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Human Cytomegalovirus Proteins PP65 and IEP72 Are Targeted to Distinct Compartments in Nuclei and Nuclear Matrices of Infected Human Embryo Fibroblasts

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Abstract The cellular distribution of the human cytomegalovirus (HCMV)-specific UL83 phosphoprotein (pp65) and UL123 immediate-early protein (IEp72) in lytically infected human embryo fibroblasts was studied by means of indirect immunofluorescence and confocal microscopy. Both proteins were found to have a nuclear localization, but they were concentrated in different compartments within the nuclei. The pp65 was located predominantly in the nucleoli; this was already evident with the parental viral protein, which was targeted to the above nuclear compartment very soon after infection. The nucleolar localization of pp65 was also observed at later stages of the HCMV infectious cycle. After chromatin extraction (in the so-called in situ nuclear matrices), a significant portion of the pp65 remained associated with nucleoli within the first hour after infection, then gradually redistributed in a perinucleolar area, as well as throughout the nucleus, with a granular pattern. A quite different distribution was observed for IEp72 at very early stages after infection of human embryo fibroblasts with HCMV; indeed, this viral protein was found in bright foci, clearly observable in both non-extracted nuclei and in nuclear matrices. At later stages of infection, IEp72 became almost homogeneously distributed within the whole nucleus, while the foci increased in size and were more evenly spread; in several infected cells some of them lay within nucleoli. This peculiar nuclear distribution of IEp72 was preserved in nuclear matrices as well. The entire set of data is discussed in terms of the necessity of integration for HCMV-specific products into the pre-existing nuclear architecture, with the possibility of subsequent adaptation of nuclear compartments to fit the needs of the HCMV replicative cycle. J. Cell. Biochem. 90: 1056–1067, 2003. © 2003 Wiley-Liss, Inc.

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Nuclear organization of the host cell plays an important role in the host–virus challenge, particularly for viruses such as herpesviruses, whose replicative cycle takes place in this cellular compartment [Lukonis and Weller, 1996; Burkham et al., 1998, 2001; Monier et al., 2000; Reynolds et al., 2001; Hutchinson et al., 2002; Sourvinos and Everett, 2002]. Since cytomegalovirus can establish different relationships with the host cell, resulting in a lytic cycle or a latent infection, it is likely that this virus has developed different kinds of strategies to exploit the same pathways and nuclear regulatory apparatus, as the cell does in physiological conditions, and/or to modify them to its own benefit.

The nuclear matrix represents a structural "milieu" for the organization of virtually all biosynthetic processes in eukaryotic cell nuclei. DNA replication, transcription, and RNA splicing were shown to occur at the level of the

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nuclear matrix within specific compartments that can be visualized as "foci" or "speckles" (reviewed in Berezney et al., 1995; Brown, 1999; Stein et al., 2000; Berezney, 2002).

This spatial organization seems to be highly important for the normal functioning of genetic apparatus. Localization of necessary proteins at the nuclear matrix and within the appropriate compartments is controlled by specific targeting signals [Barseguian et al., 2002; Lee and Skalnik, 2002]. The inability to target transcription factors to the appropriate nuclear compartment (not roughly to the nucleus) is at the origin of serious diseases [McNeil et al., 1999; Meyers and Hiebert, 2000].

In order to exploit efficiently the enzymatic apparatus of the host cell, the viral DNA, messenger RNAs, and possibly some proteins are probably incorporated in a very specific way into pre-existing nuclear compartments that are the physiological sites of cellular DNA replication and transcription, and of RNA splicing as well. Furthermore, it is likely that the nuclear architecture is modified in a certain way to fit the new functional activities imposed by the viral replication pathway.

As far as human cytomegalovirus (HCMV) is concerned, this important area of virus–host cell interaction still remains poorly explored.

This paper deals with the nuclear matrix targeting of two HCMV (AD169 strain) regulatory proteins in the course of lytic infection of MRC5 human embryo fibroblasts.

Our results show that two viral proteins with relevant functional activities, the 76 kDa immediate early gene product, and the 65–68 kDa lower matrix structural phosphoprotein, are closely and dynamically associated with specific and quite different sites of the nuclear matrix.

MATERIALS AND METHODS

Cell Culture

Monolayer cultures of MRC5 human embryo lung fibroblasts (Istituto Zooprofilattico, Brescia, Italy) were grown in Earle's modified minimum essential medium (MEM), supplemented with 1% L-glutamine, 1% non-essential aminoacids, 10% fetal calf serum, and antibiotics (10,000 U/ml penicillin, 10,000 μg/ml streptomycin) (cell culture medium and supplements were provided by Invitrogen, Milan, Italy).

Virus Infection and Titration

The reference AD169 strain (ATCC UR-538) of HCMV was used for the in vitro infection of MRC5 human embryo fibroblasts. Viral infectious titer was determined by the plaque assay, as already described [Landini et al., 1979].

The AD169 strain was used at a multiplicity of infection (MOI) of one plaque-forming unit (pfu)/cell to infect monolayer cultures of MRC5 fibroblasts, previously grown in 35 mm plastic Petri dishes or, alternatively, in 12 mm round cover-slips for 48 h.

Drug Treatment

In some experimental procedures, 50 μM cycloheximide (Sigma Aldrich, Milano, Italy) was added to the culture medium at the beginning of the infectious cycle and kept in the medium for the planned times of infection.

Preparation of Nuclear Matrices

MRC5 fibroblasts were cultured in 35 mm Petri dishes, or, alternatively, in 12 mm round glass cover-slips at low density (6×10^5 cells/dish; 2×10^5 cells/cover-slip) for 48 h, before they were infected with the above reference strain of HCMV for the planned times.

To prepare the in situ matrices, cell monolayers were rinsed twice with TM buffer (50 mM Tris-HC1 (pH 7.5), 3 mM $MgCl_2$), and incubated for 10 min on ice in TM buffer supplemented with 0.2 mM phenylmethylsulphonyl fluoride (PMSF), 0.5% Triton X-100, and 0.5 mM $CuCl_2$. After incubation, the cells were washed three times with TM buffer and then treated with 20 U/ml RNase-free DNase I (Roche Diagnostics, Milan, Italy) for 20 min at 37°C in TM buffer, in the presence of an RNase inhibitor (RNasin, Promega, Madison, WI), to a final concentration of 1,000 U/ml. The above buffer was then replaced by TM buffer supplemented with 2 M NaCl and cells incubated in this buffer for 30 min on ice. The in situ matrices obtained by this experimental protocol were washed twice with TM buffer and immediately used for detecting viral antigens by indirect immunofluorescence (IIF).

Giemsa Stain

MRC5 monolayers grown on round glass coverslips were extracted to obtain the in situ nuclear matrices, as detailed above. Nuclear

matrix preparations, as well as unextracted cells were infected with the AD169 strain of HCMV for 1, 3, and 20 h in the presence (or the absence) of cycloheximide. After the planned infectious cycles, cells were fixed with cold methanol for 10 min and stained with Giemsa working solution (1:10 in pH 7.2—buffered distilled water, from stock solution, Merck Sharp & Dohme, Rome, Italy) at room temperature for 40 min; finally they were gently rinsed twice in distilled water, then mounted.

Antibodies

The following primary antibodies were used: a purified monoclonal blend (clones 1C3 and AYM-1) reacting with the 65–68 kDa lower matrix structural phosphoprotein (pp65) of HCMV (Argene-Biosoft, France); a monoclonal antibody (MAb) specific for the 76 kDa viral immediate-early gene product (IEp72) (Argene-Biosoft); the "ANA-N" serum, a human antiserum for nucleolar antigens (The Binding Site, United Kingdom). Anti-pp65 and anti-IEp72 Mabs were diluted 1:20 and 1:30, respectively, in 0.2% bovine serum albumin (BSA) in phosphate buffered saline (PBS) [7 mM Na_2HPO_4, 1.5 mM KH_2PO_4 (pH 7.4), 137 mM NaCl, 2.7 mM KCl]. The immunoreaction was revealed by rhodamine isothiocyanate (RITC)-conjugated goat anti-mouse immunoglobulin G (IgG) (EuroClone, United Kingdom) to detect viral antigens and fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG (The Binding Site) for nucleolar proteins.

Indirect Immunofluorescence

After the planned infection and/or matrix extraction procedures, the cells were gently rinsed with cytoskeleton (CSK) buffer [10 mM Pipes (1.4-piperazinediethanesulfonic acid) (pH 6.9), 100 mM NaCl, 1.5 mM $MgCl_2$, 300 mM sucrose, and 1.2 mM phenyl-methyl-sulfonyl fluoride (PMSF)], then permeabilized and fixed with 2.5% Triton X-100 and 1% paraformaldehyde in CSK buffer, added simultaneously, and left at room temperature for 20 min [Arcangeletti et al., 1997].

The fixed cells were washed three times (5 min/wash) with PBS. Unspecific, immunoreactive sites were saturated with 1% BSA (pH 8.0) in PBS for 15 min. The cells were then incubated with primary antibodies for 1 h at 37°C, in a humid chamber. After three washes with PBS, RITC-conjugated secondary antibodies were applied alone or together with FITC-linked antibodies in the case of double label immunofluorescence, for 1 h at 37°C, in a humid chamber, and in the dark. Negative controls were processed with an identical procedure, except that the first antibody was replaced by 0.2% BSA in PBS. After incubation, the cells were washed three times with PBS. Stained cells were examined under a fluorescence microscope (Zeiss-Axiophot) or a confocal microscope (Multiprobe 2001, Molecular Dynamics, Sunnyvale, CA).

Confocal Microscopy

The images were acquired with a confocal laser scanning microscope (CLSM), based on a Nikon diaphot inverted microscope and equipped with an argon laser. We employed ×100 NA 0.75 oil immersion Planapo lenses coupled to a 100 μm pinhole. Section series were acquired with Z-step set at 0.8 μm. Samples were excited with the 488 nm laser line of CLSM, and the emission was recorded through a 510 nm primary beamsplitter and a 530 dichroic filter 30 or a 570 nm long-pass emission barrier filter for FITC and RITC signal acquisition, respectively. Image processing was performed on a Silicon Graphics Personal Iris workstation (Image Space Software; Molecular Dynamics).

RESULTS

Cytolocalization of Viral pp65 During the Replicative Cycle of HCMV in MRC5 Cells

The 65 kDa phosphoprotein (pp65) is one of the HCMV structural proteins, representing the major component of the viral particle tegument. The cellular distribution of pp65 during a lytic infectious cycle (1, 3, and 20 h post-infection (p.i.), 1 pfu/cell) in MRC5 human embryo fibroblasts is shown in Figure 1.

Firstly, the pp65 localization was studied during the early times of infection, namely at 1 and 3 h p.i. In cells with unextracted nuclei (Fig. 1a (1 h); b (3 h)), the protein was scattered within the nucleoplasm, significantly more concentrated in nuclear areas with morphological features consistent with nucleoli. A similar pattern was detected in most cells with extracted nuclear matrices at 1 h p.i. (Fig. 1d). Some cells, however, evidenced an immuno-

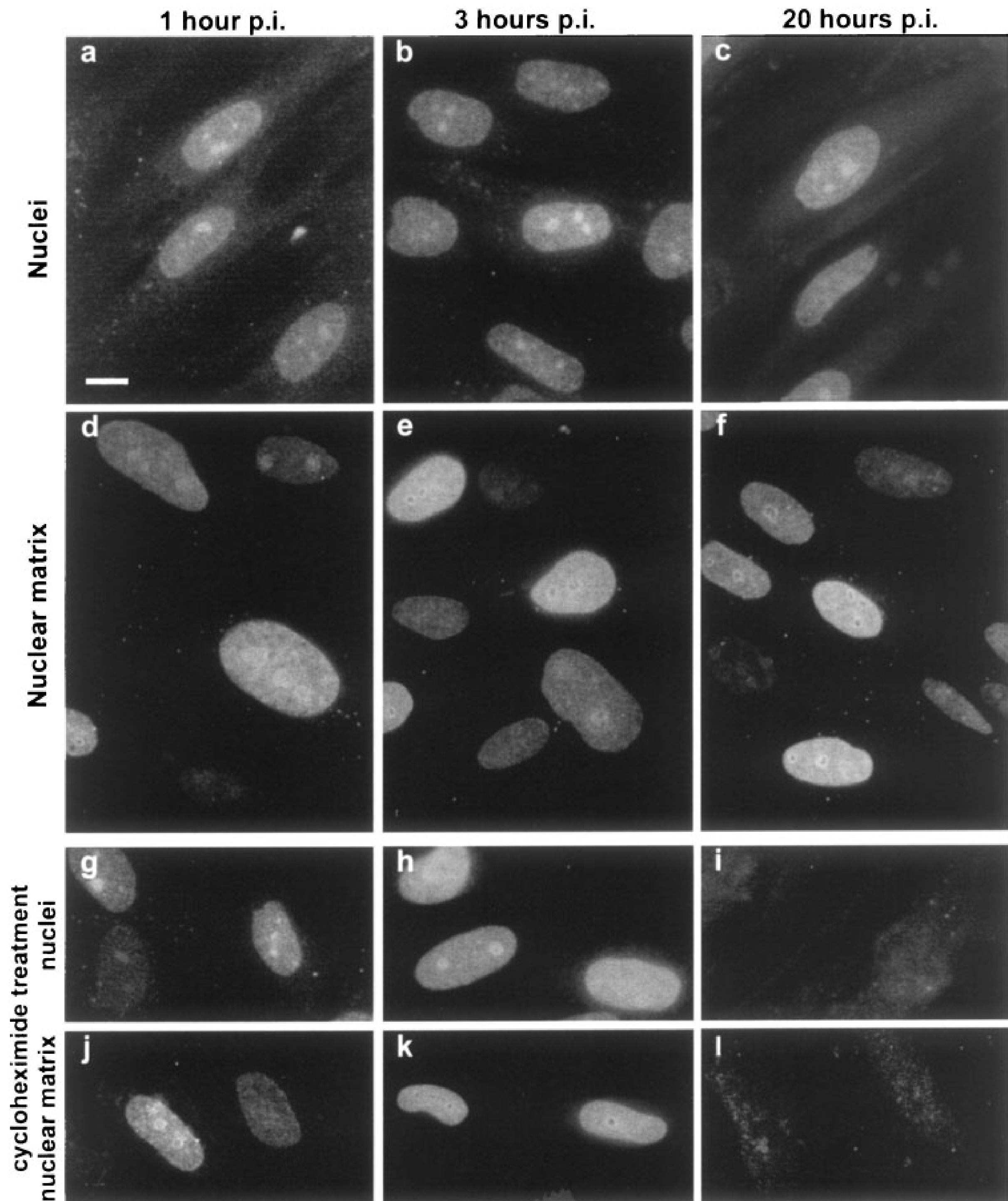

Fig. 1. Nuclear distribution of human cytomegalovirus pp65 antigen and effects of cycloheximide treatment on its expression in human embryo fibroblasts. Fibroblasts grown for 48 h in 35 mm plastic Petri dishes were infected with the AD169 strain of human cytomegalovirus at a MOI of 1 pfu/cell for 1, 3, and 20 h. At the indicated times p.i., cells were fixed and (**a**, **b**, **c**, **g**, **h**, **i**) immediately processed for immunofluorescence or, alternatively (**d**, **e**, **f**, **j**, **k**, **l**), first extracted to obtain in situ nuclear matrices, before immunoreaction was carried out. Experimental infection in g–l was performed in the presence of 50 μM cycloheximide. The fixed cell monolayers were labeled with a monoclonal antibody to pp65 viral protein. The immunoreaction was revealed with RITC-conjugated goat anti-mouse IgG. Bar = 5 μm.

fluorescence signal encircling the nucleolar-like compartments, instead of their central region. This pattern was even more accentuated at 3 h p.i. (Fig. 1e).

Cycloheximide treatment did not affect immunostaining pattern at early stages of infection in either unextracted (Fig. 1g,h) or in nuclear matrix-extracted (Fig. 1j,k) nuclei, confirming that the parental virus protein was responsible for the observed fluorescence distribution.

No significant changes in immunofluorescence pattern were detectable at 20 h p.i. in either nuclei or in nuclear matrix-extracted cells (Fig. 1c,f). However pretreatment with cycloheximide almost completely abolished pp65 immunoreactivity in both conditions, thus clearly pointing to an ex novo origin of the protein, at this infection time (Fig. 1i,l). Giemsa staining did not evidence an excess of apoptotic figures in cycloheximide-treated cells (data not shown).

Confocal Microscopy Analysis of the Nuclear Distribution of pp65 in HCMV-Infected MRC5 Fibroblasts

To demonstrate the nucleolar distribution of the pp65 immunofluorescence signal in HCMV-infected MRC5 fibroblasts, we carried out a series of double labeling experiments, using an anti-pp65 monoclonal antibody (red labeled) and a human anti-"ANA-N" serum (green labeled). Immunoreactivity was assessed in unextracted nuclei and nuclear matrices at 1, 3, and 20 h after infection. For each experimental point, two consecutive, equatorial confocal sections (white numbers 1 and 2) of a representative microscopic field are shown in Figure 2. The two channels (red, pp65; green, nucleoli) of the same field are shown in separate panels.

In unextracted nuclei, anti-pp65 (Fig. 2 panels a–c) and anti-"ANA-N" (panels a′–c′) immunofluorescence allowed us to confirm that pp65 was actually primarily located within the nucleoli. The fluorescence pattern of pp65 remained constant throughout infection, namely after 3 h (b1, b2) and 20 h (c1, c2). As for signal intensity, a slight decrease was recorded at 3 h p.i., as expected for the parental protein, present at this time of infection and gradually degraded.

In nuclear matrices, colocalization between pp65 and nucleolar proteins was complete at 1 h after infection (Fig. 2 d1, d2 and d′1, d′2), while it became rather rearranged in a perinucleolar layer at 3 h p.i. (e1, e2 and e′1, e′2), with a concomitant increase of a diffuse nuclear signal pattern. At 20 h p.i. (f1, f2 and f′1, f′2), the pp65 distribution was quite unchanged, except for a more marked accumulation of the signal at the nuclear periphery, clearly detectable in most cells.

Nuclear Distribution of Viral IEp72 During the Replicative Cycle of HCMV in MRC5 Cells by Confocal Microscopy Analysis

IEp72 is one of the major immediate-early gene products of HCMV. The nuclear distribution of IEp72 during the early stages (1 and 3 h p.i.) of HCMV infectious cycle was studied in unextracted nuclei and nuclear matrices of MRC5 fibroblasts (Fig. 3). Double label immunofluorescence experiments were done to assess the relative distribution of the viral IEp72 (Fig. 3 a–d, red) with respect to nucleoli (Fig. 3 a′–d′, green). Two consecutive, equatorial confocal sections (white numbers 1 and 2) of a representative microscopic field are shown for each experimental condition. The red (IEp72) and green (nucleoli) channels of the same field are shown in separate panels.

One hour after infection, IEp72 immunofluorescence was detectable as a few, discrete, bright spots often arranged at the periphery of unextracted nuclei (Fig. 3 a1–a2). No nucleolar overlap was detectable at this time, as shown in Figure 4 (panels a1–a′1), by superposing the indicated confocal sections of Figure 3. At 3 h after infection, an increasing number of bright IEp72 speckles was observable within the nucleoplasm (Fig. 3 b1–b2). The colocalization study of Figure 4 reveals that in this case (panel b1–b′1, see yellow/orange spots at the nucleolar level), IEp72 granules (red spots) lay within nucleoli (green) in a number of cells.

A very similar pattern was observed in nuclear matrices (Fig. 3c, d: IEp72; c′,d′: nucleoli, 1 and 3 h, respectively), concerning the IEp72: the "spots" distribution was still present, and much more pronounced at 3 h pi. Once again at this time, as observed in unextracted nuclei, a clear colocalization between the above viral protein's speckles and nucleolar areas is detectable in several cells, at the nuclear matrix level (Fig. 4, panel d1–d′1), whereas no codistribution was appreciable at 1 hour p.i. (Fig. 4, panel c1–c′1).

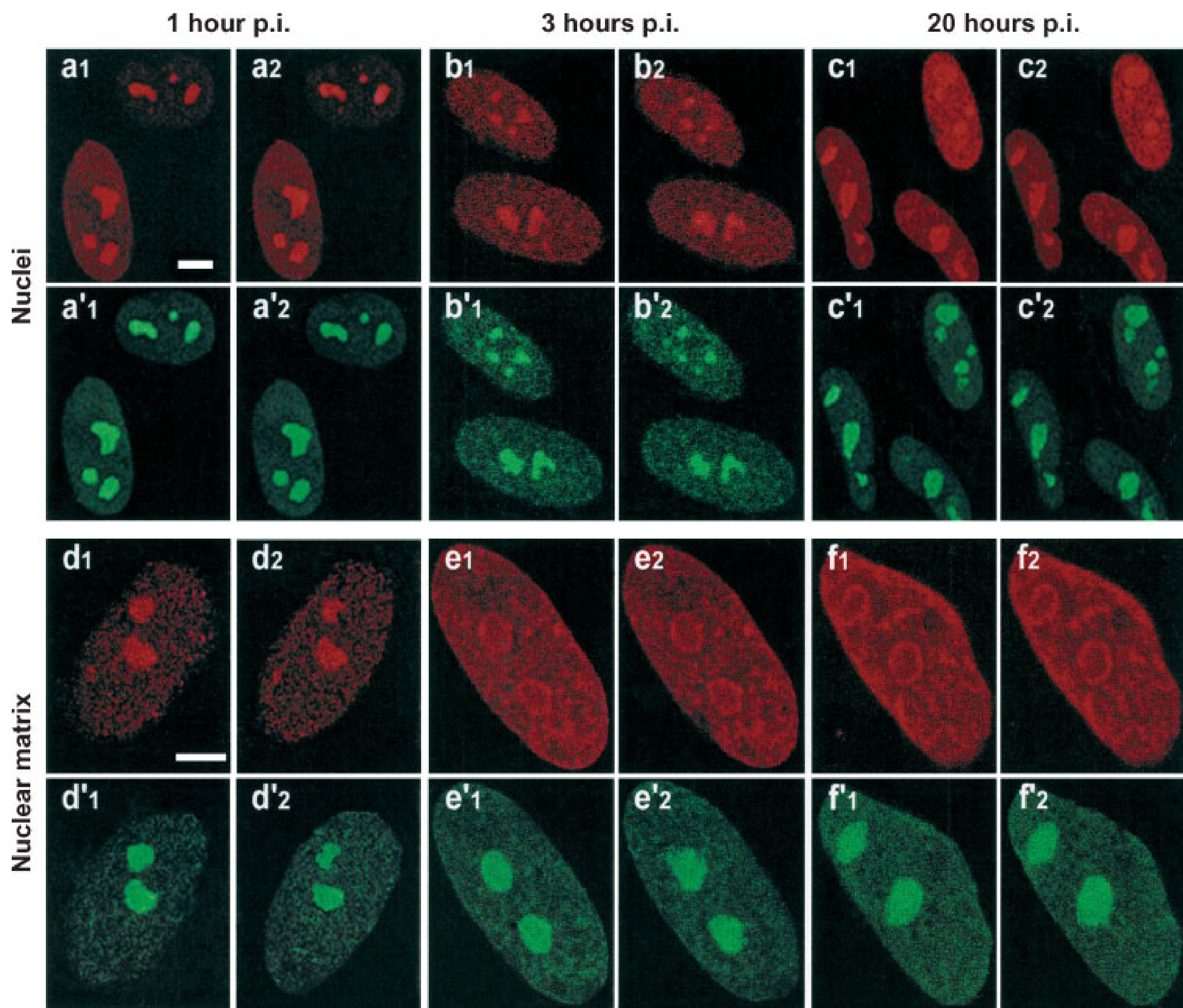

Fig. 2. Confocal microscopy analysis of the nuclear distribution of human cytomegalovirus pp65 antigen during the experimental infection of human embryo fibroblasts. Fibroblasts grown for 48 h on 12 mm round glass cover-slips were infected with the AD169 strain of human cytomegalovirus at a MOI of 1 pfu/cell for 1, 3, and 20 h. At the indicated times p.i., cells were fixed and (**a**–**c**; **a**′–**c**′) immediately processed for immunofluorescence or, alternatively (**d**–**f**; **d**′–**f**′), first extracted to obtain in situ nuclear matrices, before fixation and immunoreaction were carried out. In both cases, the fixed monolayers were simultaneously labeled with (a–f) a monoclonal antibody to pp65 and (a′–f′) an anti-nucleolar antigens serum. The immunoreaction was then revealed with (a–f, red), RITC-conjugated goat anti-mouse IgG, and (a′–f′, green) FITC-conjugated goat anti-human IgG antibodies to detect the viral antigen and nucleolar proteins, respectively. Confocal microscopy analysis was done on both, unextracted cell nuclei and nuclear matrices: two consecutive, equatorial, confocal sections (white numbers 1 and 2) of a representative microscopic field are shown. Bars (a–c) = 5 μm; (d–f) = 2 μm.

No staining was observed either in nuclei or nuclear matrices prepared from uninfected cells or from infected cells in the presence of cycloheximide (not shown).

DISCUSSION

The nuclear matrix constitutes a structural “milieu” for the spatial organization of virtually all biosynthetic processes in eukaryotic cell nuclei [Berezney et al., 1995; Berezney, 2002]. It seems obvious that virus-specific products should be incorporated somehow in the pre-existing system of nuclear compartments and hence, at least some of them should be located at the nuclear matrix. Indeed, association with the nuclear matrix of many different virus-specific proteins has been reported [Ciampor, 1988; Deppert and Schirmbeck, 1995; Yamada et al., 1997; Sanchez et al., 1998; Tanaka et al., 2002; Lethbridge et al., 2003]. Thus, it was not at all

unexpected to find both a regulatory (IEp72) and a structural (pp65) HCMV proteins in the nuclear matrix compartment of HCMV-infected cells.

The tegument protein pp65 was previously reported to be associated with the nuclear matrix of HCMV-infected cells [Sanchez et al., 1998]. However, in this previous study the pp65

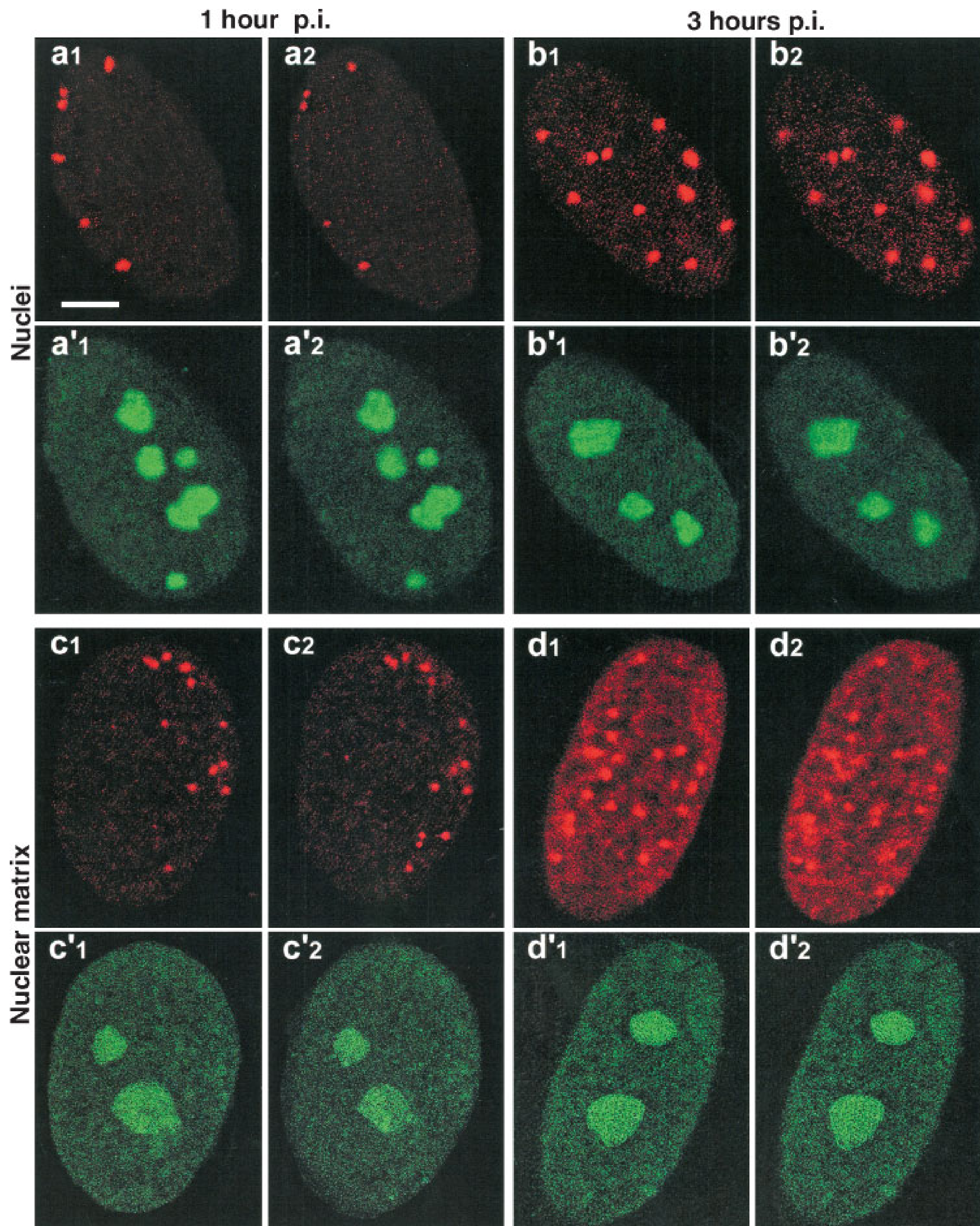

Fig. 3.

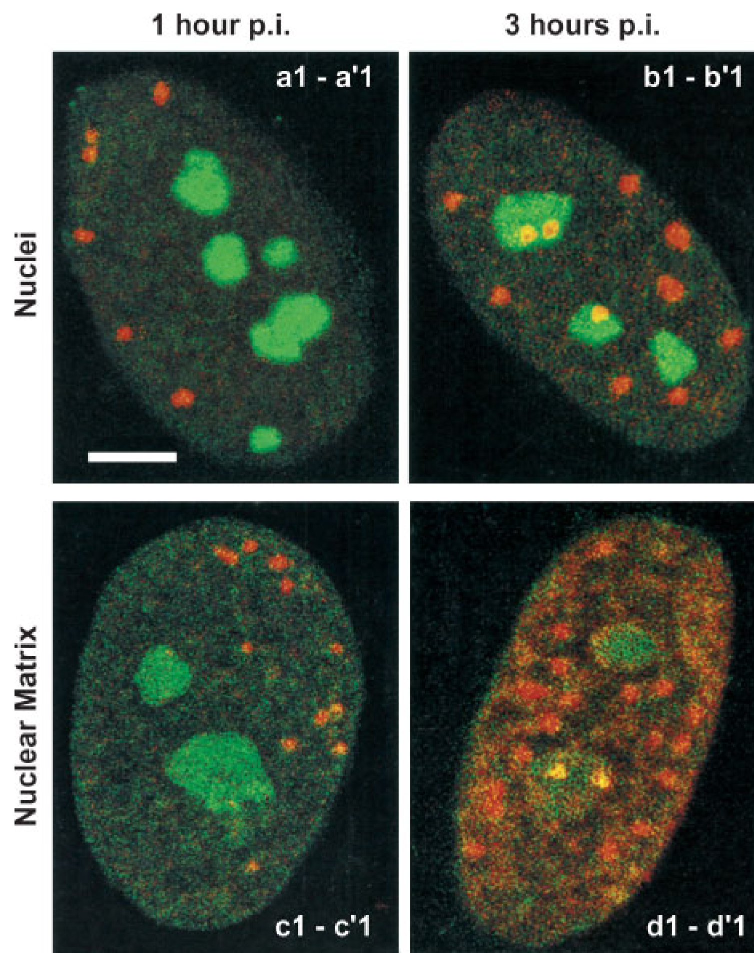

Fig. 4. Nucleolar distribution of human cytomegalovirus IEp72 immediate-early antigen during the experimental infection of human embryo fibroblasts. Confocal microscopy data shown in Figure 3 were exploited for a colocalization study between the human cytomegalovirus IEp72 antigen (red) and specific nucleolar proteins (green). The images were obtained by superimposing the red and green channels; (**a1**–**a′1**) and (**b1**–**b′1**) represent the two selected confocal sections for infected nuclei at 1 and 3 h pi, respectively (see Fig. 3). Similarly, (**c1**–**c′1**) and (**d1**–**d′1**) are the chosen in situ matrix sections at the indicated times of infection. The colocalized sites appear as orange/yellow or yellow colored areas. Bar = 2 μm.

was found predominantly in association with the nuclear lamina [Sanchez et al., 1998]. We were also able to observe a clear accumulation of the pp65 at the nuclear periphery (most likely the nuclear lamina), either in non-extracted nuclei or in nuclear matrices, particularly evident 20 h after infection. Aside from this common observation, we noted clear nucleolar targeting of the pp65, as a very characteristic and reproducible feature of the incoming virus protein, as well as of the newly formed one. The only partial coincidence of the two series (ours and the above authors') of results may be due to the fact that in the above cited study, the cytolocalization of the pp65 was analyzed at very late stages of infection, when the very high

Fig. 3. Confocal microscopy analysis of the nuclear distribution of human cytomegalovirus IEp72 immediate-early antigen during the experimental infection of human embryo fibroblasts. Cells grown for 48 h in 12 mm round glass cover-slips were infected with the AD169 strain of human cytomegalovirus at a MOI of 1 pfu/cell for 1 and 3 h. At the indicated times p.i., cells were fixed and (**a**–**a′**, **b**–**b′**) immediately processed for immunofluorescence, or alternatively (**c**–**c′**, **d**–**d′**), first extracted to obtain in situ nuclear matrices, before fixation and immunoreaction were performed. In both cases, the fixed monolayers were simultaneously labeled with (a–d) a monoclonal antibody to IEp72 and (a′–d′) an anti-nucleolar antigens serum. The immunoreaction was revealed with (a–d, red) RITC-conjugated goat anti-mouse IgG, and (a′–d′, green) FITC-conjugated goat anti-human IgG antibodies, to detect the viral antigen and nucleolar proteins, respectively. Confocal microscopy analysis was carried out on both, unextracted cell nuclei and nuclear matrices: two consecutive, equatorial, confocal sections (white numbers 1 and 2) of a representative microscopic field are shown. Bar = 2 μm.

levels of this viral antigen could largely hide the nucleolar pattern.

Another very interesting observation emerging from our study refers to the nuclear matrix association of the considered viral proteins. Specifically, the pp65 derived from the incoming viral particles was targeted to the nuclear matrix compartment, where a significant portion of this protein was concentrated first of all in the central nucleolar area (as observed in unextracted nuclei) and then redistributed in a perinucleolar layer, aside from a more diffuse nuclear pattern. It is known that, in contrast to some other HCMV tegument proteins, pp65 has a nuclear localization signal [Gallina et al., 1996]. Our data suggest that it should also harbor a nuclear matrix localization signal and, perhaps, a nucleolar targeting signal as well; to this extent, it is worth mentioning that the nucleolus includes several functional compartments, not only reflecting the dynamic process of ribosome biogenesis, but also being involved in many regulatory functions in cellular and viral mRNA and protein metabolism [Dundr and Misteli, 2001]. Our results suggest that the mode of interaction of pp65 with the molecules present in these compartments may differ, dependent on the infectious cycle stage. One cannot also exclude the possibility that pp65 interaction targets present in the central region of nucleoli are extracted in the course of nuclear matrix preparation.

The fast delivery of the incoming pp65 to nuclei and furthermore to nucleoli of infected cells suggests that this viral tegument protein may have a dual function, as they are being involved in some regulatory/signaling pathways. It is known that nucleoli are somehow involved in the control of cell growth [Rogalsky et al., 1993]. Suppression of the growth of host cells upon infection with different viruses in some cases is mediated by virus-specific proteins that are targeted to nucleoli [Miyazaki et al., 1995; Besse and Puvion-Dutilleul, 1996; Pokrovskaja et al., 2001; Chen et al., 2002]. As far as the possible function of pp65 during the early stages of HCMV infection is concerned, it may be of importance that pp65 strongly interacts with one of the cellular kinases and, perhaps, even mediate a capture of this kinase [Gallina et al., 1999]. It is also interesting that in cells transfected with the pp65-expressing vector, this protein was found to be associated with condensed chromatin throughout cell division and also with metaphase chromosomes [Dal Monte et al., 1996a]. Inhibition of pp65 expression by an antisense RNA caused arrest of HCMV replication, that occurred at early stages of infection, again in agreement with the hypothesis that pp65 plays some essential regulatory function in addition to its known function in assembly of viral capsids [Dal Monte et al., 1996b]. It should also be mentioned that pp65 is not the unique HCMV tegument protein with a suggested regulatory function. Several other HCMV tegument proteins also seem to be involved in adaptation of the host cell metabolism to the needs of HCMV replication [Liu and Stinski, 1992; Sindre et al., 2000]. Of special interest is the observation that this regulatory function of HCMV tegument proteins may be realized before the beginning of translation of HCMV-specific transcripts, i.e., that tegument proteins uptaken from viral particles play a special regulatory role in HCMV-infected cells [Sindre et al., 2000].

IEp72 is known to be a DNA-binding protein, with regulatory functions on viral gene expression [Isomura and Stinski, 2003]. Furthermore, it is able to interfere with several cellular processes, including gene regulation, cell cycle progression, signal transduction, apoptosis, interaction with specific nuclear domains, such as promielocytic leukemia (PML) bodies [Margolis et al., 1995; Zhu et al., 1995; Wilkinson et al., 1998; McElroy et al., 2000; Castillo and Kowalik, 2002]. Specifically, IEp72 was previously reported to accumulate in PML bodies and to trigger their disruption about 4 h after infection [Kelly et al., 1995; Ahn and Hayward, 1997; Muller and Dejean, 1999]. Disruption of PML bodies coincides with an organization of new nuclear compartments, i.e., the viral replication centers [Ahn et al., 1999]. In herpes simplex virus-infected cells, it has been shown that the disruption of PML bodies by a viral immediate-early protein, mediated by cellular proteasome, is a critical event that can modulate the efficiency of the lytic infection or, upon repression, hypothetically favor the evolution towards latency [Everett et al., 1998]; in a similar, though not identical way, this mechanism is suggested in case of cytomegalovirus [Xu et al., 2001]. Although we have not focused on this question in the present manuscript, preliminary immunostaining data support the notion that the IEp72 bright speckles observed in our

experimental model colocalize with the PML bodies (confirming the data in the literature), some of which are located in the nucleolus (unpublished results). Consistent with these data, two further new observations concerning IEp72 result from this study: one relates to the codistribution of IEp72 "speckles" and nucleoli, observed in several cells at early times after infection, the second to its association with the nuclear matrix.

The nucleolar targeting of IEp72 speckles is of interest, if we take into account the already mentioned nucleolar functions and, furthermore that PML too have been reported to localize within nucleoli under certain conditions, related to protein trafficking and catabolism [Mattsson et al., 2001].

Concerning the nuclear matrix–IEp72 association, it has been reported that this protein was (at least for the most part) extracted from nuclei in the course of the nuclear matrix preparation [Sanchez et al., 1998]. As in the case of pp65, the discrepancy may reflect the fact that, in the above cited work, the cytolocalization of IEp72 was studied at very late stages of HCMV infection. Nevertheless, it is important to emphasize that our results clearly demonstrate that IEp72 remains associated with the nuclear matrix at a time when the described HCMV-induced disruption of PML bodies has largely begun. Redistribution of IEp72 within the nuclear matrix compartment (rather than the loss of matrix association), occurring in connection with the disruption of PML bodies, shows that matrix association of IEp72 is important for the normal functioning of this protein both at the early and intermediate stages of the HCMV replication cycle.

Overall, these observations point to a prominent role for the nuclear matrix and the nucleolus, as HCMV interaction sites during a lytic cycle. These interactions involve at least two viral products, namely, two proteins with relevant regulatory activities and appear to be highly dynamic, depending on the phase of the infectious cycle.

Work is in progress for increasing knowledge concerning the basic nature and the functional involvement of the aforementioned nuclear compartments during the HCMV replicative cycle. Comprehension of this would provide us with important information on spatial relations and molecular mechanisms that govern nuclear function, which are necessary for the correct progression of a lytic infection.

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