Analysis of intracellular and intraviral localization of the human cytomegalovirus UL53 protein

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Human cytomegalovirus (HCMV) UL53 belongs to a family of conserved herpesvirus genes. In this work, the expression and localization of the UL53 gene product was analysed. Results obtained showed that pUL53 is a new structural protein. In infected human fibroblasts, pUL53 localizes in cytoplasmic perinuclear granular formations together with other structural viral proteins. In the nucleus, pUL53 forms patches at the nuclear periphery and co-localizes with lamin B at the internal nuclear membrane level. Immunoelectron microscopy studies have disclosed that nuclear pseudo-inclusions are labelled, whereas nucleocapsid formations within the intranuclear skein are negative. Furthermore, the mature virus particle maintains pUL53 at its tegumental level. These data suggest that pUL53 could be involved either in nucleocapsid maturation or in the egress of nucleocapsids from the nucleus to the cytoplasm through the nuclear membrane, a role compatible with the function hypothesized for UL31, its positional homologue in herpes simplex virus type 1.

Human cytomegalovirus (HCMV), a ubiquitous member of the family Herpesviridae, genus Betaherpesvirus, is associated with a wide spectrum of diseases, particularly in newborns and immunocompromised patients.

The HCMV genome (AD169 strain) has been sequenced completely and shown to contain 208 open reading frames (ORFs) predicted to encode for proteins of more than 100 aa in length; however, only a minority of these proteins have been assigned a precise function.

The aim of this work was to study the product of one of these previously uncharacterized ORFs: HCMV UL53, which is predicted to encode a basic protein of 376 aa with a molecular mass of 41.4 kDa (Chee et al., 1990). This ORF belongs to a family of conserved herpesvirus genes, which includes herpes simplex virus type 1 (HSV-1) UL31, equine herpesvirus type 1 29, Epstein–Barr virus BFLF2, varicella-zoster virus 27 and human herpesvirus type 6 U37. An initial characterization has only been attempted for the product of HSV-1 UL31, a largely insoluble protein associated with the nuclear matrix of infected cells (Chang & Roizman, 1993; Chang et al., 1997). Recently, pUL31 has been hypothesized to be involved in virus assembly and nucleocapsid maturation at the nuclear level (Shiba et al., 2000; Roller et al., 2000; Ye & Roizman, 2000; Ye et al., 2000), as it seems both to associate with the nuclear lamina and to have a role in encapsidation and envelopment (Roller et al., 2000; Yamauchi et al., 2001).

This report studied the product of UL53 both in transiently transfected cells and during virus replication. For a preliminary characterization of the protein, the entire UL53 sequence (1131 bp) was amplified from the genome of HCMV, strain AD169, using modified primers by adding a short sequence encoding a FLAG-octapeptide at its 3′ end. For eukaryotic expression, the amplified sequence was cloned into the pcDNA3 vector (Invitrogen) (named pc53flag) and was used to transiently transfet mammalian COS7 cells. pUL53 expression was analysed by immunoblot (IB) and immunofluorescence (IIF). IB was performed using the anti-FLAG M2 monoclonal antibody (Kodak) followed by ECL detection. As shown in Fig. 1(A, left panel), one band of the expected size (a protein with a molecular mass of approximately 42 kDa) was detected. IIF was performed on methanol–acetone-fixed cells with mAb (mAb FLAG) and on transiently transfected mammalian COS7 cells. pUL53 expression was analysed by immunoblot (IB) and immunofluorescence (IIF). IB was performed using the anti-FLAG M2 monoclonal antibody (mAb FLAG) (Kodak) followed by ECL detection. As shown in Fig. 1(A, left panel), one band of the expected size (a protein with a molecular mass of approximately 42 kDa) was detected. IIF was performed on methanol–acetone-fixed cells with mAb FLAG. The results obtained showed a strong nuclear localization of pUL53 (Fig. 2A, panel a).

For prokaryotic expression, pROS53flag was obtained by subcloning the UL53 sequence of pc53flag into pROS (kindly provided by G. Jahn, Tubingen, Germany), which over-expresses the antigen as a fusion protein to a truncated β-galactosidase molecule. The expression of the recombinant

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protein, rpUL53, was confirmed by IB analysis using mAb FLAG (data not shown).

rpUL53 was purified, as described by Battista et al. (1996), and injected into mice, as described by Cevenini et al. (1991), to obtain rpUL53-specific polyclonal antiserum (pAb rpUL53).

pc53flag was subjected to in vitro transcription/translation and the product was immunoprecipitated, as described by Battista et al. (1996), with pAb rpUL53 or mAb FLAG. Both antibodies reacted with a product of the expected size (42 kDa), a product that was not recognized by a mAb specific for the HCMV 72 kDa immediate-early product (data not shown). pAb rpUL53 was also tested in IB performed on cells transfected with pc53flag. A band of the expected size was obtained (Fig. 1A, right panel), while preimmune mouse serum did not recognize any specific protein (Fig. 1A, central panel). These results indicated that the antiserum specifically detected the 42 kDa UL53 product and pAb rpUL53 was, therefore, used to characterize the HCMV UL53 gene product in infected cells.

pUL53 expression was tested in human embryonic lung fibroblasts (HEL) infected with strain AD169 (1–2 p.f.u. per cell). Viral DNA synthesis was inhibited by adding foscarnet (fos) (100 µg/ml) (Astra) to the medium.

UL53 expression was analysed first by RT–PCR on total RNA, extracted with the RNAzolB kit (TEL-TEST), from mock-infected and HCMV-infected cells. Purified RNA was treated with DNase I (Boehringer Mannheim) and then used as the template for RT–PCR. As shown in Fig. 1(B), the UL53 mRNA was detectable from 48 h post-infection (p.i.), was markedly increased at 72 and 96 h p.i. and was inhibited by fos treatment, indicating a true late kinetics of expression, in agreement with data reported for its HSV-1 homologue, UL31 (Holland et al., 1984). PCR samples amplified without reverse transcription were negative.

Due to its poor solubility, pUL53 extraction from infected cells was carried out using a specific buffer for insoluble proteins (100 mM Tris–HCl, 100 mM NaCl and 0–5% NP-40); IB was performed using pAb rpUL53 followed by ECL detection. As shown in Fig. 1(C), pUL53 was found at the expected molecular mass; therefore, we concluded that the protein is not significantly post-translationally modified in infected cells.

To define the subcellular localization of pUL53 during HCMV replication, mock-infected and HCMV-infected HELF were studied by IIF using pAb rpUL53 (Fig. 2A, panels b–f). Faint staining dispersed throughout the cytoplasm was first observed at 48 h p.i. (data not shown). At 72 h p.i., the intensity of the signal increased and was associated with large cytoplasmic formations juxtaposed to the nucleus (Fig. 2A, panel e). A similar cytoplasmic localization was described previously during the late phases of the replication cycle for other HCMV structural proteins belonging to both the
Fig. 2. (A) Intracellular localization of pUL53 in transfected and infected cells. Panels a: subcellular localization of pUL53 transiently expressed in mammalian COS7 cells (48 h post-transfection), using the mAb FLAG (diluted 1:250); b–f, intracellular localization of pUL53 during HCMV replication was studied by IIF using a specific antisera (pAb rpUL53, diluted 1:350). Observations were performed under a Zeiss UV-microscope with photographic equipment. Mock-infected (panel b) and HCMV-infected HELF at 72 h (panel e), 96 h (panel f), 96 h + fos (panel d) probed with pAb rpUL53 and 96 h probed with preimmune serum (panel c) cells are shown. Magnification: panels a–e, 40 ×; f, 100 ×. (B) Co-localization of pUL53 with the tegument protein ppUL32-pp150 in human fibroblasts at 120 h p.i. was carried out by double staining. Co-staining was observed under a confocal microscope. The channel overlap was avoided using highly selective filters. Left panel: subcellular localization of pUL53 recognized by pAb rpUL53 followed by secondary fluorescein-conjugated antibody (green); central panel, the same cells reacted with the anti-ppUL32 mAb and then reacted with the secondary TRITC-conjugated antibody (red); right panel, co-localization of pUL53 with ppUL32 (yellow). Bars, 10 µm. (C) Co-localization of pUL53 (green) with nuclear lamins (red) was studied 120 h after HCMV infection in human fibroblasts. Antibodies to lamins (Novocastra Laboratories) were diluted at 1:30 and double staining was observed under a confocal microscope. Lamin A/C (left panel); lamin B (right panel). Bars, 10 µm.

tegument (pp150, pp65, pp71 and pUL25) and the envelope (gB and gH) proteins (Hensel et al., 1996; Battista et al., 1999). These condensed structures were referred to as ‘cap-like structures’ (Tooze et al., 1993) or ‘juxtanuclear structures’ (Sanchez et al., 2000) and are thought to represent subcellular compartments where the assembly of the virus progeny takes place.

Furthermore, at 96 h p.i., staining was also associated with punctate ‘patches’ localized at the nuclear periphery, similar to those already described as sites of intranuclear pp150, pp71 and pp65 accumulation (Fig. 2A, panel f) (Hensel et al., 1995, 1996; Radsak et al., 1990; Sanchez et al., 1998). The difference in nuclear localization and time of translocation between transfected and infected cells suggests a possible regulation in the nuclear uptake of pUL53.

The expression of pUL53 was abolished in the presence of
Fig. 3. (A) Intracellular localization of pUL53 studied by IEM. A low magnification view of HELF 120 h p.i. (m.o.i. of 5) is shown in panel a. Intense gold labelling is present on a very large black hole whose juxtanuclear localization is similar to that observed by IIF. In the nucleus (panels b and c), the nucleocapsid in formation (arrowheads) within the skein are unlabelled (panel b), whereas the nuclear envelope (panel c) appear to be labelled on the nucleoplasmic face. Panels (d)–(f) show pseudo-inclusions (*), enlargements of the perinuclear cisternae, whose inner and outer membranes are clearly evident after osmium fixation (panel d) with respect to samples used for immunoreaction (panels e and f). The labelling visible along the nuclear envelope is associated with the inner nuclear membrane of the pseudo-inclusions and on the outer layer of the virions in formation (arrowhead) (panel f). Magnification: panels a, 16000 ×; b, 60000 ×; c, 66000 ×; d, 20000 ×; e, 32000 ×; f, 76500 ×. N, nucleus; C, cytoplasm; NE, nuclear envelope; IM, inner nuclear membrane; OM, outer nuclear membrane; BH, black hole; DB, dense body; S, skein. (B) Intraviral localization of pUL53 studied by IEM. In the cytoplasm, DBs appear to be faintly labelled, whereas BHs are more intensely labelled (panels a and b). In the virions (panels a–d), the tegument appears uniformly electron-dense and the gold particles are scattered on its surface. When sectioned virions reveal the capsid (arrows), the labelling is less intense, whereas it appears markedly stronger when the core has been removed by section etching and the tegument is more available to antibody binding (arrowheads) (panels c and d). A similar localization of labelling is present in the extracellular virions (panels e–g). In a negatively stained virion (panel h), gold particles are visible between the capsid (arrow) and the damaged envelope (arrowhead). Magnification: panels a, 63000 ×; b, 105000 ×; c, 126000 ×; d, 82000 ×; e, 66000 ×; f, 103500 ×; g, 175000 ×; h, 118000 ×.

fos (Fig. 2A, panel d), indicating an absolute requirement of viral DNA replication for its synthesis. Staining of uninfected cells (Fig. 2A, panel b) with pAb rpUL53 and of infected HELF with preimmune serum (Fig. 2A, panel c) was always negative.

In order to check whether pUL53 co-localizes with other HCMV proteins, we performed a two-colour staining analysis, according to Battista et al. (1999), on infected HELF at 96 h p.i. pUL53 co-localizes in the cytoplasmic juxtanuclear structures.
with the tegument proteins pp150 (Fig. 2A), pp28, ppUL25 and pp65 and with the envelope glycoprotein gB (data not shown).

Since previous studies showed that nuclear granules could represent sites of virus replication, as described in HCMV-infected (Penfold & Mocarski, 1997) and HSV-1-infected (Liptak et al., 1996) cells, and in order to determine whether DNA synthesis occurred in the nuclear patches observed at 96–120 h p.i., we performed co-localization analysis of pUL53 and BrdU, as described by Penfold & Mocarski (1997). Results showed that pUL53 did not co-localize with the site of DNA replication at 24, 48, 72, 96 and 120 h p.i., demonstrating that
the pUL53-positive nuclear patches do not correspond to sites of virus replication. This was further supported by the absence of co-localization of pUL53 with other proteins involved in viral DNA replication, such as pUL57 (single-stranded DNA-binding protein) and ppUL44 (DNA polymerase processivity factor) (data not shown).

Localization of pUL53 in the nuclear patches was further analysed using antibodies against nuclear lamins A/C and B (Fig. 2C): an evident co-localization was detected, particularly with lamin B (Fig. 2C, right panel), assumed to be involved in the anchorage of the nuclear lamina to the inner nuclear membrane (Stuurman et al., 1998). This result is in agreement with data reported recently on co-localization between the nuclear lamina and the pUL53 homologue in HSV-1, pUL31, and its bound protein, pUL34 (Yamauchi et al., 2001). The possible interaction of pUL53 with nuclear laminae, already shown for other CMV proteins (Radsak et al., 1989, 1991; Sanchez et al., 1998) is a topic for future investigations.

The precise localization of pUL53 was studied by immunoelectron microscopy (IEM) on thin sections of infected HELF cells conjugated with 10 nm colloidal gold, according to Zini et al. (1999). Controls comprised samples that were not incubated with the primary antibody, incubated with the preimmune antiserum or uninfected cells incubated with pAb rpUL53.

To allow antigen detection, samples were not osmicated: under these conditions the membranes are not readily visible (Fig. 3A, compare panel d to other panels). Ultrastructural labelling was compared with IIF results. The large juxtanuclear fluorescent signal corresponds to cytoplasmic areas involved in virion maturation containing large ‘black holes’, appearing intensively gold-labelled late during infection (Fig. 3A, panel a). Black holes represent the electron-dense granular sites of accumulation/retention of overproduced viral and cellular material (Severi et al., 1988) and very likely play a role in CMV-induced cytopathology (Severi et al., 1992). They probably correspond either to the cap-like or juxtanuclear structures or mature within them during the final stages of virus replication.

Moreover, as expected on the basis of co-localization studies with lamins, a specific labelling was associated with the nucleoplasmic face of the nuclear envelope of infected cells (Fig. 3A, panel c), whereas the nucleocapsids in formation within the intranuclear skein are negative (Fig. 3A, panel b). Quantitative evaluation of pUL53 gold-labelling was performed on 30 micrographs at the same magnification by a Quantimet520 Image Analyser (Leica Instruments). The amount of specific labelling was obtained by subtracting the background values found in the extracellular spaces (< 1 gold particle/µm²). Results were statistically evaluated by Student’s t-test at a confidence level of 95% (P < 0.05).

The mean value of pUL53 gold particles counted on the nuclear membrane was 1.85 ± 0.66 gold particles/µm and 0.40 ± 0.26 gold particles/µm on the plasma membrane. This difference was statistically significant (t = 3.535; P = 0.017), indicating that there is a preferential association of pUL53 with the nuclear membrane.

Furthermore, the peculiar pseudo-inclusions (Fig. 3A, panels d–f), where nucleocapsids undergo sequential envelopment and de-envelopment crossing the nuclear membranes (Severi et al., 1979, 1988), were pUL53-immunolabelled (Fig. 3A, panels e and f) and very likely represent the late nuclear patches identified by IIF. Within these pseudo-inclusions, gold particles are localized mainly on the inner nuclear membrane and on the outer layer of virions. The quantitative evaluation of pUL53 labelling determined in the pseudo-inclusions (32.50 ± 3.51 gold particles/µm²) was compared to that determined in the cytoplasm (7.75 ± 2.50 gold particles/µm²) and in the nucleus (6.25 ± 2.50 gold particles/µm²). Results indicate that pUL53-specific gold particles are localized in the nuclear pseudo-inclusions with a frequency that is statistically significant with respect to localization in the nucleus (t = 12.183; P = 0.000) or in the cytoplasm (t = 11.487; P = 0.000). As pUL53 does not seem to be an integral membrane protein but a lamin-associated protein, it is conceivable that pUL53 is released to the nucleocapsid by the inner nuclear membrane during egress from the nucleus. Therefore, pUL53 seems to be acquired by the virus at the level of the nuclear membrane, although cytoplasmic sites of acquisition cannot be excluded, as tegument protein are supposed to be acquired at multiple sites (Hensel et al., 1995, 1996).

As shown in Fig. 3(B), pUL53 is maintained by the virus particle emerging from the nuclear pseudo-inclusions to the cytoplasm (Fig. 3B, panels a–d), indicating that pUL53 is a structural component of virions, with labelling localized in the tegument (Fig. 3B, panel c). The number of gold particles is more intense in virions from which the core has been removed by the section etching and the matrix is more available to antibody binding, as demonstrated previously for the tegument protein pUL25 (Fig. 3B, panel d) (Zini et al., 1999).

Moreover, extracellular virions appeared to be heavily labelled (Fig. 3B, panels e–g). Positive signals were localized mainly in the innermost part of the viral tegument, presumably the earliest acquired portion of the matrix.

Negative staining was performed on virus purified by sorbitol gradient (Dal Monte et al., 2001), according to Jons et al. (1996). As shown in Fig. 3(B, panel h), labelling was obtained exclusively when the envelope of the virions was damaged, confirming that pUL53 is a tegument component.

Data reported in this work suggest a role for pUL53 in the maturation of nucleocapsids or in their egress from the nucleus, as already hypothesized for other tegument proteins such as pp65, pp71 and pp150, which localize in the pUL53-like patches at the nuclear periphery (Hensel et al., 1995, 1996; Sanchez et al., 1998). This function would be compatible with the role suggested for its HSV-1 homologue, UL31 (Chang et al., 1997), which has been shown to co-localize with the nuclear lamina, whose depolymerization could provide
nucleocapsid access to envelopment sites at the inner nuclear membrane (Reynolds & Baines, 2000).

In conclusion, pUL53 is a novel HCMV structural protein localized in the tegument of the virus particle, most likely on its internal side, as it is acquired early during virus maturation at the level of the nuclear membrane. Its localization during virus replication suggests that pUL53 could be involved in either nucleocapsid maturation or egress of nucleocapsids from the nucleus through the nuclear membrane to the cytoplasm.

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