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## Nuclear trafficking of the Human Cytomegalovirus pp71 (ppUL82) tegument protein

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### Abstract

The human cytomegalovirus tegument protein pp71 localizes to the nucleus immediately upon infection, and functions to initiate viral gene expression. Analysis of a series of random insertion mutations revealed that sequences within the mid-region (MR) of pp71 are important for localization to the nucleus. Fusion of MR sequences with eGFP revealed that amino acids 94 to 300 were sufficient to target proteins to the nucleus. Random substitution mutagenesis within this domain resulted in two double-substitution mutants, pp71P203T/T223M and pp71T228M/L275Q, with a predominantly cytoplasmic localization. Disruption of nuclear targeting resulted in relocation of the fusion proteins to a distinct perinuclear region. Using tandem mass spectrometry, we determined that threonine 223 can be phosphorylated. Mutation of this residue to a phosphomimetic amino acid resulted in abrogation of nuclear targeting. These results strongly suggest that the intracellular trafficking of pp71 is regulated by phosphorylation.

### Keywords

HCMV; nuclear trafficking; phosphorylation; tegument; mass spectrometry

### Introduction

Human cytomegalovirus (HCMV), a member of the beta subgroup of herpesviruses, is a ubiquitous human pathogen, which infects 40 to 60% of the human population in developed countries (Mocarski, 2007). HCMV infection is usually asymptomatic and maintains a latent state for the lifetime of the individual. However, primary infection or reactivation of a latent infection can cause severe morbidity and mortality in immunocompromised individuals such as AIDS patients and organ transplant patients (Griffiths, 2006; Snyderman, 2006). The HCMV genome is a linear double-stranded DNA of approximately 235kb, which is enclosed in an icosahedral capsid surrounded by a tegument layer, and finally enveloped in a lipid bilayer embedded with a number of virus-encoded glycoproteins (Mocarski, 2007). HCMV gene expression is separated into three temporal phases termed immediate early (IE), early and late (Fortunato and Spector, 1999; Mocarski, 2007). Expression of immediately early genes begins

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immediately after virus entry, and encodes critical viral proteins, which regulate viral early and late gene expression (Anders, Kerry, and Pari, 2007; Mocarski, 2007).

The tegument layer of HCMV consists of at least 25 viral proteins, and the majority of the tegument proteins are phosphoproteins (Baldick and Shenk, 1996; Mocarski, 2007; Varnum et al., 2004). As might be predicted, tegument proteins can play critical roles in virus assembly and particle stability (AuCoin et al., 2006; Britt et al., 2004; Jones and Lee, 2004; Lorz et al., 2006; Munger, Yu, and Shenk, 2006; Silva et al., 2003). In addition, a number of the HCMV tegument proteins have key functions in the initiation of viral infection. For example, the tegument protein pp65 is the most abundant protein made during replication (Gibson, 1983) and is thought to be involved in immune evasion by modulating interferon signaling (Abate, Watanabe, and Mocarski, 2004; Browne and Shenk, 2003). The 559 amino acid pp71 protein, which is encoded by the UL82 gene, was the first HCMV tegument protein to be characterized as a transactivator, activating the major immediate early promoter and initiating viral gene expression (Bresnahan and Shenk, 2000; Chau, Vanson, and Kerry, 1999; Liu and Stinski, 1992). More recently, a number of other tegument proteins including pIRS1/pTRS1 (Iskenderian et al., 1996; Kerry et al., 1996; Romanowski and Shenk, 1997), pUL26 (Stamminger et al., 2002), ppUL35 (Schierling et al., 2004) and ppUL69 (Winkler, Rice, and Stamminger, 1994) have been added to the list of tegument transactivators of viral gene expression. The pIRS1/pTRS1 proteins have also been shown to assist in the evasion of the interferon response by relocalizing PKR to the nucleus (Hakki et al., 2006).

Upon fusion of the viral envelope with the cell membrane, the viral capsid rapidly localizes to the nucleus (Ogawa-Goto et al., 2003). In addition, some viral tegument proteins including pp71 are targeted to the nucleus (Hensel et al., 1995; Hensel et al., 1996), consistent with their role in initiating viral gene expression. The pp71 protein can also act with the immediate early gene products to activate early promoters of HCMV (Chau, Vanson, and Kerry, 1999), and interacts with the cellular protein hDaxx to facilitate viral transcription at nuclear domains 10 (ND10) (Cantrell and Bresnahan, 2005; Cantrell and Bresnahan, 2006; Hofmann, Sindre, and Stamminger, 2002; Ishov, Vladimirova, and Maul, 2002; Preston and Nicholl, 2006; Saffert and Kalejta, 2006). Furthermore, pp71 has been shown to enhance the infectivity of viral DNA and thereby accelerate the process of virus infection (Baldick et al., 1997). In addition, pp71 can bind to the retinoblastoma protein (Rb), resulting in its degradation and enhanced progression through the cell cycle (Kalejta, Bechtel, and Shenk, 2003; Kalejta and Shenk, 2003a; Kalejta and Shenk, 2003b), although this interaction is not important for the transcriptional activity of pp71 (Cantrell and Bresnahan, 2005).

Recent studies show that the nuclear import of pp71 is inhibited when assessed in nonpermissive cell lines such as undifferentiated THP-1 cells, suggesting that the nuclear localization of this protein plays a key role in the initiation of viral infection and that nuclear import is a regulated process (Saffert and Kalejta, 2007). However, little is known about the mechanism by which pp71 localizes to the nucleus. In addition, at the late phase of viral infection, pp71 relocates to the cytoplasm in dense perinuclear structures (Hensel et al., 1996), presumably sites of virion tegumentation. These data suggest that pp71 contains multiple subcellular targeting sequences. In the current study, we investigated the regions of pp71 responsible for nuclear accumulation of this protein. Our studies reveal that sequences within the mid region (MR) of pp71 are responsible for nuclear localization, even though this region contains no sequences matching known NLS motifs (Christophe, Christophe-Hobertus, and Pichon, 2000; Dingwall and Laskey, 1991; Macara, 2001). The disruption of sequences required for nuclear targeting within the MR domain results in protein localization to a distinct perinuclear region that may correspond to tegumentation sites. Further, our studies suggest that the phosphorylation status of the threonine residue at amino acid position 223 plays a critical role in the regulation of pp71 subcellular trafficking.

## Results

### Identification of pp71 sequences involved in nuclear trafficking

Based on the fact that pp71 localizes predominantly to the nucleus upon viral infection and in transfected cells (Hensel et al., 1996; Hofmann, Sindre, and Stamminger, 2002; Marshall et al., 2002), we reasoned that pp71 contained an intrinsic nuclear localization signal, allowing it to be actively imported into the nucleus. Initial sequence analysis of the pp71 559 amino acid open reading frame revealed a novel basic region in the C-terminus from amino acids 362 to 383 that had some similarity to nuclear localization signals (Christophe, Christophe-Hobertus, and Pichon, 2000; Dingwall and Laskey, 1991; Macara, 2001). However, preliminary experiments showed that this region is not involved in nuclear targeting (data not shown). Other than this basic region, pp71 contains no sequences with homology to known nuclear localization signals. Thus, in order to map the protein domain(s) responsible for nuclear localization of pp71, random insertion mutagenesis was performed. This approach has been used successfully to map functional domains within the major immediate early proteins (Stenberg et al., 1990) and was selected to minimize the potential impact of the mutations on protein conformation. The mutants were generated by randomly inserting a 12bp oligonucleotide linker into the pp71 open reading frame, resulting in four amino acid insertions within the amino-terminal region of pp71 at amino acids 66, 154, 191, 215, 217, and 284. The pp71 mutants were then transfected into HFF cells along with a construct expressing the HCMV IE86 protein as a marker for nuclear staining, and examined by immunofluorescence and confocal microscopy. The IE86 protein localizes exclusively to the nucleus, co-localizing with the wild type pp71 protein which exhibits predominantly nuclear staining (Figure 1A & B). Proteins with insertions at amino acids 66, 154, and 191 retained a predominantly nuclear localization, similar to the wild type pp71 protein (Figure 1A & B). However insertions at amino acids 215, 217, or 284 resulted in a diffuse cytoplasmic staining (Figure 1A & B). These results indicate that sequences around amino acids 215–284, that we term the mid-region (MR), are important for the nuclear targeting of pp71.

As pp71 activates the major immediate early promoter (MIEP) and initiates viral infection (Baldick et al., 1997; Liu and Stinski, 1992), we investigated the transcriptional activity of these pp71 mutants using a promoter-reporter assay. HFF cells were co-transfected with a CAT reporter plasmid containing the immediately early promoter region and expression plasmids containing either full-length pp71 or the random insertion mutants. Cells were assessed for CAT activity 48 hours after transfection. This study showed that the insertion at aa 66 caused an approximately 50% loss of pp71 activity (Figure 1C). In contrast, the insertions at aa's 154 and 191 resulted in a slight but not significant ( $p>0.05$ ) reduction in activity compared with the wild type protein. However insertion at aa's 215, 217, or 284 completely abrogated the activity of pp71 ( $p<0.001$ ). Similar results were obtained when pp71 enhancement of IE protein activation of the HCMV UL54 DNA polymerase promoter was assessed (data not shown). These analyses provide a functional assessment of pp71 nuclear targeting and indicate that the insertions at aa's 215, 217, and 284 disrupt nuclear targeting to the extent that transactivation is abolished. One possible explanation for the reduced activity of these pp71 mutants is that the mutations affect protein stability. To assess this, we subcloned the pp71 mutants into the pSI vector which expresses proteins under the control of the SV40 promoter. This promoter is less responsive to auto-activation by pp71 than the MIEP (data not shown) and therefore provides a more accurate measure of steady-state pp71 protein levels. Western blot analysis demonstrated that the pp71 mutants were expressed at similar levels to the wild type protein (Figure 1D). This analysis confirms that the decrease in activity that we observe is most likely the result of the change in subcellular localization, and is not related to major effects on the stability of the protein.

Analysis of the random insertion mutants suggested that a relatively large domain was involved in the nuclear targeting of pp71. We therefore decided to utilize a random site-directed domain mutagenesis approach in order to further map the sequences within the MR responsible for nuclear targeting of pp71 as described in the Materials and Methods. This strategy was used to generate pp71 mutants between aa 200–300 in the pp71 open reading frame cloned into the pSI vector. Using the random mutagenesis approach, we identified five mutants that resulted in amino acid changes: three single substitution mutants, M238I, H269Q, I287S, and two double substitution mutants, P206T/T223M, T228M/L275Q. Localization of these mutants was assessed by immunofluorescence and confocal microscopy (Figure 2A). The three single substitution mutants did not affect the nuclear targeting of pp71. In contrast, the two double substitution mutants exhibited a reduction in the nuclear accumulation of pp71, displaying a diffuse cytoplasmic fluorescence with some punctate staining, similar to that of the pp71 random insertion mutants. We then investigated the transcriptional activity of these pp71 mutants as a functional assessment of pp71 nuclear targeting using a construct containing the MIEP upstream of the luciferase reporter gene which provides for increased sensitivity over the CAT reporter. As expected, the three single substitution mutants did not significantly affect pp71 transactivation, whereas the two double substitution mutants significantly reduced the activity of pp71 ( $p < 0.05$ , Figure 2B). Again, similar results were obtained when activation of the UL54 promoter by the pp71 mutants was assessed (data not shown). These data demonstrate that nuclear localization is important for the activity of pp71. In addition, these results clearly indicate that the sequences required for nuclear localization of pp71 do not correspond to a conventional nuclear localization signal. One possibility is that interactions with a cellular protein via the mid-region of pp71 may be required for nuclear transport. Preliminary studies showed that interaction with the Daxx protein was unaffected by these mutations (data not shown), suggesting that this protein is not involved in the nuclear transport of pp71.

To determine if the mid region (MR) is sufficient for nuclear targeting, a region containing aa's 188–300 was cloned in frame with the amino-terminus of eGFP (188MR-eGFP) and the localization of the fusion protein examined (Figure 3). As expected, eGFP alone can be detected throughout the cell (Figure 3A). Surprisingly, the 188MR-eGFP fusion protein did not localize in the nucleus, but was distributed in a distinct punctate perinuclear pattern (Figure 3B) suggesting that while sequences within the MR are important for nuclear localization, these sequences are not sufficient to independently direct nuclear targeting. To assess if sequences adjacent to the MR domain participated in the nuclear localization of pp71, we generated additional eGFP fusion protein constructs containing sequences from aa 94–300 (94MR-eGFP) and 62–300 (62MR-eGFP). Confocal microscopy analysis revealed that amino acids 94 to 300 of pp71 were sufficient to mediate nuclear targeting (Figure 3C), suggesting that sequences adjacent to the MR influence recognition of the pp71 nuclear localization signal. To identify the location of the perinuclear fluorescence observed with the 188MR-eGFP fusion protein, HFF cells were transfected with this construct and co-stained with a series of subcellular markers including a panel of Golgi-related antibodies. These studies showed that the perinuclear fluorescence associated with 188MR-GFP colocalized with syntaxin 6 (data not shown) and syntaxin 11 (Figure 4A), SNAREs that are localized to the TGN-late endosomal compartment (Teng, Wang, and Tang, 2001). We also observed partial co-localization with other late TGN markers, including GS27 and Vti 1a (data not shown). This staining pattern is reminiscent of that observed for pp71 at the late times of viral infection (Figure 3E & 7A (Hensel et al., 1996)) and may correspond to the site of cytoplasmic tegumentation (Mettenleiter, 2004).

### Phosphorylation regulates pp71 trafficking

Using the random mutagenesis strategy, we identified two threonine residues that play a role in the subcellular trafficking of pp71 (Figure 2). Based on these findings, we speculated that

phosphorylation events may be involved in regulating the subcellular localization of pp71. To address this hypothesis, we purified pp71 protein from transfected HeLa cells using S-tag affinity chromatography. The resultant protein was subjected to phosphopeptide mapping using LC-MS/MS (Durkin et al., 2006). Using this approach, we detected phosphorylation of nine residues within the pp71 open reading frame (Figure 5A). Within the MR, a single residue, the threonine residue at amino acid position 223 (T223) was found to be phosphorylated. A rapid semi-quantitative assessment of the total tryptic peptides derived from the wild type pp71 protein analyzed by mass spectrometry revealed that 25% of the peptides were phosphorylated at position T223, indicating that the majority of pp71 in transfected cells was not phosphorylated at this site. To assess the effect of T223 phosphorylation on pp71 trafficking, we mutated this residue to either alanine in order to block phosphorylation, or an aspartic acid to mimic phosphorylation within the context of the full-length pp71 protein. Assessment of the T223 mutants in transfection assays showed that mutation of amino acid 223 to an alanine (T223A) resulted in a reduction of the activity of pp71 (Figure 5B). However, mutation of this residue to aspartic acid (T223D) reduced transcriptional enhancement to a level not significantly different to the promoter activity observed in the absence of pp71 ( $p = 0.67$ ). We next assessed the effect of the T223 mutations on the localization of pp71 (Figure 5C). The T223A mutant displayed an identical localization to the wild type pp71 protein, colocalizing with ND10 domains within the nucleus. In contrast, substitution of T223 with the phosphomimetic amino acid abrogated nuclear import of pp71, resulting in a punctate cytoplasmic localization. Analysis of the cytoplasmic localization of the pp71T223D protein revealed colocalization with syntaxin 11 (Figure 4B), consistent with the results obtained with the 188MR-GFP fusion protein (Figure 4A).

Analysis of the transfected cells by western blot using an antibody to the HA-tag showed lower levels of protein in cells transfected with pp71 containing either mutation of threonine 223 (Figure 5D). The decrease in protein levels could arise from the reduction in activity of the mutants, resulting in diminished auto-regulation of the SV40 promoter construct used to express pp71. However, the SV40 promoter displays minimal (approximately 2-fold) activation by pp71 which would not account for the marked decrease in protein levels observed. Alternatively, this could indicate a decrease in the stability of the protein due to a conformational change resulting from the mutation of this residue. To assess this, we inserted the SV40 nuclear localization signal (NLS) into the amino terminus of both the wild type and pp71T223D open reading frames in order to independently target the protein to the nucleus. This analysis showed that localization of the pp71T223D mutant to the nucleus via the SV40 NLS did not result in restoration of pp71 activity (Figure 6A). This finding is consistent with the phosphomimetic mutation causing a conformational change in pp71. Surprisingly, insertion of the SV40 NLS into the wild type protein significantly enhanced the activity of pp71 (Figure 6A). Thus, it is possible that the addition of the SV40 NLS enhances nuclear import of pp71, or blocks nuclear export of this protein. To confirm the nuclear localization of the pp71 proteins containing the SV40 NLS, we performed immunofluorescence studies (Figure 6B). This analysis showed that both the wild type pp71 and the pp71 T223D mutant proteins containing the SV40 NLS localized to the nucleus of transfected cells. However, both proteins displayed only partial colocalization with ND10 domains, suggesting that the SV40 NLS influences the subnuclear localization of pp71.

The trafficking of the pp71 tegument protein to the TGN-endosomal compartment when nuclear import is inhibited may be related to the finding that pp71 localizes to a punctate perinuclear region at the late stages of virus infection (Hensel et al., 1996), thought to be sites of tegumentation. To investigate this further we performed transfection/superinfection experiments to assess the localization of these pp71 mutants at the late stages of virus infection when virion morphogenesis is occurring. In our initial experiments, we confirmed that during infection, the transfected pp71 protein colocalized with endogenous pp71 (data not shown),

and that endogenous pp71 localizes adjacent to gB (Figure 7A), a marker for virus assembly compartments (Homman-Loudiyi et al., 2003; Jarvis et al., 2004; Sanchez et al., 2000). Analysis of the co-transfected cells showed that both the wild type pp71 and the pp71T223D protein localized to the cytoplasm with some evidence of colocalization with gB (Figure 7B & D), suggesting that cytoplasmic pp71 localization partially overlaps the virus assembly compartments (Homman-Loudiyi et al., 2003; Krzyzaniak, Mach, and Britt, 2007; Sanchez et al., 2000). Surprisingly, the pp71 protein containing the T223A substitution that is unable to be phosphorylated displayed a similar pattern of localization (Figure 7C), indicating that this mutant protein is capable of cytoplasmic trafficking at the late stages of virus infection. These studies indicate that viral and/or cellular proteins that may be modified by infection are important for pp71 nuclear export at the late stages of infection and suggest that nuclear import and export are controlled via different mechanisms.

## Discussion

Transport of proteins into the nucleus occurs via two primary mechanisms, either via passive diffusion or by active transport through nuclear pore complexes (Macara, 2001). Based on its molecular weight, pp71 most likely contains a nuclear targeting signal allowing it to be actively transported into nucleus. In the current study, we determined that mutations within the mid-region (MR) of pp71, consisting of sequences from aa's 200–300 disrupted nuclear accumulation of pp71, suggesting that the MR domain is important for the nuclear import of this protein. This finding is consistent with studies using a transposon mutagenesis approach demonstrating that insertions within the central domain of pp71 disrupted nuclear trafficking (Willey, S. J. & Preston, C. M., personal communication). We further show that nuclear localization of pp71 is necessary, although not sufficient, for its transcriptional activities, consistent with the mechanism of pp71 transactivation via Daxx degradation (Cantrell and Bresnahan, 2005; Cantrell and Bresnahan, 2006; Hwang and Kalejta, 2007; Saffert and Kalejta, 2006; Saffert and Kalejta, 2007). It should be noted that we observed some variability in the assessment of pp71 activity that reflects the nature of these transient transfection studies, although the mutants that displayed disruption of pp71 nuclear trafficking all exhibited significantly lower activity than the wild type protein. In addition, our studies show that the threonine residue at position 223 within the MR plays a critical role in regulating the subcellular trafficking of pp71. Mass spectrometric analysis demonstrated that T223 can be phosphorylated, and our data strongly suggests that phosphorylation at this site influences nuclear import. Other studies have reported the effects of mutations within the MR domain of pp71, as this region contains one of two Daxx interaction domains, as well as the Rb-interaction motif (Cantrell and Bresnahan, 2005; Hofmann, Sindre, and Stamminger, 2002; Kalejta, Bechtel, and Shenk, 2003). These analyses demonstrated that mutation of aa 219 from cysteine to glycine that disrupts the Rb-interaction motif (Kalejta, Bechtel, and Shenk, 2003) had no effect on virus replication, suggesting that nuclear import was unaffected by this mutation (Cantrell and Bresnahan, 2005). Likewise, deletion of the Daxx interaction domain from aa 206–213, while disrupting pp71 transcriptional activation, had no effect on nuclear import (Cantrell and Bresnahan, 2005; Hofmann, Sindre, and Stamminger, 2002). Taken together, these studies support the notion that the effect of the pp71T223D mutation on nuclear import is specific to this residue and not a generic effect of mutations within the MR domain.

Analysis of the mid region fusion proteins demonstrated that sequences from aa 188–300 were unable to independently target proteins to the nucleus. These findings suggest that while the mid region of pp71 is necessary for the nuclear trafficking of pp71, it is not sufficient to target proteins to the nucleus. Further, these findings suggest that the higher-ordered structure of the pp71 protein or amino acids outside this essential region also play a role in subcellular targeting. Together, our data suggest that the pp71 mid region contains an unconventional, non-sequential, long nuclear targeting sequence that may be conformation sensitive. However, the

mechanism by which the T223 phosphomimetic mutation affects nuclear import is currently unclear. Our current model is that phosphorylation of T223 alters the conformation of pp71, such that recognition of the nuclear import sequence is blocked. This effect is likely to be specific as other mutations and deletions within this region had no effect on pp71 nuclear import (Cantrell and Bresnahan, 2005; Hofmann, Sindre, and Stamminger, 2002). An alternative possibility is that phosphorylation of this residue affects the interaction with a cellular protein required for nuclear transport. Our preliminary experiments suggest that Daxx- interaction is not sufficient for nuclear import (data not shown). The identification of additional cellular pp71-interacting proteins would help to address this question.

A recent study has shown that nuclear import of pp71 can be inhibited upon infection of *in vitro* models of quiescent or "latent-like" infections (Saffert and Kalejta, 2007). Specifically, this study showed that nuclear import of pp71 was blocked in non-permissive THP-1 cells, and that this block contributes, at least in part, to the defect in virus replication. In contrast, pp71 nuclear import was unaffected when these cells are induced to their permissive states, suggesting that the block in pp71 nuclear import is a cell specific effect (Saffert and Kalejta, 2007). However, the precise mechanism by which pp71 nuclear import is regulated in these cell types is currently unclear. Our findings suggest that the phosphorylation of the threonine residue at position 223 may be important in this phenomenon. Specifically, our studies revealed that T223 can be phosphorylated by a cellular kinase, consistent with a cell specific effect. Analysis of the sequence surrounding T223 using Scansite software did not result in any matches to known cellular kinase recognition motifs, even when the search was conducted at a low stringency (Obenauer, Cantley, and Yaffe, 2003). Thus, it is unclear at this point which cellular kinase is involved in phosphorylating pp71 at this site. However, our findings are consistent with other studies that show both cellular and viral kinases associate with HCMV tegument proteins, and phosphorylation events modulate their function and/or localization. For example, the HCMV pp65 protein interacts with a cellular polo-like kinase, although the phosphorylation targets of this complex have yet to be defined (Gallina et al., 1999). The HCMV UL97 kinase may also be a component of the virion tegument (van Zeijl et al., 1997) and UL97 kinase activity is important for pp65 subcellular localization and virion morphogenesis (Prichard et al., 2005). Interestingly, the cellular phosphatase PP2A is also a component of HCMV particles (Michelson et al., 1996), suggesting the possibility of finely-tuned regulation of virion protein phosphorylation. Other studies demonstrated that of three differentially phosphorylated forms of the HCMV UL69 tegument protein, only one form is actually incorporated into the tegument of virion particles (Winkler and Stamminger, 1996). In addition, cyclin-dependent kinases can modulate the phosphorylation state and localization of UL69 and pp65 (Sanchez et al., 2007; Sanchez and Spector, 2006). Together, these studies demonstrate that differential phosphorylation can influence both subcellular localization and virion incorporation of the HCMV tegument proteins. In addition, HCMV infection can influence both the localization and activity of a number of cellular kinases (Hakki et al., 2006; Kudchodkar et al., 2004; Sanchez et al., 2004; Tamrakar, Kapasi, and Spector, 2005), suggesting the possibility of differential regulation of virion protein phosphorylation during the course of infection. Our findings are therefore consistent with a model whereby phosphorylation of pp71 modulates the subcellular localization of this tegument protein to enable efficient virus replication.

While our studies suggest that phosphorylation of T223 regulates the nuclear import of pp71, the mechanism that controls nuclear export at the late stages of infection is less clear. Recent studies suggest that cyclin-dependent kinases play a role in pp65 nuclear egress at the late stages of infection (Sanchez et al., 2007), and a similar mechanism may be involved in regulating pp71 cytoplasmic localization. Analysis of the 188MR-eGFP protein and the pp71T223D mutant revealed that pp71 also contains a sequence that targets proteins to a distinct perinuclear region that colocalizes with syntaxin 6 and syntaxin 11, markers of the

TGN-late endosomal compartment (Teng, Wang, and Tang, 2001). Trafficking to this region is consistent with findings that pp71 is localized to a punctate perinuclear region at late times of infection (Hensel et al., 1996), that may correspond to sites of viral tegumentation (Mettenleiter, 2004). Also of note are findings that in non-permissive cell lines where nuclear import of pp71 is blocked, the protein localizes to punctate perinuclear region (Saffert and Kalejta, 2007). The presence of a domain that enables perinuclear targeting within pp71 suggests that relocalization at the late stages of infection is an inherent property of this protein to allow for complete tegumentation during virion morphogenesis. Two models have been proposed by which nuclear tegument proteins become incorporated into the virion (Mettenleiter, 2004). Firstly, nuclear tegument proteins may associate with nascent nucleocapsids and traffic to the cytoplasm together with the nucleocapsids. Second, the viral tegument proteins may independently traffic to the cytoplasm to specific tegumentation sites where they then associate with the newly formed capsids. Our studies showing that the pp71T223D mutant protein can independently traffic to a specific cytoplasmic site that partially overlaps previously characterized virus assembly compartments would be consistent with this latter model. However, analysis of the location of the pp71T223A mutant at the late stages of infection demonstrating that viral and/or cellular proteins modified by virus infection can regulate the nuclear export of pp71 could be consistent with either model. Further analysis of these mutant proteins in the context of virus infection will be necessary to distinguish between these two models.

In summary, we have identified an unconventional non-sequential NLS sequence in the mid region of pp71 which is sufficient for targeting pp71 to the nucleus. This NLS appears to be conformational sensitive, and does not fit any known consensus motifs. In addition, sequences targeting pp71 to a distinct perinuclear region were identified. Further, our studies strongly suggest that differential phosphorylation events modulate the subcellular localization of pp71. Our observations provide novel insight into the mechanisms that regulate the trafficking of this key viral protein, and offer useful tools for a full understanding of the functions of pp71 in HCMV infection, assembly and egress.

## Materials and Methods

### Plasmids

The plasmids pSVH and pSV0d have been previously described (Stenberg et al., 1990). The plasmids pCMV71 and p760CAT were obtained from Dr. Mark Stinski. The pMIEPLuc plasmid was constructed by inserting the major immediate early promoter region from p760CAT into pGL2Basic (Promega). The plasmid pCMV71 was modified by the insertion of the influenza hemagglutinin (HA) epitope tag immediately downstream of the initiating AUG codon by overlapping PCR mutagenesis using primer HA-1 (Table 1) to generate plasmid pCMV71HA. An additional Xba I site was also introduced immediately upstream of the pp71 ORF by PCR using primer 71X-1 to generate pCMV71HAX. The resultant Xba I fragment was then cloned into pBSKS(+) (Stratagene) modified by the deletion of the Kpn I site (pBSKN71HAX). Random insertion mutants within the pp71 open reading frame were generated as previously described (Stenberg et al., 1990). Briefly, the pCMV71 plasmid was partially digested with either Hae III or Pvu II and the linear DNA isolated. A 12 nt linker containing a unique Bst BI site (5' CGCTTCGAAGCG) was ligated to the linear DNA and the plasmid recircularized. Clones that contained Bst BI sites were identified by restriction enzyme digestion and the site of insertion confirmed by complete sequencing of the pp71 open reading frame. Some insertion mutants that contained multiple copies of the linker were further digested with Bst BI, and recircularized, resulting in a single copy of the linker. Mutants were subcloned by inserting the Not I and Bsm I fragments from pCMV71 into pBSKN71HAX. The resultant



Xba I fragments containing the pp71 ORF and its mutants forms were then subcloned into the final expression vectors pSI (Promega) and pRc/RSV (Invitrogen).

Random mutagenesis of the MR domain (amino acids 198–294) was performed using the GeneMorph® II EZClone Domain Mutagenesis kit (Stratagene) using the MRS and MRAS primers (Table 1). Mid region (MR)-eGFP fusion protein constructs were generated using PCR-based mutagenesis and standard cloning. Briefly, the pp71 mid region sequences from amino acid 62, 94, and 188 to 300 were amplified using the appropriate forward primers with a common reverse primer (Table 2). The PCR products were cloned into pCR4-TOPO, sequenced and then subcloned into peGFP-N2 to generate p62MR-eGFP, p94MR-eGFP and p188MR-eGFP. Insertion of the SV40NLS into the pp71 open reading frame immediately downstream of the HA-epitope tag was performed using the Quikchange mutagenesis procedure (Stratagene) with the appropriate primers (Table 1).

### Cells and transient transfection

Human foreskin fibroblasts (HFF) were grown in minimal essential medium supplemented with 10% newborn calf serum and 1% penicillin, streptomycin and glutamine. HFFs were transfected using the DEAE-dextran method as previously described (Stenberg et al., 1989) or using Lipofectamine 2000 according to the manufacturer's directions (Invitrogen Corporation). HeLa cells (ATCC) were maintained in Iscove's media supplemented with 10% fetal calf serum and 1% penicillin and streptomycin and transfected using standard calcium-phosphate procedures.

### Reporter Assays and Western Blot analysis

HFF cells were co-transfected with either p760CAT or pMIEPLuc and the corresponding expression vectors containing either wild type or mutant pp71. Cells were harvested 48 hours post-transfection and assessed for either CAT activity or luciferase activity using the luciferase assay kit (Promega) and a TD-20/20 luminometer (Turner designs). Western Blot analysis was performed as previously described (Ciocco-Schmitt et al., 2002) using a monoclonal antibody to the HA-tag (12CA5, Roche Applied Science).

### Immunofluorescence

HFF cells were transfected and seeded onto 2-well permanox™ chamber slides or 8-chamber cover glasses pretreated with 2% gelatin (Nalge Nunc International) 24 hours post-transfection. After an additional 24 hours, HFF cells were first fixed with 4% paraformaldehyde for 10 minutes at room temperature, washed twice with PBS, and then fixed with 100% methanol for 2 minutes and washed four times with PBS. HFF cells were permeabilized with 0.1% Triton X-100 in PBS for 10 minutes at room temperature and blocked with 5% normal goat serum (NGS) in PBS for 45 minutes at 37°C. Primary antibodies were diluted in PBS containing 5% NGS, and added to HFF cells for 45 minutes including 1218 peptide antibody to IE86 (Stenberg et al., 1989), anti-HA (12CA5, Roche Applied Science) or HA-probe (Y-11, Santa Cruz Biotechnology, Inc.) to pp71 and anti-PML (PG-M3, Santa Cruz Biotechnology, Inc.). Cells were washed 3 times with PBS containing 0.1% BSA and incubated with fluorochrome-conjugated secondary antibodies (goat anti-rabbit Alexa Fluor® 546, anti-mouse Alexa Fluor® 488, Molecular Probes) diluted in 5% NGS in PBS as indicated by the manufacturers for 45 minutes at 37°C. Following three washes with 0.1% BSA in PBS, the cells were washed 2 times with sterile water, dried in a fume hood, and finally mounted with cover glass using vecta-shield mounting medium (Vector Laboratories, Inc.) and assessed by confocal microscopy using a Zeiss LSM 510 confocal microscope and the images analyzed using Metamorph. Identification of the cytoplasmic localization of pp71 was performed by co-staining with a panel of antibodies to Golgi-related markers (BD Biosciences). Nuclei were stained using the TOTO3 dye (Invitrogen Corporation) according to the manufacturer's

directions. Immunofluorescence of infected cells was performed at 72 or 96 hours post-infection by the AD169 strain using a monoclonal antibody to gB kindly provided by Dr. W. J. Britt (University of Alabama School of Medicine, Birmingham), a polyclonal antibody to the gB protein provided by Dr. T. Compton (Novartis Institutes for BioMedical Research) or an antibody to pp71 provided by Dr. R. F. Kalejta (University of Madison-Wisconsin).

### S-tag protein purification and Mass Spectrometry

The pBSKN71HAX plasmid was subjected to a two-step mutagenesis procedure (Quikchange, Stratagene) using the S-tag1 and S-tag2 primers (Table 1) to introduce the 15 amino acid sequence corresponding to the S-tag (Hackbarth et al., 2004). The resultant Xba I fragment was then subcloned into pSI to generate pSI71S. This plasmid was then transfected into HeLa cells using a standard calcium-phosphate procedure. Approximately  $1.5 \times 10^7$  transfected cells were harvested and the cell nuclei isolated as previously described (Kerry, Priddy, and Stenberg, 1994), except for the addition of 1 mM sodium vanadate in the buffers. The resultant nuclear extracts were then subjected to S-tag affinity chromatography essentially as described by the manufacturer (Novagen). Bound proteins were eluted with  $1 \times$  SDS-PAGE Gel Loading Buffer and treated for 20 min at 95°C to reverse the formaldehyde cross-links. Proteins were then separated on a 4–20% SDS-PAGE gel and visualized with a Bio-Safe Coomassie staining kit (Bio-Rad).

Protein bands were excised from 1-D polyacrylamide gels. Gel slices were cut into 1–2 mm cubes; washed 3X with 500  $\mu$ l Ultra-pure water and incubated in 100% acetonitrile for 45 minutes. Samples were reduced with 50 mM DTT at 56°C for 45 minutes then alkylated with 55 mM iodoacetamide for 1 hour at room temperature. The material was dried in a speed-vac, rehydrated in a 12.5 ng/ $\mu$ l modified sequencing grade trypsin solution (Promega, Madison, WI) and incubated in an ice bath for 40–45 min. The excess trypsin solution was then removed and replaced with 40–50  $\mu$ l of 50mM ammonium bicarbonate, 10% acetonitrile, pH 8.0 and the mixture was incubated overnight at 37°C. Peptides were extracted 2X with 25  $\mu$ l 50% acetonitrile, 5% formic acid and dried in a speed-vac. Digests were resuspended in 20  $\mu$ l Buffer A (5% Acetonitrile, 0.1% Formic Acid, 0.005% heptafluorobutyric acid) and 3–6  $\mu$ l were loaded onto a 12-cm  $\times$  0.075 mm fused silica capillary column packed with 5 $\mu$ m diameter C-18 beads (The Nest Group, Southboro, MA) using a N2 pressure vessel at 1100 psi. Peptides were eluted over 55 minutes, by applying a 0–80% linear gradient of Buffer B (95% Acetonitrile, 0.1% Formic Acid, 0.005% HFBA) at a flow rate of 150  $\mu$ l/min with a pre-column flow splitter resulting in a final flow rate of ~200 nl/min directly into the source. In some cases, the gradient was extended to 150 minutes to acquire more MS/MS spectra. A LTQ™ Linear Ion Trap (ThermoFinnigan, San Jose, CA) was run in an automated collection mode with an instrument method composed of a single segment and 5 data-dependent scan events with a full MS scan followed by 4 MS/MS scans of the highest intensity ions. Normalized collision energy was set at 35, activation Q was 0.250 with minimum full scan signal intensity at  $1 \times 10^5$  with no minimum MS<sup>2</sup> intensity specified. Dynamic exclusion was turned on utilizing a three minute repeat count of 2 with the mass width set at 1.0 m/z. Sequence analysis was performed with TurboSEQUENT™ (ThermoFinnigan, San Jose, CA) or MASCOT (Matrix Sciences, London GB) using an indexed viral subset database of the non-redundant protein database from National Center for Biotechnology Information (NCBI) web site (<http://www.ncbi.nlm.nih.gov/>).

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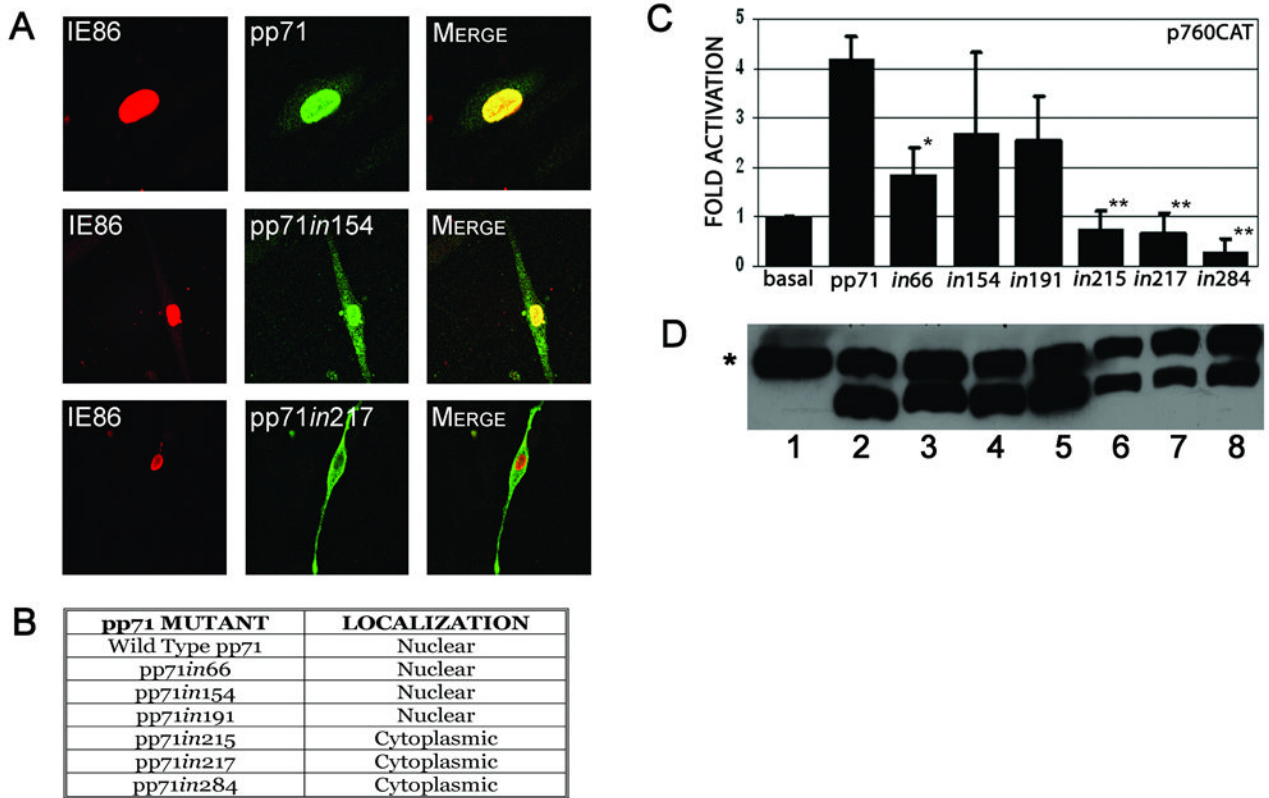
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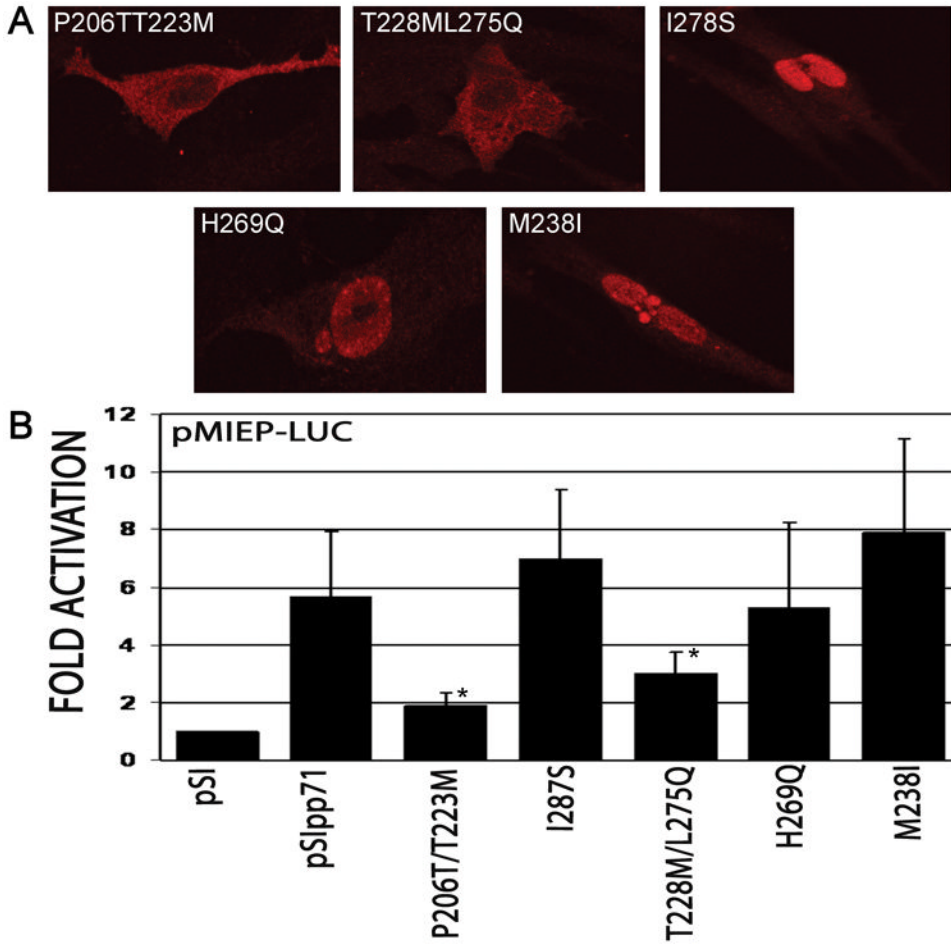
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**FIGURE 1.**

**A & B. Subcellular localization of pp71 insertion mutants.** HFF cells were co-transfected with constructs expressing the indicated pp71 insertion mutants and pMCRS86, and processed for immunofluorescence and confocal microscopy 48 hours after transfection using primary antibodies to the HA epitope tag of pp71 and IE86, with goat anti-mouse Alexa Fluor® 488 and anti-rabbit Alexa Fluor® 546 as secondary antibodies. The pp71 proteins are shown as green fluorescence, and IE86 shown as red fluorescence. The pp71in154 and pp71in217 proteins are shown as representatives of the insertion mutant phenotypes as indicated in B.

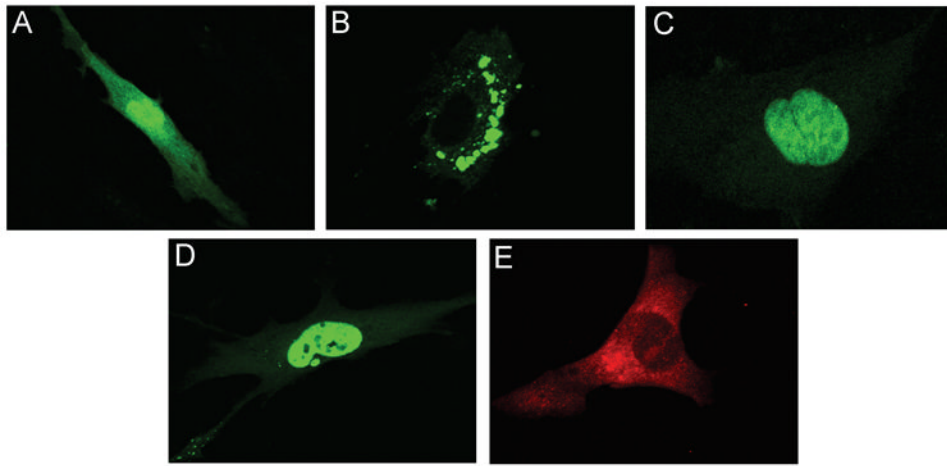
**C. Transcriptional activity of pp71 insertion mutants.** HFF cells were co-transfected with p760-CAT and pCMV71HAX or the indicated insertion mutants. Cells were harvested 48 hours post-transfection and assessed for CAT activity. Data is the average and standard deviation of a minimum of three experiments, normalized to promoter activity in the absence of pp71. Symbols: \* significantly different from wild type,  $p < 0.01$ ; \*\* significantly different from wild type,  $p < 0.001$ .

**D. Western blot analysis of the pp71 insertion mutants.** HeLa cells were transfected with the following plasmids: pSI (lane 1), pSI71HAX (lane 2), pSI71HAXin66 (lane 3), pSI71HAXin154 (lane 4), pSI71HAXin191 (lane 5), pSI71HAXin215 (lane 6), pSI71HAXin217 (lane 7) or pSI71HAXin284 (lane 8). Cells were harvested at 48 hours post-transfection and the extracts subjected to western blot analysis using an antibody to the HA-epitope tag. Symbol: \* non-specific band used as a loading control.



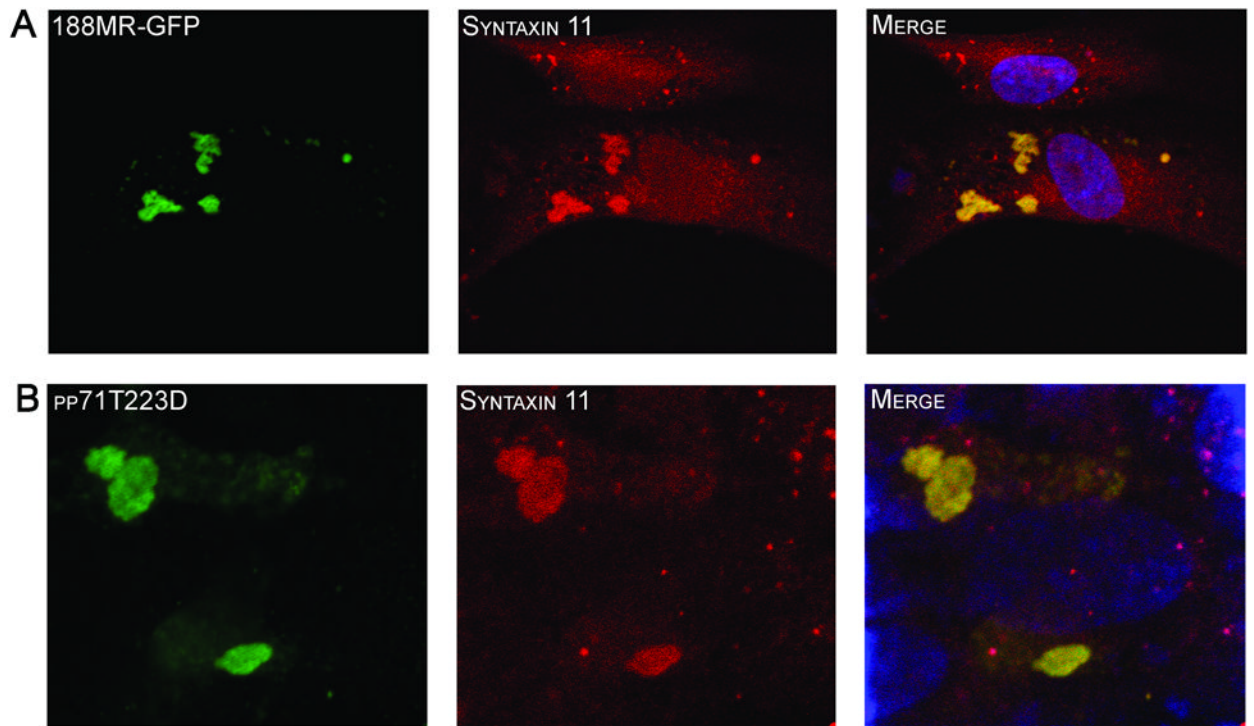
**FIGURE 2.**  
**A. Subcellular localization of pp71 mutants generated by random mutagenesis.** HFF cells were transfected with constructs expressing the indicated pp71 mutants, and processed for immunofluorescence and confocal microscopy 48 hours after transfection using antibodies to the HA epitope tag. **B. Transcriptional activity of pp71 mutants.** HFF cells were transfected with pMIEP-LUC and pSI71HAX or the indicated mutants. Cells were harvested 48 hours post-transfection and assessed for luciferase activity. Data is the average and standard deviation of a minimum of three experiments, normalized to promoter activity in the absence of pp71. Symbol: \* significantly different from wild type,  $p < 0.05$ .





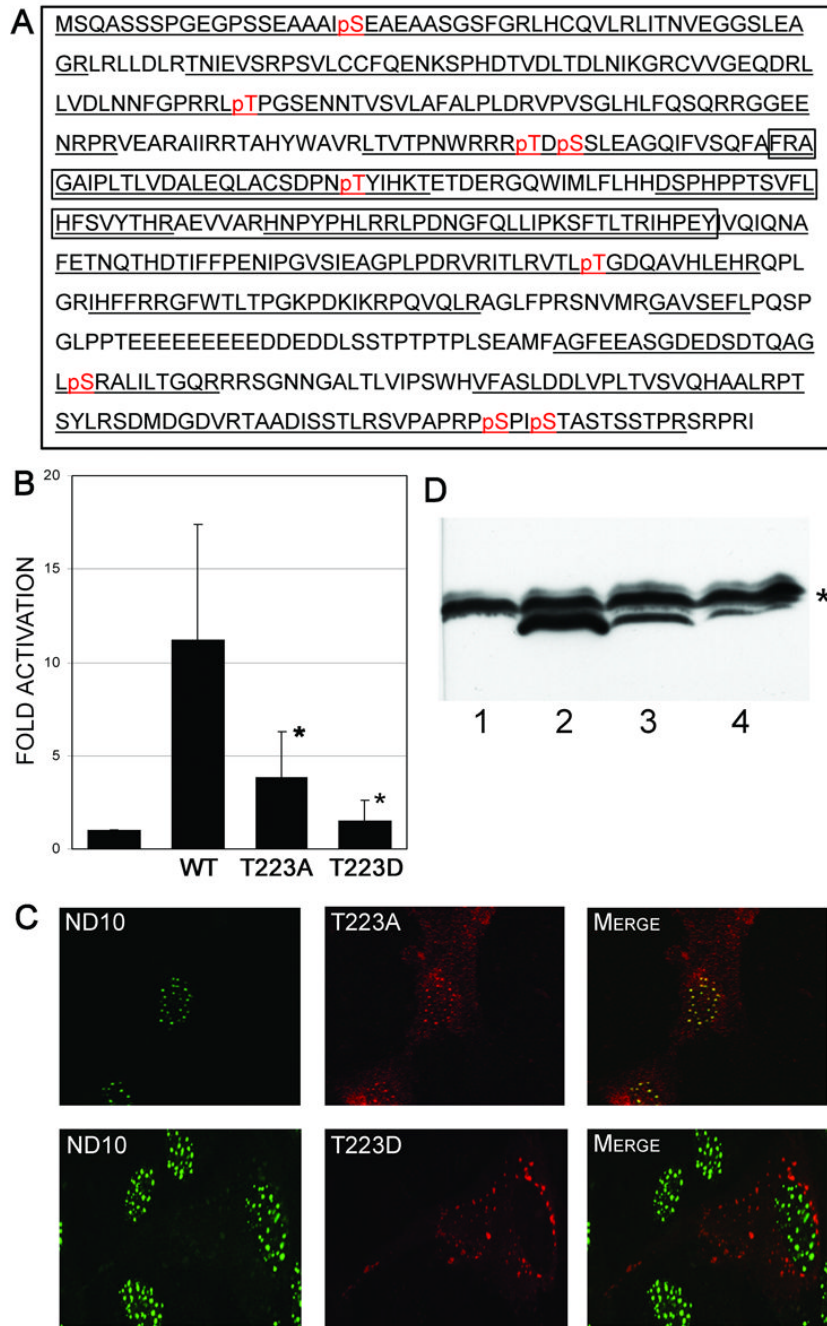
**FIGURE 3. Subcellular localization of MR-eGFP fusion proteins**

HFF cells were transfected with peGFP-N2 (A), p188MR-eGFP (B), p94MR-eGFP (C), or p62MR-eGFP (D) and processed for confocal microscopy 48 hours after transfection. HFF cells infected with HCMV were processed for immunofluorescence at 72 hours post-infection and subjected to immunofluorescence using a monoclonal antibody to pp71 (E).



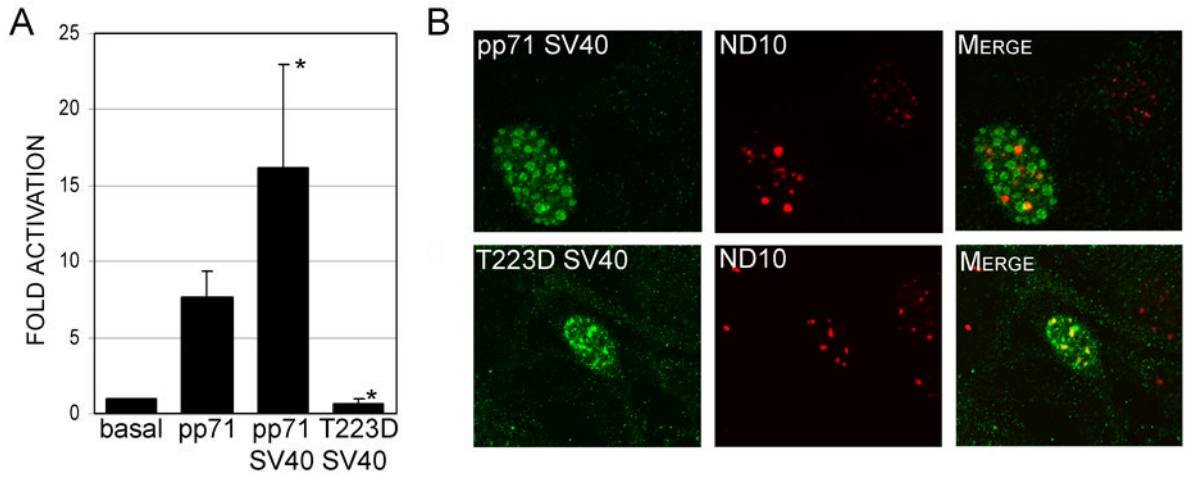
**FIGURE 4. Characterization of pp71 cytoplasmic localization**

HFF cells were transfected constructs expressing p188MR-eGFP (A) or pp71T223D (B) and processed for confocal microscopy 48 hours after transfection. Cells transfected with the construct expressing pp71T223D were stained using an antibody to the HA-epitope tag (green) and both were costained with an antibody to syntaxin 11 (red). Nuclei were stained with TOTO3 dye (blue).

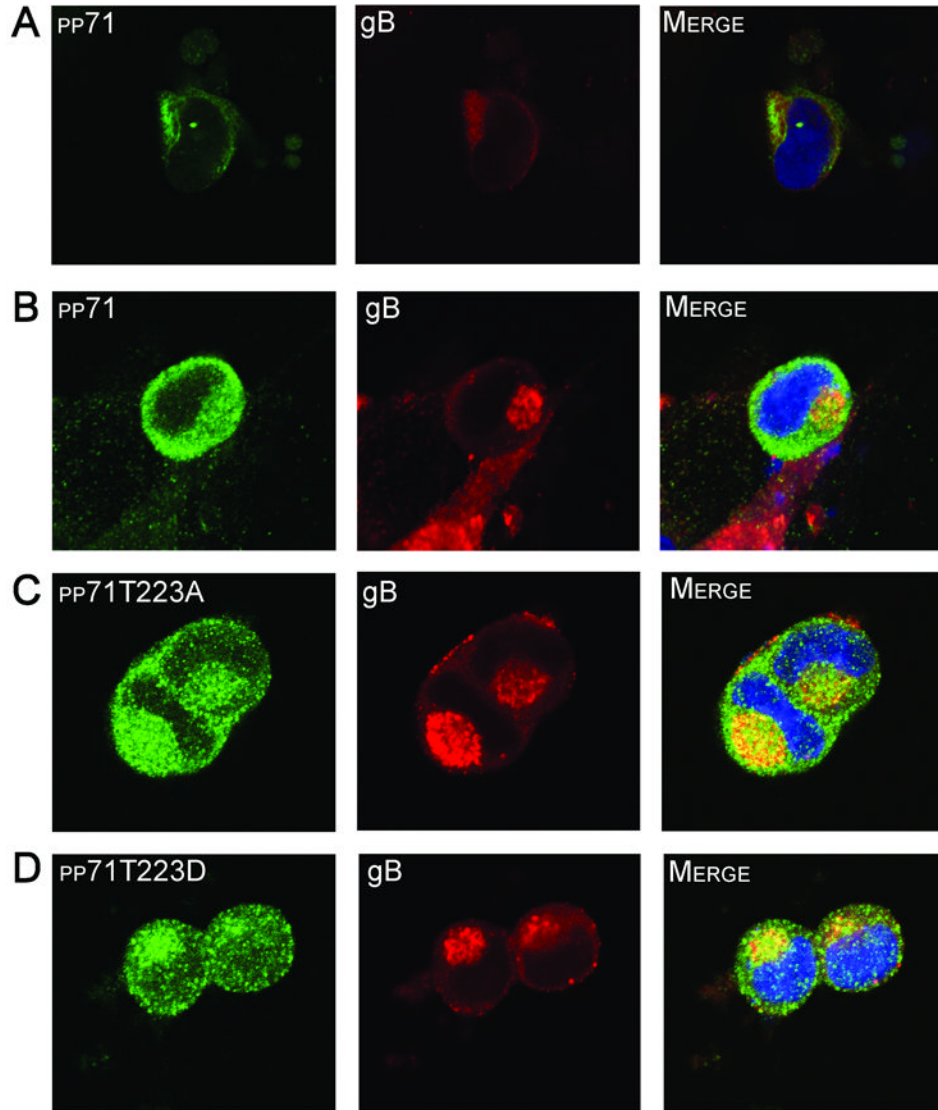


**FIGURE 5.**  
**A. Phosphopeptide map of pp71.** A compilation of the results obtained with MS/MS analysis of phosphorylation sites of the pp71 tegument protein. The total analyzed sequence is underlined, and the phosphorylated residues are indicated in red. The region corresponding to the Mid-Region (MR, aa 200–300) is boxed. **B. Transcriptional activity of pp71 phosphorylation site mutants.** HFF cells were transfected with pMIEP-LUC and pSI71HAX or the indicated mutants. Cells were harvested 48 hours post-transfection and assessed for luciferase activity. Data is the average and standard deviation of a minimum of three experiments and is expressed relative to the activity of the wild type (WT) pp71 protein. Symbol: \* significantly different from wild type,  $p < 0.05$ . **C. Subcellular localization of pp71**

**phosphorylation site mutants.** HFF cells were transfected with constructs expressing the indicated pp71 mutants, and processed for immunofluorescence and confocal microscopy 48 hours after transfection using antibodies to the HA epitope tag and nuclear ND10 domains. The pp71 proteins are shown as red fluorescence, and ND10 domains shown as green fluorescence. **D. Western Blot analysis of pp71 phosphorylation site mutants.** HeLa cells were transfected with constructs expressing eGFP (lane 1), pp71 (lane 2), pp71T223A (lane 3), or pp71T223D (lane 4). Cells were harvested at 48 hours post-transfection and the extracts subjected to western blot analysis using an antibody to the HA-epitope tag. Symbol: \* non-specific band used as a loading control.

**FIGURE 6.**

**A. Transcriptional activity of pp71 mutants containing the SV40 NLS.** HFF cells were transfected with pMIEP-LUC and pSI71HAX or the indicated mutants. Cells were harvested 48 hours post-transfection and assessed for luciferase activity. Data is the average and standard deviation of a minimum of three experiments and is expressed relative to the activity of the wild type (WT) pp71 protein. Symbol: \* significantly different from wild type,  $p < 0.01$ . **B. Subcellular localization of pp71 mutants containing the SV40 NLS.** HFF cells were transfected with constructs expressing the indicated pp71 mutants, and processed for immunofluorescence and confocal microscopy 48 hours after transfection using antibodies to the HA epitope tag and nuclear ND10 domains. The pp71 proteins are shown as green fluorescence, and ND10 domains shown as red fluorescence.



**FIGURE 7. Subcellular localization of pp71 at the late stages of virus infection**

HFF cells were infected with HCMV strain AD169 (A) and fixed at 96 hours post-infection. The cells were then subjected to immunofluorescence using a monoclonal antibody to pp71 to or a polyclonal antibody to gB. Alternatively, HFF cells were transfected with constructs expressing pp71 (B), pp71T223A (C), or pp71T223D (D) and subsequently infected with HCMV strain AD169. Cells were fixed at 96 hours post-infection and subjected to immunofluorescence using an antibody to the HA epitope tag of pp71 (green) and the gB protein (red). Nuclei were stained with TOTO3 dye (blue).

Primers used for mutagenesis. Mutated sequences are underlined. For primers used in PCR mutagenesis procedures, only the forward primer sequence is shown.

Table 1

| Primer Name  | Primer Sequence   | Purpose                                    |
|--------------|---|--|
| HA-1         | 5'-CCGCCCTTCGCAATGTACCCATACCATACGATGTTCCAGAITTACGGTCTCAGGCATCGTCC | Introduce HA-Tag sequence                  |
| 71X-1        | 5'-TTGCCGGAACCTTCTAGACCTCCACCGAAGAC                               | Introduce Xba I site 5' of pp71 ORF        |
| MRS          | 5'-GCAGATCTTTGTGAGCCAGT   | Amplify MR for random mutagenesis          |
| MRAS         | 5'-TGACTCGGGATGTA TGCCG   | Amplify MR for random mutagenesis          |
| pp7162MR-For | 5'-CTCGAGTCTCTGCTGTTTTC   | Introduce Xho I site for eGFP-62MR cloning |
| pp7162MR-N   | 5'-CTCGAGGAACCTTCGTGCCACCAATGGAGGTG                               | N-terminal primer for 62MR-eGFP cloning    |
| pp7194MR-N   | 5'-CTAAACATCAAGGAGCTCACCAATGGTGGCG                                | N-terminal primer for 94MR-eGFP cloning    |
| pp71188MR-N  | 5'-GCAGAACCGAGCTCACCAATGGAGG                                      | N-terminal primer for 188MR-eGFP cloning   |
| pp71MR-Rev   | 5'-GTACCCGGGATGTA TGCC  | Introduce Xma I site for MR-eGFP cloning   |
| S-tag1       | 5'-CGCCTTCGCAATGAAAGAAACCCTGCTGCGAAAATTTCTCAGGCATCGTCC            | Introduce 8 aa of the S-tag sequence       |
| S-tag2       | 5'-GCTGTAAATTTGAACCGCAGCACATGGACTGCTCAGGCATCGTCC                  | Introduce 7 aa of the S-tag sequence       |
| SV40         | 5'-GATGTTCCAGATTACGGCTCCAAAGAAAGAGAAAGGTTGCTCAGGCATCGTCCCTCG      | Introduce the SV40 NLS                     |