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CELL BIOLOGY

Immunocytochemical Reorganization of the Nucleolus in Human Embryo Fibroblasts Infected with Cytomegalovirus *in vitro*

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It is known that the nucleolus is a large structural domain of the cell nucleus, whose main function is the biogenesis of ribosomes [1]. In addition, several recently published data suggest that the nucleolus *per se* or its individual proteins may also be involved in the processes that are not related to ribosome formation (e.g., virus infection development [2]). This assumption is confirmed by the fact that proteins of different viruses, including herpes viruses [3–6], adenovirus [7], human immunodeficiency virus (HIV) [8], human papillomavirus (HPV16) [9], and coronavirus IBV [10], are located not only in the nucleus, but also in the nucleolus of the infected cell. Moreover, some viral proteins can directly interact *in vivo* or *in vitro* with specific nucleolar proteins, such as B23/nucleophosmin [7], C23/nucleolin [10], RNA polymerase I-specific transcription factor, and SL1 [11]. Some data are also indicative of accumulation of specific nucleolar proteins outside of the nucleolar regions of nucleoplasm, where viral DNA is replicated and nucleocapsids are assembled [12]. However, in general, the role of the nucleolar apparatus in the productive development of virus infection is poorly understood. Almost nothing is known on the response to virus infection of a specific nucleolar protein, fibrillarin (34–36 kDa), which plays a key role in the early processing of primary rRNP transcripts [1, 13]. The fibrillarin dynamics during cell infection with cytomegalovirus has not been studied thus far.

Human cytomegalovirus (CMV) is classified with large DNA-containing viruses of the herpes virus β family [14]. Its genome may encode more than 200 proteins, which, depending on the beginning of their expression, are divided into intermediate-early, early, and late proteins. The expression of the intermediate-early proteins begins several (four to six) hours after the infection. One of the best studied intermediate-early CMV proteins is phosphoprotein IEp72 (pp 72, UL123, 68–72 kDa), which plays a key role in the viral DNA expression [14]. The expression of early proteins begins later (one to two days after infection). The products of their expression are mainly involved in the viral DNA replication. One of early CMV proteins is phosphoprotein pp65 (UL83, 65 kDa) contained in the tegument (the virion coat formed by different proteins and RNA). The majority of late genes encode the structural viral proteins [14].

Earlier, when studying the response of the nucleolar apparatus to the development of CMV infection in human embryo fibroblasts *in vitro*, we discovered that, at the early stages of infection, rDNA transcription in the infected cells is enhanced compared to intact cells. In this study, we attempted to determine whether this process is related to the migration of a CMV protein into the nucleolus. For this purpose, we studied the localization of the IEp72 and pp65 proteins at different stages of infection development. Simultaneously, we studied the dynamics of the nucleolar protein fibrillarin. The results obtained demonstrate that, beginning from the early stages of infection, the CMV protein pp65 is located in the nucleoli and that the nucleolar protein fibrillarin is accumulated in the nucleoplasmic regions containing the pp65 and IEp72 proteins. Apparently, the function of fibrillarin in these regions is different from that in the nucleolus.

This study was performed with human embryo fibroblasts (HEF) (the Laboratory of Tissue Cultures of the Ivanovsky Research Institute of Virology, Russian Academy of Medical Sciences) at the 10th to 17th passages *in vitro*. The cells were grown in the mixture of Eagle's MEM and medium 199 (1 : 1) supplemented with 10% fetal calf serum (PanEco, Russia). The cells were transplanted onto cover slips (at an initial density of 8×10^4 to 10^5 cells/ml) and a day later infected with the CMV strain AD169 (kindly provided by Dr. D. Emanuel, United States) with an infection multiplicity of one to five plaque-forming units per cell. The cells were then placed into virus-free medium and cultured until mass cell death (which, at the specified

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infection multiplicity, occurred on the day 3 to 4 after infection). Uninfected HEF cells cultured under the same conditions served as the control. To detect the localization of CMV antigens, we used mouse monoclonal antibodies against proteins IEp72 (dilution, 1 : 50) and pp65 (dilution, 1 : 100), which were obtained earlier in the Laboratory of Cell Engineering of the Ivanovsky Research Institute of Virology. The nucleolar protein fibrillarin was detected using autoimmune serum K56 [15].

For immunocytochemical analysis, the cells were fixed in a 3% paraformaldehyde (PFA) (Sigma, United States) solution prepared in phosphate saline buffer (PBS) by the standard procedure [15]. The cells were incubated with antibodies against the former protein at 37°C for 45 min, washed with PBS buffer, and incubated with competent antibodies against the latter protein conjugated with the fluorochrom FITC (dilution, 1 : 100, Sigma) or TRITC (dilution, 1 : 200, Sigma) at 37°C for 30 min. For double staining of proteins IEp72 and pp65, the cells preliminarily fixed and treated with the detergent as described above were incubated with antibodies against the former protein at 37°C for 45 min, washed with PBS, and incubated with FITC-labeled antibodies against mouse immunoglobulins at 37°C for 30 min. Thereafter, the cells were washed again with PBS, fixed in 3% PFA for 10 min, and incubated with the antibodies against the latter protein and with TRITC-labeled antibodies against mouse immunoglobulins. All antibody solutions were prepared in PBS supplemented with 3% dry skim milk. Nuclear chromatin was additionally stained with the fluorochrom DAPI (1 μg/ml, Sigma, United States) for 10 min. The preparations were then embedded into Moviol (Calbiochem, United States).

RNase A treatment of the preparations was performed as follows. The fixed cells were incubated in a PBS solution of RNase A (1 mg/ml; Sigma, United States) for 60, 90, and 120 min at 37°C [15]. Subsequent procedures of cell immunolabeling and preparation of specimens were performed as described above. The specimens were viewed and photographed using an Opton III microscope (Carl Zeiss, Germany) with a 100× lens and an Olympus C3030Z camera (Olympus, Japan). The images were processed using the Adobe Photoshop 4.0 software.

Staining of HEF cells with antibodies against proteins IEp72 and pp65 at different time intervals after infection showed that both proteins were locaed predominantly in the nuclei of the infected cells. Because the expression of the IEp72 protein began earlier than the expression of the pp65 protein [4], the preparations contained cells whose nuclei exhibited positive staining for IEp72 but not for pp65 (data not shown). The IEp72 and pp65 proteins were not only evenly distributed over the nucleoplasm, but also formed more homogeneous and bright aggregates ("viral fields") inside the nucleus that were often stained with DAPI brighter than the remaining nuclear chromatin (Figs. 1a–1c). The sizes of these aggregates progressively increased as the infection developed. On the day 3 to 4 after infection, they could occupy up to a half of the total nuclear area (Figs. 1d–1f). In addition, the pp65 protein was also detected in the nucleoli (Figs. 1a–1c) and in discrete inclusions contained in the cytoplasm (Fig. 1e). None of the specimens contained the IEp72 protein in the nucleoli. In uninfected cells, the IEp72 and pp65 proteins were not detected at all (data not shown). In infected cells at the early stages of the infection, the nucleolus staining for fibrillarin insignificantly differed from the control: in the nucleolus, fibrillarin was located in discrete sites corresponding to the dense fibrillar component (Figs. 1a, 1b) [15]. However, in contrast to the control, the antibodies against fibrillarin also stained, in many cells, the nucleoplasm, local regions within the nucleus (Figs. 1a, 1b), and the discrete structures in the cytoplasm (Fig. 1d), the location of which coincided well with the location of the pp65 protein (Fig. 1e). At later stages of infection development, which preceded the cell death (day 4), the staining of the nucleoli for fibrillarin became less bright than on days 1 and 2. Conversely, immunostaining of the "viral fields" with antibodies against fibrillarin increased (data not shown). This effect is indicative of fibrillarin redistribution from the nucleolus to the nucleoplasm.

Earlier, we found that staining of the nucleolus with antifibrillarin antibodies is blocked after the treatment of cells with HeLa RNase A [15], which indirectly confirms the relationship between fibrillarin and RNA in the control [13]. To determine whether these properties are retained after the cell infection with CMV, the control and infected HEF cells were treated with RNase A and then stained with antibodies against fibrillarin and pp65 (Figs. 1d–1f). We discovered that a 1.5–2-h incubation with RNase A (1 mg/ml) completely blocked the nucleolus staining for fibrillarin in the control and infected HEF cells. However, immunostaining of extranucleolar zones under the same conditions was still observed (Figs. 1d, 1e). The extranucleolar regions of the nucleus were still stained for the pp65 protein (Fig. 1e).

According to the data published recently, a significant number of viral proteins belonging to the herpes virus family not only is located in the nucleolus of the infected cell, but also contains specific signal nucleolar sequences (NoLS). This group of proteins includes the intermediate-early HSV-1 viral oncoprotein, ICP27, which is essential for viral DNA replication [3]; UL3, a HSV-2 viral protein, with an unknown function [6]; and MEQ, an MDV avian viral oncoprotein, which is apparently a trans-activator that regulates gene expression of both the virus and the host cell [5]. In this work, we demonstrated that the CMV protein pp65 is also located in the infected cell nucleolus beginning from the initial stages of infection.

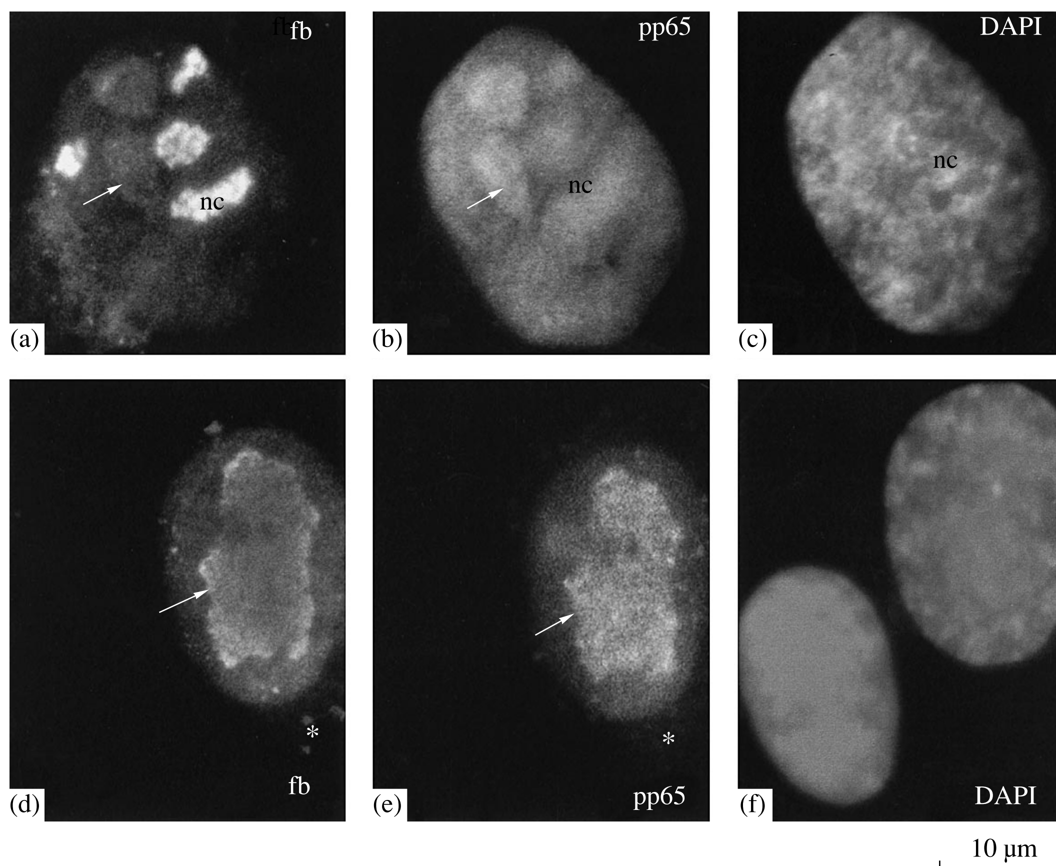

Fig. 1. Immunolocalization of the CMV protein pp65 and nucleolar protein fibrillarin in human embryo fibroblasts at the (a–c) early (days 1–2) and (d–f) late (days 3–4) stages of infection development. (a–c) Colocalization of (a) fibrillarin and (b) pp65 in the nucleoli (nc), "viral fields" (shown with arrows), and in the nucleoplasm. In the nucleoli, fibrillarin is visualized as more discrete and bright sites than in the extranucleolar regions. (c) The nucleolar region stained with DAPI is less bright than the other nuclear chromatin. (d–e) RNase A treatment of the infected (upper right) and uninfected (lower left) cells. In the infected cell, (d) fibrillarin and (e) pp65 protein are retained in the "viral fields" (shown with arrows) and in the discrete cytoplasmic inclusions (shown with asterisks). In the uninfected cell, (d) fibrillarin and (e) pp65 are not detected. (f) Nuclear chromatin is stained with DAPI, the edges of nuclei are clearly seen. Designations: nc, nucleolus; fb, fibrillarin.

It is known that the pp65 phosphoprotein is contained in the viral tegument; however, its role in the CMV infection development has not been clarified yet [14]. Taking into consideration the nucleolar location of pp65, it can be assumed that this protein is involved in the regulation of the synthesis of the ribosomes of the host cell. This assumption is indirectly confirmed by the fact that pp65 occurrence in the cell coincides with activation of rDNA transcription in the infected HEF cells, which was demonstrated earlier. As mentioned above, many viral proteins regulating virus infection development, such as HSV-1 proteins ICP27 [3] and Us11 [4], HIV proteins Rev and Tat [8], and HTLV-1 protein Rex [17], are also located in the nucleolus.

The nucleolar protein fibrillarin is one of the most evolutionarily conserved eukaryotic proteins. It is an inherent C/D component of small nucleolar ribonucleoprotein particles *U3*, *U8*, and *U14* and is involved in posttranslational 2'-O-methylation and processing of pre-rRNA [1]. In normal cells, fibrillarin is located predominantly in the nucleolus and only in trace amounts in the nucleoplasm [1]. The results of this study show that CMV infection changes the specific location of fibrillarin and causes its accumulation in the extranucleolar regions ("fields") containing viral proteins IEp72 and pp65. Unlike nucleolus, fibrillarin in these regions is present in the state insensitive to the RNase A treatment. This may be explained in two ways: either extranucleolar fibrillarin is present in a free (RNA-unbound) state or, unlike the pool of nucleolar fibrillarin, the complex of fibrillarin with RNA formed outside of the nucleolus is inaccessible for RNase A. Anyway, it is most likely that, outside of the nucleolus, fibrillarin functions are other than pre-rRNA processing. It is known that the central part of the fibrillarin molecule contains a domain comprised of approximately 90 amino acids, which displays universal RNA-binding activity [13]. In view of this, it can be assumed that, in the nucleoplasmic "fields," fibrillarin is involved in viral mRNA processing. The finding that

the RNase A-resistant pool of fibrillarin is present not only in the nucleus, but also in the cytoplasm (where it colocalizes with the pp65 protein (Figs. 1d, 1f)) is suggestive of the existence of these proteins in the form of a stable macromolecular complex.

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