The products of human cytomegalovirus genes UL23, UL24, UL43 and US22 are tegument components

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We have investigated the human cytomegalovirus (HCMV) US22 gene family members UL23, UL24, UL43 and US22. Specific antibodies were generated to identify pUL23 (33 kDa), pUL24 (40 kDa) and pUL43 (48 kDa), while pUS22 was identified by monoclonal antibody HWLF1. A C-terminally truncated UL43 product (pUL43t; 21 kDa) produced by a deletion mutant was also investigated. The UL24 and UL43 genes were expressed with early-late (γ1) and true-late (γ2) kinetics, respectively. Immunoblot and immuno-EM studies demonstrated that pUL23, pUL24, pUL43 and pUS22 were virion tegument components. Immunofluorescence and immuno-EM studies showed that pUL23, pUL24, pUL43 and pUL43t were located in cytoplasmic protein aggregates, manifesting two forms: complex juxtanuclear structures and smaller, membrane-bound aggregates resembling dense bodies. The complex-type aggregate is a putative site of particle maturation. Because pUL43t was present in protein aggregates, but under-represented in virus particles compared to pUL43, it was concluded that N-terminal sequences target pUL43 to protein aggregates and that C-terminal sequences are important for incorporation into particles. Since three other US22 family products (pUL36, pTRS1 and pIRS1) are documented tegument components, at least seven of the twelve US22 family genes encode tegument proteins, suggesting that the products of the remaining five genes might be similarly located. These findings demonstrate a common biological feature among most, if not all, US22 family proteins and implicate the family in events occurring immediately after virus penetration.

Introduction

Human cytomegalovirus (HCMV), a member of the Betaherpesvirinae, has a large double-stranded DNA genome (230 kbp) containing 208 ORFs that potentially encode proteins (Chee et al., 1990). We are investigating the HCMV US22 gene family with the view to comparing the functions provided by individual members. The family contains 12 members (UL23, UL24, UL28, UL29, UL36, UL43, TRS1, IRS1, US22, US23, US24 and US26), each encoding products having at least one of four conserved amino acid sequence motifs (Kouzarides et al., 1988; Efstathiou et al., 1992). The roles of these motifs in protein function are not known. US22 genes are also coded by other members of the Betaherpesvirinae, including human herpesviruses 6 and 7 (HHV-6 and HHV-7), murine cytomegalovirus (MCMV), rat cytomegalovirus (RCMV) and tupaia herpesvirus (TuHV) (Gompels et al., 1995; Nicholas, 1996; Megaw et al., 1998; Rawlinson et al., 1996; Vink et al., 2000; Bahr & Darai, 2001). Each virus has a similar number of US22 family genes, suggesting that the family plays an important role in the betaherpesvirus life-cycle.

Relatively few US22 gene family members have been investigated. The HCMV US22 gene itself is expressed with early kinetics and specifies a nuclear/cytoplasmic protein of unknown function, which is secreted into the extracellular medium (Mocarski et al., 1988). The UL36 gene encodes a potent inhibitor of Fas-mediated apoptosis that operates via interference with caspase 8 activation (Skaletskaya et al., 2001). In transient transfection assays, the IRS1 and TRS1 gene products (pIRS1, pTRS1) enhance expression from the viral UL44 and UL54 promoters, when operating in concert with the major immediate-early (IE) gene products IE1 (72 kDa) and IE2 (86 kDa) (Stasiak & Mocarski, 1992; Kerry et al., 1996). Similarly, pTRS1 and pIRS1 cooperate with the virion transactivating protein pUL69 to enhance transcription from
the HCMV major IE promoter (Romanowski & Shenk, 1997). In analogous transient transfection studies, members of the HHV-6 US22 gene family (U3, DR7, U16 and U25) trans-activated the HIV-1 LTR promoter (Mori et al., 1998; Geng et al., 1992; Kashanchi et al., 1994; Nicholas & Martin, 1994). These various findings suggest that at least some members of the US22 family encode products with gene regulatory functions.

We have begun our studies of the HCMV US22 gene family by investigating the US22, UL23, UL24 and UL43 gene products. We have included in our investigation a deletion mutant virus (∆UL42/UL43) that makes a C-terminally truncated version of pUL43 (pUL43t) (Dargan et al., 1997). The deletion abrogates expression of gene UL42 and removes 3’-terminal coding sequences from UL43. The function provided by pUL42 is unknown, but it has some characteristics of a membrane protein. In the mutant the UL43 ORF terminates three codons downstream from the deletion point producing a truncated product consisting of the first 187 amino acids of the 423 amino acid product. Wild-type pUL43 contains all four US22 family amino acid sequence motifs, but only the first motif and a partial copy of the second are retained in pUL43t (Dargan et al., 1997).

We find that UL24 and UL43 are expressed with early-late (γ1) and true-late (γ2) kinetics, respectively, and that pUS22, pUL23, pUL24 and pUL43 are components of the virion tegument. pUL23, pUL24 and pUL43 are located in cytoplasmic protein aggregates which appear to be sites of virion maturation. pUS22, however, was distributed throughout the cell. pUL43 N-terminal amino acid sequences are involved in translocation to protein aggregates, whereas C-terminal sequences are required for efficient incorporation into particles.

Methods

■ Cell lines. Human fibroblast cells (HFFF-2), grown in Dulbecco’s MEM supplemented with 10% foetal calf serum (DMEM/F), were used throughout.

■ Viruses. The HCMV strain AD169 (wild-type) and AUL42/UL43 (deletion mutant) viruses were described previously (Dargan et al., 1997).

■ PCR primers/cloning. Primers for UL23, UL24 and UL43 ORFs were amplified by PCR from the HCMV (AD169) HindIII Y fragment (Oram et al., 1982), the HCMV AD169 cosmid fragments (Cos 65; nucleotides 23495–60934) and the cosmid fragment Cos 15 (Dargan et al., 1997), respectively, using oligonucleotide primers. The 5’ primers for UL23, UL24 and UL43 were 5’ CGGAATTCCTGCAATGCTGGA-TCAAGGACTTCTC 3’, 5’ AATGAAATTCGACGATATGCA- GGAGACCCGGGGACGTTAT 3’ and 5’ GGAATTCAGTCG- ACATGGGAAGACCCGGGGACGTTAT 3’, respectively. The 3’ primers for UL23, UL24 and UL43 were 5’ TATTCTCGAGTCACCGGGGTGCTGA- CGTCCTTGGGGCA 3’ and 5’ CGCGTCGACTCCCATCACACG- AAAGAG 3’, respectively. In each case the 5’ primer had EcoRI (GAATTC) and SalI (GTCGAC) linker sequences immediately upstream of the ATG initiation codon (in bold), while the 3’ primer had a XhoI (CTCGAG) linker sequence immediately downstream from the stop codon (complement in bold). The UL23, UL24 and UL43 PCR products (0–9, 1–1 and 1–2 kbp, respectively) were digested with EcoRI and XhoI, recovered from agarose gels, and cloned in-frame into GST fusion vectors (UL23 and UL24 into pGEX-6P-1 and UL43 into pGEX-6P-3; Pharmacia). The sequences of the UL23, UL24 and UL43 ORFs in the GST fusion vectors were confirmed by DNA sequencing.

■ Antibodies. pGEX/UL23, pGEX/UL24 and pGEX/UL43 GST fusion plasmids were transfected into E. coli (strain BL-21) which were treated with 0.1 mM IPTG at 37 °C for 4 h. The fusion proteins were largely insoluble and were purified from inclusion body preparations by SDS–PAGE, then recovered from gel slices by electro-elution. The preparations were then used to immunize rabbits (50 µg per immunization) or mice (20 µg per immunization) to generate polyclonal or monoclonal antibodies, respectively. Antipeptide antibodies (PtdAb) were also raised in rabbits (50–100 µg per injection) against synthetic branched peptides derived from amino acid sequences at the C terminus of UL23 (ADDELQHDVGYP) and UL24 (DPVRDVIYGNHSFR).

The anti-US22 (HWLF1) and anti-UL99 (pp28) mAbs were obtained from Santa Cruz Biotechnology Inc., and the anti-8 (CMV-023-40154) mAb from Capricorn Products Inc. The AP33 control mAb, directed against the hepatitis C virus E2 protein, was the gift of Dr A. Patel (Glasgow).

■ Western immunoblots. Polypeptides were electro-transfered to Hybond ECL membrane (Amersham). The blot was treated with blocking buffer [PBS containing 0.005% Tween 20 (PBST) and 5% dried milk powder] for 2 h at 37 °C, washed five times with PBST (3 min per wash) and incubated with primary antibody (1:500 dilution of pAbs, or PtdAbs, prepared in PBST containing 1% BSA, or mAbs as undiluted hybridoma cell culture medium) for 2 h at 37 °C. The membrane was then washed, incubated with the secondary antibody (HRP-conjugated donkey anti-rabbit or goat anti-mouse IgG, as appropriate) for 1 h at 37 °C, washed for the final time, and then treated with ECL-reagents (Amersham) and exposed to film.

■ Immediate-early, early and late HCMV proteins. For IE proteins, cells were maintained in the continuous presence of 200 µg/ml cycloheximide (CHX) from 1 h prior to infection until 18 h post-infection (p.i.). Cells were infected with AD169 at an m.o.i. of 10 p.f.u. per cell, and actinomycin D (Act D) was added to the cultures at 18 h p.i., to a final concentration of 5 µg/ml for 30 min. CHX was removed by three washes with DMEM/F containing 5 µg/ml Act D, and the cultures incubated for 3 h at 37 °C. For early (E) proteins, HFFF-2 cells were infected at 10 p.f.u. per cell and grown in the continuous presence of 300 µg/ml phosphonoacetic acid (PAA) for 72 h. Late proteins (L) were prepared similarly, except that no drugs were added and the infected cell extract was harvested at 96 h p.i.

■ Immunofluorescence. Cells grown on glass coverslips were either mock-infected or infected with AD169 or ∆UL42/UL43 at 1 p.f.u. per cell. Cells were processed for immunofluorescence as described by Sanchez et al. (2000). The fixed, permeabilized cells were incubated with blocking buffer (20% normal goat serum in PBS) for 45 min at 37 °C and then incubated with primary antibody (anti-UL24 mAb 116, anti-UL43 mAb 92 or control mAb AP33) for 60 min at 37 °C. After washing, the cells were then treated with FITC-conjugated anti-mouse secondary antibody (45 min at 37 °C), fixed with 2% paraformaldehyde in PBS for 10 min at room temperature (t.t.), mounted in anti-fade buffer (Citifluor) on glass slides and viewed under UV illumination.

The HCMV tegument contains a strong Fc-binding receptor (Stannard & Hardie, 1991). To control for non-specific Fc-binding, treatments with the second antibody alone, and with a non-HCMV control mAb (AP33)
having the same IgG1 subtype as the UL23, UL24 and UL43 mAbs, were included.

- **Virus particle purification and negative-stain immunogold electron microscopy.** Extracellular AD169 or ΔUL42/UL43 particles were purified by banding on glycerol–potassium tartrate gradients (Irmiere & Gibson, 1983). Particle numbers were determined by direct counting in the EM. Capsid/ tegument and envelope fractions were prepared by treatment of virions with 1.0% Triton X-100 in PBS for 30 min at 4 °C, followed by centrifugation (13,000 r.p.m. for 15 min at r.t. in an MSE microfuge). For immunogold EM investigation, particles were adsorbed on Parlodion-coated nickel EM grids and then treated for 5 h at r.t. with primary antibody, control mAb AP33 or PBS alone. After washing, the particles were treated with goat anti-mouse IgG conjugated to gold particles (Nanoprobes Inc.). Following further washing the preparations were negatively stained with phosphotungstic acid and examined in a JEOL 100S electron microscope.

- **Thin-section immunogold EM.** HFFF-2 cells were infected with AD169 or ΔUL42/UL43 at an m.o.i. of 5 p.f.u. per cell. At 96 h p.i., the cells were scraped into PBS, pelleted in a BEEM capsule (TAAB laboratories) and fixed with 2.5% glutaraldehyde in PBS. The cell pellet was dehydrated through a series of increasing ethanol concentrations up to 100% and then permeated with acrylic resin (Unicryl) for 8 h at r.t. The cell pellet was embedded in fresh resin, which was then polymerized by exposure to UV light at —15 °C for 4 days. Thin cell sections (70–80 nm) were cut and treated with primary antibody, control mAb AP33 or PBS alone for 5 h at r.t. After washing, the sections were treated with goat anti-mouse IgG conjugated to gold particles. The sections were fixed with osmium tetroxide vapour for 2 h at r.t., stained with uranyl acetate (saturated solution in 1:1 ethanol–water), counter-stained with lead citrate, and examined in a JEOL 100S electron microscope.

**Results**

**Identification of the UL23, UL24 and UL43 gene products**

Monoclonal and polyclonal antibodies were raised against GST/UL23, GST/UL24 and GST/UL43 fusion proteins and anti-peptide antibodies were raised in rabbits against pUL23- and pUL24-related branched peptides. In order to identify the UL23, UL24 and UL43 protein products, the GST fusion proteins were cleaved with Pre-Scission protease (Pharmacia) to yield the viral protein, which was then identified in immunoblots by reaction with the cognate set of prepared antibodies. Representative immunoblots are shown in Fig. 1(a).

Proteins of 48 kDa (pUL43) and 74 kDa (residual GST/UL43) were identified by anti-UL43 mAb 92; proteins of 33 kDa (pUL23) and 59 kDa (residual GST/UL23) were recognized by anti-UL23 PtdAb 683; and proteins of 66 kDa (residual GST/UL24), 44 kDa and 40 kDa were recognized by anti-UL24 pAb 675. The sizes of pUL43 (48 kDa) and pUL23 (33 kDa) determined by SDS–PAGE closely correspond to those predicted from the DNA sequences (47703 and 32,950 Da respectively) (Dargan et al., 1997; Chee et al., 1990). The predicted size of the pUL24 product is 40,186 Da, which corresponds to the major band detected by the anti-UL24 antibody. Since the 44 kDa band was not detected in infected cell extracts it is likely that this represents a bacterial protein contaminant which is also recognized by pAb 675.

**Temporal class of UL24 and UL43 gene expression**

HFFF-2 cells were either mock-infected or infected with AD169 at an m.o.i. of 10 p.f.u. per cell and grown under conditions for synthesis of IE, E or L viral proteins. pUL24 (40 kDa) was detected in immunoblots in the late protein extract and in minor amounts in the early extract. pUL43 (48 kDa), however, was detected only in the late protein extract, indicating that pUL43 is expressed with true-late (γ1) gene kinetics. pUL43 (48 kDa), however, was detected only in the late protein extract, indicating that pUL43 is expressed with true-late (γ2) gene kinetics (Fig. 1b). These temporal class designations were confirmed by time-course experiments; pUL24 was detected from 12 h p.i., and pUL43 not detected before 60 h p.i. (data not shown). The sizes of pUL24 and pUL43 made in infected cells corresponded closely to the sizes of the proteins produced in bacteria (Fig. 1a), suggesting that neither protein is extensively modified.

The kinetics of pUL23 expression could not be determined since it was never detected by Western immunoblotting of
Fig. 2. pUL24, pUL43, pUL43t and pUS22 are associated with purified preparations of NIEPs, virions and dense bodies. Immunoblots of protein extracts of AD169 or ΔUL42/UL43 (pUL43t) derived NIEP, virion and dense bodies probed with anti-UL24 (mAb 116), anti-UL43 (mAb 92) and anti-US22 (HWLF1). Greater amounts (10-fold) of virion and dense body protein extract (1 \times 10^{10} particle equivalents) were loaded in experiments to detect pUL43t and pUS22, but insufficient NIEPs were generated to allow for the increased loading. The locations of marker proteins are indicated.

We have also consistently found pUL23 to be associated with purified virions (see Figs 3 and 4), demonstrating that the UL23 gene is expressed in infected cells. Like its full-length counterpart, pUL43t (21 kDa) corresponds closely to its predicted size (20993 Da; Chee et al., 1990), and contains the epitope recognized by mAb 92. However, ΔUL42/UL43 particles contained lower relative amounts of pUL43t, suggesting inefficient incorporation of the truncated protein into the particle compared with pUL43. To demonstrate the presence of pUL43t or pUS22 in virions and dense bodies, 10-fold more protein extract was loaded than for the pUL24 and pUL43 immunoblots (Fig. 2).

Anti-UL24 mAb 116 and anti-UL43 mAb 92 both identified an additional NIEP-specific band running below pUL24 and pUL43 in SDS–PAGE gels (Fig. 2). The identity of these proteins is not clear, but it is likely that they represent cross-specific antibody reactions with contaminating cellular proteins. However, the possibility that they represent NIEP-specific cleavage products of pUL24 and pUL43 cannot be excluded.

To confirm that pUS22, pUL23, pUL24, pUL43 and pUL43t were indeed particle proteins and not simply associated with co-purifying infected cell material, banded particles were investigated by immuno-gold, negative-stain EM. Particles treated with the negative-control mAb (AP33), the secondary antibody alone or particles with intact envelopes were not labelled. pUL23, pUL24, pUL43, pUL43t and pUS22 were detected in tegument material adhering to capsids (Fig. 3) and in matrix material issuing from dense bodies with damaged envelopes (data not shown).

To confirm that pUS22, pUL23, pUL24 and pUL43 were indeed components of the tegument, the virus envelope was removed from AD169 virions by treatment with detergent. After centrifugation, the pelleted capsid/t tegument and soluble envelope fractions were probed by immunoblotting. pUL23, pUL24, pUL43 and pUS22 were each detected in intact virions and were retained in the capsid/t tegument fraction following solubilization of the viral envelope (Fig. 4). pUL23 appeared to be present in the particle in very low abundance. As before, 10-fold more virion protein extract was used in experiments to detect pUL23 and pUS22. To assess efficient de-envelopment of the particles, immunoblot membranes were routinely stripped and re-probed with a control antibody directed against the gB envelope protein (Fig. 4). Since the proteins comprising the capsid shell have been identified (reviewed by Butcher et al., 1998), pUS22, pUL23, pUL24 and pUL43 are deduced to be tegument proteins.

Intracellular location of pUL23, pUL24, pUL43 and pUL43t

HFFF-2 cells grown on glass coverslips were either mock-infected or infected with AD169 or AUL42/UL43 at an m.o.i. of 0-1 p.f.u. per cell and processed for UV immunofluorescent microscopy at 96 h p.i., using anti-UL24 mAb 116 or anti-

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<td>97K</td>
<td>pUS22 (76K)</td>
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HCMV tegument proteins

Fig. 3. Immunogold labelling of virus particles. Negatively stained AD169 (a, c, d, e and f) and ΔUL42/UL43 (b) virions were probed with (a and b) anti-UL43 (mAb 92), (c) UL23 (mAb266), (d) anti-UL24 (mAb116), (e) anti-US22 (mAb HWLF1) and (f) control non-HCMV mAb (AP33) and then with an anti-mouse secondary antibody conjugated to 10 nm (a, b, c, d and f) or 5 nm (e) gold particles. The location of gold-tagged target proteins in the micrographs is shown by the small black dots. Capsid structures are 100 nm.

UL43 mAb 92 as probes. Virus-infected cells treated with control mAb AP33 (Fig. 5a), or mock-infected cells treated with mAb AP33, anti-UL24 mAb 116 or anti-UL43 mAb 92 (not shown), exhibited only background fluorescence. In infected cells, pUL24 and pUL43 were located in a discrete juxtanuclear compartment in the cytoplasm (Fig. 5c, d) where they exhibited a punctate staining pattern (Fig. 5e, f). In cells infected with ΔUL42/UL43, pUL43t was also located in the juxtanuclear structure but was additionally present within bodies distributed throughout the cytoplasm (Fig. 5b).

Immunofluorescence studies using the anti-UL23 mAb 266 did not show a clear or consistent fluorescence over background level that would permit the location of pUL23 to be determined. pUS22 has been reported to be present in both the nucleus and the cytoplasm of infected cells (Mocarski et al., 1988).

The intracellular location of pUL23, pUL24, pUL43 and pUL43t in infected cells was further investigated by thin-section immuno-gold EM (Fig. 6). Each of these proteins was largely contained within cytoplasmic protein aggregates of two morphological forms. One was bounded by a membrane and resembled dense bodies (Fig. 6a, b). The membrane-bound aggregates were generally smaller, predominated in ΔUL42/UL43-infected cells and were more widely distributed throughout the cytoplasm than in AD169-infected cells, where they were largely confined to the perinuclear region. The other form was observed in AD169-infected cells as large complex structures (up to about 2 μm in diameter) located close to the nuclear membrane and appeared to lack an enclosing membrane (Fig. 6c–f). The fluorescent juxtanuclear structures (Fig. 5) and the protein aggregates appear to be the same structures. The complex-type aggregates were characterized by the presence...
of spherical microvesicles (40–50 nm diameter) with a concentric double ring appearance (Dalton, 1975) (Fig. 6c–f) embedded within the aggregate. Non-enveloped, tegumented virus particles with or without a DNA core were frequently associated with complex-type aggregates, suggesting acquisition of tegument at that site (Fig. 6c, f). The small membrane-bound aggregates were not associated with virus particles, were apparently devoid of microvesicles (Fig. 6a, b), and appeared to be derived from the complex-type aggregates by envelopment of the matrix (Fig. 6d).

**Discussion**

Antibodies were generated to identify the proteins specified by the HCMV US22 gene family members pUL23 (33 kDa), pUL24 (40 kDa) and pUL43 (48 kDa). pUL24 and pUL43 were made with early-late (γ1) and true-late (γ2) gene kinetics, respectively. These findings are in accord with the kinetics of transcript expression: both UL23 and UL24 transcripts are rare early-late (γ1) messages (P. Akter & A. Davison, personal communication) while the UL43 transcript is late (Chambers et al., 1999). Individual US22 family members fall into different classes with respect to gene expression: UL36, TRS1 and IRS1 are IE (α) genes (Tenney & Colberg, 1991; Romanowski & Shenk, 1997; Chambers et al., 1999); US22 and US26 are early (β) genes (Mocarski et al., 1988; Chambers et al., 1999), and UL23, UL24, UL29 and UL43 are late (γ1 and γ2) genes (Chambers et al., 1999; P. Akter & A. Davison, personal communication, and this report), suggesting that the family per se might operate during each phase of viral gene expression.

In immunofluorescence experiments, pUL43 and pUL24 were located in a cytoplasmic juxtanuclear structure resembling that described by Sanchez et al. (2000) and suggested to be a site of virus particle tegumentation and maturation. EM studies correlated the fluorescent juxtanuclear structure with numerous protein aggregates containing pUL23, pUL24, pUL43 and pUL43t. The complex-type aggregate appeared to lack a limiting membrane, but microvesicles were embedded in the matrix suggesting a possible association with the post-Golgi
network. Virus particles were frequently located at the peripheral surface of complex aggregates.

Cytoplasmic protein aggregates are well-documented in HCMV-infected cells (Severi et al., 1992) and have been shown to contain tegument proteins [pp150 (UL32), pp65 (UL83) and pp28 (UL99)] and virus envelope glycoproteins [gB (UL55), gH (UL75) and gp65] (Landini et al., 1987; Hensel et al., 1995; Sanchez et al., 2000). To establish a link with the structures reported by others, we have confirmed that pp28 was present in protein aggregates (data not shown). Since the
Fig. 6. pUL23, pUL24, pUL43 and pUL43t are present in cytoplasmic protein aggregates. Electron micrographs of thin sections of HFFF-2 cells infected with AD169 (a, c, d, e and f) or ΔUL42/UL43 (b) and harvested at 96 h p.i. Cell sections were probed with (a–d) anti-UL43 (mAb 92), (e) anti-UL24 (mAb 116) or (f) anti-UL23 (mAb 266) and subsequently treated with anti-mouse antibody conjugated to 10 nm (a, b, c and d) or 5 nm (e and f) gold particles. (a) pUL43 in small membrane-
pUL23, pUL24 and pUL43 tegument proteins are almost exclusively located in protein aggregates, our data support the view of Sanchez et al. (2000) that the juxtanuclear structure is a site of HCMV tegumentation. However, as pUS22 was present in all cell compartments (not shown) we cannot rule out the possibility that pUS22 is acquired at a different site.

Biochemical and electron microscopy experiments confirmed the presence of pUS22, pUL23, pUL24 and pUL43 in the virion tegument. Incorporation of pUL43 into particles was greatly reduced relative to pUL43, suggesting that pUL43 C-terminal sequences are important for efficient incorporation of pUL43 into the virus particle, although we cannot exclude the possibility that the UL42 gene, also deleted in ΔUL42/UL43, may also be involved. Since pUL43t was present in protein aggregates, sequences located in the N-terminal half of pUL43 must be involved in targeting pUL43 to these structures.

In addition to US22, UL23, UL24 and UL43, three other HCMV US22 family proteins (pUL36, pTRS1 and pIRS1) are documented tegument components (Patterson & Shenk, 1999; Romanowski et al., 1997). Thus, seven of the twelve US22 family genes encode tegument proteins, raising the expectation that the remaining five family members also code tegument components. This important finding demonstrates, for the first time, a common biological feature of most, if not all, US22 family members and implicates the family in events that occur during the early stages of infection. It is unlikely that US22 family tegument proteins perform a crucial role in virion architecture since pUL36, pIRS1 and pUL43 at least are dispensable (Patterson & Shenk, 1999; Jones & Muzithras, 1992; Dargan et al., 1997). Rather, the family probably facilitates the initial stages of infection. This does not, however, exclude the possibility of additional functions, performed at different stages of the virus replication cycle, as implied by their different kinetics of gene expression.

The reported functions of the few US22 family tegument proteins investigated so far are in keeping with a role during the initial stages of infection: pUL36 exhibits anti-apoptotic activity (Skaletskaya et al., 2001), pTRS1 and pIRS1 cooperate with the pp69 tegument protein to transactivate the major IE promoter (Romanowski & Shenk, 1997), while pUL43 and its MCMV homologue M43 appear to be involved in cell tropism (Brown et al., 1995; Xiao et al., 2000).

Our future work will investigate the functions supplied by pUL23, pUL24 and pUL43 during the early stages of infection and will address the possibility that these tegument proteins are transiently located in the nucleus immediately after infection. Failure to detect these proteins in the nucleus between 72 h and 96 h p.i. argues against a direct involvement in gene regulation at the level of transcription at late times. Nevertheless, several mechanisms can be invoked to account for an indirect effect of US22 family tegument proteins on host or viral gene regulation. They might interact with cell proteins in the cytoplasm that are directly involved in gene regulation or that operate as part of an intracellular signalling pathway, or they might influence transcript stability or translation. Alternatively, they might operate by binding to, or otherwise inhibiting the function of, cellular proteins involved in pathways leading to a cellular antiviral defence mechanism.

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References


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