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Redistribution of Microtubules and Golgi Apparatus in Herpes Simplex Virus-Infected Cells and Their Role in Viral Exocytosis

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Earlier studies have shown that the Golgi apparatus was fragmented and dispersed in herpes simplex virus 1-infected Vero and HEp-2 cells but not in human 143TK$^-$ cells, that the fragmentation and dispersal required viral functions expressed concurrently with or after the onset of DNA synthesis (G. Campadelli-Fiume, R. Brandimarti, C. Di Lazzaro, P. L. Ward, B. Roizman, and M. R. Torrisi, Proc. Natl. Acad. Sci. USA 90:2798–2802, 1993), and that in 143TK$^-$ cells, but not Vero or HEp-2 cells, infected with viral mutants lacking the U_L20 gene virions were glycosylated and transported to extracellular space (J. D. Baines, P. L. Ward, G. Campadelli-Fiume, and B. Roizman, J. Virol. 65:6414–6424, 1991; E. Avitabile, P. L. Ward, C. Di Lazzaro, M. R. Torrisi, B. Roizman, and G. Campadelli-Fiume, J. Virol. 68:7397–7405, 1994). Experiments designed to elucidate the role of the microtubules and of intact or fragmented Golgi apparatus in the exocytosis of virions showed the following. (i) In all cell lines tested (Vero, 143TK$^-$, BHK, and Hep-2) microtubules underwent fragmentation particularly evident at the cell periphery and then reorganized into bundles which circumvent the nucleus. This event was not affected by inhibitors of viral DNA synthesis. We conclude that redistribution of microtubules may be required but is not sufficient for the fragmentation and dispersal of the Golgi apparatus. (ii) In all infected cell lines tested, nocodazole caused fragmentation and dispersal of the Golgi and a far more extensive depolymerization of the microtubules than was seen in untreated, infected Vero or HEp-2 cells. Taxol precluded the depolymerization of the microtubules and fragmentation of the Golgi in both infected cell lines. Neither nocodazole nor taxol affected the exocytosis of infectious virus from Vero, HEp-2, or 143TK$^-$ cells infected with wild-type virus. We conclude that the effects of nocodazole or of taxol are dominant over the effects of viral infection in the cell lines tested and that viral exocytosis is independent of the organization of microtubules or of the integrity of the Golgi apparatus. Lastly, the data suggest that herpes simplex viruses have evolved an exocytic pathway for which the U_L20 protein is a component required in some cells but not others and in which this protein does not merely compensate for the fragmentation and dispersal of the Golgi apparatus.

This report concerns the relationship between two phenomena associated with the replicative cycle of herpes simplex virus 1 (HSV-1). The first event is the fragmentation and dispersal of the Golgi apparatus in Vero, HEp-2, and, to a lesser extent, BHK cells productively infected with wild-type virus (5). For these cells immunofluorescence studies have shown that markers associated with the Golgi apparatus are dispersed throughout the cytoplasm and are no longer localized in a few tight clusters in the vicinity of the nucleus. Electron microscopic studies have shown that in these cells the Golgi stacks typical of uninfected cells are replaced by clusters of distended vacuoles. In contrast, the localization of the Golgi markers in infected 143TK$^-$ cells cannot be differentiated from that in the uninfected cells, and electron microscopic observations have verified the presence of intact, albeit at times distended, Golgi stacks (5). The Golgi apparatus plays a key role in the processing of oligosaccharide chains of the numerous viral glycoproteins in the course of the exocytosis of virions from infected cells (4, 19), and therefore the role of the fragmentation and dispersal of the Golgi apparatus in the biology of the virus appears to be a significant issue.

The second phenomenon concerns the exocytosis of virus from infected cells. In cells productively infected with wild-type HSV-1, capsids are assembled in the nucleus and become enveloped at the inner nuclear membrane, encased in transport vesicles, and transported through the cytoplasm to the extracellular space (4, 19). During this transit virions become modified by enzymes derived from either the intact (143TK$^-$ cells) or the fragmented and dispersed (Vero cells, HEp-2 cells, etc.) Golgi apparatus. A curious observation which led to these studies is that in Vero and HEp-2 cells infected with an HSV-1 mutant lacking the U_L20 gene virions accumulate in the space between the inner and outer membranes and are not transported to the extracellular space (1a, 2, 25). In contrast, virions were exported in 143TK$^-$ cells infected with U_L20^- virus, albeit less efficiently than in cells infected with wild-type virus (2). The central question is whether the fragmentation and dispersal of the Golgi apparatus and the block in the exocytosis of the U_L20^- virus are causally related or are consequences of independent events.

Electron microscopic studies indicate that the Golgi apparatus of uninfected cells appears to be composed of stacks of flattened cisternae which colocalize with the microtubule organizing center, from which microtubules radiate. Fragmentation and dispersal of Golgi apparatus in uninfected cells are

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commonly associated with depolymerization of microtubules (12). The causal relationship between the fragmentation of Golgi and the depolymerization of microtubules has been established in the absence of drugs for cells undergoing mitosis (13, 14) and for cells exposed to agents like nocodazole and colchicine, which bind tubulin heterodimers and inhibit their polymerization and thus induce progressive depolymerization (18, 22). The relationship is supported by studies of taxol, a drug which binds to microtubules (7) and induces the assembly of tubulin into microtubules (20) and thus blocks the normal microtubule dynamics and cell division (21). When added upon removal of nocodazole, taxol prevents repolymerization of microtubules and the reassembly of the Golgi (18). A key question which we have attempted to answer is whether the fragmentation of the Golgi apparatus is related to the modification of the organization of microtubules in infected cells. While changes in the organization of microtubules during HSV infection were reported previously (8, 16), these studies did not deal with temporal aspects of microtubule reorganization or the effect of microtubule reorganization on the structure of the Golgi apparatus or on release of virions from infected cells.

We report two key findings. First, the organization of microtubules is indeed altered in infected cells. Specifically, early in infection microtubules become fragmented; this is particularly evident at the periphery of the cell. This event is independent of viral DNA synthesis and is followed by the reformation of microtubules into bundles which appear to surround the nucleus. Inasmuch as the fragmentation of the Golgi occurs in Vero cells but not in infected 143TK$^-$ cells, whereas the depolymerization of microtubules occurs in both cell lines, we conclude that the depolymerization of the microtubules may be required but is not sufficient to account for the fragmentation and dispersal of the Golgi apparatus.

The second finding of key interest is that the extreme depolymerization of microtubules and attendant fragmentation of the Golgi caused by nocodazole or the stabilization of microtubules caused by taxol has no appreciable effect on the exocytosis of wild-type virus. We conclude that the requirement for U_L20 protein for exocytosis of virions from some cell lines is due to as yet unknown factors and not solely to the fragmentation and dispersal of the Golgi apparatus.

MATERIALS AND METHODS

Cells and viruses. Baby hamster kidney (BHK) cl 21/13, HEp-2, 143 thymidine kinase-minus (143TK$^-$), and Vero cells were grown in Dulbecco's modified Eagle's medium supplemented with either 10% newborn calf serum (BHK cells) or 5% fetal calf serum (Vero, HEp-2, and 143TK$^-$ cells). Wild-type HSV-1(F) (10) and the recombinant R7032 lacking the glycoprotein E (gE) gene (15) were described elsewhere. R7032 was used in immunofluorescence studies to avoid nonspecific binding of immunoglobulin G (IgG) to the Fc receptor encoded by gE.

Drugs. All drugs tested in these studies were from Sigma (St. Louis, Mo.), and they were used at the following final concentrations: nocodazole (dissolved in dimethyl sulfoxide), 20 μM; taxol (dissolved in methanol), 20 μg/ml; and cycloheximide and phosphonoacetic acid (PAA), 50 and 300 μg/ml, respectively. Nocodazole and taxol were added to the medium 4 to 5 h after the end of virus adsorption (1 h).

Antibodies. Mouse monoclonal antibody to β-COP (9), the Golgi coat protein of non-clathrin-coated vesicles, was used at a 1:100 dilution; polyclonal rabbit antiserum to HSV-1 glycoprotein B (gB) was used at a 1:400 dilution; and mouse monoclonal antibody to α-tubulin was purchased from the Radiochemical Center, Amersham, United Kingdom. Goat anti-mouse IgG Texas red-conjugated antibody was from Jackson Immuno Research Laboratories, and swine anti-rabbit IgG fluorescein-conjugated antibody was from DAKO (Glostrup, Denmark).

Immunofluorescence studies. BHK, HEp-2, 143TK$^-$, and Vero cells, grown on glass coverslips for 24 h, were infected or mock infected with R7032. At times after infection indicated in Results, cells were fixed with methanol for 4 min at −20°C, rinsed with phosphate-buffered saline (PBS), reacted for 60 min at room temperature with monoclonal and polyclonal antibodies diluted in PBS, rinsed extensively with PBS, reacted for 40 min with secondary antibodies conjugated to fluorescein isothiocyanate or rhodamine, rinsed once with PBS and once with distilled water, and mounted with Mowiol 40-88 (Hoetsch, Frankfurt, Germany).

Electron microscopic studies. Cells were fixed in 2% glutaraldehyde in PBS for 60 min at 4°C, postfixed with 1% osmium tetroxide, en bloc stained with uranyl acetate (5 mg/ml), dehydrated in acetone, and embedded in Epon 812. Thin sections were examined either unstained or poststained with uranyl acetate and lead hydroxide.

Determination of virus yield. Replicate HEp-2, 143TK$^-$, and Vero cell monolayer cultures were exposed to 3 PFU of HSV-1(F). After 90 min of exposure at 37°C, the inoculum was removed and the cultures were rinsed three times and overlaid with Dulbecco's modified Eagle's medium containing 1% fetal calf serum. Nocodazole and taxol were added from 3 h after infection until harvesting. At 3 h (zero time) and 24 h after infection, cells and medium were harvested separately. Cells were resuspended in a volume identical to that of the medium and disrupted by sonication. Virus titers were determined on Vero cells by plaque assay.

RESULTS

Effect of HSV-1 infection on the organization of microtubules. In the first series of experiments we examined the organization of microtubules at various times after HSV-1 infection by reacting fixed uninfected and infected Vero, HEp-2, BHK, or 143TK$^-$ cells with antibody to α-tubulin. Since wild-type HSV-1 strains encode a strong Fc receptor (gE by itself [3] or in combination with glycoprotein I [gI] [11]) that binds IgG and masks even strong signals, these studies were done with the recombinant virus R7032. Except in animal studies and in polarized cells, no difference between this gE$^-$ virus and its wild-type parent, HSV-1(F), has been noted. The results were as follows.

(i) In uninfected cells microtubules assembled in bundles of fibers in a highly ordered fashion. They originated from the microtubule organizing center (e.g., in 143TK$^-$ cells [see Fig. 2A, arrows]) and concentrated around the nucleus. Their free ends radiated outward toward the cell membrane, forming a network (Fig. 1A and D and 2A and D). In some cells (e.g., Vero cells) the networks tended to crisscross (Fig. 1D). In spindle-shaped cells (e.g., BHK cells) the filaments formed parallel bundles encompassing the length of the cell (Fig. 1A).

(ii) In infected cells microtubules underwent a two-stage reorganization. In the first stage (Fig. 1B and E and 2B and E), between 6 and 12 h after infection, the long bundles of microtubules fragmented into short, disorganized fibers which tended to point in all directions and were particularly evident at the cell periphery (Fig. 1B and E and 2B and E, arrows). In the vicinity of nuclei the microtubules acquired an amorphous appearance (Fig. 1B and E and 2B). The microtubule organizing centers were no longer apparent.

(iii) The second change occurred subsequently and culminated, at approximately 16 h after infection, in the reassembly of microtubules in more ordered structures which differed from those of uninfected cells (Fig. 1C and F and 2C and F). Specifically, microtubules again formed bundles, but these were arranged in ring-like structures around the nucleus. From these ring-like structures microtubules radiated to the cell periphery in a crisscross fashion (Fig. 2F, arrows). Particularly in rounded, shrunken cells, the microtubules formed condensed, amorphous rings or quasispherical structures around the nucleus. These two consecutive changes occurred in all four cell lines tested, i.e., Vero, HEp-2, BHK, and 143TK$^-$. It should be stressed again that R7032 did not differ from HSV-1(F) except that in cells infected with the latter virus the immunofluorescence due to the interaction with the Fc receptor was additionally detectable.

One objective of these studies stemmed from the observation that the Golgi apparatus was fragmented and dispersed in infected Vero or HEp-2 cells but not in 143TK$^-$ cells (5) and

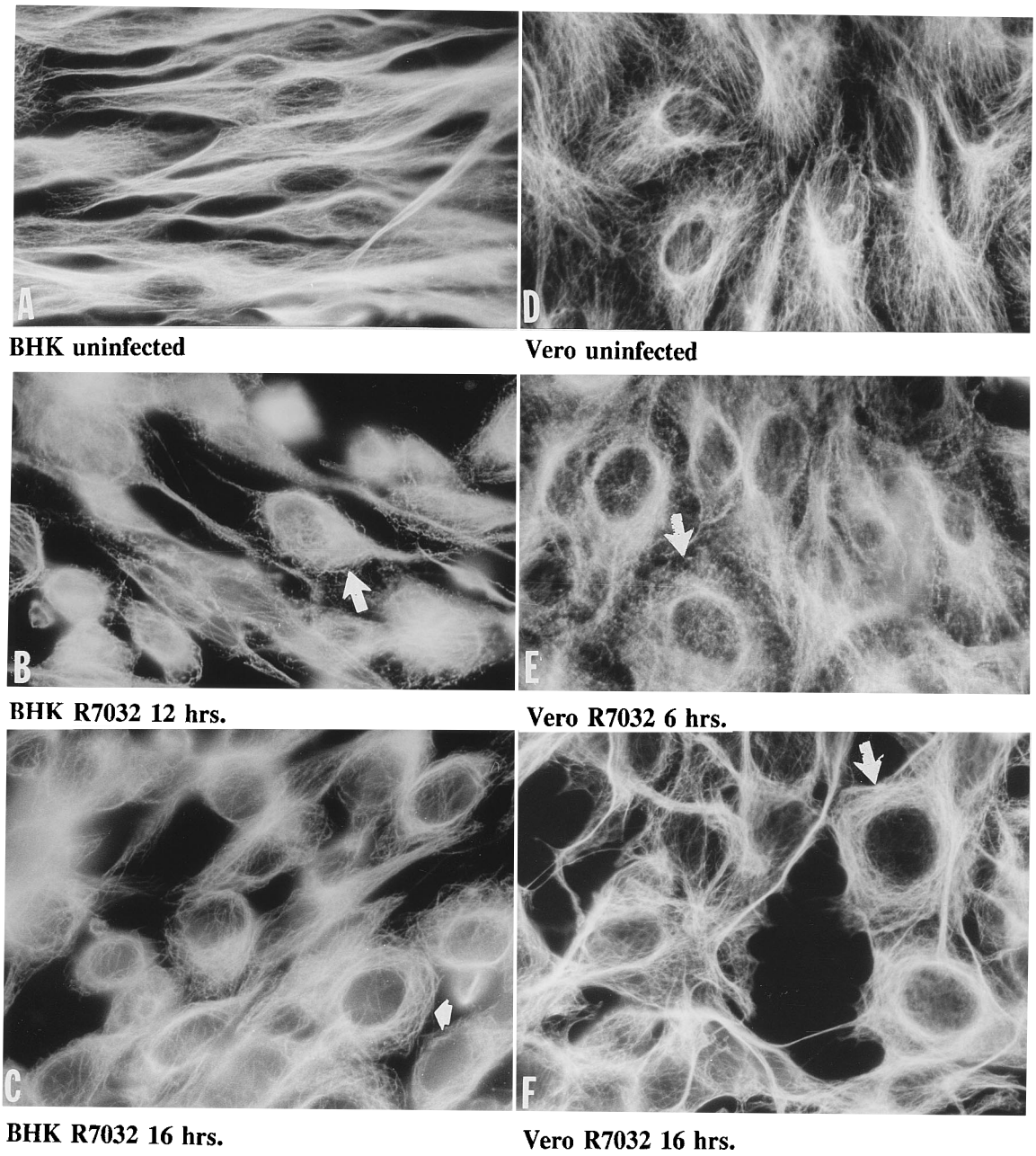


FIG. 1. Fluorescence staining with monoclonal antibodies to α-tubulin of BHK (A to C) and Vero (D to F) cells uninfected or infected with gE$^-$ recombinant R7032. Infected cells were fixed at the indicated times after infection. In panels B and E arrows point to the cell periphery, where microtubule fragmentation is more apparent. In panels C and F arrows point to bundles of reorganized microtubules forming circles around the nucleus.

the expectation that the microtubule organization of infected 143TK$^-$ cells would differ from that of infected Vero and HEp-2 cells. This was not the case inasmuch as we could not differentiate the changes in the organization of microtubules in the cell lines that we examined by fluorescence microscopy. We conclude that the rearrangement of microtubules after infection as observed by microscopy does not fully account for the differences in the structure and distribution of the Golgi apparatus of different infected cell lines.

The reorganization of microtubules is caused by an early HSV-1 function. Earlier studies have shown that the fragmentation and dispersal of the Golgi apparatus was caused by a late function inasmuch as it did not occur in infected cells treated with PAA at concentrations sufficient to inhibit viral DNA synthesis (5). To determine whether the reorganization of the microtubules was similarly dependent on a late viral function, cells were treated with PAA, from the time of exposure to virus to the time at which cells were fixed (7 or 10 h after infection). The fixed infected cells were reacted by double immunofluorescence with monoclonal antibody to tubulin and polyclonal antibody to gB. The results were as follows.

(i) As illustrated in Fig. 3C, the distribution of microtubules appeared altered in PAA-treated Vero cells relative to that in uninfected cells (Fig. 1D) and essentially resembled that seen

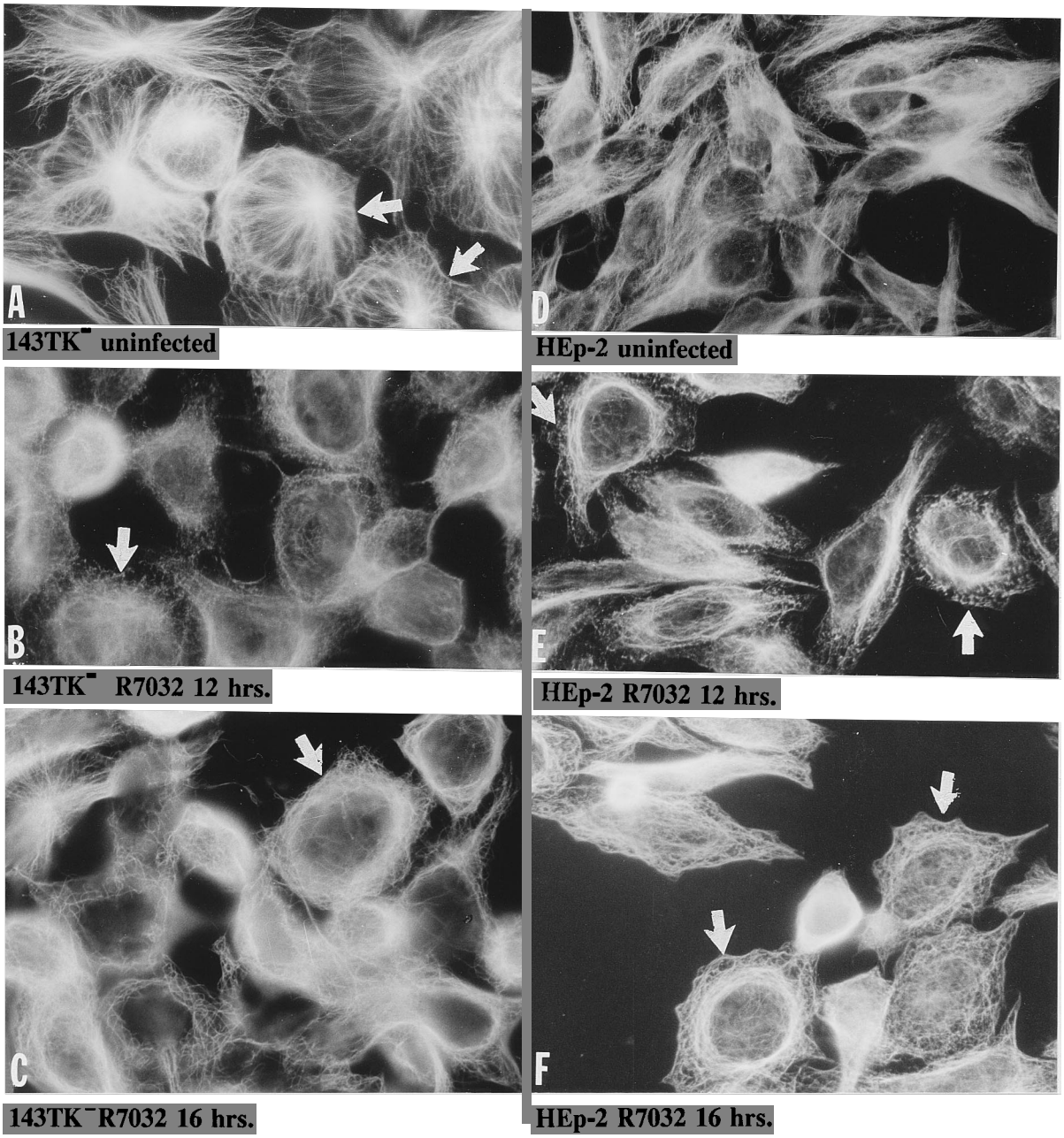


FIG. 2. Fluorescence staining with monoclonal antibodies to α-tubulin of 143TK⁻ (A to C) and HEp-2 (D to F) cells uninfected or infected with R7032. Infected cells were fixed at the indicated times after infection. Arrows in panel A point to the microtubule organizing centers. Arrows in panels B and E point to the cell periphery, where microtubule fragmentation is more apparent. In panels C and F arrows point to bundles of reorganized microtubules forming circles around the nucleus. In panel F arrows point to microtubules which appear to underlie the plasma membranes.

in infected untreated cells fixed at the same times after infection (Fig. 3A). A more limited fragmentation of microtubules at the cell periphery was the sole differential feature between PAA-treated and untreated infected cells.

(ii) Earlier studies have shown that in infected cells viral glycoproteins, including gB, localize to the nuclear and plasma membranes, to reticular cytoplasmic structures, and to the Golgi apparatus (4, 6, 19, 23). In addition, gB is part of the envelope of virions in transit to the extracellular space, but the localization of virion glycoproteins cannot be differentiated from that of membrane glycoproteins by immunofluorescence. The localizations of gB in untreated and PAA-treated infected cells differed somewhat. In untreated cells (Fig. 3B, arrows) gB localized to small structures dispersed throughout the cytoplasm in addition to the reticular network and the nuclear membranes. In PAA-treated cells, gB localized to a few large irregularly shaped structures larger than those observed in untreated cells (Fig. 3D). These structures were intracellular as determined by phase microscopy even though they appeared in photomicrographs to be situated on top of the cells. In earlier studies we found that gB colocalizes, in part, with β-COP (1), a coatomer of Golgi-derived vesicles (9). Current studies sug-

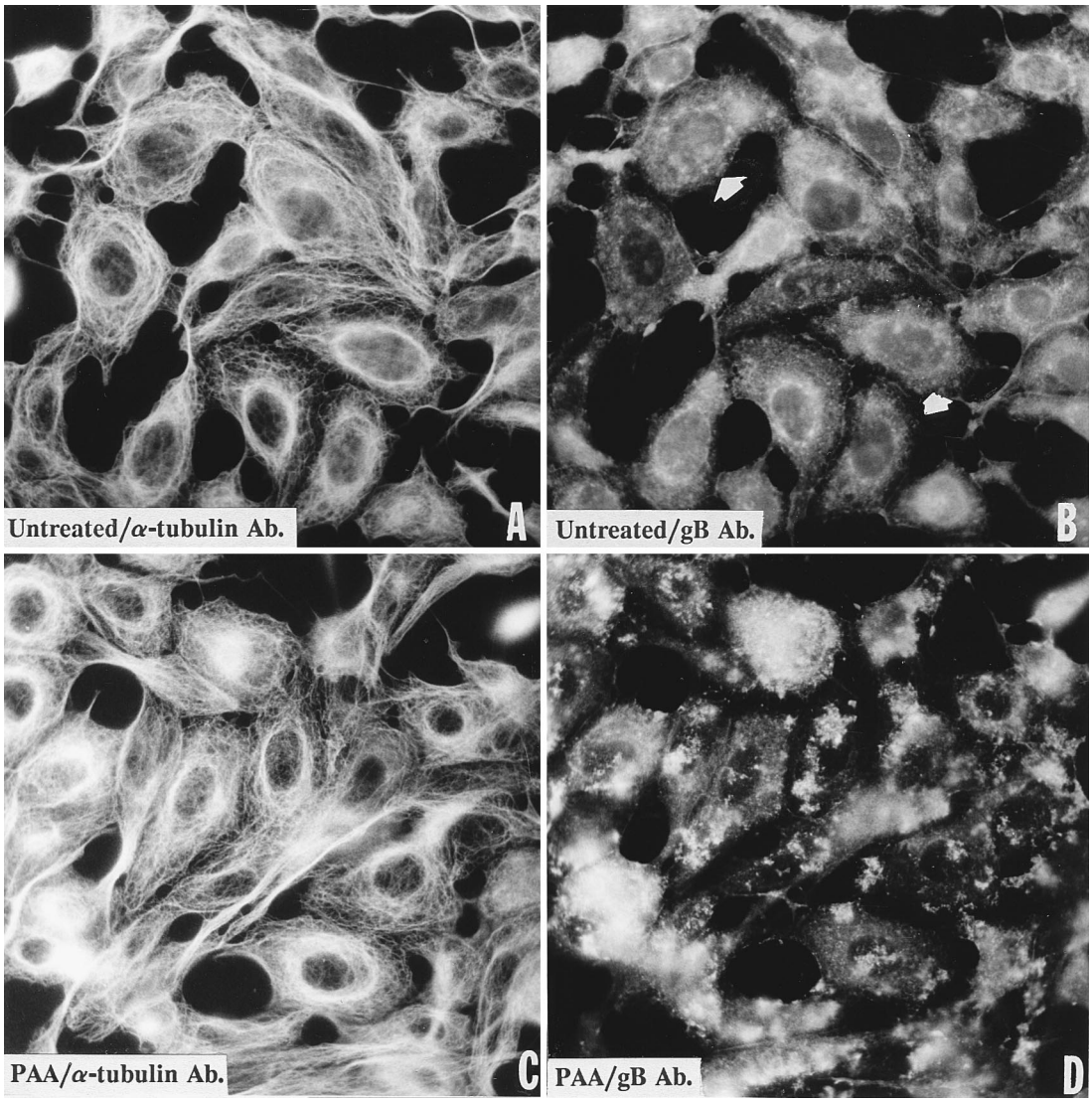


FIG. 3. Double immunofluorescence staining with monoclonal antibodies (Ab.) to α-tubulin (A and C) and a polyclonal antibody to gB (B and D) of R7032-infected Vero cells untreated (A and B) or treated with PAA (C and D). Cells were fixed at 10 h after infection. Arrows in panel B point to condensed spots of staining with antibody to gB very likely representing fragmented Golgi stacks. The irregularly shaped structures reacting with gB antibody shown in panel D were cell associated as determined by phase-contrast microscopy.

gest that the differential distribution of gB may reflect the colocalization of the viral glycoproteins with fragmented and dispersed or intact Golgi in untreated and PAA-treated cells, respectively.

We conclude from these studies that the fragmentation of the long bundles of microtubules is an event triggered by early functions, whereas the fragmentation and dispersal of the Golgi apparatus is a late viral function.

Effect of cycloheximide on the late reorganization of microtubules. To ascertain if the second stage in the reorganization of microtubules required the expression of proteins made late in infection, cells were exposed to cycloheximide from 8 to 16 h after infection. The organization of microtubules in cells exposed to cycloheximide did not differ substantially from those in untreated cells in that microtubules formed a prominent ring-like bundle around the nucleus and the fragmentation of microtubules at the periphery of the cell—a hallmark of the early stage in microtubule reorganization—was no longer visible (data not shown). The results are consistent with those obtained with cells treated with PAA and suggest that reorganization of microtubules observed late in HSV infection was set in motion by events which occurred early in the reproductive cycle. They also suggest that reorganization of microtubules does not require de novo synthesis of tubulin.

Effect of HSV infection on the organization of microfilaments. In order to determine whether the fragmentation of microtubules is accompanied by a breakdown of microfilaments, the organization of actin microfilaments in HSV-1-infected Vero cells was analyzed by immunofluorescence staining with fluorescein-labeled phalloidin. The results (not shown) did not reveal any fragmentation in microfilament organization in HSV-infected cells.

Effects of nocodazole and taxol on the organization of microtubules in infected cells. Two compounds which affect microtubules in opposite manners were tested in another series of experiments. Nocodazole causes depolymerization of microtu-

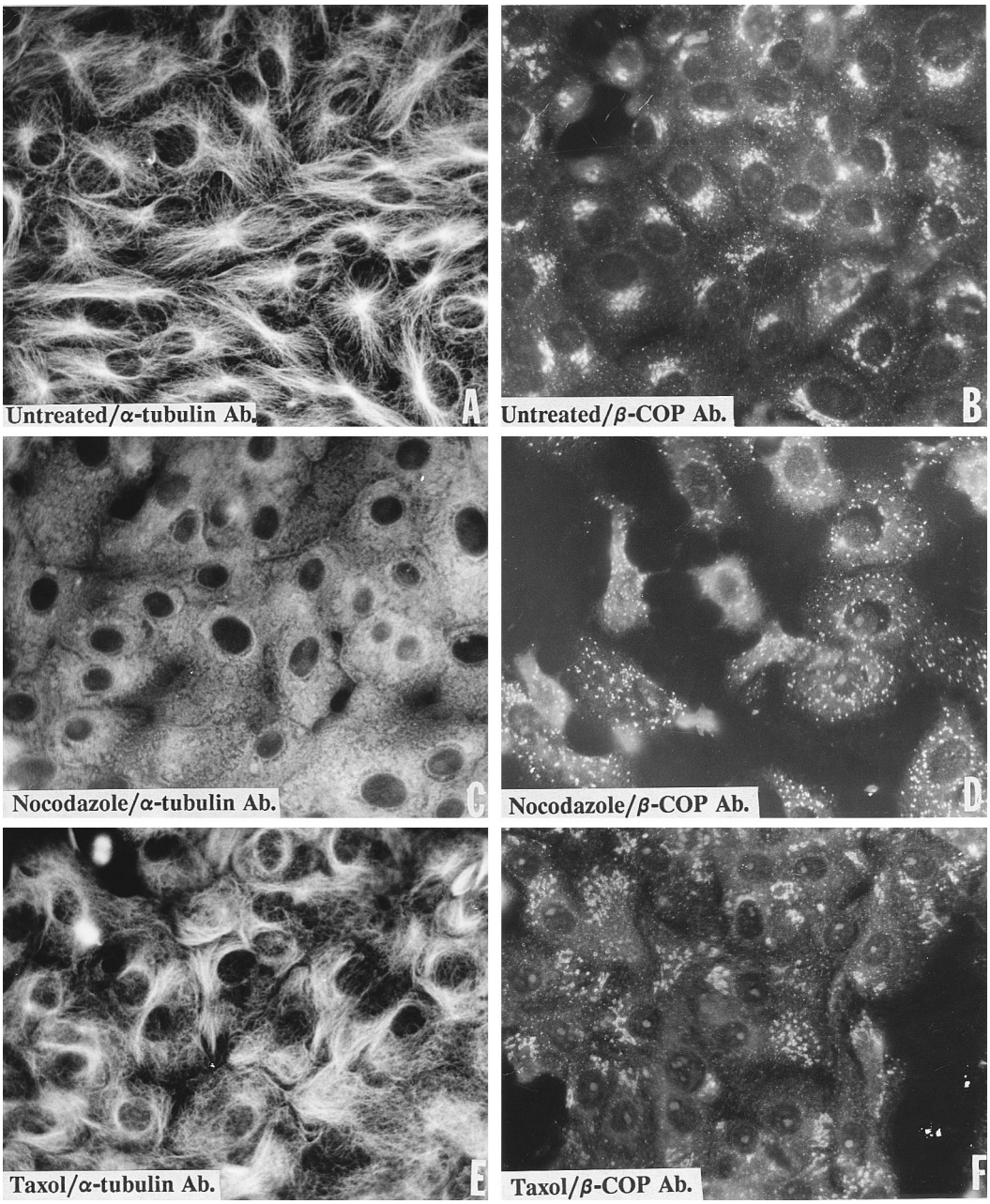


FIG. 4. Immunofluorescence staining with monoclonal antibodies (Ab.) to α-tubulin (A, C, and E) or β-COP (B, D, and F) of uninfected Vero cells untreated or treated with nocodazole (C and D) or taxol (E and F) from 4 h after virus adsorption.

bules, and concurrently, the drug induces a fragmentation and dispersal of the Golgi apparatus throughout the cytoplasm (22). Taxol stabilizes microtubules and prevents repolymerization of microtubules and the reassembly of the Golgi in cells treated with the drug upon removal of nocodazole (18).

In the first series of experiments we compared the alterations in microtubule organization induced by HSV with those induced by nocodazole or taxol in uninfected cells. Concomitantly, changes in the Golgi-associated proteins were monitored by staining with antibodies to the Golgi-associated protein β-COP. The results were as follows.

(i) The effect of nocodazole on the organization of uninfected-cell microtubules was much more dramatic than that induced by HSV-1 infection. In nocodazole-treated Vero cells the bundles of microtubules were no longer detectable and α-tubulin was localized diffusely and amorphously throughout the cytoplasm (Fig. 4C). The complete collapse of microtubules was accompanied by a dramatic fragmentation and dispersal of the Golgi apparatus as deduced from the distribution of β-COP (Fig. 4D). In contrast, in untreated cells β-COP was localized in structures which formed an arch or partial ring typical of the distribution of the Golgi apparatus (Fig. 4B). The fragmentation of microtubules and of the structures associated with β-COP was also seen in infected HEp-2, 143TK$^-$, and BHK cells (data not shown).

(ii) Taxol had a less dramatic effect than nocodazole. Bun-

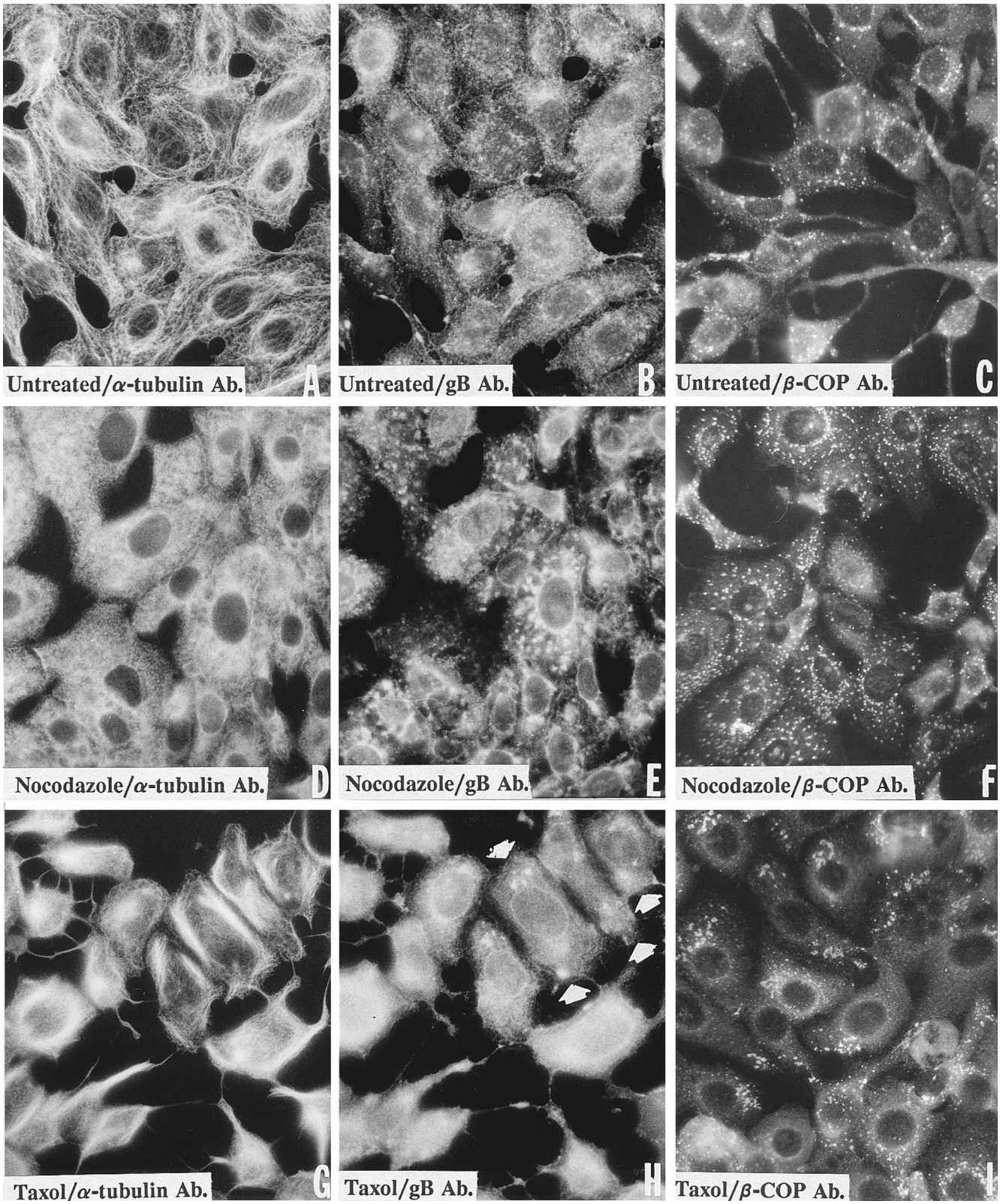


FIG. 5. Double immunofluorescence staining with monoclonal antibodies (Ab.) to α-tubulin (A, D, and G) or gB (B, E, and H) and staining with antibodies to β-COP (C, F, and I) of R7032-infected Vero cells untreated or treated with nocodazole (D to F) or taxol (panels G to I) from 4 h after virus adsorption. Arrows in panel H point to polar or bipolar areas of intense staining, similar to those apparent in cells stained with monoclonal antibody to β-COP as shown in panel I, which very likely represent a Golgi apparatus.

dling of microtubules and loss of typical architecture were the most common effects of the drug (Fig. 4E). β-COP was localized to one or two masses per cell which were characterized by a less compact structure and a more peripheral position than the localization of β-COP in untreated cells (Fig. 4F).

In the second series of experiments we investigated the effects of nocodazole or taxol on the organization of microtubules of HSV-1-infected cells and especially whether exposure to nocodazole or taxol affected the HSV-1-induced reorganization of microtubules. In these experiments the fixed infected cells were reacted with both monoclonal antibody to α-tubulin and polyclonal antibody to gB. As shown in Fig. 5D, nocodazole induced in infected cells the same dramatic depolymerization of microtubules seen in uninfected cells. In fact, the distribution of α-tubulin in HSV-infected Vero cells exposed to nocodazole could not be differentiated from that in uninfected cells (compare Fig. 5D with Fig. 4C). The distribution of β-COP also could not be differentiated from that seen in nocodazole-treated uninfected cells (compare Fig. 5F with Fig. 4D). gB was localized diffusely to a reticular network, to nuclear membranes, and to small, dot-like structures dispersed throughout the cytoplasm (Fig. 5E). The distribution of the dot-like structures was consistent with that of β-COP. It is remarkable that cells took on a flat rather than a rounded-up appearance. The results shown for Vero cells were obtained also with 143TK$^-$, HEp-2, and BHK cells (data not shown).

Exposure of infected Vero cells to taxol prevented the HSV-induced fragmentation of microtubules (Fig. 5G). Moreover, microtubule bundling similar to that seen in uninfected cells was also seen in infected cells treated with taxol (compare Fig. 5G with Fig. 4E). β-COP localized to one or two large, diffuse structures located at the periphery of the cell (Fig. 5I). This localization differed markedly from that seen in untreated infected cells, which consisted of fine fragments dispersed throughout the cytoplasm (Fig. 5C), and essentially resembled that seen in taxol-treated uninfected cells (compare Fig. 5I with Fig. 4F). The distribution of gB (Fig. 5H) resembled that of β-COP and differed from the distribution of the glycoprotein in untreated infected cells (compare Fig. 5H with Fig. 5B).

We conclude from these experiments that the effects of nocodazole or taxol on microtubule assembly were dominant over those induced by HSV infection. Both compounds prevented the alterations induced by HSV infection. Specifically, nocodazole induced a complete microtubule depolymerization, an effect morphologically more dramatic than the fragmentation induced by HSV infection. The depolymerization of microtubules was accompanied by the fragmentation and dispersal of the Golgi even in those cells, like 143TK$^-$, in which the Golgi apparatus remains intact after HSV infection. Conversely, the microtubule-stabilizing compound taxol prevented the HSV-induced fragmentation of microtubules apparent at the periphery of the cell and, in Vero cells, also prevented the fragmentation and dispersal of the Golgi apparatus. Consistent with the conclusions stated above, the complete collapse of microtubules induced by nocodazole prevented the typical rounding up of infected cells after infection with HSV-1.

Effect of nocodazole or taxol on Golgi ultrastructure. In another series of experiments we examined the effects of taxol and nocodazole on the Golgi apparatus at the ultrastructural level in both uninfected and infected cells.

Electron microscopic studies of HSV-1(F)-infected Vero cells (Fig. 6a) showed several clusters of vacuoles in the perinuclear area of the cell. As previously described (23), the vacuoles were approximately 500 nm in diameter and in many instances contained two or more virions. Typical Golgi stacks were only rarely observed, in contrast to the case with uninfected Vero cells (data not shown), in which the classical intact Golgi apparatus was readily detected. Occasionally, vacuoles appeared in continuity with Golgi fragments or isolated cisternae (Fig. 6a, arrow).

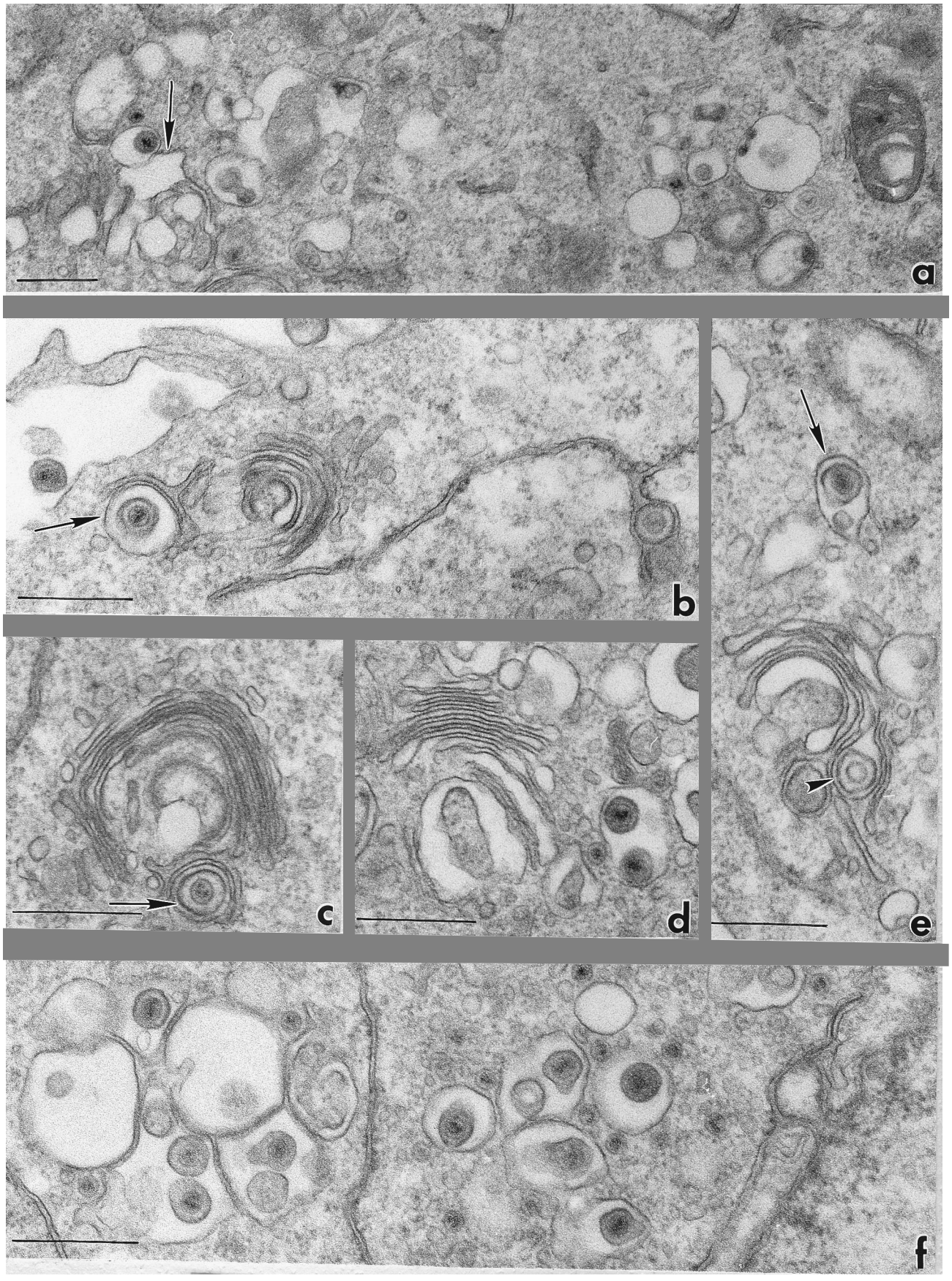
Ultrastructural examination of taxol- or nocodazole-treated cells revealed no major difference between uninfected (data not shown) and infected cells, except for the presence of virions, and was consistent with the results of immunofluorescence analyses presented above. In infected Vero cells exposed to taxol typical Golgi apparatus characterized by stacks of cisternae with *cis* transpolarity was easily detected (Fig. 6b to e). Virions were frequently observed inside vesicles of 200 to 250 nm in diameter in close proximity to Golgi cisternae (Fig. 6b, c, and e, arrows). Less frequently, virions were observed in larger vacuoles (Fig. 6d) containing two or more virions, similar to those visible in untreated infected cells. Occasionally, enveloped virions were found inside the dilated rims of Golgi cisternae (Fig. 6e, arrowhead). Infected Vero cells exposed to nocodazole (Fig. 6f) could not be readily differentiated from untreated infected cells. Clusters of vacuoles containing virions were frequently seen, whereas intact Golgi stacks were very rarely observed.

We conclude that the appearance and distribution of the Golgi marker seen by immunofluorescence in infected Vero cells treated with taxol correlate with the presence of intact Golgi stacks as seen at the ultrastructural level. Conversely, the redistribution of the Golgi marker throughout the cytoplasm in infected Vero cells treated with nocodazole corresponds to fragmented, dispersed Golgi at the ultrastructural level.

Effect of microtubule depolymerization or stabilization on virion egress. The finding that in some cell lines (Vero and HEp-2) the Golgi apparatus is fragmented and dispersed following HSV infection raised the question of whether the efficiency of viral exocytosis is related to the state of the Golgi apparatus in infected cells. The experiments described above indicated that for HSV-1-infected cells nocodazole induced the depolymerization of microtubules and the fragmentation and dispersal of the Golgi apparatus in all cells tested, i.e., including the 143TK$^-$ cells shown previously to retain the structure of the Golgi apparatus during HSV-1 infection. These experiments also led to the conclusion that taxol stabilized microtubules. To test whether the conformation of microtubules and the integrity of the Golgi apparatus specifically correlated with viral exocytosis, monolayer cultures of Vero, HEp-2, and 143TK$^-$ cells were infected with the wild-type parent virus HSV-1(F). At 24 h after infection and incubation at 37°C the cells and extracellular medium were harvested independently and assayed for virus titer as described in Materials and Methods. The results (Table 1) show that neither nocodazole nor taxol significantly affected the release of HSV-1(F) in any of the three cell lines tested. In HEp-2 cells both compounds induced a sevenfold reduction in HSV-1(F) yield—both cell associated and extracellular, so that the ratio of extracellular virus to cell-associated virus remained unchanged.

DISCUSSION

Alterations in microtubule architecture in HSV-1-infected cells. As noted in the introduction, the breakdown of the Golgi apparatus which occurs during mitosis (13, 14) and as a result of exposure to certain drugs is linked to depolymerization of microtubules (18, 22). Conversely, repolymerization of microtubules results in reassembly of Golgi stacks (18). We report that in HSV-1-infected cells the microtubules undergo at least two discernible changes in architecture. The first involves par-


a
b
c
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f

FIG. 6. Morphological appearance of HSV-1(F)-infected Vero cells. (a) Untreated cells showed clusters of vacuoles, occasionally containing enveloped virions. The arrow points to a vacuole in continuity with a cisterna presumably derived from Golgi fragmentation. (b, c, and e) In taxol-treated infected cells, typical Golgi stacks are visible in close proximity to vesicles containing enveloped virions. The arrowhead in panel e points to an enveloped virion inside a Golgi cisterna. (d) Rarely, larger vacuoles containing two or more virions were observed near Golgi stacks. In nocodazole-treated cells, clusters of vacuoles tightly juxtaposed, containing enveloped virions, were present. Magnifications are as follows: panel a, ×28,000; panel b, ×43,500; panel c, ×49,000; panel d, ×45,000; panel e, ×43,500; and panel f, ×46,000.

tial fragmentation of microtubules particularly apparent at the cell periphery, where the microtubular network appears to lose connections to the plasma membrane. This depolymerization is not nearly as extensive as that seen in cells treated with nocodazole. In the second step, the microtubules form bundles which surround the nucleus and in effect enable the cell to assume a nearly spherical shape. Two observations were unexpected and impact on our conclusions. First, the fragmentation of microtubules is an early event independent of the onset of viral DNA synthesis. Conversely, the breakdown and dispersal of the Golgi apparatus is an event dependent on the onset of viral DNA synthesis and, we assume, on the expression of viral late or γ_2 genes (5). The temporal dissociation of the reorganization of microtubules and of the fragmentation and dispersal of the Golgi stacks argues that as yet unknown factors are required for the latter event. This hypothesis is supported by the second observation that whereas the fragmentation and reorganization of microtubules in 143TK$^-$ cells cannot be differentiated from that occurring in Vero cells, the two cell lines exhibit dramatically different effects on the integrity and distribution of the Golgi stacks following HSV infection. These results do not permit us to conclude that the fragmentation of the Golgi is due solely to the depolymerization of microtubules. Given the evidence linking the integrity of the Golgi apparatus to that of microtubule organization, we have to conclude that in HSV-infected cells the depolymerization of the microtubules may be required but it is not sufficient for the fragmentation of the Golgi apparatus to occur.

It is of interest that the effects of nocodazole and taxol on the architecture of microtubules and the Golgi are dominant over the changes induced by HSV infection. At least two hypotheses may account for these observations. First, the effects of nocodazole and of taxol take place more rapidly and preempt the effects of viral gene expression. Second, both nocodazole and taxol act through the same biochemical pathways as viral gene products, and they either have additive effects (nocodazole) or preclude the effects (taxol) of viral gene expression.

TABLE 1. Effect of nocodazole or taxol[a] on virus release from infected cells

Cell line and treatment[b]	HSV-1(F) yield[c]		E/C ratio[d]
	Cell associated (10^8 PFU)	Extracellular (10^7 PFU)	
Vero cells			
None	20	4	0.02
+Noc	10	5	0.05
+Tax	20	4	0.02
143TK$^-$ cells			
None	20	0.8	0.004
+Noc	10	1	0.01
+Tax	6	0.4	0.007
HEp-2 cells			
None	40	50	0.12
+Noc	6	6	0.10
+Tax	6	8	0.13

[a] Nocodazole and taxol were added to culture medium at 4 h after adsorption.
[b] +Noc, nocodazole added; +Tax, taxol added.
[c] At 24 h after infection.
[d] Ratio of extracellular virions to cell-associated virions.

Effects of alterations in microtubule architecture and of Golgi breakdown and dispersal on the release of HSV from the cell. The effects of changes in Golgi structure on transport of membrane-bound or secretory proteins that move along the exocytic pathway have been the subject of extensive investigations. These effects include, for example, those of Golgi fragmentation induced by drugs such as nocodazole and the redistribution of Golgi enzymes to the endoplasmic reticulum induced by brefeldin A. The fragmentation and dispersal of the Golgi stacks has been reported to lower the efficiency of protein export in some studies (22) but not in others (17, 24). As mentioned in the introduction, in HSV-infected cells the Golgi apparatus plays a role in the processing of viral glycoproteins and the exocytosis of virions. The significance of the HSV-1-induced Golgi fragmentation is demeaned by the observation that both cells with fragmented Golgi (e.g., Vero and HEp-2) and cells with intact Golgi (e.g., 143TK$^-$) can release wild-type virions into the extracellular space. Indeed, differences between Vero and 143TK$^-$ cells with respect to viral exocytosis were observed only in cells infected with the U_L20^- mutant. One experimental approach to assess the roles of Golgi fragmentation and dispersal and of microtubule reorganization in the release of virions was to investigate the effects of taxol and nocodazole. As noted above, taxol prevented the HSV-induced alteration of microtubules, whereas nocodazole induced complete depolymerization in all infected cell lines tested. The effects of Golgi fragmentation on virion release are best demonstrated in 143TK$^-$ cells exposed to nocodazole and in Vero and HEp-2 cells exposed to taxol. From these results we conclude the following. (i) Depolymerization of microtubules does not adversely affect virion release, and therefore viral egress is independent of the organization of microtubules. (ii) We have not established a detrimental role of the fragmentation and dispersal of the Golgi stacks on the exocytosis of virions from infected cells. (iii) The failure of U_L20^- virus to egress from Vero cells might be primarily due to the absence of cell-specific factors and not solely to the breakdown and dispersal of the Golgi apparatus.

The significance of these results stems from the prediction that the exocytosis of virions in transport vesicles through the Golgi involves the cellular cytoskeleton and a Golgi apparatus of a defined structure. Our studies revealed that neither of these expectations is correct. One hypothesis under consideration is that herpesviruses have evolved gene products which in combination with selected cellular elements form an efficient exocytic pathway independent of the state of the Golgi apparatus. In such a system a fragmented, dispersed Golgi apparatus might even simplify the task of the viral gene products in ensuring efficient processing of viral glycoproteins and transport of virions from the infected cell. U_L20 protein appears to be a component of the viral exocytic machinery, and as initially proposed, proteins functionally related to U_L20 protein may be present in some cells (e.g., 143TK$^-$ cells) but not others (e.g., Vero and HEp-2 cells).

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