 7% of the cells which were positive for gp350. Representative data is shown in Fig. 4D. In no case were nucleocapsids or enveloped virions observed in EBV(gp110−)$^+$ LCLs in any intracellular or extracellular compartment, a result that is consistent with a block in normal assembly and maturation of infectious virions.

Conclusion. Previous studies have shown that gp110 is essential for either EBV entry or assembly but is not essential for EBV-driven proliferation of latently infected, transformed B lymphocytes (15). Two essential roles can be proposed for gp110 in EBV infection and lytic replication, one in entry of virus into cells and one in assembly and maturation of EBV virions. Recent work with the gB gene of HSV type 1 (HSV-1) has demonstrated that it is also an essential gene for the production of infectious virus (4). In those studies, virus could be propagated in a noncomplementing cell line but was not infectious, demonstrating a role for gB only in viral entry and not in virion assembly (4). The experiments performed in the present study utilizing EBV(gp110−)$^+$ LCLs address the paradox of the role of gp110 in EBV lytic replication. In this study, the tail domain of gp110 was analyzed by the construction of three deletion mutants with 16, 41, or 56 amino acids of the gp110 tail domain removed. We focused on the tail domain because of its potential role in virion assembly and/or budding and in the transport of virions through the nuclear membrane. The two larger deletions, DM(1-816) and DM(1-801), were not retained in the ER or the nuclear membrane. This observation

may be a result of the loss of the four basic arginine residues at amino acids 836 to 839, which could function as a nuclear or ER retention signal (9, 13, 18, 20). Interestingly, a similar motif in HSV-1 gB in the same location (Fig. 1), consisting of three arginines and one lysine, does not appear to be important for gB localization to the nuclear membrane and ER; rather, a portion of the hydrophobic domain from amino acids 774 to 795 has been shown to be sufficient for nuclear membrane and ER localization (8).

None of the gp110 tail deletion mutations were able to complement EBV(gp110$-$)$^+$ LCLs. In light of previous results with HSV-1 gB, in which deletions of 27 and 41 amino acids from the gB tail resulted in the production of viable virus, the mutations in the gp110 tail domain define a novel domain essential for the production of infectious virus which is not contained in HSV-1 gB. Electron microscopy of EBV (gp110$-$)$^+$ LCLs demonstrated the absence of nucleocapsids in the nucleus and of enveloped virions in any cellular compartment. Taken together, the data in this study suggests that gp110 may have evolved functions different from those of gB homologs in the alphaherpesviruses. Little is known about the envelopment of herpesvirus nucleocapsids, but the long cytoplasmic tail of gp110, the longest of the identified EBV glycoproteins, may provide signals that are responsible for the assembly of EBV nucleocapsids and their egress from the nucleus of an EBV-infected cell.

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