Biologic and Immunologic Effects of Knockout of Human Cytomegalovirus pp65 Nuclear Localization Signal

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Received 9 January 2009/Returned for modification 20 March 2009/Accepted 9 April 2009

The human cytomegalovirus (CMV) pp65 protein contains two bipartite nuclear localization signals (NLSs) at amino acids (aa) 415 to 438 and aa 537 to 561 near the carboxy terminus of CMV pp65 and a phosphate binding site related to kinase activity at lysine-436. A mutation of pp65 with K436N (CMV pp65mII) and further deletion of aa 537 to 561 resulted in a novel protein (pp65mIINLSKO, where NLSKO indicate NLS knockout) that is kinaseless and that has markedly reduced nuclear localization. The purpose of this study was to biologically characterize this protein and its immunogenicity compared to that of native pp65. Unlike the native CMV pp65, following either DNA- or recombinant adeno-associated virus-based transduction of CMV pp65mIINLSKO into cells in vitro, the first observation of pp65mIINLSKO expression was in the cytoplasm and pp65mIINLSKO was expressed at higher levels than the native protein. The CMV pp65mIINLSKO mRNA was more abundant earlier than CMV pp65 mRNA (at 4 h and 8 h, respectively), but the half-lives of the proteins were the same. This modification altered the antigenic processing of CMV pp65 in vitro, as measured by the improved efficiency of cytotoxic killing in a pp65mIINLSKO-transduced human HLA A*0201 target cell line. In HHDII mice expressing HLA A*0201, pp65mIINLSKO was as immunogenic as CMV pp65. By RNA microarray analysis, expression of the CMV pp65mIINLSKO had less of an effect on cell cycle pathways than the native CMV pp65 did and a greater effect on cell surface signaling pathways involving immune activity. It is concluded that the removal of the primary NLS motif from pp65 does not impair its immunogenicity and should be considered in the design of a vaccine.

A major immunodominant protein of human cytomegalovirus (CMV) is the tegument protein CMV pp65 (UL83) (15, 16, 23, 36). The biologic function of CMV pp65 is unclear, but as a nucleotropic protein which enters the nucleus immediately after infection (10, 28, 31, 33), CMV pp65 binds to polo-like kinase 1 (PLK-1), an enzyme important in mitosis (11), and it is likely that the protein has specific effects on cell cycle events (14, 22, 30). Despite this potential for cell toxicity, CMV pp65 has been proposed to be a critical antigen in any anti-CMV vaccine (17, 32). CMV pp65 has been shown to have protein kinase activity (8, 39), and a mutation at a critical phosphate binding site (CMV pp65mII) removed the kinase activity without altering the antigenicity (39). UL83 is considered an early-late gene, with synthesis beginning between 12 and 24 h after infection, during which time the protein product accumulates in the nucleus. However, at late times after infection, this CMV pp65 is exported back to the cytoplasm by means of the exportin system (31). CMV pp65 contains elements of the prototypic nuclear localization signal (NLS) in which arginine and lysine predominate within a bipartite motif in which short regions of basic amino acids are separated by 10 or more nonconserved amino acids (13, 24, 35). The nuclear localization signals of CMV pp65 consist of at least two such motifs located in the carboxy-terminal region of the polypeptide (33). One of these (termed the A-B motif by Schmolke et al. [33]) but simplified to region A in this paper) is a classic bipartite signal located at amino acids (aa) 415 to 438, in which two arginine- and lysine-rich motifs are separated by 18 aa. When this region A is deleted, however, there is very little change in nuclear localization, indicating that there are other components to the NLS. A second NLS of CMV pp65 consists of a basic region of amino acids between aa 537 and 561; this region was termed the C-D motif by Schmolke et al. (33) and is termed region B in this paper. When this region was deleted by Schmolke et al. (33), there was a more drastic reduction in the nuclear localization of CMV pp65, suggesting that this is the dominant NLS. However, the combined deletion of both the A and the B regions leads to the more complete inhibition of nuclear localization (33).

Although CMV pp65 has been the prototypic antigen for the demonstration of CMV-specific T-cell immunity (2, 17, 34), it is likely that other proteins of CMV will be necessary for the development of a vaccine that generates humoral and cellular protection. CMV pp65-specific T-cell responses have been used for the development of other immunotherapeutic approaches to the control of CMV infection (4, 19, 20). Because of the importance of CMV pp65 in a vaccine strategy, we have explored the effects of mutating elements of the protein in ways that preserve the class I-restricted cytotoxic T-cell epitopes while removing biologic signals that could have effects on normal cells. For example, we have demonstrated the immunogenicity of a kinaseless CMV pp65 in which the phosphate binding lysine-436 is mutated (8). By use of this approach, a phase I trial of an anti-CMV vaccine by using a mutation at this same active site has been completed (38).
However, these mutations of CMVpp65 remain nucleotropic and, thus, potentially toxic to normal cellular processes. This current study shows that the removal of the nucleotropic properties of CMV pp65 resulted in earlier RNA and protein synthesis posttransfection and abundant accumulation in the cytoplasm, without alteration of the immunogenicity of the protein.

**MATERIALS AND METHODS**

**Plasmid constructs.** Native CMV pp65 (pp65N) was derived from CMV strain Towne DNA, as described previously (25), and inserted into plasmid Bsp65 18.1. This served as a template in which K436N, a phosphate binding site, was mutated, creating a kinaseless CMV pp65 (pp65MII) (39). By using a DNA expression plasmid (pVAXintApp65mII), this mutation was shown to have no detectable effect on viral nuclear localization or immunogenicity (8, 39). NLS motif B (NLS-B) was then removed from pVAXintApp65mII by a bidirectional reverse PCR with the following primers: 5′-TGGCGGACGCGGCTGCCCATACG (positions 1601 to 1622) and 5′-GACCCACGTCCACTCAGACACGCGAC (positions 1741 to 1764). The PCR cycles were 94°C for 1 min, followed by 18 cycles of 94°C for 30 s, 55°C for 1 min, and 68°C for 10 min. For the parental DNA template, pVAXintApp65mII, was then digested with DpnI. The PCR product was recircularized by ligation to obtain pVAXintApp65mII-NLSKO (KO representing knockout), from which NLS-B was deleted (Fig. 1). By using Escherichia coli DH5α competent cells, transformed clones were isolated and were confirmed to contain the NLSKO deletion by sequencing. The resulting plasmid, pCMVAAV-NLSKO, was then purified with a ViralKit purification kit (Virapur, LLC, San Diego, CA), and the viral titer was measured both by the enumeration of the CMV pp65-positive plaques in transduced HT1080 cells stained with a CMV pp65-specific monoclonal antibody (Vector Laboratories Inc., Burlingame, CA) and by real-time PCR.

**Culture cell and transfection.** The MRC-5 and HT1080 cells were grown in Dulbecco’s modified Eagle’s medium with a low glucose concentration supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT), 2 mM t-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA). HEK293 cells were maintained in DMEM with a high glucose concentration with 10% FBS and 2 mM L-glutamine. HeLa cells were grown in RPMI with 10% FBS, 2 mM t-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were plated overnight and were transiently transfected by use of a calcium phosphate coprecipitation technique (Cell-Phect transfection kit; Pharmacia Biotech, Piscataway, NJ), according to the manufacturer’s protocol. For rAAV transduction, MRC-5 or HT1080 cells were treated with permisive medium containing 40 mM hydroxyurea and 1 mM sodium butyrate for 5 to 6 h and were then transduced with rAAV for 16 to 48 h at a multiplicity of infection (MOI) of 10. At this MOI, the transduction efficiency was 80 to 100%. Three transduction experiments were performed for each condition.

**Indirect immunofluorescence and immunoperoxidase staining.** The cells were plated on coverslips and transfected for 48 h, after which the coverslips were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde on ice for 20 min, and then permeabilized with 0.2% Triton X-100 in ×1 PBS for 10 min at room temperature. After a thorough rinsing with PBS containing 0.05% Triton X-100 and 1% bovine serum albumin, the cells were incubated with primary anti-CMV pp65 monoclonal antibody (Vector Laboratories Inc.) at a 1:200 dilution in PBS containing 0.5% Tween 20 (PBST) for 1 h at 37°C, washed, and then incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin G (IgG) specific for Fab (Sigma, St. Louis, MO) at 37°C for 30 min at a 1:160 dilution in PBST. During the last wash, 4,6-diamidino-2-phenylindole (Sigma) in PBS (1:6,000) was added to stain the nuclei. Finally, the excess fluid was drained, the coverslip was mounted onto the slide, and photomicrographs were taken with an Olympus IX81 PA camera with Image Pro Plus 5.1 film (Media Cybernetics Inc.).

**Total RNA extraction and reverse transcription-PCR (RT-PCR).** Total RNA from mock- or rAAV-transduced cells was extracted by using an RNasy minikit (Qiagen, Valencia, CA), according to the manufacturer’s instructions. One microgram total RNA was digested with DNase at 37°C for 1 h, and half of the RNA was converted to cDNA by using primer oligo(dT)12-18 (Invitrogen) and SuperScript II reverse transcriptase (Invitrogen). The remaining RNA was used as a negative control without reverse transcriptase. One-tenth of the cDNA was subjected to PCR amplification with HotStart Taq DNA polymerase (Invitrogen). The PCR conditions were as follows: denaturation at 95°C for 2 min, followed by 40 cycles of 57°C for 30 s, 72°C for 30 s, and 95°C for 30 s. The primers for CMV pp65 amplification were L1 (5′-CAAAGAGGCAGCAGCTCATTACACAGCAGA-3′) and L2 (5′-CCAGCTGACCTCTGAGTCTGTC-3′), and those used for β-actin were 5′-GAAGATTCCAgGCGAAGCTGACCA-3′ and 5′-GCCCTGTCACCTGGAACCCACGCG-3′ (40). The PCR products were resolved by electrophoresis on a 1.5% agarose gel containing ethidium bromide, and the bands were visualized under UV light.

To achieve better sensitivity and for quantitation, the same cDNA was also ampliﬁed by real-time quantitative PCR with a 7900 HT sequence detection system (Applied Biosystems, Foster City, CA). The primers used were: ppp65F (5′-CACGGATCCCTGGAAGCTGACCA-3′) and ppp65R (5′-CCACGGGACCTGACGATACG-3′), and the probe was 5′-6-carboxyfluorescein–6-carboxyfluorescein–6-carboxytetramethylrhodamine–3′. One microliter of each cDNA, 10 nl primer, and 10 nl probe mixture with the TaqMan universal PCR master mixture (Applied Biosystems) were ampliﬁed in a 30-μl reaction mixture. The results were analyzed with the software SDS2.3, provided with the ABI Prism 7900 HT sequence detection system (Applied Biosystems).

**Western blotting.** The rAAV- or mock-transduced cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], and 1 mM EDTA containing 1 mM phenylmethylsulfonyl fluoride and 20 mM iodoacetamide plus protease inhibi-
tors [1 μg/ml], as described below. The protein concentrations were determined with a bichinchoninic acid protein assay kit (Pierce, Rockford, IL). Equal amounts of protein were resolved by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a Hybond-ECL nitrocellulose membrane (Amersham, Piscataway, NJ). The membrane was blocked in 5% nonfat milk in PBS for 1 h at 37°C and incubated with primary antibody overnight at 4°C. After four washes with PBS, the blot was incubated with goat-anti mouse antibody conjugated to horseradish peroxidase (Sigma) at room temperature for 1 h in PBS; and then detected with an enhanced chemiluminescence (ECL) Western blotting analysis system (Amersham). Briefly, the blot was submitted to the ECL detection reagent for 1 min and was then exposed to Hyperfilm, which was developed in a Kodak M35X-OMAT processor. Epon Perfect 4490 Photo was used to scan the individual bands by densitometry.

**Pulse-chase analysis.** MRC-5 cells were infected with rAAV pp65N, rAAV pp65mII, or rAAV pp65mINLSKO at an MOI of 10 or 24 for 24 h and then rinsed twice and incubated for 1 h with methionine- and cysteine-free DMEM (Invitrogen) supplemented with 10% dialyzed fetal calf serum (Gibco-BRL) to which penicillin, streptomycin, glutamine, [L-35S]methionine, and [L-35S]cysteine were added. The cells were then washed and the medium was replaced with complete DMEM containing 5 mM cysteine and 5 mM methionine. At various time points, the cells were washed with ice-cold PBS and then were lysed in RIPA buffer. After a brief centrifugation at 14,000 × g for 10 min at 4°C, the cell lysate was pretreated with mouse IgG (Sigma) for 10 min, followed by incubation with 50 μl protein A-Sepharose fast-flow bead slurry (50%; GE Healthcare) at 4°C for 30 min. The supernatant was collected and immunoprecipitated by incubation with monoclonal anti-CMV pp65 antibody (Vector Laboratories Inc.) and then with 100 μl protein A beads for 3 h. After three washes with buffer A (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.5% Nonidet P-40, 0.1% SDS), the pellets were analyzed on a 10% SDS-polyacrylamide gel, dried, exposed to a PhosphorImager screen overnight, and analyzed with a Typhoon 9410 scanner (Amersham) and ImageQuant software.

**In vitro cytotoxicity assay.** Antigen presentation by the HLA A2-expressing MRC-5 cells was monitored by the standard 4-h [3]Cr release assay, as described previously (10). MRC-5 cells were transduced with rAAV and were used 48 h later as target cells in triplicate wells. Cells of human CD8+ T-lymphocyte (CTL) clone 3.3F4, specific for the HLA A*02-restricted CMV pp65 495 epitope, were used as effector T lymphocytes (5) to lyse the MRC-5 cells expressing the CMV epitope.

**Mouse immunization.** Eight- to 10-week-old HHDII mice transgenic for the HLA A*0201 molecule (27) were immunized by priming the mice with DNA. This was followed, 4 weeks later, with a booster with rAAV (9). In short, 50 μg of pVAXpp65mII, or control pVAXintA, in addition to 50 μg of pVAX granulocyte-macrophage colony-stimulating factor (pVAX-GM-CSF), were used for priming. This was followed, 4 weeks later, with a boost of 1 × 10^6 to 1 × 10^7 infectious unit (IU) of rAAV pp65mINLSKO, rAAV pp65mII, or rAAV LacZ, as required by the experiment. The spleenocytes were collected 20 to 30 days after the last injection and were processed either for ex vivo CTL detection or for in vitro stimulation (IVS) by using autologous blasts loaded with the CMV pp65_495 epitope.

**CTL detection in splenocytes collected from immunized HHDII mice.** The standard method of CTL detection in spleenocytes collected from immunized HHDII mice consisted of a 4-h chromium release assay after one IVS at day 6 and a second IVS at day 12 postcollection, as described previously (8). In addition, an enzyme-linked immunospot (ELISPOT) assay was performed at day 5 after stimulation by using in each well 5 × 10^5 cells that had been incubated overnight on a mixed cellular ester membrane 96-well plate that had previously been coated with anti-γ interferon (anti-IFN-γ; BD Pharmingen, Franklin Lakes, NJ). Spots were detected by incubation with biotinylated anti-IFN-γ, followed by streptavidin-peroxidase conjugate (Vector Laboratories Inc.) and then with 100 μl protein A beads for 3 h. After three washes with buffer A (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.5% Nonidet P-40, 0.1% SDS), the pellets were analyzed on a 10% SDS-polyacrylamide gel, dried, exposed to a PhosphorImager screen overnight, and analyzed with a Typhoon 9410 scanner (Amersham) and ImageQuant software.

**Microarray data accession number.** The data discussed in this report have been deposited in NCBI’s Gene Expression Omnibus (GEO) database (6), are accessible through GEO series accession number GSE14347, and may be viewed at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE14347.

**RESULTS**

**Removal of NLS from CMV pp65 protein.** The expression of the wild-type (WT) CMV pp65 protein in a transfected cell is restricted to the nucleus at early times and is detectable in the cytoplasm at late times (for a review, see reference 31). In an attempt to minimize the nuclear localization of the CMV pp65 protein and obtain primarily cytoplasmic expression, the NLS motifs were modified or removed from the CMV pp65 gene sequence. Motif A (location, aa 415 to 438) had already been mutated at the K436N site to remove the putative kinase domain II and was not further changed in the construct (Fig. 1, pp65mII) (39). The motif B (location, aa 537 to 561) was removed from the carboxy end of CMV pp65, resulting in a shorter form of the CMV pp65 (1 to 532 aa), called pp65mINLSKO (see Fig. 1).

**Cytoplasmic expression of pp65mINLSKO and quantification by Western blotting.** The truncated pp65mINLSKO DNA was inserted into the expression vector pVAX and into rAAV and tested for protein expression. Expression in transfected HeLa cells is shown in Fig. 2, for which the expression vector pVAXpp65mINLSKO (Fig. 2B) and control vector pVAXpp65mII (Fig. 2A) were used. As expected, the alteration of the NLS motifs resulted in the cytoplasmic localization of the CMV pp65 protein, whereas CMV pp65mII localized to the nucleus. However, there were still stained nuclear cells with the deleted NLS motif at 48 h posttransfection (40% nucleated cells versus 60% cytoplasmic cells). The same was true for CMV pp65 expressed in rAAV-transduced MRC-5 cells (Fig. 2E and F). Although the stain was predominantly cytoplasmic with the removal of the NLS motif, there was still some nuclear localized CMV pp65 protein. The transduction efficiency was 80 to 100% at an MOI of 10.
Western blot analysis showed that the amount of CMV pp65 was increased in cells expressing pp65mIINLSKO, and to quantify this, serial dilutions of transduced MRC-5 cell extracts were separated by PAGE, as shown in Fig. 3. MRC-5 cells were transduced with rAAV pp65mII, rAAV pp65N, and rAAV pp65mIINLSKO for 48 h and lysed; and twofold dilutions of the lysate were analyzed by Western blotting. As shown in Fig. 3, pp65mIINLSKO yielded a CMV pp65-specific band that was present at an eightfold greater amount than either the pp65N or the pp65mII band (compare 1/8 and 1/64 serial dilutions, respectively). Of note, pp65N and pp65mII yielded the same amount of CMV pp65. The actin protein was detected at up to 1/32 serial dilutions, which meant that less actin was present in the cellular lysate of the pp65mIINLSKO-transduced cells.

Time required for CMV pp65mII and CMV pp65mIINLSKO expression. The kinetics of the CMV pp65-specific mRNA and protein appearance could explain the relative abundance of pp65mIINLSKO in transduced cells. To check this, MRC-5 cells were transduced with rAAV pp65mII and rAAV pp65mIINLSKO and collected at 2, 4, 8, 16, 24, and 48 h postinfection. The input rAAV DNA was confirmed by quantitative PCR for all transductions, and no sample contained rAAV in an amount that varied by more than 10% of the amount of input rAAV, as measured by determination of the number of genome copies (gc) in each cell (data not shown). The mRNA was purified and amplified by RT-PCR for both CMV pp65- and actin-specific sequences, as described in Materials and Methods. The mRNA in the cells transduced with pp65mIINLSKO first appeared at 4 h postinfection and was present at 100 gc/µl, but only 10 gc/µl pp65mII and pp65N RNAs was present. As shown in Fig. 4A and B, the levels of pp65mII and pp65N reached 100 gc/µl 8 h later. Following the same pattern, the appearance of CMV pp65, as detected by Western blotting, first occurred at 16 h for pp65mIINLSKO and 24 h for pp65mII (Fig. 4C and D). This disparity in the time to the appearance of RNA and protein and the relative increase in the amount of CMV pp65, was observed in A293 cells and MRC-5 cells, but only the results from MRC-5 cell transduction are shown.

Stability of pp65mIINLSKO, pp65mII, and pp65N proteins. To determine if the abundance of the pp65mIINLSKO protein could be explained by an increased protein stability in the cell, pulse-chase experiments were performed. MRC-5 cells transduced for 24 h were pulsed with [35S]cysteine and [35S]methionine for 1 h; and then the radiolabeled compound was chased with complete medium and the cells were collected at 0, 1, 4, 6, 8, and 24 h. As shown in Fig. 5, the pp65mII, pp65mIINLSKO, and pp65mIINLSKO proteins exhibited no difference in stability, even though more pp65 protein was present at the start of the chase with NLSKO (Fig. 5A). Counts, obtained with ImageQuant software, were normalized to 100 at time zero and are shown in Fig. 5B. All three pp65 proteins were extremely stable in transduced MRC-5 cells, and 50% degradation was not reached by 24 h.

Comparison of pp65mIINLSKO with pp65N by RNA microarray analysis. Finally, the question of whether pp65mIINLSKO had specific effects on the gene expression of transduced cells compared with the effects of native CMV pp65 was raised. Total RNAs were collected from each MRC-5 cell transduced for 48 h with rAAV IntA, rAAV pp65N, or rAAV pp65mIINLSKO and processed individually, in triplicate. A total of nine Affymetrix slides (GeneChip Human Gene, version 1.0, ST array) were hybridized with the processed product. Statistical analysis of the genes differentially expressed between each of the samples identified 989 genes, and the clustering diagram of these genes is shown in Fig. 6.
The vector-only array was used as an rAAV reference for the other two constructs. Three distinct clusters were defined according to the downregulation of cellular genes by both pp65N (WT) and pp65mIINLSKO (KO) (176 probe sets representing 108 annotated genes in the David Bioinformatics Resources software; see the green cluster in Fig. 6), upregulation by pp65N but downregulation by pp65mIINLSKO (110 probe sets representing annotated 80 genes in the David Bioinformatics Resources software; see the blue cluster in Fig. 6), and upregulation of cellular genes by both constructs (703 probe sets representing annotated 389 genes in the David Bioinformatics Resources software; see the red cluster in Fig. 6). Table 1 shows the gene families affected in each cluster, as determined by the use of the David Bioinformatics Resources software. The enrichment score is a measure of the difference in P values defining differences within the cluster of gene families induced during transduction by native CMV pp65 and the NLS mutant. The green cluster (see the green bar in Fig. 6) indicated that 108 genes were downregulated, and within this group, 62 genes were involved in cellular metabolism and 34 genes were involved with nuclear proteins. In addition, the ZNF zinc finger family of proteins, the retinoblastoma binding protein 8 (RBBP8), and the E2f transcription factor were among the genes downregulated by both CMV pp65 and the NLSKO mutant. A blue cluster of genes that were upregulated by pp65N and downregulated by pp65NLSKO involved 28 genes of the mitotic cell cycle; 8 genes involved with chromosome segregation; 5 genes involved in the cell cycle check point; and...
others arbitrarily assigned to functions such as intracellular localization (17 genes), transport (11 genes), and ATP binding (15 genes). Here, the highest enrichment score was seen (13.65), confirming a role of CMV pp65 in cell cycle regulation, nuclear localization, and transport (14, 22, 30). Among the genes involved in this cluster were PLK-1, KIFxx (kinesin family), CDCA2 (cell division cycle associated 2), CCNB2 (cyclin b2), TOP2A (topoisomerase [DNA] II), and ABCA13/5 in the ATP-binding cassette transporter family.

The red cluster was the largest and contained 389 genes upregulated by both pp65N and pp65mIINLSKO. The enrichment score was 14.83. These genes comprised the glycoprotein families (143 genes); signaling pathways (102 genes); and also immune pathways, such as the complement pathway (5 genes), the humoral immune response (9 genes), the immunoglobulin domain (21 genes), and the response to stimulus (27 genes) (Fig. 6; Table 1). The immune response genes upregulated in this cluster included several which exhibited immunoglobulin-like structures, such as HLA-DQA2, CD1C, CD86, CD3e, CEACAM1, IGSF9B; other proteins involved in innate immunity; and the complement pathway proteins (C1QB, C2, C1QC, CR2, C4BPA).

**Peptide presentation as measured by a ^51Cr release assay in vitro.** Since pp65mIINLSKO was more abundant in the cytoplasm, the next question was whether this immune response could be shown by increased antigen processing and presentation on the HLA of target cells. The human CD8+ CTL clone (clone 3.3F4), specific for pp65 495 peptide presentation on the HLA A*0201 molecule (5), was used to determine the surface expression of the pp65 495 peptide. MRC-5 cells expressing HLA A*0201 were transduced with rAAV pp65intA, rAAV pp65N, rAAV pp65mII, and rAAV pp65mIINLSKO. The cells obtained at effector cell/target cell ratios of 1:1, 3:1, and 10:1 are shown in Fig. 7. Statistical significance, as determined by analysis of variance, was reached for all groups by comparison of the results with those for the control vector, rAAV IntA. The level of the significant difference for positive control MRC-5 cells loaded with peptide pp65 495 was the same as that

### TABLE 1. Description of cluster families in pp65 WT versus NLSKO

| Gene family affected (n = 108 genes) | WT and KO | No. of genes | Enrichment score |
|-------------------------------------|------------|--------------|-----------------|
| Cellular metabolism                 | 62         | 3.34         |
| Nucleus                             | 34         | 1.65         |
| Zinc finger C2H2                     | 7          | 1.65         |
| Regulation of transcription         | 17         | 1.65         |
| Apoptosis                           | 8          | 0.63         |
| Protein kinase                      | 5          | 0.77         |
| Cell cycle                          | 4          | 0.38         |

| Gene family affected (n = 80 genes) | WT and KO | No. of genes | Enrichment score |
|-------------------------------------|------------|--------------|-----------------|
| Mitotic cell cycle                  | 28         | 13.65        |
| Cell cycle                          | 28         | 13.65        |
| Chromosome segregation              | 8          | 5.84         |
| Cell cycle checkpoint               | 5          | 4.87         |
| Microtubule cytoskeleton            | 7          | 3.43         |
| Localization                        | 17         | 3.43         |
| Transport                           | 11         | 3.43         |
| ATP binding                         | 15         | 3.57         |
| Chromosome                          | 11         | 2.12         |
| Nuclear protein                     | 33         | 2.37         |
| Cellular process                    | 57         | 2.37         |

| Gene family affected (n = 389 genes) | WT and KO | No. of genes | Enrichment score |
|-------------------------------------|------------|--------------|-----------------|
| Glycoprotein                        | 143        | 12.32        |
| Signal                              | 102        | 12.32        |
| Secreted                            | 58         | 5.61         |
| Signal transduction                 | 76         | 4.43         |
| Plasma membrane                     | 97         | 4.38         |
| Cell differentiation                | 52         | 4.72         |
| Complement pathway                  | 5          | 2.47         |
| Humoral immune response             | 5          | 2.47         |
| Immunoglobulin domain               | 21         | 2.18         |
| Response to stimulus                | 27         | 2.72         |
| Immune response                     | 26         | 2.47         |
| Immunoglobulin-like fold            | 25         | 2.18         |
| Adaptive immune response            | 9          | 2.47         |
| Innate immune response              | 6          | 2.47         |
| Regulation of T-cell proliferation  | 5          | 1.38         |

*A* WT is pp65N; KO is pp65mIINLSKO. ↓, downregulation; ↑, upregulation.

*b* Number of genes and gene families determined with David Bioinformatics Resources software.

*c* The enrichment score is the geometric mean of each gene’s *P* value in the cluster of the gene family.
as that for the MRC-5 cells transduced with pp65mIIINLSKO (P < 0.001); the level of the significant difference for MRC-5 cells transduced with pp65N and pp65mII was lower (P < 0.05).

Prime-boost vaccination in HHDII mice. pp65mIIINLSKO was further evaluated in vivo in HHDII mice transgenic for HLA A*0201. The vaccination consisted of a prime-boost strategy, as described for the pp65mII constructs (9). In this study, the mice were primed with DNA consisting of 50 μg pVAXpp65mIIINLSKO or 50 μg pVAXpp65mII to which 50 μg pVAXGM-CSF was added as the adjuvant. Four weeks later, they were boosted with serial dilutions ranging from 1 × 10^1 to 1 × 10^3 IU rAAV expressing the same CMV pp65 mutant genes. Twenty days later, the splenocytes were tested by the chromium release assay after 6 days (IVS I) or 12 days (IVS II) of stimulation in culture with autologous blast cells loaded with the pp65mIIINLSKO-specific peptide (Fig. 8). The response to pp65mIIINLSKO reached 100% 51Cr release with a low vaccine dose of rAAV (10 to 100 IU) 6 days after IVS (Fig. 8, right panel, IVS I) and was down to 80% at day 12 after IVS (Fig. 8, right panel, IVSII). The CTL immune response was fairly similar whether pp65mIIINLSKO or pp65mII was used (Fig. 8, right panel, IVS I) and was down to 80% at day 12 after IVS (Fig. 8, right panel, IVSII). The CTL immune response was fairly similar whether pp65mIIINLSKO or pp65mII was used (Fig. 8, right panel, IVS I) and was down to 80% at day 12 after IVS (Fig. 8, right panel, IVSII).

In addition to the 51Cr release assay, CTL responses were also detected by the ELISPOT assay for the IFN-γ response after in vitro stimulation and by an intracellular cytokine assay to measure the number of cells releasing IFN-γ in CD8+ cells by fluorescence-activated cell sorter analysis. This is shown in Fig. 9 for mice (two mice in each group) immunized with DNA followed by a booster of 10 IU rAAV pp65mII or rAAV pp65mIIINLSKO. In summary, it was confirmed that the two forms of CMV pp65 have similar immunogenicities.

**DISCUSSION**

Since CMV pp65 is a likely component of an eventual anti-CMV vaccine, it might become necessary to produce a protein with intact immunologic domains but little remaining biologic function. Little is known about the biologic properties of CMV pp65, but once it localizes to the nucleus, it does bind to PLK-1 and thus could have effects on the cell cycle (11).

We have previously created a kinaseless CMV pp65 with the K436N mutation (pp65mII) for use in a vaccine, and the resultant pp65mII appeared to have a nuclear localization and immunogenicity similar to those of native CMV pp65 (8). A similarly designed CMV DNA vaccine is currently in clinical trials (38). On a theoretical basis, a kinaseless CMV pp65 from which NLS is deleted would likely further improve the safety of an anti-CMV vaccine. Thus, this work was motivated by the need to determine if removal of the nuclear localizing properties of CMV pp65 influenced either its antigenicity or its immunogenicity.

Because the C-terminal portion of the protein is rich in cytotoxic T-cell epitope sequences (7, 15), we were able to alter only the K436/NLS-B region in order to preserve these potential epitopes. Yet, removal of NLS-B from pp65mII in either a DNA expression vector or rAAV encoding pp65mIIINLSKO was more efficiently lysed in vitro by CTLs than the unmodified CMV pp65. More importantly, the use of these expression vectors in a vaccine model showed that the ability of pp65mIIINLSKO to induce cellular immunity was as good as that of the native protein.

An unexpected effect of the deletion of NLS-B was the effect on transcription and expression of CMV pp65. RNA specific for CMV pp65 appeared earlier in the deletion vectors than in control CMV pp65, and the protein was detected approximately 8 h before control CMV pp65 was detected. These effects were seen with multiple cell types. The reason for the
enhancement of CMV pp65 expression after NLS-B deletion is not known, but on the basis of the concomitant enhancement of CMV pp65-specific RNA levels, it is possible that nuclear localization of the protein affects RNA transcription by a feedback loop, which has been shown for other systems (12, 21). This deserves further study with CMV pp65 to better understand the role of this abundant structural protein in CMV infection.

There is no explanation for why the level of expression of the CMV pp65 NLS mutant was greater than that of native CMV pp65. We explored the proteins for regions which could alter protein degradation, such as motifs rich in amino acids proline, glutamic acid, serine, and threonine (PEST), which are known to be associated with reduced protein half-lives (29). A PEST motif, KAESTVAPEDETDDEDNDEL, exists at aa 457 to 477 and is unlatched in both the native and the mutant CMV pp65 forms. Pulse-chase experiments with the 35S-labeled protein confirmed that there were no differences in the stabilities of the CMV pp65 form with the NLS deletion and the control CMV pp65. There appear to be no differences in CMV pp65 degradation that would explain the variation in the heightened protein levels associated with the NLSKO mutant.

Since pp65N was sequestered in the nucleus and pp65mIINLSKO was mainly found in the cytoplasm, we investigated the potential effects of these two proteins on cellular RNA expression. An Affymetrix gene microarray was used to hybridize cDNA from cells transduced with either native CMV pp65 or the NLSKO mutant for 48 h.

A potentially important observation is that the genes upregulated by WT pp65 and downregulated by NLSKO included genes of the cell cycle clusters, including genes such as PLK-1, UBE2C (ubiquitin-conjugating enzyme e2C), CCNB2, and KIF4x. Some of these genes have already been associated either with viral infection events or with binding to CMV pp65 (11, 31). One cannot know at this time if this change would translate into a safer vaccine, but it seems likely that the downregulation of families of mRNA related to the cell cycle and mitotic events could be protective. This will require further investigation, but clearly, pp65mIINLSKO differs from native CMV pp65 in this regard and could support application to improved anti-CMV vaccine design.

The upregulation of cellular genes by both the pp65 WT and the NLSKO genes also deserves attention, especially because of its number: 389 human genes were identified by the mRNA microarray to be upregulated. This cluster is characterized by numerous families of genes in the signaling, secretion pathway, and glycoprotein gene families. The impact of pp65 on inflammatory gene expression has been shown by others (1, 3). By comparing by DNA array analysis the effect of a pp65-deficient mutant virus with that of a virus with WT pp65 on fibroblast cells, those investigators showed that there was a much stronger induction of many IFN responses and proinflammatory chemokine RNAs in the absence of pp65. It is possible that these alterations in gene expression explain the immunodominance of CMV pp65 (18, 37) but also suggest a much larger role of the nuclear localization signal of pp65 than was previously anticipated.

In summary, the removal of the NLSs from CMV pp65 can be accomplished without significant alteration of the immunogenicity of the protein. In the absence of the nuclear localization of CMV pp65, there is modification of cellular events which appear to influence the appearance mRNA and the amount of protein expressed. However, the ability of the protein to be processed and antigenically presented to the class I system is actually enhanced by these changes, and the immunogenicity of the mutant CMV pp65 in a vaccine model is comparable to that of WT CMV pp65. These data suggest that the NLSKO mutation of CMV pp65 be evaluated in future candidate CMV vaccination strategies.

ACKNOWLEDGMENT

This study was supported in part by U.S. Public Health Service grant AI58148 (to J.A.Z.) from the National Institutes of Health. We are grateful to Don J. Diamond for providing the 3.3F4 cells and to the Functional Genome Core facility at the Beckman Research Institute of the City of Hope, in particular, Ning Ye for her excellent technical expertise in microarray technology.

REFERENCES
1. Abate, D. A., S. Watanabe, and E. S. Mocarski. 2004. Major human cytomegalovirus structural protein pp65 (ppUL83) prevents interferon response factor 3 activation in the interferon response. J. Virol. 78:10995–11006.
2. Berencsi, K., Z. Gyalai, E. Gonczol, S. Pincus, W. J. Cox, S. Michelson, L. Karli, C. Meric, M. Cadot, J. Zahradnik, S. Starr, and S. Plotkin. 2001. A canarypox vector-expressing cytomegalovirus (CMV) phosphoprotein 65 induces long-lasting cytotoxic T cell responses in human CMV-seronegative subjects. J. Infect. Dis. 183:1171–1179.
3. Browne, E. P., and T. Shenk. 2003. Human cytomegalovirus UL83-coded pp65 virion protein inhibits antiviral gene expression in infected cells. Proc. Natl. Acad. Sci. USA 100:11439–11444.
