Intracellular localization and expression of the human cytomegalovirus matrix phosphoprotein pp71 (ppUL82): evidence for its translocation into the nucleus

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A polyclonal antiserum, raised against a pp71 fusion protein, was prepared in order to investigate the biosynthesis and localization of the matrix protein pp71 of human cytomegalovirus (HCMV), the UL82 gene product, during the HCMV infectious cycle in human fibroblasts. Transcription of the pp71-specific bicistronic 4.0 kb mRNA and pp71 biosynthesis exhibited a biphasic pattern during one round of the HCMV infectious cycle, with a first peak at 12 h and a second at 72 h post-infection (p.i.). Cycloheximide treatment of infected human fibroblasts revealed that the presence of pp71 in total cell extracts prior to 3 h p.i. was due to the input virus inoculum. Transcription of the two specific pp71 mRNAs commenced 5–7 h p.i. as shown by Northern blot analysis of total cellular RNA. Western blot analysis of isolated nuclei and indirect immunofluorescence experiments indicated that pp71, like the major tegument protein pp65, is present in the nucleus shortly after infection as well as during the late phase of viral morphogenesis. Also, after transient transfection of UL82 into U373MG cells, pp71 was found to be present in the nucleus of the transfected cells. By immunogold labelling, pp71 was detected in the nucleoplasm in association with nucleocapsids in electron-dense nuclear skein structures at late stages of the infection cycle. These findings suggest functions of pp71 in viral maturation in addition to that as an early transactivator of viral gene transcription described recently.

Introduction

Diseases induced by human cytomegalovirus (HCMV), a beta herpesvirus, can range from congenital malformation in newborns and pneumonitis in immunocompromised adults to subclinical infection in normal healthy individuals (Alford & Britt, 1993).

After infection, the viral DNA genome of 230 kb undergoes a sequential, highly regulated expression of the viral genes that ultimately leads to replication of progeny virus in permissive cell types (Mocarski, 1993). Although the individual stages of the infectious cycle have been delineated, many details of their regulation remain unresolved. After viral infection, immediate early (IE) genes are first transcribed without de novo protein synthesis (Wathen & Stinski, 1982). Early gene expression occurs after IE protein synthesis but before viral DNA replication. Late genes (yL-genes), which require viral DNA replication for their maximal expression, have been demonstrated, as well as true late genes (yL-genes) that have an absolute requirement for DNA replication prior to expression (Depto & Stenberg, 1989). The majority of the late mRNAs code for the structural components of the virus (Wathen & Stinski, 1982).

HCMV morphogenesis starts in the nucleus with the assembly of early and late nucleocapsids, referred to as A, B and C capsids (Mocarski, 1993; Rixon, 1993). The egress of viral nucleocapsids from the nucleus is mediated by transient envelopment at the inner and fusion of the temporary viral envelope with the outer nuclear membrane (transport budding) (Radsak et al., 1991; Eggers et al., 1992; Rixon, 1993). Finally, the envelopment of naked nucleocapsids in the cytoplasm occurs in association with the tubular early endosome (maturational budding), a network of fine tubules in the vicinity

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of the Golgi complex and around the centriole (Tooze et al.,
1993).

Regarding the regulation of both transport and matu-
ration budding of the nucleocapsids, it has been postu-
lated that specific interactions are involved between structural
components of nucleocapsids and viral envelope proteins in
nuclear and endosomal membranes. Precise information, how-
ever, on where and how the various viral tegument proteins
are assembled during virion maturation is still lacking. Also,
the exact structural and functional relevance of the different
tegment proteins is largely unknown (Spaete et al., 1994).

Several proteins, particularly phosphoproteins, have been
identified in the viral tegument, which lies between the capsid
and the final envelope, and were readily recognized as
abundant structural components (Gibson, 1991). Two phos-
phorylated tegument proteins, pp71 and pp65, are encoded by
two viral genes, UL82 and UL83, respectively, that are located
adjacent to each other in the viral genome (Nowak et al., 1984;
Pande et al., 1984; Roby & Gibson, 1986; Rüger et al., 1987).
pp71 may be translated from either the bicistronic 4·0 kb
mRNA that is controlled by the pp65 promoter and also
encodes pp65, or from the monocistronic 1·9 kb mRNA that
codes for pp71 alone and is controlled by the pp71 promoter
(Rüger et al., 1987). Protein kinase activity has been associated
with pp65 (Britt & Auger, 1986; Somogyi et al., 1990) whereas
pp71 has been identified as a transactivator of IE gene
expression in transfected cells. (Liu & Stinski, 1992). pp65 is
transported to the nucleus of HCMV-infected cells immedi-
ately after infection and can be detected in significant quantities
before the appearance of IE1 and IE2, although pp65 has no
activity as a transactivator (Weiner et al., 1985; Revello et al.,
has been presented that pp65 is non-essential for the growth of
HCMV in human fibroblasts as demonstrated by deletion of
the pp65 gene in the HCMV strain AD169 (Schmolke et al.,
1995a).

pp71 has been shown to stimulate transcription from viral
and cellular promoters containing upstream ATF or AP-1 sites
and recently it has also been reported that pp71 can activate
the major IE enhancer of HCMV in synergy with the tegument
protein UL69, a homologue of herpes simplex virus (HSV)
ICP27, (Liu & Stinski, 1992; Winkler et al., 1994). However, it
has not been shown directly that pp71 is localized in the
nucleus of infected cells.

In order to analyse how pp71 may be involved in the
HCMV infection cycle, we investigated the in vivo tran-
scription and intracellular localization of the UL82 gene
product, pp71, during the HCMV infectious cycle in human
fibroblasts using a specific polyclonal anti-pp71 antiserum
against a glutathione-S-transferase (GST)–pp71 fusion protein.
Evidence is presented that this viral structural protein is
translocated into the nucleus immediately after infection and
that during the late infectious cycle pp71 assembly with viral
nucleocapsids occurs in the infected cell nucleus.

Methods

■ Cells and virus. Human primary foreskin fibroblasts (HFF) were
cultivated in Eagle's minimum essential medium (MEM; Gibco) supple-
mented with 1% MEM non-essential amino acids (Gibco), 1% MEM
vitamins (Sigma), 200 U/ml penicillin plus 10 μg/ml streptomycin and
10% fetal calf serum (FCS). HFF were infected with the HCMV strain
AD169 (Gupta et al., 1977) at m.o.i. of 1–3 for 60 min.

U373MG-pp65-RSV cells expressing the tegument protein pp65 in
the nucleus were obtained by stable transfection of U373MG cells with
the expression plasmid pp65-RSV (Greffe et al., 1992).

■ Antibodies. The mouse monoclonal antibody (MAb) against pp150
(XP1) as well as the anti-pp150 polyclonal antiserum (XP1 antiserum)
were kindly provided by Dr M. Böker (Behringwerke, Marburg,
Germany). The XP1 antiserum was produced in rabbits immunized with
a recombinant β-galactosidase–pp150 fusion protein (XP1, Scholl et al.,
1988). The mouse MAb pps3-27 against the immediate early protein IE1
(pps2) was kindly provided by Dr W. Britt (Birmingham, Alabama, USA).
The polyclonal antiserum BgE1 was raised in rabbits immunized with
a recombinant GST–pp65 fusion protein (Hensel et al., 1995).

For prokaryotic expression of a pp71–GST fusion protein containing
the protein product of a defined portion of pp71, the BglII/BglII fragment
(1310 bp) of the cosmid pCM1007, containing HindIII fragments b and
c (Fleckenstein et al., 1982) was transferred into pGEX-3X (Pharmacia).
The construct was introduced into Escherichia coli JM101 and the GST
fusion protein was isolated from the induced cultures according to
the manufacturer's instructions. Rabbit immunizations with the pp71 fusion
protein were performed according to the scheme of Harlow & Lane
(1988) using ABM-S or ABM-N (Linaris) as adjuvant. Specific antibodies
against pp71 (BgL2) were affinity-purified from positive antisera with
the antigen bound to nitrocellulose strips essentially as described by
Harlow & Lane (1988).

■ Plasmid construction and transfection analysis. For transfection
analysis, the UL82 reading frame encoding pp71 was cloned into the
eukaryotic expression vector pCB6 (Brewer, 1994), kindly provided
by Dr M. Stinski (Iowa City, Iowa, USA).

Cloning of the eukaryotic expression plasmid pRC-150ST/S was
done in several steps. In the first step, the 2·1 kb SstI fragment (bp –83
to +2020) of the UL32 open reading frame encoding pp150 was excised
from the cosmid pCM1017 (Fleckenstein et al., 1982) and ligated into
the Bluescript vector KS(--) (Stratagene) in order to clone a 2·1 kb
HindIII–Apal fragment, representing bp –83 to +1993 of UL32, into
the eukaryotic expression vector pRC/CMV (Invitrogen) giving rise to
pRC-150/SP. In a second step, another construct, pBS/Eco/S, containing
a 3·5 kb EcoRI fragment of UL82 (bp +337 to +3873) at the EcoRI site
in the Bluescript vector KS(--) was used to excise a 2·1 kb Apal
fragment (bp +1993 to +3873) of UL32. This fragment was then inserted into
the Apal site of pRC-150/SP resulting in the construct pRC-150ST/S which
contains bp –83 to +3873 of the pp150 gene in sense orientation under
the control of the HCMV IE promoter. Transcription and translation of
the construct using the TNT coupled reticulocyte lysate system (Promega)
resulted in a product of 150 kDa which was recognized by immuno-
blotting with the anti-pp150 polyclonal serum XP1 (data not shown).

For transient expression of pp71, U373MG glioblastoma cells were
transfected with 2 μg DNA by the calcium phosphate coprecipitation
technique (Ausubel et al., 1989). For transient expression of pp150,
U373MG cells were transfected with pRC-150ST/S using liposomes
(Lipofectamine reagent; Gibco) according to the manufacturer's
instructions. Briefly, 2 x 10⁶ cells were incubated with a mixture of 5 μg
DNA and 5 μl liposomes in medium for 5 h. The expression of the
recombinant proteins was examined by immunostaining 2 days after transfection.

- **Radioactive labelling of infected cultures.** Cells were labelled with [35S]methionine/[35S]cysteine (50 μCi/ml; DuPont NEN) in methionine/cysteine-free MEM plus 2% FCS for 3 h. Cells were then chased by incubation with MEM plus 2% FCS for 1 h prior to cell harvesting and preparation of cell extracts.

- **Purification of viral particles.** For the isolation of viral particles, extracellular virions and dense bodies were sedimented from the medium of infected cultures at 6 days post-infection (p.i.) at 100,000 g for 2 h at 4 °C. Purification of viral particles was carried out by centrifugation through a 15% sucrose cushion in TN-buffer (50 mM-Tris-HCl, pH 7.4) at 100,000 g for 2 h at 4 °C.

- **Cell fractionation and preparation of extracts.** Total extracts of cell pellets were obtained by lysis, sonication and sedimentation of insoluble material in NP40 lysis buffer (150 mM-NaCl, 1% w/v NP40, 50 mM-Tris–HCl, pH 8.0, 100 U/ml aprotinin, 1 mM-PMSF).

- **Immunoprecipitation and immunoblotting.** Aliquots of cell extracts of identical TCA-insoluble radioactivity were pretreated with Protein A-Sepharose CL-4B beads for 2 h at 4 °C, with the respective antisera. Immunocomplexes were incubated with Protein A-Sepharose CL-4B beads for 90 min, at room temperature, and collected by centrifugation. Following seven washing cycles of the beads with PBS containing 1% (v/v) NP40 and 0.1% (w/v) SDS, boiling and reduction, the precipitates were subjected to SDS–PAGE followed by fixation and fluorography of the dried slab gels.

- **Immunofluorescence and visualization.** Cells were fixed and permeabilized with methanol/acetone (1:1) or methanol fixed for 10 min at -20 °C.

**Results**

**Analysis of UL82 gene expression in human fibroblasts**

HFF cells were infected with HCMV and whole-cell RNA was isolated at various times after infection during one round of HCMV replication. The RNA was analysed by Northern blot hybridization using the 1.3 kb BglII/BglII DNA fragment of UL82 generated from the cosmid pCM1007 (Fleckenstein et al., 1982) as a specific probe. As shown in Fig. 1, two pp71-specific mRNAs, the monocistronic 1.9 kb transcript and the bicistronic 4.0 kb mRNA, coding for pp65 and pp71, were detected as early as 5 h p.i., although the signals were very weak. At 5 and 7 h p.i., the relative quantities of the 1.9 and the 4.0 kb pp71-specific RNAs were comparable, whereas at all time points after 7 h p.i., the relative quantity of the 4.0 kb transcript was much higher than that of the 1.9 kb mRNA. Interestingly, the 4.0 kb mRNA levels reached a first peak at 12 h p.i., then declined followed by a second higher peak at 24 h p.i.
72 h p.i., thus exhibiting a biphasic pattern during the observed single round of the HCMV infectious cycle. Although the 1.9 kb mRNA was present at 5–7 h p.i., it remained barely detectable at 12 and 24 h p.i., but then its level slightly increased and reached its maximum at 72 h p.i.

The time course of biosynthesis of pp71 closely correlated with that of pp71-specific steady-state RNA levels. This is apparent from the results of immunoprecipitation experiments that are shown in Fig. 2. pp71 biosynthesis was already detectable at 12 h p.i.; at 24 h p.i., the relative amount of labelled pp71 appeared to decline to half of the levels observed at 12 h p.i., followed by a second increase at 48 h p.i., and maximum expression at 72 h p.i. During later stages of the viral cycle, the tegument protein pp65 was always co-precipitated whatever antiserum (Bgl2 pre-immune serum, anti-pp150 antiserum XP1 or anti-pp71 Bgl2 antiserum) was applied because of non-specific binding of pp65 to Protein A-Sepharose.

These results indicate a temporal correlation between fluctuations of pp71 biosynthesis and of the concentration of its respective bicistronic 4.0 kb mRNA and suggest a rapid turnover of the mRNA during the first 24 h p.i.

**Detection of pp71 in cell extracts immediately after viral entry**

In order to investigate whether pp71 is present in the immediate early phase due to uptake from virus inoculum, HFF cells were cultured in the presence of cycloheximide (CHX; 100 µg/ml) starting at 1 h before and extending treatment for various time periods after infection (1, 3, 5, 7, and 12 h p.i.). Cell extracts harvested at the respective time points were separated by PAGE and analysed by immunoblotting using polyclonal antisera against pp71 (Bgl2), pp65 (Bge1) or a MAb for the detection of immediate early viral antigen (IE1; p72; p63-27). As shown in Fig. 3 (a and b), the tegument proteins pp65 and pp71 were detected at all chosen time points p.i., demonstrating a relatively constant protein quantity up to 12 h p.i. Both antibodies, Bgl2 and especially Bge1, showed a weak cross-reactivity with a cellular protein. Confirming our previous experiments (Hensel et al., 1995), another tegument protein, pp150, could not be found at these respective time points using the polyclonal anti-pp150 antiserum (XP1) (data not shown). Synthesis of IE1 protein was completely inhibited up to 7 h p.i. by CHX (Fig. 3d). In the absence of CHX, IE1 protein expression started 5 h p.i. as shown in Fig. 3 (c). The three protein bands detected by the anti-IE1 antibody are splice variants of IE1 (Mocarski, 1993). Thus, the presence of pp71 at 1 and 3 h p.i. was due to the uptake of viral inoculum.

**Accumulation of pp71 in the nucleus**

To further address the cellular localization of pp71 in HFF cells before the appearance of immediate early protein...
expression, AD169-infected HFF cells were subjected to cell fractionation 1 and 3 h.p.i. in order to obtain nuclear extracts (N) and postnuclear supernatants (PNS). Gel electrophoretic separation of mock-infected (M) or HCMV-infected cell lysates (L), PNS (P) and nuclear extracts (N) was followed by immunoblotting with the right-hand side, the positions of molecular mass markers on the left.

Our previously reported studies revealed that the major tegument protein of virions, pp150, was localized in the nucleoplasm during the late stages of the HCMV infectious cycle in HFF, suggesting that association of nucleocapsids with this tegument protein had already occurred in the nucleus (Hensel et al., 1995). In order to investigate whether newly synthesized pp71 is also present in the nucleus immediately after viral entry, nuclei of uninfected and infected HFF were isolated and fractionated into a soluble nuclear extract (SN) and an insoluble nuclear pellet (P) containing nuclear membranes, DNA, nucleoli and nuclear viroplasm (Severi et al., 1988, 1992). Gel electrophoretic separation of nuclear extract (SN) and pellet (P) fractions was followed by immunoblotting with BgE1 (anti-pp65), XP1 (anti-pp150) and BgL2 (anti-pp71) in succession. As shown in Fig. 5, not only the tegument proteins, pp65 and pp150, but also pp71 were detectable in the soluble nuclear extract as well as in the nuclear pellet fraction 48 h.p.i. and with relatively larger amounts at 72 h.p.i. Lanes 1 and 2 show total cell lysates (L) at 72 h.p.i., and total extracellular viral particles (V), respectively, harvested 6 days p.i.

Thus, the tegument protein is not only present in the nucleus immediately after viral entry but also following its de novo synthesis a portion of pp71 is found during the later stages of the infectious cycle, when it is associated with either nuclear insoluble structures or the viroplasm. As expected, a greater portion of pp71 is present in the cytoplasm at 48 and 72 h.p.i. where the envelopment of viral particles takes place (Fig. 6g–h).

**Subcellular localization of pp71 by immunofluorescence**

In order to further confirm our results obtained from the cell fractionation experiments, uninfected and HCMV-infected fibroblasts were subjected at various time points p.i. to indirect immunofluorescence with the anti-pp71 antiserum (BgL2). Human immunoglobulin G (IgG) can bind to the tegument of HCMV virions by the Fc portion of the molecule (Stannard & Hardie, 1991). Therefore, in order to avoid non-specific binding, F(ab)₂ fragments of BgL2 (anti-pp71) were generated by proteolytic digestion of IgGs (BgL2) with pepsin according to standard methods (Harlow & Lane, 1988). The immunofluorescence staining using either the affinity-purified antiserum or the F(ab)₂ fragments gave identical results at early and late time points p.i. (data not shown), so, only the antiserum was applied in the following experiments.
Fig. 6. Subcellular localization of the tegument proteins pp71 and pp65 in HCMV-infected HFF revealed by indirect immunofluorescence using pp71-specific polyclonal antibodies BgL2 (1:100) and pp65-specific polyclonal antibody BgEI (1:100) and secondary rhodamine-conjugated anti-rabbit F(ab)2. (a) 1 h p.i., BgL2; (b) mock-infected, BgL2; (c) 1 h p.i., BgE1; (d) mock-infected, BgE1; (e) 12 h p.i., BgL2; (f) 24 h p.i., BgL2; (g) 48 h p.i., BgL2; (h) 72 h p.i., BgL2. The exposure time was varied depending on the fluorescence intensity. The bar marker represents 5 µm.

Fig. 6(a) and (c) compare the cellular localization of pp71 and pp65, respectively, at 1 h p.i. Both proteins in this immediate early phase of infection were associated with the nucleus and were distributed as a punctate or floccular staining in the nucleoplasm. Fig. 6(b) and (d) compare the immunofluorescence staining of pp71 and pp65, respectively, on uninfected human fibroblasts. It is evident that both antibodies also stain some structures in uninfected nuclei, particularly the anti-pp65 antibodies, but these reactions can still be estimated as background stainings.

In Fig. 6(e-h) the immunofluorescence staining of pp71 is shown in human fibroblasts at different time points after HCMV infection. After 12 h p.i., the immunofluorescence staining (BgL2) had changed dramatically compared to the immediate early period (Fig. 6e). In addition to the nuclear staining, granular staining of the cytoplasm was visible which was concentrated around the nucleus. As shown in Fig. 6(f), the cytoplasmic staining became more concentrated and was limited to a defined region around the nucleus 24 h p.i. But some cells, which reacted pp71-positive at 12 h as well as later at 72 h p.i., did not show any visible staining at 24 h p.i. (data not shown), reflecting the biphasic course of pp71 expression in human fibroblasts. During the late phase of the infection cycle at 48 and 72 h p.i. (Fig. 6g and h, respectively), the specific cytoplasmic staining with the granular condensed localization around the nucleus increased progressively. It probably represents the pericentriolar region, where viral envelopment takes place (Tooze et al., 1993).

In order to investigate whether the nuclear targeting of pp71 was independent of other viral proteins, transient transfection analyses were carried out. For this, UL82, encoding pp71, was cloned into the vector pCB6. Driven by the HCMV immediate early promoter, this construct, termed pCBpp71, expressed pp71 after transient transfection of U373MG cells. For the detection of pp71, the rabbit antiserum BgL2 was used in indirect immunofluorescence analyses (Fig. 7a-c). The pp71 was found to be exclusively located in the cell nucleus indicating that nuclear transport of the protein was independent of other viral proteins. Staining was predominantly found in the form of dots and large globular structures within the nucleus. In contrast, transfection of UL83, the ORF of pp65, did not result in this nuclear pattern, instead, pp65 was evenly distributed throughout the nucleus (Schmolke et al., 1995b) (Fig. 7f). These results suggest that pp71 is targeted to the nucleus when either delivered to the cytoplasm via infection or when newly synthesized within the cell.

In order to control the specificity of the anti-pp71 antibody, two additional experiments were conducted to rule out the possibility of cross-reactivity with tegument proteins pp65 and pp150. U373MG cells, stably transfected with the plasmid pp65-RSV, expressed tegument protein pp65 that was localized in the nucleus of the cell as shown by indirect immunofluorescence using polyclonal antiserum BgL2 (Fig. 7j) (Grete et al., 1992). In comparison, staining of the stably pp65-RSV-transfected cells with the anti-pp71 antiserum BgL2 did not result in any nuclear staining (Fig. 7d). In parallel, the nuclei were stained by Hoechst 33258 (Fig. 7e, g). In the second control experiment, U373MG cells were transiently transfected with the construct pRC-150ST/S containing the pp150 gene (UL32). The transfected cells were further analysed by indirect immunofluorescence using either anti-pp150 or anti-pp71 antiserum. As shown in Fig. 7(i and k) pp150 is expressed
Fig. 7. For legend see facing page.
in these cells, predominantly in the cytoplasm and around the nuclear membrane. The BgL2 anti-pp71 antibody did not react with transiently expressed pp150 (Fig 7h, j). These results strongly indicate that the anti-pp71 antibody did not cross-react with either the tegument protein pp65 or pp150. So, the observed co-precipitations of pp65 and pp150 using anti-pp71 antibody in Fig. 2 appears to be due to protein–protein interactions between the tegument proteins.

**Localization of pp71-specific staining in infected fibroblasts by immunoelectron microscopy**

To define more precisely the localization of pp71 in the cytoplasm and, in particular, in the nucleus, immunoelectron microscopy was performed on thin sections of infected HFF cultures 96 h.p.i. As shown in Fig. 8, the polyclonal anti-pp71 antiserum (BgL2) reacted with Unicryl-embedded cells and produced little background reaction. The pp71 pre-immune serum did not result in any gold labelling of cells or viral particles when using the same working conditions as for BgL2. In the nucleoplasm (Fig. 8a), nucleocapsids were found to be immersed in an electron-dense skein-like structure. Some of the developing nucleocapsids were stained by the antibody at the outer protein scaffold. In the cytoplasm, viral particles, vacuoles or vesicles and cytoplasmic dense bodies were found in close association with each other forming large ‘black holes’ that were shown to be positive for pp65 (Landini et al., 1987; Severi et al., 1988, 1992). Fig. 8(b) shows such a ‘black hole’. Interestingly, there was pp71-specific gold-labelling at the edge of these structures and around some vesicles or vacuoles which were fused with the aggregate. Cytoplasmic virions with a pp71-positive stained tegument were observed (Fig. 8b). Fig. 8(c and d) show extracellular viral particles after cytoplasmic virus maturation and fusion with the plasma membrane. Specific gold label was found, in general, at the inner part of the viral tegument. Extracellular and cytoplasmic dense bodies, with the exception of the ‘black holes’, did not react with the pp71-specific antiserum as demonstrated in Fig. 8(d).

**Discussion**

Using our pp71 antiserum we have been able to determine the intracellular destination of the pp71 protein derived from the infecting virus and the pattern of de novo expression of the protein during the HCMV infectious cycle. The results suggest a multifunctional role for this protein. The antiserum provided both biochemical and cytochemical evidence that exogenously derived pp71 is transported into the nucleus after infection, as is newly synthesized endogenous pp71. Furthermore, the observed biphasic time course of pp71 expression during one HCMV infection cycle suggests a bifunctional role of this viral protein in the nucleus: firstly during the immediate early and early phases as a transactivator of gene expression and secondly during progeny maturation during the late phase of virus replication.

In the first part of the present study, we have demonstrated that expression of the pp71 gene, UL82, starts during the early phase of HCMV infection. This has also been shown for the gene UL83 that codes for the tegument protein pp65 which is expressed at low levels early in the infection cycle and is greatly amplified after DNA replication (Geballe et al., 1986; Depto & Stenberg, 1989). Transcription of the two pp71-specific mRNAs clearly starts 5–7 h after HCMV infection in human fibroblasts. Comparison of pp71-specific mRNA kinetics with the kinetics of the de novo synthesis of pp71 protein suggests that it is more likely that the bicistronic 4.0 kb mRNA functions as the template for pp71 translation at least during the early phase of HCMV morphogenesis. The bicistronic mRNA showed the biphasic time course that parallels the course of the de novo synthesized pp71 protein. The 1.9 kb pp71-specific mRNA might be used as an additional transcript for pp71-translation at the late stages of HCMV maturation in order to guarantee large amounts of pp71 structural protein for incorporation into progeny virions.

We found pp71 in the nucleus of infected cells even prior to the pp71 gene expression that occurs early in HCMV replication. 1 and 3 h.p.i. after virus entry, most of the detected pp71 protein had accumulated in the nucleus. Therefore, our findings strongly suggest that pp71 was translocated into the nucleus immediately after virus entry and breakdown of input virus.

Given the fact that pp71 is transported into the nucleus, it has to be assumed that a nuclear-targeting sequence is present in its amino acid sequence. A possible bipartite consensus nuclear-targeting sequence exists between amino acids 366 and 382 (RRGFWTLTPKPNKIKKR). This functional consensus signal motif is defined as two basic amino acids, a spacer
Fig. 8. Ultrastructural localization of the tegument protein pp71 in the nucleoplasm (a), the cytoplasm (b) and extracellular viral particles (c, d) of HCMV-infected cells 96 h p.i. by immunogold labelling using pp71-specific polyclonal antiserum Bgl2. (a) pp71 positively stained nucleocapsids (arrows) are embedded in a electron-dense skein-structure in the nucleoplasm. In some gaps of the skein-structure, accumulations of immunogold (arrowheads) can be observed that seem to be associated with nucleocapsid precursors. (b) In the cytoplasm, vesicles and vacuoles that are going to fuse with a 'black hole', are stained at their surfaces (arrowheads), whereas virions reacted with their tegument (arrow). (c and d) Extracellular NIEPs (non-infectious enveloped particles) and virions (arrows) were found to be pp71-positive whereas dense bodies (stars) were pp71-negative. Bar markers represent 0.1 µm.
region of any ten amino acids and a basic cluster in which three out of the next five amino acids must be basic (Dingwall & Laskey, 1991). Specific deletion mutagenesis of this possible nuclear-targeting sequence should help to determine whether this sequence is essential and sufficient for transport of pp71 into the nucleus in human fibroblast cells.

The rapid targeting to the nucleus of pp71 from infecting virus particles must be of functional significance. Liu & Stinski (1992) previously demonstrated, in vitro, that pp71 trans-activates gene expression. It potently stimulates transcription from the major immediate early promoter (MIEP) as well as from cellular promoters containing upstream binding sites for the eukaryotic transcription factors AP-1 or ATF (Lee et al., 1987; Montminy & Bilazikian, 1987).

In this context it is interesting to note that pp71 colocalizes with discrete nuclear domains after transient expression. Given the fact that pp71 is also a transcriptional transactivator, it is tempting to speculate that this viral protein is targeted to certain locations within the nucleus involved in transcription. However, detailed analysis of the structures containing pp71 is required.

Not only exogenous, but also endogenous, newly synthesized pp71 protein is translocated into the nucleus. During the late and final phase of extensive production of viral structural components and maturation of progeny, the tegument protein pp71 is also found in the nucleoplasm along with the other two major tegument proteins, pp150 and pp65, and is associated with nucleocapsids (Landini et al., 1987; Severi et al., 1992; Revello et al., 1992; Hensel et al., 1995). Therefore, pp71 is another candidate protein involved in the protein–protein interactions that occur between nucleocapsid and inner nuclear membrane and is necessary for the transport budding of nucleocapsids through the nuclear membrane (Eggers et al., 1992; Tooze et al., 1993).

The transfection experiments further indicated that pp71 can be translocated into the nucleus in the absence of any viral proteins. Given the fact that pp71 was found to be exclusively located in the cell nucleus after transient transfection, it could be assumed that also in vivo all newly synthesized pp71 has to be compartmentalized into the nucleus before its packaging into virions and that pp71 requires association with nucleocapsids or other tegument proteins in order to leave the nucleus again.

During the immediate early phase, as demonstrated by the direct translocation of uptake pp71 in the nucleus, and during the early phase of virus replication, as indicated by the first peak of maximum pp71 expression, the protein may act as an optional transactivator of gene expression that shortens the onset of IE gene expression and enhances, in general, the gene expression of viral and cellular genes, essential for virus replication. At the late phase of viral maturation, however, nuclear pp71 may be essential for the correct assembly of nucleocapsids, as indicated by its association with nucleocapsids in the nuclear skein structure, or for the transport of nucleocapsids through the nuclear membranes. Another possibility is that pp71 is involved in the cytoplasmic envelopment of nucleocapsids at cisternae of the tubular endosome. Alternatively, the reason for maximum expression of pp71 during the late phase could simply be the packaging of sufficient transactivating pp71 protein for the next infection cycle. Further experiments that investigate pp71 protein–protein interactions, should help to determine the function of pp71 during HCMV morphogenesis.

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