

Mitochondrial distribution and function in herpes simplex virus-infected cells

Takayuki Murata, Fumi Goshima, Tohru Daikoku, Kyoko Inagaki-Ohara, Hiroki Takakuwa, Keisuke Kato and Yukihiro Nishiyama

Laboratory of Virology, Research Institute for Disease Mechanism and Control, Nagoya University School of Medicine, Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan

In this study, mitochondria migrated to a perinuclear region in the cytoplasm in herpes simplex virus (HSV)-infected cells. HSV infection did not promote the expression of cytochrome c oxidase subunit 2 but did promote that of stress-responsive HSP60, both of which are known to be components of mitochondria. The levels of cellular ATP and lactate and mitochondrial membrane potential were maintained for at least 6 h but decreased at the late stage of infection. It was also found that the UL41 and UL46 gene products, both of which are known to be tegument proteins, accumulated in the perinuclear region. The clustering of mitochondria and the accumulation of tegument proteins were completely blocked by the addition of nocodazole and vinblastine. These results suggest that mitochondria respond to the stimulation of HSV infection, migrating with tegument proteins along microtubules to a site around the nucleus, and maintain function until at least the middle stage of infection.

Mitochondria are well-known multifunctional organelles of which eukaryotic cells usually contain several hundred, widely spread throughout the cytoplasm. Although mitochondria have their own genome, transcription–translation equipment and replication system, nuclear-encoded gene products are indispensable for mitochondria in order to function and, conversely, eukaryotic cells could not survive without mitochondria. They have thus evolved very intimate and complicated communication systems, some parts of which remain elusive.

This study focused on the effects of herpes simplex virus (HSV) infection on mitochondria. Since mitochondria play prominent roles in supplying energy, regulating calcium levels and controlling apoptotic cell death, processes that are critically important for cell mortality, it is important to study virus-

mediated mitochondrial changes. Recently, it has been reported that mitochondria migrate to the virus assembly site in African swine fever virus (ASFV)-infected cells, where the respiratory function of mitochondria is activated (Rojo *et al.*, 1998). This study suggested that mitochondria are altered to supply energy effectively for the virus morphogenetic process. It has also been shown that the herpesvirus saimiri-encoded Bcl-2 homologue prevents apoptosis by maintaining mitochondrial membrane potential and inhibiting the release of cytochrome *c* into the cytosol (Derfuss *et al.*, 1998). As for HSV, it was previously reported that synthesis of mitochondrial proteins in infected cells decreases progressively, dropping to about 60% (Latchman, 1988; Lund & Ziola, 1986). Tsurumi & Lehman (1990) have reported that mitochondrial RNA polymerase appears in extracts of HSV-infected cells as a result of infection-induced disruption of the mitochondrial membrane, followed by leakage of the enzyme into the cytoplasm. In addition, Lund & Ziola (1985) have observed that mitochondria in cells infected with HSV-1 exhibit a gradual decline in the initial Ca²⁺ uptake rate, dropping to 65% of the control rate at the end of the 12 h lytic cycle.

We first examined changes in mitochondrial subcellular localization by using immuno-fluorescent microscopy. In mock-infected Vero cells, rod-like mitochondria were distributed throughout the cells (Fig. 1*a*). When Vero cells were infected with HSV-2 186, they appeared to gather in the perinuclear region of the cytoplasm at 6 h post-infection (p.i.) (Fig. 1*d*), and sometimes formed a ring-like structure (Fig. 1*g*). The shape of mitochondrial particles in HSV-infected cells became more unclear. Similar observations were made in HSV-1 (KOS)-infected Vero and HEp-2 cells (not shown).

It has been shown that some tegument proteins accumulate in the perinuclear region of the cytoplasm (Elliott & O'Hare, 1999; Daikoku *et al.*, 1998). In order to determine the relationship between the subcellular distributions of mitochondria and tegument proteins, cells were stained with a mouse anti-mitochondrion monoclonal antibody (Fig. 1*a, d, g*) and a rabbit anti-UL41 serum (Fig. 1*b, e, h*). In mock-infected cells, no significant fluorescence was detected for the UL41 gene product and mitochondria were widely scattered in the cytoplasm. In HSV-infected Vero cells, mitochondria migrated

Author for correspondence: Yukihiro Nishiyama.

Fax +81 52 744 2452. e-mail ynishiya@med.nagoya-u.ac.jp

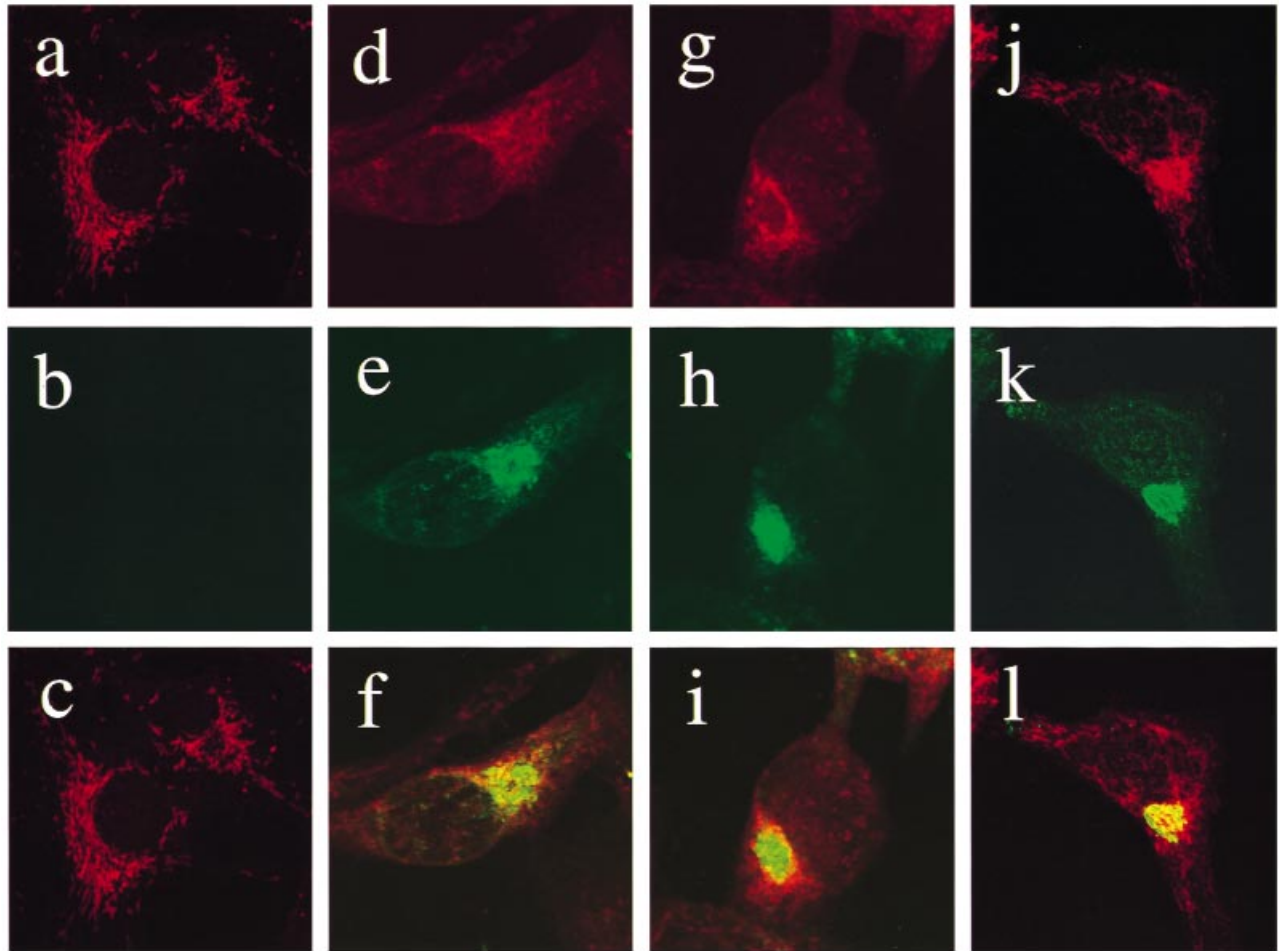


Fig. 1. Confocal microscopic images of mock-infected (*a-c*) or HSV-2-infected cells (*d-l*) showing mitochondria (*a, d, g, j*; rhodamine), UL41 (*b, e, h*; FITC) and UL46 (*k*; FITC) proteins and merged regions (*c, f, i, l*). Vero cells on coverslips maintained at 37 °C were fixed with cold acetone for 5 min at 6 (*d-f*), 9 (*j-l*) and 15 (*g-i*) h p.i. After air drying, cells were treated with 20% human serum for 1 h at room temperature to block non-specific antibody reaction. Cells were then reacted with mouse anti-mitochondrion antibody (Biogenesis, clone AE1) and rabbit anti-UL41 or anti-UL46 antisera (prepared by immunizing rabbits with purified 6 × His-tagged UL41 or UL46 protein) for 30 min at 37 °C, rinsed extensively with PBS and reacted with FITC-conjugated goat anti-rabbit immunoglobulin and rhodamine-conjugated goat anti-mouse immunoglobulin. Cells were then examined with an Axioplan2 confocal microscope (Zeiss). Fluorescent images were obtained with 488 and 568 nm bandpass filters for excitation of FITC and rhodamine, respectively.

to the perinuclear region, where the UL41 gene product appeared in the centre of the assembled mitochondria. The UL46 gene product, another tegument protein, gave similar staining patterns in HSV-infected cells (Fig. 1*j, k, l*).

The appearance of assembled mitochondria reminded us of the stress-responsive changes caused by heat shock (Collier *et al.*, 1993). It has been shown that some stress-responsive cellular genes are induced by HSV infection (Notarianni & Preston, 1982; La Thangue & Latchman, 1988). We therefore performed Western blot analysis of the chaperonin HSP60, which resides exclusively in mitochondria and plays a major role in the folding and assembly of proteins conveyed from the cytoplasm into the mitochondria (Martin *et al.*, 1995). HEp-2 cells were mock infected or infected with HSV-2 186 at an m.o.i. of 5. Western blotting was carried out as described

previously (Daikoku *et al.*, 1993). As expected, infection with HSV-2 resulted in significant induction of HSP60 (Fig. 2*a*).

We next examined the level of cytochrome *c* oxidase subunit 2 (COX2), which is one of the COX subunits encoded by the mitochondrial genome (Capaldi, 1990). COX catalyses the transfer of electrons from reduced cytochrome *c* to molecular oxygen, with a concomitant translocation of proteins across the mitochondrial inner membrane (Capaldi, 1990). When HEp-2 cells were infected with HSV at an m.o.i. of 5, the quantity of COX2 did not change until 6 h p.i. and was slightly reduced at 12 h p.i. (Fig. 2*b*).

In order to estimate mitochondrial energy supply function, the content of ATP was determined by the ATP assay system (Toyo ink. Co.), in which luciferin-luciferase reactions were used, since these reactions reflect ATP content. From 3 to 9 h

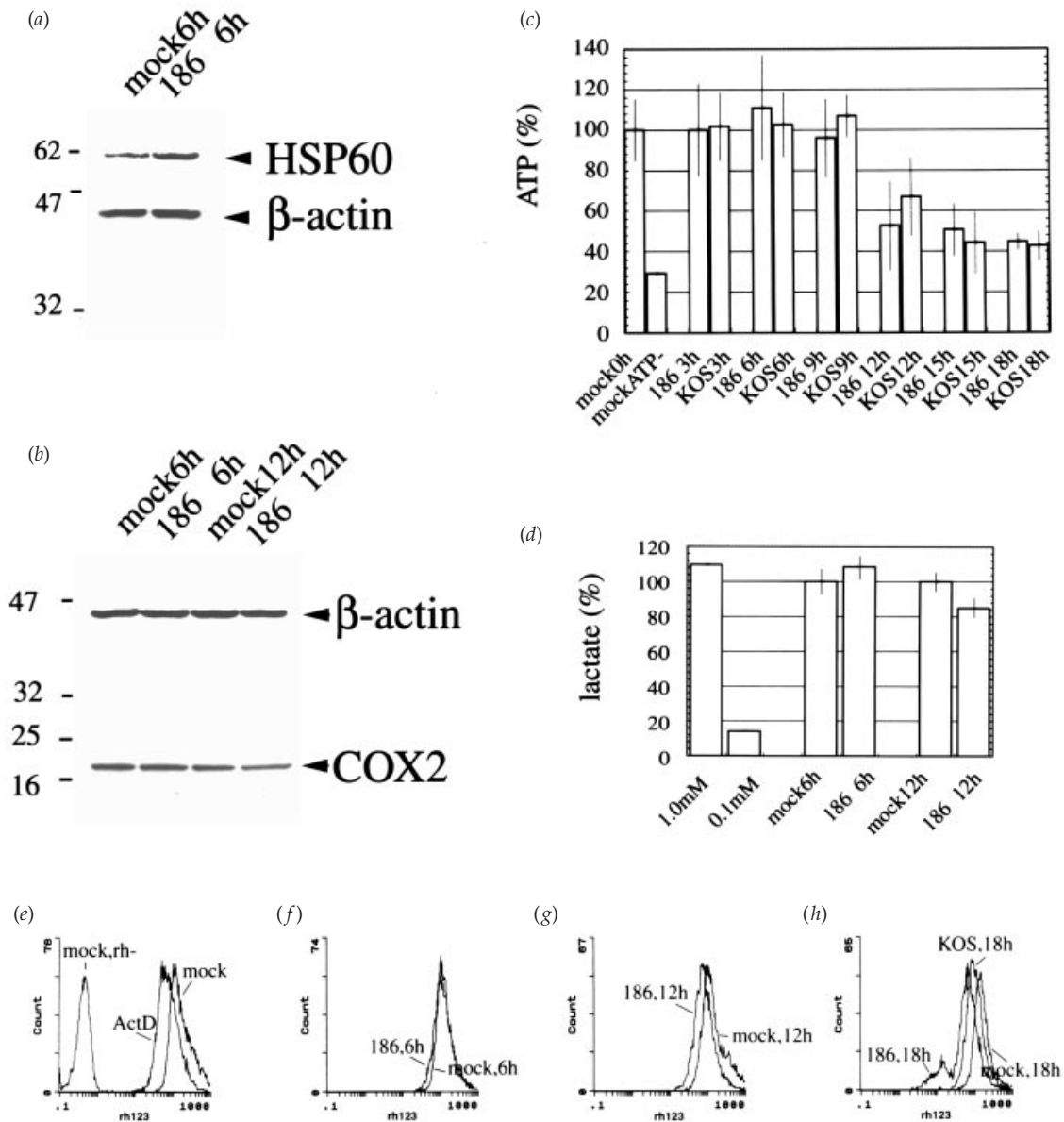


Fig. 2. Mitochondrial function in HSV-infected cells. (a)–(b) Immunoblot analysis of infected cells. The antibodies used were mouse monoclonal antibodies against HSP60 (a), β -actin (a, b; used as a loading control) and COX2 (b), obtained from Affinity Biogenesis (clone 4B9/89), Sigma (clone AC-15) and Molecular Probes (clone 12C4-F12), respectively. (c) Levels of cellular ATP. HEp-2 cells were infected with HSV-2 186 or HSV-1 KOS at an m.o.i. of 5 and, at the times indicated p.i., cellular ATP was measured with an ATP assay system. A standard curve was made and the data obtained fell within the linear region. Means of the ATP quantification data from three independent experiments are expressed relative to the mean level of mock-infected cells at 0 h p.i. (mock 0h); bars represent SD. As a negative control, three independent cell samples were treated with ATP-depletion cocktail (25 mM sodium azide and 25 mM 2-deoxyglucose) (Dasgupta & Wilson, 1999) for 1 h and ATP was measured (mock ATP-). (d) Relative lactate levels in the culture medium. HEp-2 cells were infected with HSV-2 186 at an m.o.i. of 5 at 37 °C for 90 min. The medium was then changed to fresh medium and incubated at 37 °C. At 6 or 12 h p.i., the medium was collected after centrifugation, precipitated with TCA and supernatants were assayed for lactate levels by using a kit (Sigma). A standard curve was made and linearity was confirmed. Each bar represents the mean and SD of three independent experiments. As controls, triplicate independent lactate standards (1.0 and 0.1 mM) were also measured. The mean lactate level in mock-infected cells at 6 h p.i. (mock 6h) was defined as 100%. (e)–(h) Measurements of mitochondrial inner membrane potential. HEp-2 cells were mock infected or infected with HSV-2 186 and HSV-1 KOS at an m.o.i. of 5. At 6 (f), 12 (g) or 18h (h) p.i., cells were stained with rhodamine (10 μ M). As controls (e), mock-infected cells (mock, rh-) and actinomycin-treated (ActD, 1 μ g/ml) cells are also shown.

p.i., HEp-2 cells infected with HSV maintained the level of ATP (100 and 102, 111 and 103 and 96 and 106% of mock infection levels, in 186 and KOS cells at 3, 6 and 9 h p.i., respectively), but ATP declined significantly from 12 h p.i. (53 and 67, 51 and 44, 45 and 42% at 12, 15 and 18 h p.i.) (Fig. 2*c*).

Cells can also generate ATP by glycolysis, starting with glucose and converting it into lactate. Although the reactions are carried out in the cytoplasm and not in mitochondria, production of lactate would be an indirect measure of the capacity of mitochondria to generate ATP. Analysis of the lactate secreted into the cell culture medium showed no significant increase or decrease at 6 h p.i. (Fig. 2*d*), suggesting that mitochondria maintained their energy supply function until the middle stage of infection.

Proton translocation by the electron transport system results in the highly negative potential of the inner mitochondrial membrane. Since rhodamine 123 is an aromatic cation, it distributes electrophoretically into the mitochondrial matrix across the inner membrane in response to membrane potential. Agents known to depolarize or deenergize mitochondria, such as uncouplers or respiratory inhibitors, decrease rhodamine 123 fluorescence of mitochondria in cells (Johnson *et al.*, 1981). Fluorescence is also reduced when cells are involved in apoptotic cell death processes (Shimizu *et al.*, 1996). Hence, we used rhodamine 123 to monitor mitochondrial membrane potential. The results of flow cytometric analysis of rhodamine 123 in HSV-infected HEp-2 cells are shown in Fig. 2(*e*)–(*h*). At 6 h p.i., membrane potential was not altered or was slightly reduced by HSV infection (Fig. 2*f*). However, at 12 and 18 h p.i., HSV infection resulted in a significant decrease in membrane potential (Fig. 2*g, h*). The small peak of HSV-2-infected cells at 18 h p.i. was thought to represent dead cells, since a certain population of HEp-2 cells infected with HSV-2 are known to be apoptotic (Koyama *et al.*, 1998). As a control, actinomycin D-treated cells, in which apoptosis was markedly induced, were prepared so that we were able to learn the relative level of the reduction in HSV-infected cells (Fig. 2*e*). We therefore conclude that, while HSV infection caused a significant reduction in membrane potential in the late stage of infection, the potential was maintained until at least 6 h p.i.

There is a consensus that mitochondria are scattered or transported in association with cytoskeletal elements such as microtubules or intermediate filaments. Microtubules have been shown to play a central role in the transportation of mitochondria (Heggeness *et al.*, 1978; Miller & Lasek, 1985), although intermediate filaments are also reported to be involved in mitochondrial migration (Collier *et al.*, 1993). In order to clarify which structures were crucial for the accumulation of mitochondria and tegument proteins in HSV-infected cells, we used the following inhibitors: nocodazole and vinblastine to disassemble the tubulin network and taxol to stabilize microtubules. At 9 h p.i., cells were fixed with acetone and then mitochondria and UL41 were stained by

rhodamine and FITC, respectively. In mock-infected cells (Fig. 3*a*), no green fluorescence was detectable but a number of small red dots representing mitochondria were observed in the cytoplasm. The addition of DMSO, which was used to dissolve the drugs, did not affect migration of the UL41 protein or mitochondria (Fig. 3*b*). Nocodazole (Fig. 3*c*) and vinblastine (Fig. 3*d*) inhibited the migration of the UL41 product and mitochondria. In the presence of these drugs, the UL41 gene product was distributed in the cytoplasm as small dots and there was no apparent interaction between the distributions of mitochondria and UL41 protein. The subcellular localization of UL41 protein in HSV-2-infected cells that were treated with nocodazole and vinblastine (Fig. 3*c, d*) was similar to that in cells in which UL41 was singly expressed (Fig. 3*f*). According to the PSORT program (Nakai & Horton, 1999), the UL41 gene product is predicted to reside in microbodies (peroxisomes) or lysosomes in animal cells. It seems that the UL41 protein localized in microbodies or lysosomes as small dots in UL41-transfected cells, which suggests that other viral proteins may be required to facilitate accumulation of the UL41 protein to the region along the microtubule network. In contrast to the effects of nocodazole and vinblastine, treatment with taxol, which stabilizes microtubules, resulted in the migration of the UL41 protein and mitochondria to the region around the nucleus, although some mitochondria were left in the cytoplasm (Fig. 3*e*). Control experiments showed that the microtubule network was disassembled effectively by nocodazole (Fig. 3*h*) and was stabilized and thickened by taxol (Fig. 3*i*), while treatment with the solvent, DMSO, had no effect on the microtubule network (Fig. 3*g*).

The present study demonstrates that mitochondria were transported to a region in the cytoplasm to one side of the nucleus and formed a ring-like structure in HSV-infected cells. In ASFV-infected cells (Rojo *et al.*, 1998), the migration of mitochondria to the virus assembly site occurs when virus morphogenesis is under way and quite a high percentage of the mitochondria surrounding virus factories show a condensed structure, with a marked condensation of the cristae, the characteristic state of actively respiring mitochondria. An explanation for this observation is that mitochondria are altered to supply energy effectively for the virus morphogenetic process. In HSV-infected cells, mitochondria migrated and surrounded the tegument UL41 protein and accumulated in a region around the nucleus. How do the clustered tegument proteins and mitochondria contribute to HSV replication? Although we could not observe prominent activation of mitochondrial function, we presume that the gathering of mitochondria may play a role in the replication of HSV. There is general agreement that the inner lamella of the nucleus is the site of the initial envelopment, but it is still unclear when and where tegument proteins attach to capsids (Roizman, 1996). Rixon *et al.* (1992) suggested a model in which at least some of the tegument proteins are incorporated into the virion not in the nucleus, but in the cytoplasm. Recently, Elliott & O'Hare

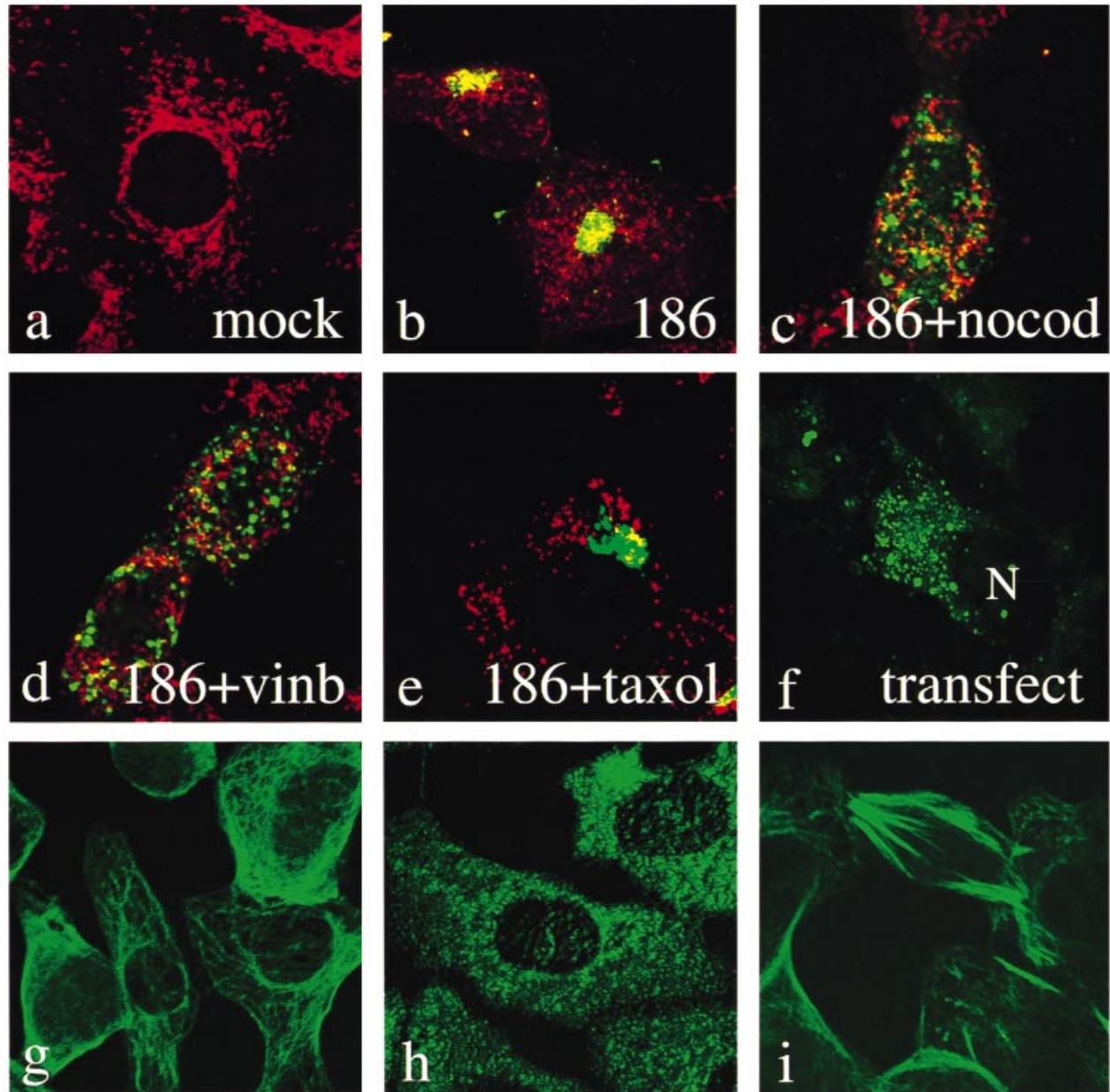


Fig. 3. Effects of microtubule-disrupting drugs on the migration of tegument proteins and mitochondria. Vero cells were mock infected (*a*) or infected with HSV-2 186 (*b–e, g–i*) for 90 min, followed by treatment with DMSO (*b, g*), nocodazole (nocod; 2 μ M) (*c, h*), vinblastine (vinb; 10 μ M) (*d*) or taxol (20 μ M) (*e, i*). At 9 h p.i., cells were fixed and reacted with anti-mitochondrion and anti-UL41 antibodies after blocking with human serum. Cells were then stained and examined with a confocal microscope as described in the legend to Fig. 1. Fluorescent images were obtained with 488 and 568 nm bandpass filters for excitation of FITC (UL41) and rhodamine (mitochondria), respectively. The staining pattern of tubulins from the same samples are shown in (*g*), (*h*) and (*i*). (*f*) COS-1 cells were transfected with pCDNAUL41 by using lipofection and, at 24 h post-transfection, cells were fixed with acetone and reacted with the anti-UL41 antiserum. N, Nucleus.

(1999) showed strong evidence that tegument assembly occurs in the cytoplasm. The process of maturation, including the incorporation of tegument proteins, may require an effective supply of energy.

Microtubules are one of the cytoskeletal structures, consisting of tubulin and expanding from the centre to the periphery of the cell. Incoming HSV is transported to the

nucleus along microtubules (Sodeik *et al.*, 1997). Our results suggest that both the UL41 gene product and mitochondria were transported to the region around the nucleus along microtubules in HSV-infected cells. Some reports have pointed out the involvement of intermediate filaments in the migration of mitochondria, but we observed an inhibition of transport of mitochondria and UL41 protein with the addition of

microtubule-disassembling drugs (Fig. 3 *c, d*). Hence, we conclude that microtubules play an essential role in gathering mitochondria and UL41 protein.

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