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A Specific Subform of the Human Cytomegalovirus Transactivator Protein pUL69 Is Contained within the Tegument of Virus Particles

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The polypeptide encoded by the open reading frame UL69 of human cytomegalovirus (HCMV), which is homologous to the immediate-early regulator ICP27 of herpes simplex virus, has recently been identified as a transactivator protein that exerts a broad stimulatory effect on gene expression (M. Winkler, S. A. Rice, and T. Stamminger, J. Virol. 68:3943–3954, 1994). Here, we provide evidence that pUL69 is a phosphorylated tegument protein of HCMV. This finding could be demonstrated by Western blot (immunoblot) analyses with purified virions and a specific antiserum against pUL69. These experiments revealed that one phosphorylated subform of the three pUL69 polypeptides that are synthesized in infected fibroblast cells is contained within the HCMV virion. After the treatment of purified virions with detergents, pUL69 could not be detected within the membrane fraction, suggesting that it is either a capsid or a tegument protein. Its presence within dense bodies, however, shows that pUL69 is a constituent of the viral tegument.

Human cytomegalovirus (HCMV), a member of the beta subgroup of herpesviruses, is a major human pathogen causing severe disease in newborns and immunocompromised patients. It is an enveloped virus with a tegument surrounding the nucleocapsid and thus has the typical structure of a herpesvirus (24).

Of these virion components, the tegument has the most poorly understood structure. According to electron micrographs it forms a spherical shell around the capsid (25). The protein composition has been mainly defined by the principle of exclusion (24). If a virion protein could not be located to the envelope or to the capsid it was assigned to the tegument. Like the structure, the function of the tegument is also largely unknown. Existing data suggest that some tegument proteins play a role in gene regulation which might influence infectivity, especially at low multiplicities of infection (1). For example, in herpes simplex virus (HSV), the prototype alphaherpesvirus, at least three proteins with a function in transcriptional regulation, the so-called VP16, ICP4, and ICP0 proteins, were identified within the tegument (4, 38, 39). Of those, VP16 is essential for the structure of the virus; in addition, this protein appears to be important for efficient viral replication and pathogenicity in certain animal model systems (1, 36). The tegument of other alphaherpesviruses also contains at least one protein with a regulatory function, usually a VP16 homolog (14, 19, 22).

VP16 of HSV directs the induction of immediate-early (IE) gene expression through specific DNA elements in the IE gene promoters; this is mediated via an interaction with cellular transcription factors on infection (26). The IE phase is the first step in a gene expression cascade that herpesviruses establish inside a cell, followed by the early and late phases (5, 8, 9, 17, 34, 35). In contrast to the relatively weak IE promoters of alphaherpesviruses, the expression of IE genes in betaherpesviruses is driven by strong constitutive enhancers (2, 6, 12, 33). Despite this strong constitutive activity, there has long been evidence that structural components of the virion may be able to further increase transcription from these enhancer elements (29, 31). Subsequent studies established that one of the polypeptides contained within the tegument of virions, the so-called upper matrix protein pp71 (ppUL82), is able to transactivate the IE-1/2 enhancer-promoter of HCMV, suggesting that this protein is involved in the initiation of IE gene expression (16).

In an effort to further characterize the regulation of the HCMV replicative cycle, we were searching for *trans*-acting proteins of HCMV in addition to the well-characterized IE proteins. We chose the open reading frame (ORF) UL69 of HCMV because it is a positional homolog of HSV ICP27, which is essential for HSV replication and appears to be necessary for switching from early to late gene expression (28). In accordance with this, we found that pUL69 could function as a transactivator of several viral and cellular promoters (37). However, expression analysis showed that, in contrast to the IE protein ICP27, pUL69 is expressed with early-late kinetics during the HCMV replicative cycle. This finding raised the question of whether pUL69 is incorporated into the virus particle, as described for some other early-late proteins of HCMV.

To investigate this, virions were isolated from HCMV-infected fibroblast cells via density gradient centrifugation (10, 32), and Western blot (immunoblot) analyses were performed in order to detect pUL69. Cell extracts from infected (at 72 h postinfection) and uninfected fibroblasts were used in parallel as positive and negative controls, respectively. As shown in Fig. 1A, lanes 1 to 3, a rabbit antiserum which has previously been shown to react specifically with pUL69 (37) could detect UL69 proteins in virions and HCMV-infected cells but not in uninfected cells. No specific reactivity was seen with a preimmune serum (Fig. 1A, lanes 7 to 9). To assess the purity of the virion preparation we did immunoblot analysis using a monoclonal antibody directed against IE2 proteins of HCMV (monoclonal antibody 2.9.5) (21). Several IE2 polypeptides are expressed during the late phase of the HCMV replicative cycle but are not contained within virions (21). As can be seen in Fig. 1A, lanes 4 to 6, three IE2 proteins with sizes of 40, 63, and 86 kDa

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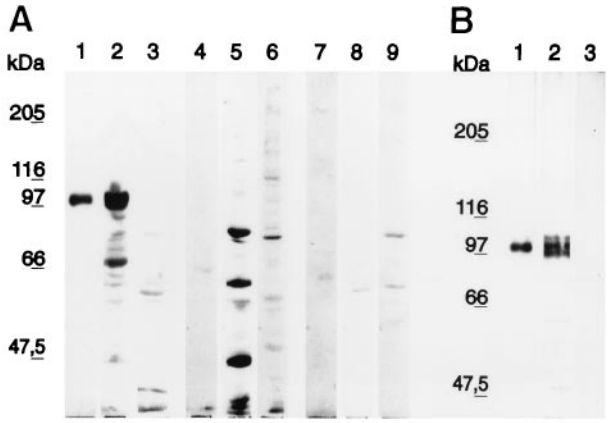

FIG. 1. Detection of UL69 protein of HCMV in purified virus particles by Western blot analysis. (A) Western blot analysis with specific anti-UL69 antiserum (lanes 1 to 3), IE2-specific monoclonal antibody 2.9.5 (lanes 4 to 6) or preimmune serum (lanes 7 to 9). Proteins were separated by SDS–10% high-bis-PAGE. Lanes: 1, 4, and 7, purified HCMV particles; 2, 5, and 8, extracts from HCMV-infected fibroblast cells (at 72 h postinfection); 3, 6, and 9, extracts from mock-infected fibroblast cells. (B) Western blot analysis with specific anti-UL69 antiserum (lanes 1 to 3). Proteins were separated by SDS–6% high-bis-PAGE. Lanes: 1, purified virus particles; 2, extracts from HCMV-infected fibroblast cells (at 72 h postinfection); 3, extracts from mock-infected fibroblast cells. The sizes of molecular mass markers are shown on the left.

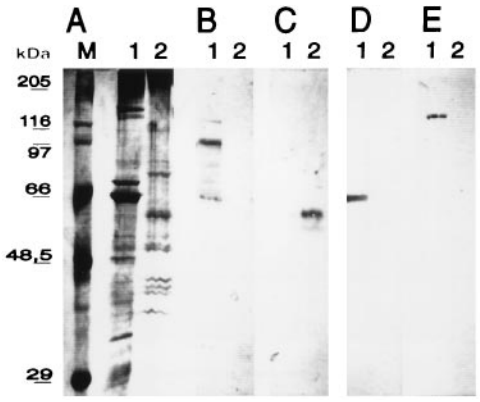

FIG. 2. Detection of various HCMV polypeptides by Western blot analysis after detergent treatment of purified virus particles and separation of solubilized and nonsolubilized proteins. (A) Silver-stained SDS–high-bispolyacrylamide gel; (B) Western blot analysis with specific anti-UL69 antiserum; (C) monoclonal antibody 27-156 (30) against the glycoprotein gp58; (D) monoclonal antibody 28-77 (3) against the tegument protein pp65; (E) monoclonal antibody 28-4 (27) against the major capsid protein of HCMV. Lanes: M, molecular mass markers; 1, insoluble proteins after detergent treatment of virions; 2, solubilized proteins after detergent treatment of virions. The sizes of molecular mass markers are shown on the left.

could be detected in infected fibroblasts but not in virions or in uninfected cells. This excludes the possibility that the pUL69 signal in virions is due to a contamination of the virion preparation with cellular material.

On careful inspection of the immunoblots we detected only one distinct form of the UL69 protein in virions, while at least three were seen in infected cells (Fig. 1A, lanes 1 and 2) (37). To show this more precisely, we separated proteins from infected cells and virions using a 6% high-bispolyacrylamide gel (10) followed by Western blot analysis with the specific UL69 antiserum (Fig. 1B). Comparison of proteins contained within virions and infected cells revealed that only the middle, 110-kDa form of pUL69 could be observed within virions (Fig. 1B, lanes 1 and 2). Thus, one specific subform of pUL69 appears to be incorporated into virus particles.

We next wanted to determine the localization of pUL69 within the virus particle. First, a detergent treatment of purified virions, which should result in a separation of capsid and tegument proteins from polypeptides contained within the envelope, was done (15). After incubation of virions in the presence of the detergents Nonidet P-40 and deoxycholate, the viral material was centrifuged in order to pellet the insoluble capsid and tegument proteins. Afterwards, polypeptides contained within the supernatant and pellet were separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). On inspection of silver-stained gels, typical tegument proteins such as the phosphoprotein pp65 or pp71 were detected in the pellet, whereas a totally different protein pattern could be observed in the supernatant (Fig. 2A, lanes 1 and 2). In order to assess the purity of the obtained fractionation, we performed Western blot experiments using monoclonal antibodies directed against typical envelope, tegument, or capsid proteins. As shown in Fig. 2C to E, the tegument protein pp65 and the major capsid protein (pUL86) could exclusively be detected in the pellet fraction (Fig. 2D and E, lanes 1), while the glycoprotein gp58 was contained only within the supernatant fraction (Fig. 2C, lane 2). Using the specific rabbit antiserum directed against pUL69, we found that the UL69 protein cofractionated with the capsid and tegument proteins in the pellet (Fig. 2B, lane 1). Therefore, pUL69 is not associated with the virion envelope but, instead, is located within the rather tightly associated capsid-tegument structure.

To resolve the question of whether pUL69 is located within the tegument or the capsid, we made use of the observation that dense bodies, which are defective viral particles produced during high-multiplicity infection of fibroblasts, are devoid of capsid proteins (10, 11, 15). HCMV virions and dense bodies were purified via two consecutive gradient centrifugations (10, 32). After separation of the proteins contained within virions and dense bodies by SDS–high-bis-PAGE (10), the purity of the particles was assessed by silver staining of the respective gel (Fig. 3A). In parallel, the protein composition was investigated by immunoblot analysis. Using a monoclonal antibody directed against the major capsid protein of HCMV, we could detect a signal only within the virion fraction (Fig. 3B, lane 1). This finding demonstrated that the dense body fraction was almost free of virions (Fig. 3B, lane 2). After removal of the monoclonal antibody against the major capsid protein by incubation in a buffer containing 1% SDS, the same filter was reprobed with the rabbit serum directed against pUL69. As shown in Fig. 3C, pUL69 could be detected both in virions and in dense bodies. Therefore, the UL69 protein is not located in the HCMV capsid structure. On the basis of these results we conclude that pUL69 is a novel tegument protein of human cytomegalovirus.

Since most characterized tegument proteins of HCMV are phosphoproteins we questioned whether pUL69 is also modified by phosphorylation. For this investigation, HCMV (at 48 h postinfection) and mock-infected fibroblast cells were metabolically labeled for 3 h with either [^{35}S]methionine or $^{32}P_i$. Cells were then lysed in radioimmunoprecipitation assay buffer and used in immunoprecipitation experiments. The immune serum against pUL69 precipitated three polypeptides with sizes of 105, 110, and 116 kDa from lysates of ^{35}S-labeled HCMV-infected cells that could not be detected with the preimmune serum (Fig. 4, lanes 3 and 7). Interestingly, only the 110- and 116-kDa UL69 proteins could be detected in ^{32}P-labeled lysates (Fig. 4, lane 11). While the intensities of the

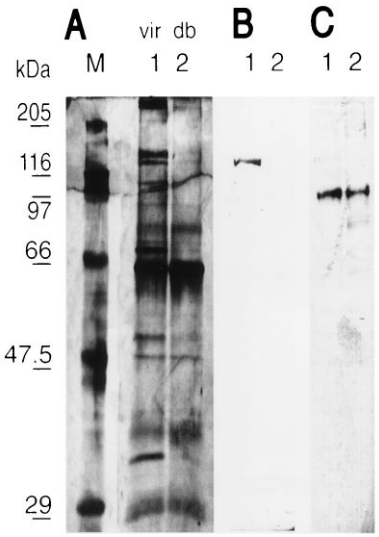

FIG. 3. Western blot analysis of dense bodies and virions. (A) Silver-stained SDS–high-bispolyacrylamide gel; (B) Western blot analysis with monoclonal antibody 28-4 against the major capsid protein of HCMV; (C) Western blot analysis with specific anti-UL69 antiserum. Lanes: M, molecular mass markers; 1, HCMV virions (vir); 2, HCMV dense body particles (db). The sizes of molecular mass markers are shown on the left.

^{32}P-labeled polypeptides were approximately equal (Fig. 4, lane 11), the band formed by the 116-kDa ^{35}S-labeled polypeptide was less intense than that of the faster migrating 110-kDa form (Fig. 4, lane 7). This suggests that the 110- and 116-kDa UL69 polypeptides differ in their extent of phosphorylation, whereas the 105-kDa form corresponds to a nonphosphorylated or considerably less phosphorylated form of pUL69.

In this study we demonstrate that the regulatory protein pUL69 of HCMV is a constituent of the virus particle. Investigations on the virion localization of pUL69 were initiated because of the abundant expression of this protein at late times of the HCMV replicative cycle (37). The evidence for a virion

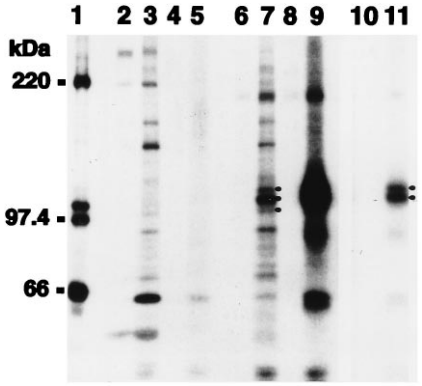

FIG. 4. Immunoprecipitation of UL69 polypeptides from infected cells lysed in radioimmunoprecipitation assay lysis buffer. Human fibroblast cells were labeled with either [^{35}S]methionine (lanes 2, 3, 6, and 7) or $^{32}P_i$ (lanes 4, 5, and 8 to 11) from 48 to 51 h after mock infection (lanes 2, 4, 6, 8, and 10) or after infection with HCMV strain AD169 (lanes 3, 5, 7, 9, and 11). Immunoprecipitates were prepared with preimmune serum (lanes 2 to 5) or anti-UL69 immune serum (lanes 6 to 9). Lanes 10 and 11 show a shorter exposure of lanes 8 and 9, respectively. The various forms of pUL69 that could be detected after immunoprecipitation analysis of labeled cell lysates are indicated by the closed circles to the right of lanes 7 and 11. Lane 1 contains a molecular mass standard. The sizes of molecular mass markers are shown on the left.

localization is based primarily on the immunological detection of pUL69 in purified virions with a rabbit antiserum which has previously been demonstrated to react specifically with pUL69 (37). We could exclude that the pUL69 signal in virions was due to a contamination with viral proteins from infected cells as IE2 polypeptides, which are also expressed in abundant amounts during the late phase of the HCMV replicative cycle (21), could not be detected in purified virus particles.

A further confirmation of the virion localization was the finding that only one specific subform of pUL69 was observed in purified virions. Instead, at least three forms of the UL69 protein are synthesized in HCMV-infected cells (37). We would have expected to detect all forms of pUL69 in the virion preparation if contaminating proteins from infected cells were responsible for the signal. However, only the 110-kDa subform of the UL69 protein was present. As all three proteins can be produced by the isolated expression of the UL69 ORF in COS-7 cells (37), the 110-kDa subform must be derived by posttranslational modification. In vivo labeling of infected cells with $^{32}P_i$ together with immunoprecipitation experiments demonstrated that the two more slowly migrating forms of pUL69 are clearly modified by phosphorylation. Thus, the incorporation into virions might be controlled by a specific phosphorylation of the UL69 protein. A similar role of posttranslational modifications in the packaging of virion proteins has been proposed for the virion host shutoff protein of HSV type 1, which also exists in various subforms that differ in their extent of phosphorylation (23).

Like the virion regulatory proteins of the other herpesviruses, the UL69 protein of HCMV could be localized within the tegument of virus particles. There are two lines of evidence for this. First, the UL69 protein could not be solubilized by detergent treatment of virions as has been demonstrated for envelope proteins (15). Instead, it remained associated with the rather stable particles consisting of capsid and tegument proteins (7). Second, a comparison of virions and dense bodies showed that pUL69 is contained within both types of particles, whereas the major capsid protein is found only within virions (10, 15). While it is suggested that dense bodies do not have a full complement of tegument polypeptides, the important point here is that dense bodies are absolutely devoid of capsid proteins (10). This finally shows that pUL69 must be a constituent of the tegument of HCMV.

The regulatory properties of pUL69 have first been demonstrated for a homologous early promoter, the UL112/113 promoter of HCMV, and several heterologous promoters such as the Rous sarcoma virus long terminal repeat (37). As a virion component we were interested to see whether it could also activate IE gene expression, in particular, the IE1/2 enhancer/promoter of HCMV. Cotransfection experiments revealed approximately 10-fold stimulation of the IE-1/2 enhancer/promoter by pUL69 (37a), which is approximately the same strength of activation as that reported for another virion-associated regulatory polypeptide, the tegument protein pp71 (16). Therefore, pUL69 constitutes a second regulatory protein within the virion which is able to augment expression from this enhancer containing promoter region. However, the exact contribution of both pUL69 and pp71 to the initiation of viral replication in various cell types remains to be determined.

The characterization of the UL69 ORF was initially undertaken because it is a positional homolog of ICP27 of HSV, which is an essential regulatory protein (28). As the positional homologs of other herpesviruses have also been identified as regulators of gene expression, one could assume that these proteins have a conserved function within the viral replicative cycle. However, on initial characterization of pUL69 we found

differences in the expression patterns and the transacting properties between pUL69 and ICP27 (37). We now add a further difference, as pUL69 is located within virions, whereas ICP27 is not (39). This result fits into an emerging picture of functional and structural variations between these positional homologs, as recent reports demonstrate differences between ICP27 and the varicella-zoster virus homolog, the protein encoded by ORF 4 (ORF4), which could also be detected in varicella-zoster virus particles (13, 18, 20). Therefore, these proteins may serve as a model for the understanding of regulatory protein evolution during the emergence of different herpesvirus species.

In summary, we have defined a novel tegument protein with transacting properties and extended the differences between pUL69 and ICP27. Further studies will have to show the importance of pUL69 by use of viral mutants and will shed more light on the role of pUL69 in the HCMV replicative cycle and the biological function of this family of homologous proteins.

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