Expression of human cytomegalovirus ppUL83 (pp65) in a stable cell line and its association with metaphase chromosomes

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Astrocytoma cells were transfected with an expression vector for the structural phosphoprotein ppUL83 (pp65) of human cytomegalovirus (HCMV). Once made, pp65 showed the same characteristics as naturally produced protein and was stable for several days in astrocytoma cells, as it is during HCMV replication in fibroblasts. In permanently transfected cells, pp65 was homogeneously dispersed in the cell nucleus and showed a preferential methanol-stable association with condensed chromatin throughout cell division. Furthermore, pp65 could be detected bound to metaphase-arrested chromosomes in pp65-expressing astrocytoma cells, and in fibroblasts and astrocytoma cells during productive virus infection.

Human cytomegalovirus (HCMV), which belongs to the beta subgroup of herpesviruses, is a cause of morbidity and mortality in congenitally infected children and in immunosuppressed patients. The 230 kb HCMV genome has more sequence complexity than most other herpesviruses and has the capacity to encode more than 200 polypeptides (Chee et al., 1990). The virions are pleomorphic and measure between 150 and 300 nm in diameter. They are composed of an internal nucleocapsid and an external envelope. A fibrous, granular array of proteins is interfaced between the capsid and the envelope. This region is referred to as the matrix or tegument. The tegument of herpesviruses has been shown to contain a number of functionally important proteins (Roizman, 1993). The HCMV tegument is formed by at least seven proteins (Spaete et al., 1994) of which ppUL32 (pp150) and ppUL83 (pp65) together compose 35% of the protein mass of virions. pp65 is also the major constituent of the viral dense bodies, particles that lack viral DNA and nucleocapsid but consist of tegument (Gibson, 1983) as well as cellular proteins (Michelson et al., 1996; Landini & Ripalti, 1982; Mar et al., 1981).

pp65 is encoded by the 5’-terminal part of an abundant bicistronic 4 kb mRNA (Somogyi et al., 1990; Liu & Stinski, 1992; Dal Monte et al., 1996) which is produced efficiently both early and late in infection. Large quantities of pp65 are translocated to the cell nucleus during the first minutes after uptake of the tegument proteins from infecting viral particles. This process is mediated by two dominant nuclear localization signals and additional targeting sequences (Schmolke et al., 1995a). It is likely that this event also occurs in vivo as pp65 can be found in up to 1% of the nuclei of polymorphonuclear leukocytes of immunocompromised patients who have HCMV viraemia (van der Bij et al., 1988; Grefte et al., 1992) in the absence of immediate early protein expression and of any detectable RNA synthesis from UL83.

While the rapid nuclear transport and protein kinase activity of pp65 (Michelson et al., 1984; Michelson et al., 1985; Somogyi et al., 1990) indicate that it may have an important function in the initiation of viral gene expression and replication, recent results indicate that pp65 is dispensable for HCMV replication in cell culture (Schmolke et al., 1995b) despite the fact that pp65 antisense RNA blocks HCMV replication (Dal Monte et al., 1996).

In other viral systems, such as herpes simplex virus, genes that are not essential for replication in vitro play a key role in multiplication of the virus, its cell-to-cell transfer, complementing cellular functions lost as a consequence of viral replication, fine-tuning of viral gene expression, overcoming the host’s response to infection and virus-induced cytopathogenicity (for review see Ward & Roizman, 1994).

In this work we stably expressed pp65 in eukaryotic cells and showed that it preferentially localizes to condensed chromatin and is associated with chromosomes throughout mitosis. Human foreskin fibroblasts (FSF) were grown in minimal essential medium and 10% fetal calf serum (FCS). Human astrocytoma cells (U373MG, originally obtained from ATCC) were grown in Dulbecco’s modified Eagle’s medium.
Monte virus was grown and titrated as previously described (Dal originally obtained from ATCC) was used for infection. The (DMEM) and 5% FCS. The Ad169 strain of HCMV (VR538, originally obtained from ATCC) was used for infection. The virus was grown and titrated as previously described (Dal Monte et al., 1996).

The pp65 gene (UL83) from plasmid pp65-RSV (gift from G. Jahn, Tübingen, and B. Plachter, Erlangen, Germany) was placed under the control of the mouse mammary tumour virus (MMTV) promoter which is inducible with glucocorticoid (Ostrowski et al., 1984). The Poul–Xhol fragment from pMAM (Clontech) was ligated to the Poul and Xhol fragment of pMAMneo (Clontech), thereby eliminating the RSV promoter in pMAMneo and yielding plasmid pMneo. The Xhol–Xhol fragment of RSV-65, containing the ppUL83 ORF, was inserted into the Nhel and Xhol site of pMneo, yielding plasmid pM65.

Plasmid pCT-TK-GR 3-795; generously provided by M. F. Thiery (Pasteur Institut, Paris, France) and described by Wieland et al. (1991), expresses the active glucocorticoid receptor (GR).

To establish cells expressing pp65, U373MG astrocytoma cells were chosen for stabilization, since they are permissive for HCMV replication (Dudos et al., 1989) and therefore should contain all the factors necessary for HCMV productive infection. For permanent transfections, \(5 \times 10^6\) cells were seeded in 25 cm\(^2\) flasks in DMEM + 5% FCS. Twenty-four hours later cells were transfected using the calcium phosphate technique, with 0.5 \(\mu\)g of plasmid DNA composed of a mixture of GR plasmid and pM65 at a ratio of 20:1 so as to ensure expression of GR to enhance the activity of the MMTV promoter which drives UL83. As controls, U373MG cells were co-transfected with the same ratio of pMAMneo and the GR expression vector. Two days later, cells were trypsinized and seeded in DMEM + 5% FCS at 5 \(\times 10^4\) cells per well of 48-well plates in the presence of 400 \(\mu\)g/ml of G418. Within 3 to 4 weeks of selection colonies appeared. Cells were screened by immunofluorescence using the monoclonal antibody (Mab) F6b developed in our laboratory and described in Michelson et al. (1984).

After dexamethasone (DEX; \(10^{-6}\) M) (Sigma) stimulation, U-M65 cells showed the presence of pp65 in almost 100% of the nuclei. No reactivity was detected in control cells (U-Mneo) (Fig. 2a, i). U-M65 cells were analysed for the expression of pp65 as a function of time after DEX stimulation by Western blot using Mab NEA9220 (DuPont). DEX stimulation for 3 h was sufficient for abundant pp65 to be detected (results not shown). With 3 h of DEX stimulation, we followed the appearance of pp65 as a function of time after stimulation. As can be seen in Fig. 1 (a), pp65 could be detected 1 h after stimulation, but maximum levels required 12 h of incubation.

pp65 produced in U-M65 cells was compared with pp65 present in purified viral particles as well as pp65 produced during natural infection in both fibroblasts and astrocytoma cells. Protein extracts from the following preparations were studied by Western blot: purified HCMV particles; FSF and astrocytoma cells infected with 1 p.f.u. per cell and harvested 72 h (for FSF) or 5 days (for U373MG) post-infection (p.i.); and DEX-stimulated and mock-stimulated U-M65 cells. Blots were reacted with Mab 9220. As shown in Fig. 1 (b), the molecular mass of pp65 produced in U-M65 cells is identical to that detected in purified virus and in both types of infected cells. Persistence of pp65 following its initial synthesis was studied after DEX stimulation for 3 h by pulse-labelling cells with \([\text{35}S]\)-methionine for 1 h and chasing for 1 to 5 days (Fig. 1c). Protein concentrations were measured with the Bio-Rad protein assay and equal amounts of protein were immunoprecipitated with Mab F6b (final dilution 1/150 of the extract). pp65 made during the 1 h pulse-labelling period was still detected at comparable levels 3 days later and at a reduced level 4 and 5 days later. Thus, pp65 is rapidly induced by DEX treatment and persists for several days once made. These kinetics are similar to the behaviour of pp65 produced during HCMV infection of fibroblasts (Michelson et al., 1984). U-M65 cells have stably

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\begin{array}{ccc}
\text{Time after DEX treatment} & \text{pp65} \\
\text{kDa} & 0 & 1 & 3 & 6 & 12 & 24 \\
\hline
80.4 & \text{~} & \text{~} & \text{~} & \text{~} & \text{~} & \text{~} \\
55.7 & \text{~} & \text{~} & \text{~} & \text{~} & \text{~} & \text{~} \\
\end{array}
\]

\[
\begin{array}{ccc}
\text{kDa} & 1 & 2 & 3 & 4 & 5 & \text{ns} \\
\hline
80.4 & \text{~} & \text{~} & \text{~} & \text{~} & \text{~} & \text{~} \\
55.7 & \text{~} & \text{~} & \text{~} & \text{~} & \text{~} & \text{~} \\
\end{array}
\]
expressed pp65 in approximately 100% of the cells for more than 20 months.

During microscopic examination of immunofluorescence preparations, we consistently observed that approximately 3% of the cells contained pp65-positive mitotic figures (Fig. 2a, i–vi). All stages of cell division (prophase, pro-metaphase, metaphase, anaphase, telophase and cytodieresis) were positively stained with a MAb specific for pp65, suggesting that pp65 specifically binds to condensed chromatin and chromosomes of mitotic cells.

To confirm this interpretation, DEX-stimulated U-M65 cells were treated with colcemid (0.05 μg/ml; Sigma) for 8 h in DMEM-5% FCS in order to induce metaphase-arrest. Cultures were vigorously shaken to dislodge the metaphase-arrested cells. The supernatant cells were pelleted, washed, swollen in hypotonic medium (0.035 M-KCl in distilled water) and deposited on glass slides by cytocentrifugation. The cells were fixed with methanol–acetone (3:1) or acetone alone (depending on the MAb used for staining metaphase-arrested chromosomes). Chromosomes were stained with the pp65-specific MAb NEA9220 (DuPont) or F6b (previously developed in our laboratory). Under these experimental conditions pp65-positive metaphase-arrested chromosomes could be observed in almost all metaphase-arrested cells (Fig. 2b, i). As a positive control we used a cell line expressing IE1 (kindly made available by J. L. Davignon, Toulouse, France) and positive staining of chromosomes was detected as previously described by LaFemina et al. (1989) (Fig. 2b, ii). We also colcemid-treated another cell line (pTR/SUL44) recently obtained in our laboratory (F. Campanini and others, unpublished). This cell line expresses ppUL44, the 52 kDa DNA-binding protein (also known as ICP36) in the cell nuclei. No chromosome staining was observed. Furthermore, pp52 staining of the chromosomes was prevented, producing a 'negative staining' effect (Fig. 2b, iii) as already shown for SV40 large T antigen (LaFemina et al., 1989).

To verify whether pp65-binding to chromosomes also occurred during HCMV productive replication, astrocytoma cells or fibroblasts (10⁵ cells per well of 24-well plates) were seeded and treated with colcemid (as described above) the night before infection. During the last hour of colcemid treatment, 50 μg/ml cycloheximide (CH) was added. Medium was drawn off gently and replaced with virus (5 p.f.u. per cell). Cells were centrifuged for 5 min at 2500 r.p.m. at room temperature then incubated for 30 min at 37°C. Cells were dislodged with PBS+EDTA, washed once with PBS and processed as described above. MAb F6b was used for pp65 and MAb E13, which reacts with HCMV IE1 and IE2 proteins (a gift from M. C. Mazeron, Paris, France) was used to determine the absence of immediate early antigen expression. Clear pp65-binding to chromosomes was observed in astrocytoma cells (data not shown) and in fibroblasts (Fig. 3a) 30 min after contact between virus and cells in the presence of cycloheximide. Furthermore, pp65 binding to chromosomes was also detected at 72 h.p.i. in both astrocytoma cells (data not shown) and fibroblasts (Fig. 3b) after infection with 50 p.f.u. per cell after 6 h of colcemid treatment.

In conclusion, we describe here the first example of a permanent cell line (astrocytoma cells) which expresses HCMV ppUL83 (pp65). The protein has the same electrophoretic mobility as pp65 present in purified virus particles and in HCMV-infected cells (both fibroblasts and astrocytoma cells) indicating that pp65 produced in astrocytoma cells, even in the absence of other viral gene products, undergoes post-transcriptional modification identical to that occurring during natural CMV infection. Once pp65 is produced, it remains homogeneously dispersed in the nuclei of permanently transfected cells for several days, indicating stable binding with nuclear material. This behaviour is similar to what happens during HCMV infection of fibroblasts (Michelson et al., 1984).

In pp65-expressing cells we observed a preferential methanol-stable association of pp65 with condensed chromatin when cells were undergoing mitosis. This was confirmed by the finding that pp65 could be detected bound to chromosomes that were arrested in metaphase by colcemid treatment. Therefore, pp65 binding to chromosomes seems a natural event which does not require any other product of the viral genome. pp65-binding to chromosomes was also seen during HCMV productive replication both 30 min after contact between virus and cells, as well as at late times of the replication cycle. This indicates that pp65 binding to chromatin occurs throughout natural infection from the very beginning (pp65 of the input virus) to the advanced phases of virus replication (neo-produced pp65). However, it should be pointed out that after infection only a very few cells could be arrested in metaphase by colcemid treatment in comparison with uninfected fibroblasts. This is probably due to the fact that CMV arrests the cell cycle in the G2/M phase (Jault et al., 1995).

Chromosome-binding is not common to all the other viral proteins which localize to the nucleus. We have shown that ppUL44 (pp52), a DNA-binding protein essential for HCMV replication (Ripatti et al., 1995), does not bind to chromosomes.

The binding of a viral protein to metaphase chromosomes has been reported previously for HCMV IE1 (LaFemina et al., 1989) and Epstein–Barr virus (EBV) EBNA-1 (Rawlins et al., 1985; Reisman et al., 1985). Interestingly, pp65 shares 57-14% similarity (31% identity) over 56 amino acids with EBNA-1. In particular, the DNA-binding region of EBNA-1 (aa 460–618) shares 40% identity with the C-terminal half of pp65.

What is the significance of pp65 binding to metaphase chromosomes? The EBNA-1 protein is thought to be involved in maintaining the plasmid state of EBV genomes during latency (Rawlins et al., 1985; Reisman et al., 1985) and has also been implicated in the uniform segregation of EBV genomes into daughter cells (Rawlins et al., 1985). Nothing is known about the significance of the association between HCMV IE1 and metaphase chromosomes. Several studies have shown that
Fig. 2. Expression of pp65 as detected by immunofluorescence. (a) Immunofluorescence of pp65-expressing cells. Cells were DEX-stimulated and stained with the pp65-specific MAb F6b. (i) U-Mneo cells. The following pp65-positive mitotic figures were detected: (ii) a nucleus in prophase; (iii) a nucleus in pro-metaphase; (iv) two nuclei in metaphase; (v) a nucleus in anaphase; (vi) a nucleus in cytodieresis. Arrows indicate the mitotic figures. (b) Association of pp65 with condensed chromosomes of metaphase-arrested cells. Cultures of U-M65 cells (i), A1B (astrocytoma cells permanently transfected with IE1) (ii) and pRC/SUL44 cells expressing ppUL44 (iii) were grown for 8 h in the presence of colcemid, and then prepared as described in the text. Cells were fixed with methanol-acetone and stained with MAb 9220 (i), MAb E13 specific for IE1 (ii) and a MAb specific for ppUL44 (CH13) (iii).
HCMV infection can result in a statistically significant increase in the frequency of chromosome aberration (chromosome breaks, exchanges and deletions) in permissive cells as well as in abortively infected peripheral blood lymphocytes (AbuBakar et al., 1988; Lüleci et al., 1980) and this may be significant in the induction of birth defects. pp65-binding to dividing chromosomes might play an important role in chromosome aberrations. This possibility is now being tested.

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References


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