Expression and Characterization of a Novel Structural Protein of Human Cytomegalovirus, pUL25

MARIA CONCETTA BATTISTA, GIOVANNA BERGAMINI, MARIA CRISTINA BOCCUNI,
FABIO CAMPANINI, ALESSANDRO RIPALTI, AND MARIA PAOLA LANDINI*

*Department of Clinical and Experimental Medicine, Division of Microbiology,
University of Bologna, St. Orsola Hospital, Bologna, Italy*

Human cytomegalovirus (HCMV) UL25 has recently been found to encode a new structural protein that is present in both virion and defective viral particles (C. J. Baldick and T. Shenk, J. Virol. 70:6097–6105, 1996). In the present work a polyclonal antibody was raised against a prokaryotic pUL25 fusion protein in order to investigate the biosynthesis and localization of the UL25 product (pUL25) during HCMV replication in human fibroblasts. Furthermore, pUL25 was transiently expressed in its native form and fused to the FLAG epitope, in COS7 and U373MG cells, in order to compare the properties of the isolated protein and that produced during infection. Immunoblotting analysis revealed a group of polypeptides, ranging from 80 to 100 kDa, in both transfected and infected cells; in vivo labeling experiments with infected cells demonstrated they are posttranslationally modified by phosphorylation. The transcriptional analysis of the UL25 open reading frame combined with the study of pUL25 biosynthesis showed true late kinetics for this protein in infected human fibroblasts. By indirect immunofluorescence both recombinant and viral pUL25 were detected exclusively in the cytoplasm of transfected or infected cells. Interestingly, pUL25 was shown to localize in typical condensed structures in the perinuclear region as already observed for other HCMV tegument proteins. Colocalization of ppUL99 in the same vacuoles suggests that these structure are endosomal cisternae, which are proposed to be a preferential site of viral particle envelopment. Our data suggest that pUL25 is most likely a novel tegument protein and possibly plays a key role in the process of envelopment.

Human cytomegalovirus (HCMV) is a ubiquitous betaherpesvirus which can be associated with a wide spectrum of diseases, particularly in newborns and immunocompromised persons. The virion has the hallmark structural features of the herpesvirus family, consisting of an icosahedral capsid which contains a large double-stranded DNA genome (240 kb) located in a central core. The nucleocapsid is embedded in an amorphous layer called the tegument which fills the space between the nucleocapsid and the envelope. Both the structure and function of the tegument are largely unknown, although it contains approximately 40% of the virion protein mass (17), most of which is made up of phosphoproteins (13). The major tegument constituents are pUL83 (the lower matrix protein) (for nomenclature of HCMV proteins, see reference 32), of 65 kDa (24), which constitutes 90% of the dense-body protein mass (17); pUL32 (basic phosphoprotein), of 150 kDa (18), which appears to be most tightly associated with the capsid (14); and pUL82 (the upper matrix protein), of 71 kDa (16, 24), which is a transcriptional activator that is able to upregulate the HCMV immediate-early promoter (21). A less abundant tegument protein, which is absent from dense bodies (20, 28), is pUL99 (28 kDa) (19, 23, 29). More recently identified tegument proteins are p130 (pUL56) (3, 5, 14), p212 (high-molecular-weight protein), pUL48 together with pUL47 (high-molecular-weight-protein-binding protein, 110 kDa) (1, 5, 14), and finally a subform of the transactivator protein pUL69, the homologue of herpes simplex virus ICP27 (35, 36).

Recently, a novel structural protein of HCMV has been identified as the product of the gene UL25 (1, 7). The UL25 open reading frame (ORF) (EMBL accession no. X17403) shows significant homology with the human herpesvirus 6 (HHV-6) U14 gene (1), and the HHV-7 homologue of HHV-6 U14 has been shown to encode a tegument protein (33).

The goal of our study was to characterize the product of HCMV UL25. Following fusion with the FLAG epitope, it was transiently expressed in mammalian cells and analyzed by both immunoblotting (IB) and indirect immunofluorescence (IIF). In order to compare the characteristics of the protein expressed in isolation with those of its viral counterpart, a polyclonal antibody (PAb) specific for pUL25 was prepared and used to study the biosynthesis as well as the posttranslational modifications of the protein during the HCMV infection cycle. The subcellular localization of pUL25 was investigated by IIF and compared with that of another tegument protein, ppUL99. The results provided useful suggestions about the possible localization of pUL25 in endosomal vacuoles of infected cells and in the tegument of viral particles.

MATERIALS AND METHODS

Cells and virus. Human astrocytoma cells (U373MG) and COS7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum. Human embryonic lung fibroblasts (HEL) were cultivated as monolayers in Eagle's minimum essential medium supplemented with 10% fetal calf serum. Infections were performed on 70 to 80% confluent monolayers of HEL cells with HCMV strain AD169 at a multiplicity of infection of 1 to 2 for 60 min. DNA synthesis was inhibited by adding foscarnet to the medium after adsorption at a concentration of 100 μg/ml.

PCR and construction of expressive plasmids. The construction of UL25 expression plasmids is illustrated in Fig. 1a. For prokaryotic expression, the entire UL25 sequence (1,968 bp) was amplified from the genome of HCMV strain AD169 by using the primers 25-CK-up (5′-AGCAAACGAGAAGAATT CATCGAGGCGTCGC-3′) and 25-CK-low (5′-TGTTTAGTCCCACGAATTC CCACAGTATTCCC-3′). The construct pE-25CK82 was obtained by cloning the amplified sequence into the EcoRI site of the vector pCKS (kindly provided by G. T. Maine, Abbott Laboratories, North Chicago, Ill.) between the 5′ and 3′

* Corresponding author. Mailing address: Department of Clinical and Experimental Medicine, Division of Microbiology, University of Bologna, St. Orsola Hospital, Via Massarenti 9, 40138 Bologna, Italy. Phone: 39-51-341652. Fax: 39-51-341632. E-mail: viroland@med.unibo.it.
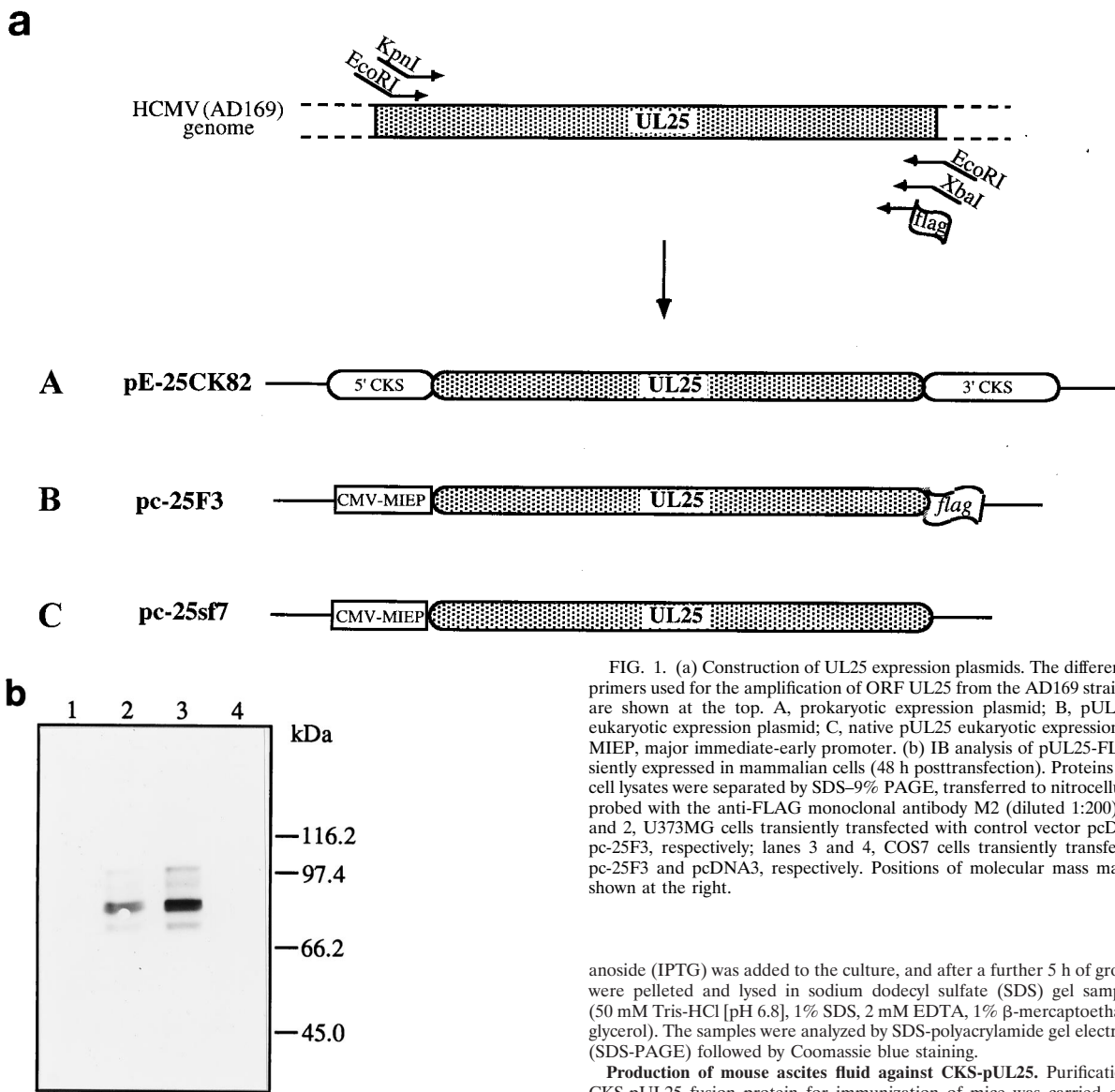
a

b

FIG. 1. (a) Construction of UL25 expression plasmids. The different pairs of primers used for the amplification of ORF UL25 from the AD169 strain genome are shown at the top. A, prokaryotic expression plasmid; B, pUL25-FLAG eukaryotic expression plasmid; C, native pUL25 eukaryotic expression plasmid. MIEP, major immediate-early promoter. (b) IB analysis of pUL25-FLAG transiently expressed in mammalian cells (48 h posttransfection). Proteins of whole-cell lysates were separated by SDS–9% PAGE, transferred to nitrocellulose, and probed with the anti-FLAG monoclonal antibody M2 (diluted 1:200). Lanes 1 and 2, U373MG cells transiently transfected with control vector pcDNA3 and pc-25F3, respectively; lanes 3 and 4, COS7 cells transiently transfected with pc-25F3 and pcDNA3, respectively. Positions of molecular mass markers are shown at the right.

anoside (IPTG) was added to the culture, and after a further 5 h of growth, cells were pelleted and lysed in sodium dodecyl sulfate (SDS) gel sample buffer (50 mM Tris-HCl [pH 6.8], 1% SDS, 2 mM EDTA, 1% β-mercaptoethanol, 10% glycerol). The samples were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue staining.

Production of mouse ascites fluid against CKS-pUL25. Purification of the CKS-pUL25 fusion protein for immunization of mice was carried out as described earlier (2). Briefly, proteins from *E. coli* extracts were separated on preparative 9% polyacrylamide gels and negatively stained with 0.3 M CuCl₂. The band of interest was cut out from gels and electroeluted in dialysis membranes (Spectrum, Laguna Hills, Calif.) in SDS-PAGE running buffer. The protein was then concentrated and extensively washed in Centricon-50 filters (Amicon, Beverly, Mass.) with phosphate-buffered saline before injection into mice. The protocol used for immunization has been previously described (6). Briefly, 50 μg of purified proteins was emulsified with an excess of complete Freund's adjuvant and injected on days 0, 7, 14, 21, and 28 into the peritoneal cavities of BALB/c mice, which were treated with pristane (2,6,10,14-tetramethylpentadecane; Sigma, St. Louis, Mo.) on day 6. This procedure induced the development of antibody-producing ascites tumors; ascites fluids were collected by peritoneal paracentesis with a sterile gauge needle.

IB. For IB analysis, whole-cell lysates were obtained by treatment with Nonidet P-40 (NP-40) lysis buffer (20 mM Tris [pH 9], 300 mM NaCl, 10% glycerol, 2 mM EDTA, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride, 0.5 U of aprotinin per ml). Aliquots containing identical amounts of proteins were separated by SDS-PAGE on 9% gels and subsequently electrotransferred to nitrocellulose sheets (Bio-Rad). These were then subjected to indirect immunostaining with pUL25-specific ascites fluid diluted 1:500 or with anti-FLAG monoclonal antibody M2 (Kodak, New Haven, Conn.) diluted 1:200. Antibody binding was detected with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) and IgM and visualized by 4-chloro-1-naphthol staining.

IIF. For IIF, HCMV-infected HEL or transfected U373MG and COS7 cells were treated as previously described (27). Briefly, cell fixation was performed '[both with methanol-acetone (3:1) for 30 min at −20°C and with paraformaldehyde (4%) for 30 min at 4°C. Fixed cells were incubated with PAb CK25

sequences encoding the bacterial protein CMP-keto-3-deoxyoctulosonic acid synthetase (CKS) (4) under the control of the *lacZ* promoter. For eukaryotic expression, two different plasmids were constructed: pc-25F3, where the UL25 sequence was modified by adding a short sequence encoding a FLAG octapeptide at the 3' end, and pc-25sf7, expressing the native pUL25 without modifications. The upper primer used for both amplifications was 25-pc-up (5'-GAGCC GAAGGTACCACAGCAGAAGAGAGGATGTCGTCGC-3'), containing the *Kpn*I site upstream from the start codon. Lower primers were 25*flag* (5'-TCAC TTGTCATCGTCGTCCTTGTAGTCGCAACAGTATTCCCCGCT-3') and 25-pc-low (5'-GCTGTTTCTAGACACCATCAGCAACAGTATTCCC-3'), with the latter containing the *Xba*I site downstream from the stop codon. Both amplified sequences were cloned into the vector pcDNA3 (Invitrogen, Carlsbad, Calif.) under the control of the major immediate-early promoter of HCMV.

Transient transfections of mammalian cells. For transient expression of pUL25, U373MG and COS7 cells were transfected by electroporation with pc-25F3 and pc-25sf7. For this purpose cells were trypsinized, washed, and resuspended in DMEM without supplementation; 5 μg of plasmid DNA was added to 8 × 10⁶ cells in 400 μl of DMEM and electroporated at 270 V and 960 μF in a cuvette (4-mm gap) with a Gene-Pulser electroporation apparatus (Bio-Rad, Richmond, Calif.). At 48 h posttransfection, cells were analyzed for expression of pUL25.

Induction of CKS-pUL25 fusion protein. The CKS-pUL25 fusion protein was produced in *Escherichia coli* Top10F′ following transformation with pE-25CK82 plasmid. Overnight cultures were diluted 1:10 with fresh medium and grown at 37°C to an optical density at 600 nm of 1; 1 mM isopropyl-β-D-thiogalactopyr-
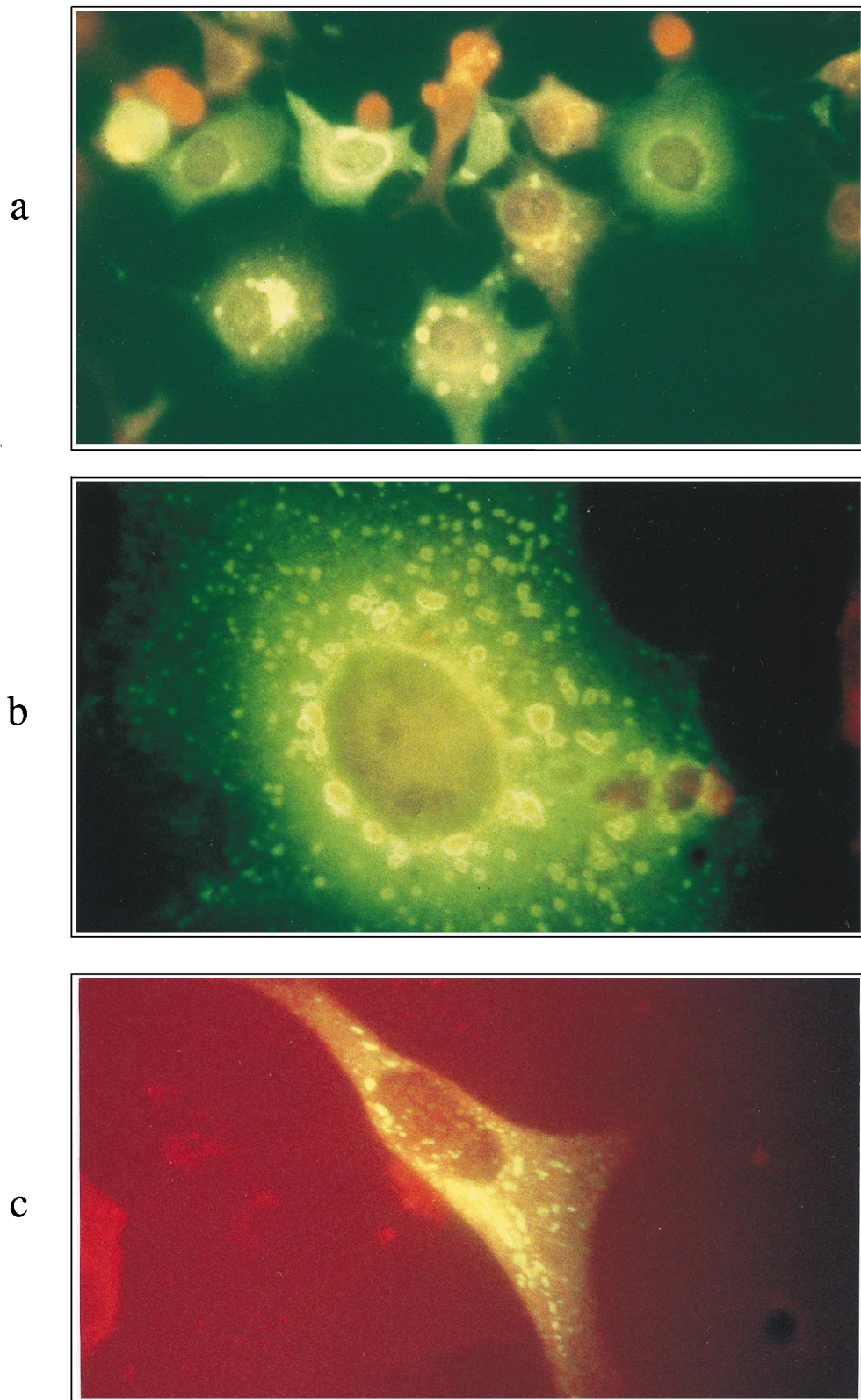
FIG. 2. IIF analysis of pUL25-FLAG transiently expressed in mammalian cells (48 h posttransfection), using the anti-FLAG monoclonal antibody M2 (diluted 1:200). (a and b) COS7/pc-25F3; (c) U373MG/pc-25F3. Magnification, ×40 (a) and ×100 (b and c).
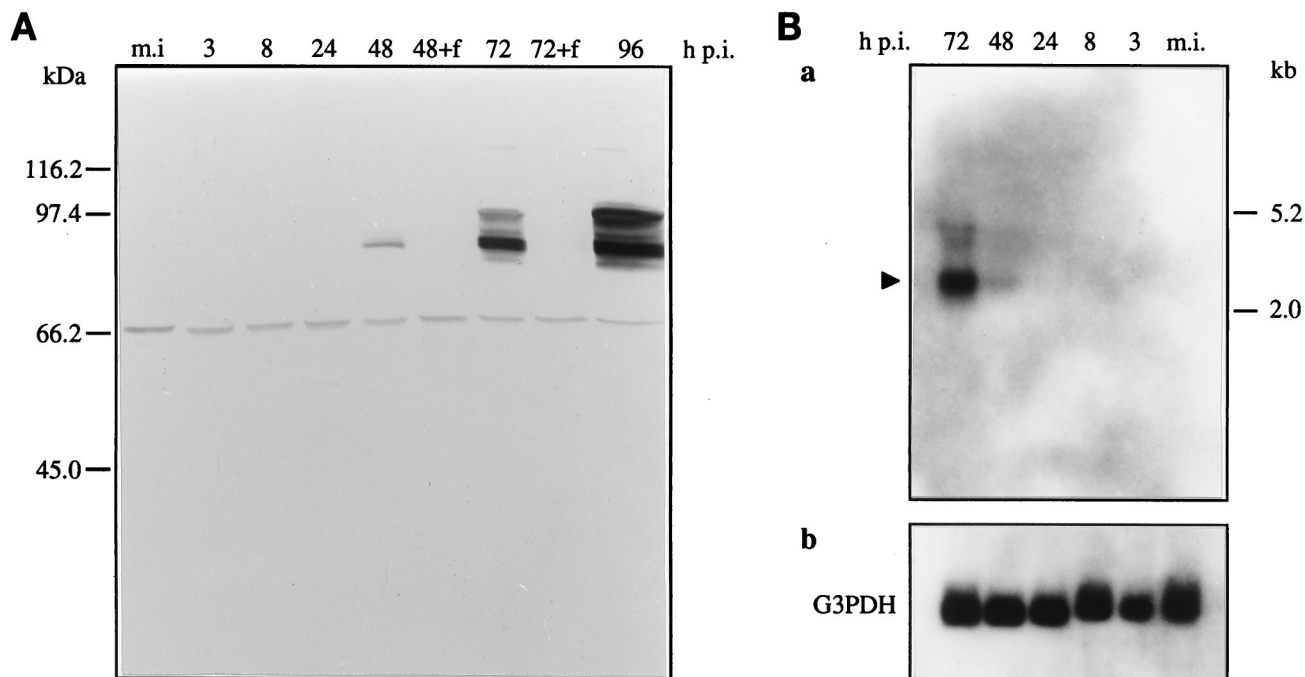
FIG. 3. (A) Time course of pUL25 expression during the HCMV replication cycle, determined by IB analysis. Whole-cell lysates of mock-infected (m.i.) and HCMV-infected HEL cells at various times after infection were separated by SDS–9% PAGE, and the proteins were then transferred to nitrocellulose and immunostained with the mouse ascites fluid PAb CK25 (diluted 1:500). +f, samples treated with foscarnet (100 µg/ml) after adsorption of virus. Positions of molecular mass markers are shown on the left. (B) Northern blot analysis of the UL25 transcript. Total RNA was prepared from mock-infected (m.i.) and HCMV-infected HEL cells at various times after infection by using an RNAzol B kit. Aliquots of 15 µg of total RNA were subjected to electrophoresis through a 1% agarose gel containing 2.2 M formaldehyde and then transferred to nylon. The resulting filter was hybridized with a ^{32}P-labeled probe of the entire UL25 sequence (a) and rehybridized with a ^{32}P-labeled G3PDH gene probe (b). The location of the 2.4-kb UL25 transcript is indicated by the arrowhead. The molecular sizes of 28S and 18S human rRNAs are indicated on the right.

ascites fluid (diluted 1:400) or anti-FLAG M2 antibody (diluted 1:200) for 1 h at 37°C in a humid chamber. After three washes with phosphate-buffered saline, secondary fluorescein-conjugated goat anti-mouse IgG (Cappel, Organon Teknika Corp., Chester, Pa.) was added to the cells. The cells were then counterstained with Evans blue and observed under a Zeiss UV-microscope with photographic equipment.

Double staining was performed by incubating cells (i) with PAb CK25 followed by secondary fluorescein-conjugated antibody as described above, (ii) with an unlabeled goat anti-mouse IgG Fab-specific antibody (diluted 1:30) in order to saturate ascites IgG Fab (Sigma-Aldrich), and (iii) with a mouse monoclonal antibody specific for ppUL99, P2G11 (20) (diluted 1:20), followed by a secondary TRITC (tetramethylrhodamine isothiocyanate)-conjugated goat anti-mouse Fab-specific antibody (diluted 1:30) (Sigma-Aldrich). To check the specificity of rhodamine staining, the TRITC-conjugated antibody was added directly after saturation in the absence of P2G11.

Northern blotting. Total RNA was prepared from mock-infected and HCMV-infected HEL cells at different times after infection by using the RNAzol B kit (TEL-TEST, Friendswood, Tex.) according to the manufacturer's instructions. Purified RNA (15 µg) was separated on 1% agarose gels containing 2.2 M formaldehyde and transferred to Hybond nylon sheets (Amersham, Little Chalfont, England). These were incubated for 1 h at 58°C in prehybridization buffer (15% formamide, 6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 1× Denhardt's 150 µg of calf thumus DNA per ml) and hybridized in the same buffer overnight with a double-stranded DNA probe of the entire UL25 sequence labeled with [α-^{32}P]dCTP by using a random-primed DNA labeling kit (Boehringer, Mannheim, Germany). Before autoradiography, the blotted membranes were washed three times with 2× SSC–0.1% SDS at room temperature, then with 0.2% SSC–0.1% SDS at 58°C, and finally with 0.1× SSC–0.1% SDS at room temperature. To monitor the amount of analyzed RNA, the same blot was subsequently stripped with hot water and reprobed with a ^{32}P-labeled glyceraldehyde-3-phosphate dehydrogenase (G3PDH) probe.

In vivo phosphorylation and immunoprecipitation. To determine whether pUL25 is phosphorylated, mock-infected and HCMV-infected HEL cells were radiolabeled for 24 h (from 48 to 72 h postinfection [p.i.]) with either ^{33}P$_i$ or [^{35}S]cysteine (50 µCi/ml). For immunoprecipitation, whole-cell extracts were prepared by solubilization in a 300 mM NaCl buffer X (20 mM Tris-HCl [pH 8], 300 mM NaCl, 10% glycerol, 1 mM CaCl$_2$, 0.5 mM MgCl$_2$, 2 mM EDTA, 0.5% NP-40, 0.1 mM phenylmethylsulfonyl fluoride, 0.5 U of aprotinin per ml) and shaking for 20 min at 4°C. Following centrifugation at 8,000 × g, the supernatants

were diluted 1:1 with a 50 mM NaCl buffer X and incubated for 90 min at room temperature on a rocking platform with either PAb CK25 or 9220 antibody (anti-ppUL83). Immunocomplexes were subsequently precipitated by adding protein A–Sepharose CL-4B (Pharmacia LKB, Uppsala, Sweden) and incubated for 15 min under the same conditions. The immunocomplexes were then centrifuged (700 × g) and washed once with 50 mM NaCl buffer X and three times with
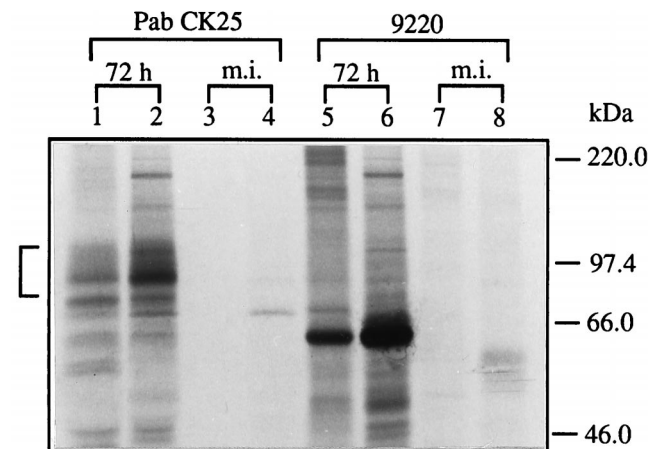

FIG. 4. In vivo phosphorylation of mock-infected (m.i.) and HCMV-infected human fibroblasts. Labeling was performed with either ^{33}P$_i$ (lanes 1, 3, 5, and 7) or [^{35}S]cysteine (lanes 2, 4, 6, and 8) from 48 to 72 h after infection with HCMV strain AD169. Cell lysates were immunoprecipitated with either the mouse ascites fluid PAb CK25 or anti-pUL83 (pp65) monoclonal antibody 9220. The bracket on the left shows the group of polypeptides specifically detected by PAb CK25. Positions of molecular mass markers are shown on the right.
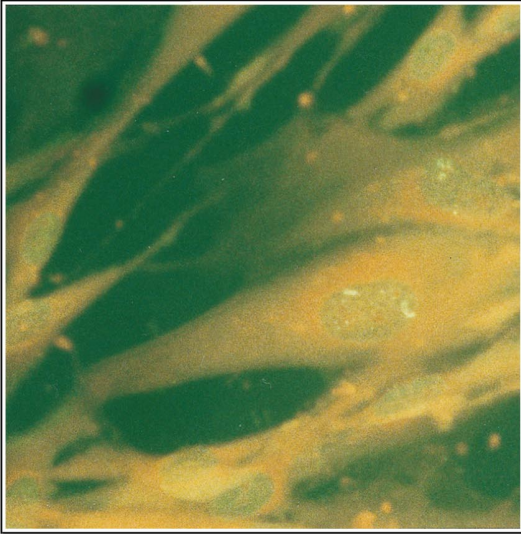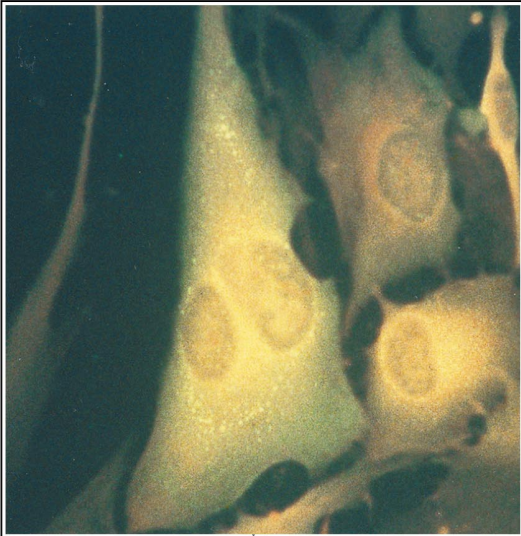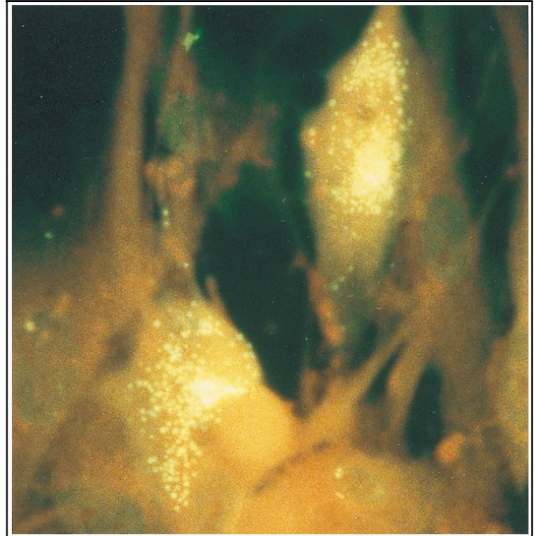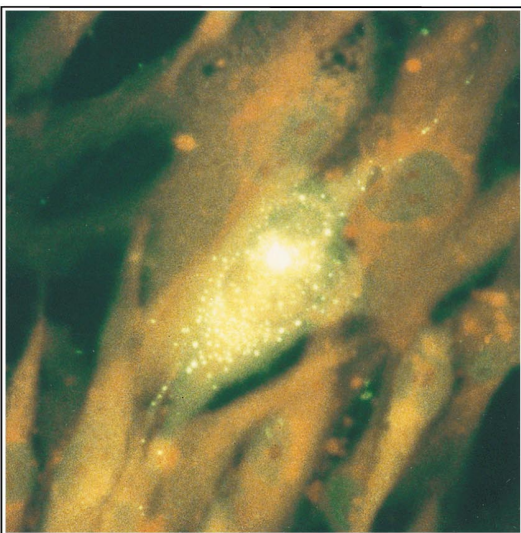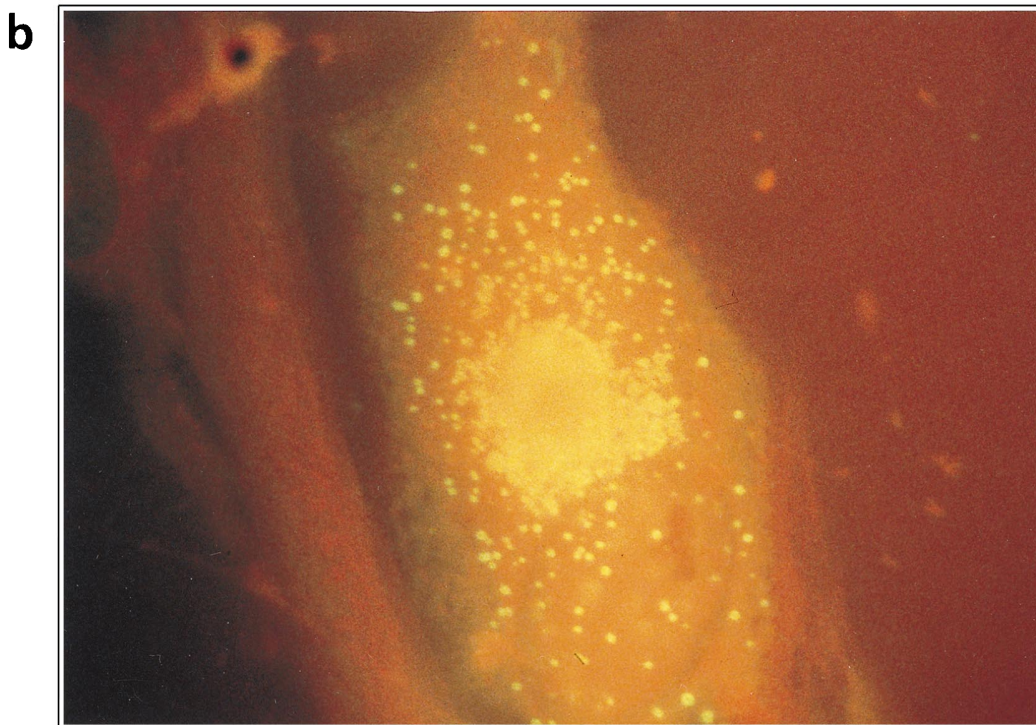
m.i.
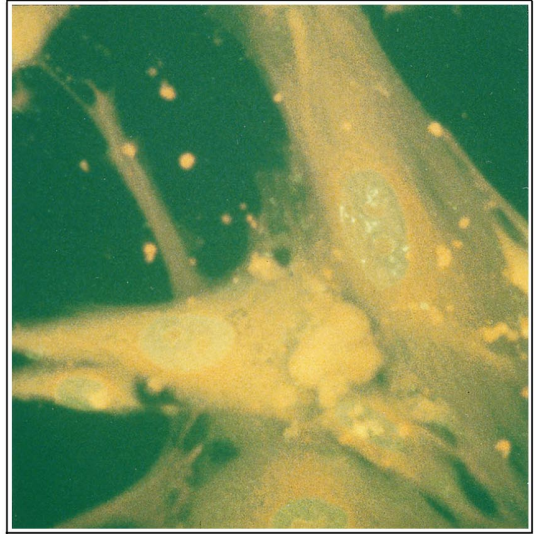
24 h

48 h

72 h

96 h

FIG. 5. (a) Subcellular localization of pUL25 during the HCMV replication cycle by IIF analysis of mock-infected (m.i.) and HCMV-infected HEL cells, using the mouse ascites fluid PAb CK25 (diluted 1:400). Magnification, ×40. (b) Association of pUL25 with cap-like structures. IIF analysis of HCMV-infected human fibroblasts (96 h p.i.) with PAb CK25 is shown. Magnification, ×100.

a 200 mM NaCl buffer X. Finally they were resuspended in SDS gel sample buffer, separated by SDS-PAGE, and analyzed by autoradiography.

RESULTS

Analysis of transiently expressed pUL25-FLAG. In order to specifically detect the product(s) of UL25, a short sequence (24 nucleotides [nt]) encoding a FLAG peptide was inserted at the 3′ end of the UL25 sequence (1,968 nt) by using a suitable lower primer for amplification of the gene from the genome of the AD169 strain (Fig. 1a). The amplified sequence was inserted between the *Kpn*I and *Eco*RV cloning sites of pcDNA3 vector under the control of the HCMV major immediate-early promoter. The resulting construct (pc-25F3) was used to transiently transfect COS7 and U373MG cells. The cell lysates were analyzed by Western blotting with the M2 monoclonal antibody, specific for the FLAG peptide, at 48 h posttransfection. Several bands were detected in lysates of both cell types (Fig. 1b): two very close and more intense bands at approximately 85 to 87 kDa and three other less intense bands at approximately 80, 92, and 100 kDa. The predicted molecular mass for pUL25 is 73.5 kDa, which is lower than the molecular masses of the bands we detected. Posttranslational modifications could account for these differences.

Subcellular localization of pUL25-FLAG. To define the subcellular localization of pUL25-FLAG, COS7 and U373MG cells transfected with pc-25F3 were analyzed by IIF with M2 anti-FLAG monoclonal antibody. The results (Fig. 2) showed an exclusively cytoplasmic localization of pUL25 in both cell types. Furthermore, the staining was mainly associated with very irregular granules or vesicle-like structures. The significance of this peculiar pattern of staining has not been further analyzed, although we speculate that it could be due to an

abundant production of the protein, which might therefore accumulate in cytoplasmic vesicles to be extruded.

Prokaryotic expression of pUL25 and reactivity of anti-pUL25 mouse ascites fluid. In order to obtain the amounts of pUL25 antigen necessary for antibody production in mice, the expression plasmid pE-25CK82 was constructed by inserting the amplified UL25 sequence in frame between the 5′ and 3′ portions of the bacterial CKS gene under the control of the *lacZ* promoter in the pCKS vector. A fusion protein (CKS-pUL25) with a molecular mass of 115 kDa (40 kDa of CKS and approximately 75 kDa of pUL25) was successfully expressed (data not shown). CKS-pUL25 was purified by electroelution and intraperitoneally injected into pristane-treated BALB/c mice for immunization. COS7 cells transiently expressing pUL25-FLAG were used to monitor antibody titers in ascites fluid by both IB and IIF. The reactivity obtained with anti-pUL25 ascites fluid (PAb CK25) was identical to that obtained with M2 antibody (data not shown), thus demonstrating that PAb CK25 could specifically detect pUL25. Moreover, the same results were also obtained by both IB and IIF for the native pUL25, obtained by transient transfection of COS7 cells with pc-25sf7 (data not shown), thus showing that the C-terminal octapeptide FLAG represented a suitable tool to study this protein.

Time course of viral pUL25 expression. In order to investigate the biosynthetic kinetics of pUL25, we examined the expression of this protein during the HCMV replication cycle in human fibroblasts by IB with the mouse ascites PAb CK25. For this purpose, six time points during the HCMV replication cycle were chosen: one at immediate-early phase (3 h p.i.), two during the early phase (8 and 24 h p.i.), and three during the late phase (48, 72, and 96 h p.i.). Figure 3A shows the results
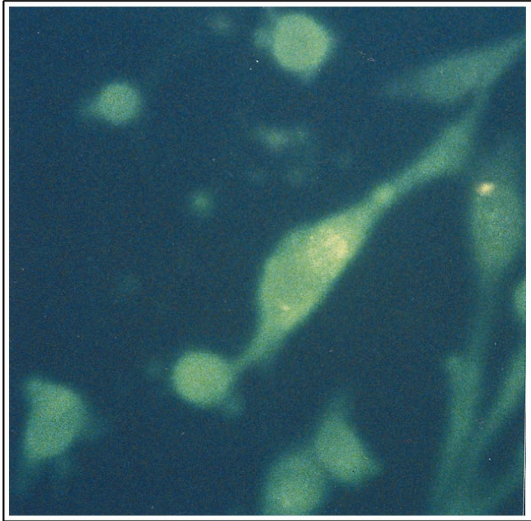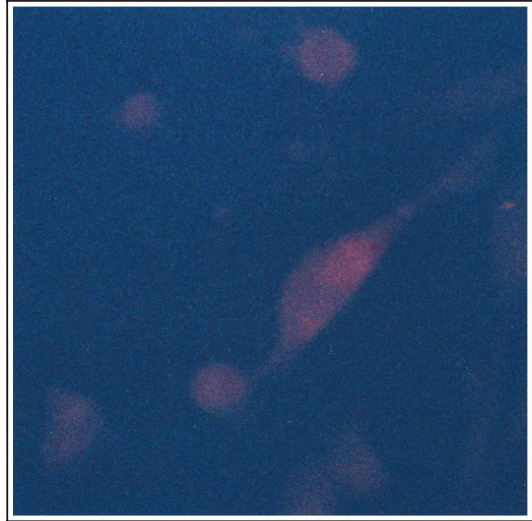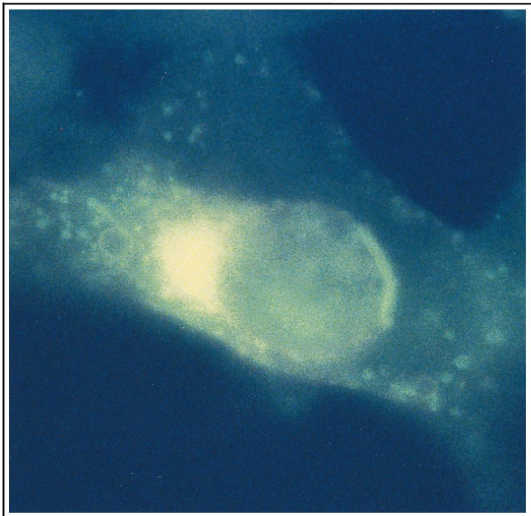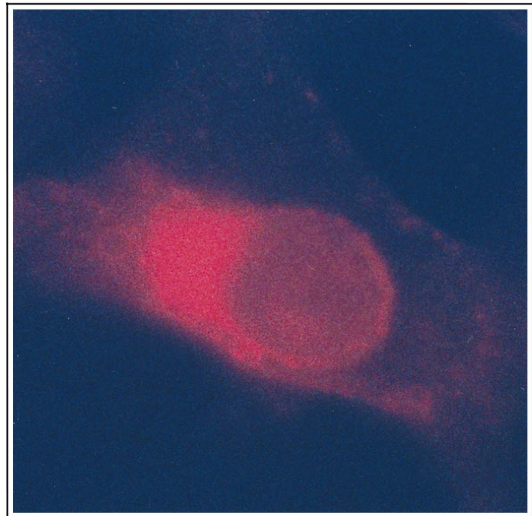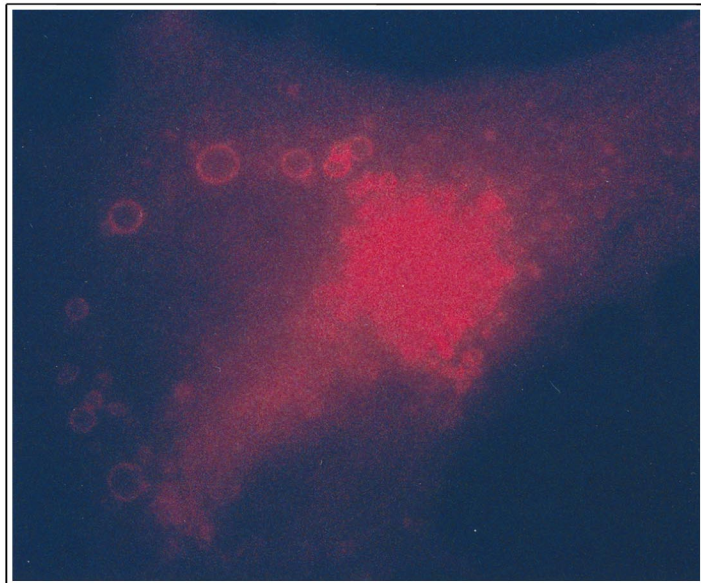
that we obtained. A specific band at approximately 85 kDa was first detected at 48 h p.i. The expression increased at later times, when additional bands, almost identical to those observed for the recombinant protein, pUL25-FLAG, appeared (the difference of approximately 0.9 kDa due to the FLAG peptide was almost undetectable). They were an upper band very close to that corresponding to 85 kDa, two less intense bands at approximately 80 and 92 kDa, and finally a band with an approximate molecular mass of 100 kDa expressed at higher levels at 96 h p.i. Among the multiple forms, only a signal corresponding to 85 to 87 kDa was detected by IB analysis of purified viral particles (data not shown).

The time course of pUL25 expression suggested a late kinetics for this protein; moreover, treatment of infected cells with foscarnet indicated an absolute requirement of DNA replication for pUL25 synthesis (Fig. 3A).

A band corresponding to a molecular mass of 66 kDa was also detected in both infected and mock-infected cells; since it decreased at higher dilutions of antibody, we hypothesized that it is a cell protein with an antigenic determinant(s) in common with pUL25. Further investigations are required to identify the cell protein responsible for this cross-reaction.

Transcriptional analysis of UL25. Whole-cell RNA was isolated from HCMV-infected human fibroblasts at the same time points chosen for the biosynthetic analysis of pUL25 expression. The RNA was analyzed by Northern blotting with the entire UL25 sequence as a specific probe. Sequence analysis revealed the presence of a TATA box consensus sequence 70 nt upstream from the start codon of the UL25 ORF and a poly(A) signal 85 nt downstream from the stop codon. The expected size for this transcript is 2.4 kb. As shown in Fig. 3B, a specific mRNA of comparable size was first observed at 48 h p.i., although the signal was very weak. It increased at later times (the pattern obtained at 96 h p.i. was identical to that observed at 72 h p.i. [data not shown]). No transcription was detectable at earlier times, thus showing a strict correspondence with the late kinetics of pUL25 expression and the absence of any posttranscriptional regulation. Two weaker bands of approximately 4 and 5 kb were also observed; since a double-stranded probe was used for hybridization, this RNA species could correspond to a polycistronic transcript from the opposite strand of the viral genome. This would be in agreement with the presence next to UL25 of several ORFs in the opposite orientation.

Posttranslational modifications of pUL25. The difference between the predicted and observed molecular masses of pUL25 suggested a posttranslational modification. The marked hydrophilicity based on the amino acid sequence and the lack of typical transmembrane sequences, as already pointed out by Dallas and coworkers (8), led us to rule out the possibility that pUL25 was a glycoprotein and to consider that most probably it was modified by phosphorylation. This would be in agreement with the homology of UL25 with HHV-7 U14, which encodes a phosphoprotein localized in the viral tegument (33). To investigate whether pUL25 is phosphorylated, we metabolically labeled HCMV-infected and mock-infected HEL cells with either $^{33}P_i$ or [^{35}S]cysteine; lysates of radiolabeled cells

were then immunoprecipitated (Fig. 4). PAb CK25 precipitated a group of polypeptides which resembled those previously observed for pUL25 in Western blotting, in both ^{35}S- and ^{33}P-labeled lysates (Fig. 4, lanes 1 and 2). The same bands could not be detected in lysates precipitated with a monoclonal antibody specific for another viral protein, pUL83 (Fig. 4, lanes 5 and 6). The considerable correspondence between the ^{35}S and ^{33}P radiolabeling patterns obtained with PAb CK25 indicated that all UL25 polypeptides are modified by phosphorylation.

Subcellular localization of viral pUL25. In order to define the subcellular localization of pUL25 during the HCMV replication cycle, mock-infected and HCMV-infected HEL were subjected to IIF with PAb CK25 at the same time points at which the cells were also analyzed by IB. Figure 5a shows the results obtained starting at 24 h p.i. (at 3 and 8 h p.i., only a low level of staining was observed near the plasma membrane, probably due to the viral inoculum [data not shown]). A background staining was present in the nuclei of uninfected cells, probably due to a cross-reacting cell protein, as already hypothesized from the IB analysis. However, higher dilutions of antibody reduced this to minimum level. No specific nuclear signal was detectable at any time of the HCMV replication cycle, thus confirming the cytoplasmic localization of pUL25, as already established for the recombinant pUL25-FLAG. A positive staining was first observed at 48 h p.i., although it was quite dispersed throughout the cytoplasm. At later times the signal intensity increased and was associated with granular formations that were especially condensed near the nucleus (Fig. 5B).

A similar pattern was previously described for two other HCMV tegument phosphoproteins, pp150 (pUL32) and pp65 (pUL83), during the late phases of the replication cycle, and these condensed structures were referred to as cap-like structures (16). Another virion tegument phosphoprotein, pp28 (UL99), localizes in cytoplasmic vacuoles which contain endosomal markers (30). In order to check whether these vacuoles correspond to the granular formations observed for pUL25, we performed a two-color staining analysis with PAb CK25 and P2G11 (a monoclonal antibody directed against ppUL99). Figure 6 shows colocalization of the two proteins in the same cytoplasmic compartments at 104 h p.i.

DISCUSSION

In this study we have described the characterization of the product of HCMV ORF UL25, pUL25, a protein previously identified by Baldick and Shenk as a structural component of all three forms of viral particles, although present in larger amounts in dense bodies (1). The UL25 ORF is 1,968 nt in length and encodes a 656-amino-acid protein with an estimated molecular mass of 73.5 kDa. We analyzed the properties of the protein both in transient transfections and during viral replication. IB analysis showed the synthesis of multiple polypeptides, ranging in molecular mass from 80 to 100 kDa in both transfected and infected cells. A similar pattern, i.e., multiple bands ranging from 87 to 110 kDa, was observed for the

FIG. 6. (a) Subcellular localization of ppUL99 in human fibroblasts at 104 h after HCMV infection. Cells were incubated with the monoclonal antibody P2G11 and than with the secondary TRITC-conjugated antibody. (b) Subcellular localization of ppUL25 in HEL at 104 h p.i. I, an optical filter allowing the view of the secondary fluorescein-conjugated antibody, which specifically recognizes the ascites fluid PAb CK25, was used. II, subcellular localization of ppUL99 in the same cell, with an optical filter allowing the view of the TRITC-conjugated antibody, which specifically recognizes the monoclonal P2G11. Green and red staining coincide, thus showing that in this phase of the infection cycle, ppUL25 and ppUL99 colocalize in the same subcellular compartment. (c) Cells incubated as for panel b except for the omission of the monoclonal antibody P2G11. Thus, the green filter showed the localization of ppUL25 (I), while the red filter showed that no red staining was present (II), proving that the TRITC-conjugated antibody recognized exclusively the monoclonal P2G11.

murine CMV (MCMV) UL25 product in MCMV-infected cells stained with a specific monoclonal antibody (10).

Our data showed that two bands at 85 and 87 kDa were more abundant than the others, even though the 100-kDa band was strongly expressed at 96 h p.i. IB analysis of purified viral particles showed a single signal corresponding to the intense bands at 85 and 87 kDa, thus indicating that among all the products only these two polypeptides are components of the viral structure. The roles of the other pUL25 polypeptides, unincorporated in virions, remain undetermined. Interestingly, similar findings were reported for another structural protein, pUL69 (36), for which the authors hypothesized that a specific phosphorylation might control the incorporation into virions. This could also be speculated for pUL25, since, like pUL69, it is phosphorylated as demonstrated by radioimmunoprecipitation following parallel labeling of infected cells with $^{33}P_i$ and [^{35}S]cysteine.

Both the transcriptional analysis of the UL25 ORF and the time course of pUL25 biosynthesis indicated a late expression for this gene; furthermore, lack of expression of pUL25 in the presence of foscarnet, an inhibitor of viral DNA synthesis, demonstrated that pUL25, like its MCMV homologue (8), is a true late protein.

Analysis by IIF of subcellular localization of pUL25 proved that the protein localizes exclusively in the cytoplasm in both transient expression and viral infection, thus demonstrating that its cytoplasmic retention is not due to any other viral factors. The cytoplasmic staining was characterized by a grainy consistence, similar to that observed for the murine pUL25 (10). However, the pattern of intracellular localization for pUL25 was quite different in infected cells than in transiently transfected cells; while the granular formations observed in transfected COS7 and U373MG cells were very irregular in size and shape and might be assimilated into vesicle-like structures (Fig. 2), the granules observed in infected human fibroblasts were much more regular and seemed to be condensed in a spot near the nucleus. The subcellular localization of pp28 was similar to that of ppUL25 (Fig. 6). Double staining of infected cells for both proteins showed a colocalization in the same cytoplasmic vacuoles, which were previously shown to contain endosomal markers (30). These endosomal vesicles were hypothesized to be the location of cytoplasmic maturation of virus particles (15), according to the demonstration by Tooze and coworkers (34) that the majority of the progeny virus particles are wrapped by endosomal cisternae derived from tubular early endosomes, which accumulate around the centrioles.

The tegument is certainly the least understood portion of the virion structure; both its function and its acquisition are largely unknown. The majority of tegument proteins are translocated into the nucleus (12, 15, 16, 29, 31, 35), suggesting that the association of the tegument with the nucleocapsid starts within this cellular compartment. However, the final steps of this process should take place in the cytoplasm, where the envelopment of nucleocapsids occurs (34), as further suggested by colocalization of pp28 and the major envelope glycoprotein gB in the same population of cytoplasmic vacuoles (30). Here we demonstrated that ppUL25 also is present in the same subcellular compartments.

The envelopment is most likely mediated by a specific recognition between a viral protein(s) residing within the tegument and a viral protein(s) present in the membrane of the tubular endosomes (34). We suggest that the tegument proteins which are expressed exclusively in the cytoplasm and during the late phases of virus replication are the best candidates for this role, being probably the last to be incorporated.

Furthermore, pUL25, unlike the other tegument proteins known so far (15, 20, 21), is detected in each of the three forms of virus particles (1), which are all surrounded by a similar envelope. For all of these reasons, we think that further investigation into this protein, focusing on its possible interactions with envelope proteins, should provide useful insights into the acquisition and function of the HCMV virion tegument.

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