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Microtubule- and Dynein-Dependent Nuclear Trafficking of Rhesus Rhadinovirus in Rhesus Fibroblasts

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We investigated the role of microtubules in rhesus rhadinovirus (RRV) nuclear trafficking in rhesus fibroblasts. Intact microtubules and microtubule dynamics are required for RRV trafficking to perinuclear regions. RRV trafficking was reduced by an inhibitor of the dynein motor and overexpression of dynamitin. Furthermore, RRV particles are colocalized with microtubules and dynein proteins. These results highlight the important roles of microtubules and dynein-dynactin complexes in the transport of RRV particles to nuclei during primary infection.

Kaposi's sarcoma-associated herpesvirus (KSHV) is a gammaherpesvirus associated with Kaposi's sarcoma (KS), primary effusion lymphoma (PEL), and multicentric Castleman's disease (MCD) (17). Because KSHV infection is restricted to humans, animal models are useful for studying KSHV and its related malignancies (34). Rhesus rhadinovirus (RRV) is closely related to KSHV (1, 7, 41). RRV naturally infects rhesus macaques and induces PEL- and MCD-like malignancies under experimental conditions (29, 36, 48).

RRV virions enter rhesus fibroblasts (RFs) primarily through clathrin-mediated endocytosis (49). Following internalization, the incoming virions have to pass the diffusional barrier in the cytoplasm in order to deliver viral genomes to nuclei (15, 43). Because of the crowded space in cytoplasm and unspecific targeting, viral trafficking is unlikely to rely on random diffusion (11, 38). Consequently, viruses often hijack cellular cytoplasmic transport machineries to achieve fast transport to nuclei during early steps of infection. In particular, a number of viruses exploit microtubules and microtubule-dependent motors to move from cell peripheries to nuclei (2, 5, 8, 24, 25, 27, 35, 42–45). KSHV infection is mediated by either actin or microtubule cytoskeletons, depending on the cell types (16, 32). Whether RRV entry and trafficking are also regulated by cytoskeletons remains unclear.

Microtubules are highly dynamic and unstable polymers undergoing rapid cycles of polymerization/depolymerization (18, 21, 30). We examined the role of microtubules in RRV trafficking following their depolymerization or stabilization with chemical inhibitors. A red fluorescent protein (RFP)-labeled RRV (RRV-RFP) was used to directly track RRV trafficking (49). Nocodazole depolymerizes microtubules by binding to β-tubulin and preventing formation of interchain bonds (19). RF cells pretreated with nocodazole (Sigma, St. Louis, MO) for 1 h were inoculated with RRV-RFP for 4 h in the presence of nocodazole. As shown in Fig. 1A, microtubules were effectively disrupted by nocodazole. The extent of microtubule disruption was intensified with increasing concentrations of nocodazole. Compared with the untreated control, nocodazole significantly inhibited RRV trafficking to the nuclei (Fig. 1B). The numbers of RRV particles docked at each nucleus significantly decreased in a dose-dependent fashion following nocodazole treatment (Fig. 1C and D).

Paclitaxel (originally named "taxol") binds to β-tubulin N terminus and promotes formation of highly stable microtubules (28). At 1 and 5 μg/ml, paclitaxel (Sigma) slightly increased the assembly of microtubules (Fig. 1E). When paclitaxel was used at 25 μg/ml, strong fibers were observed. Treatment with paclitaxel significantly inhibited RRV trafficking (Fig. 1F). The numbers of RRV particles docked at each nucleus were reduced in a dose-dependent manner following paclitaxel treatment (Fig. 1G and H). We also measured the viability of the cells using a propidium iodide (PI) labeling kit (Roche, Nutley, NJ). Neither nocodazole nor paclitaxel treatment increased the number of PI-positive cells (data no shown). Together, these results indicate that both intact microtubule networks and microtubule dynamics are required for RRV trafficking to perinuclear regions, and RRV particles might be transported along microtubules during trafficking.

Microtubules are formed with relatively stable minus ends emanating from microtubule organization center (MTOC) near nuclei and dynamic plus ends facing toward the cell membrane (33). Cargo movement on microtubules is bidirectional and is driven by microtubule-associated molecular motors, such as dynein and kinesin (18). Kinesin is a plus-end-directed microtubule motor, which moves cargos toward the cellular periphery, while dynein is minus end directed and is responsible for transporting cargos to MTOC. Both motors are ATPases, and their movements are powered by ATP hydrolysis (10, 14, 20, 23).

Cytoplasmic dynein is a protein complex responsible for retrograde movements of intracellular cargos and organelles, including viruses, along microtubules that serve as cellular conveyor belts. Dynein is composed of 4 major components: the heavy chain, the intermediate chain, the light intermediate chain, and the light chain (22, 46). Since dynein is involved in the trafficking of many viruses (6, 9, 13, 43, 47), we determined whether RRV transport along microtubules is dynein dependent. We used erythro-9-[3-(2-hydroxynonyl)]adenine (EHNA) (Sigma), an in-

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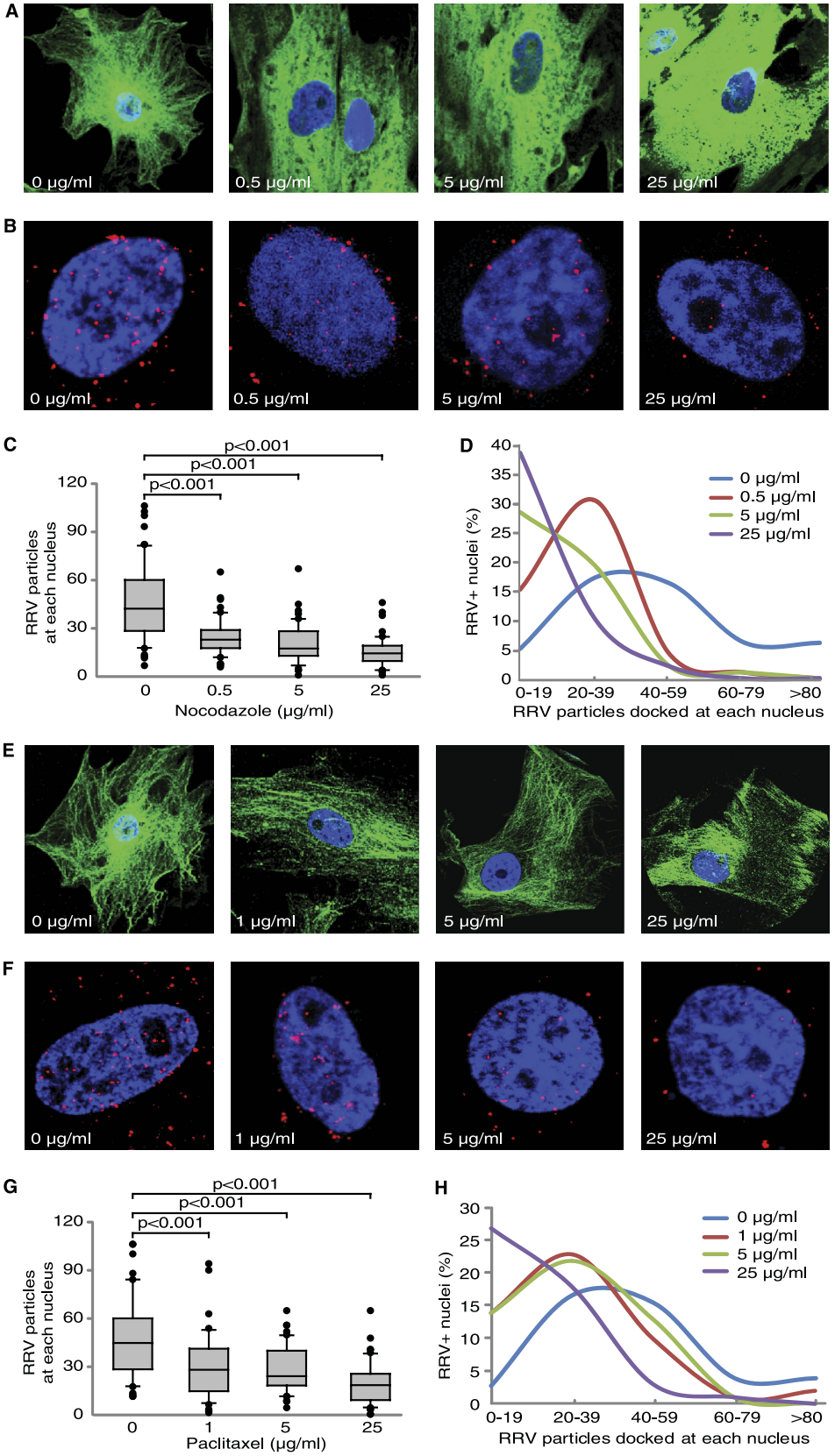

FIG 1 Disruption of microtubule networks with nocodazole or inhibition of microtubule dynamics with paclitaxel blocks RRV trafficking in RFs. (A and E) Effect of nocodazole and paclitaxel on microtubule networks. RFs were treated with increasing concentrations of nocodazole (A) or paclitaxel (E) and stained with an anti-β-tubulin antibody. Microtubule networks were disrupted by nocodazole but stabilized by paclitaxel. (B and F) Effect of nocodazole and paclitaxel

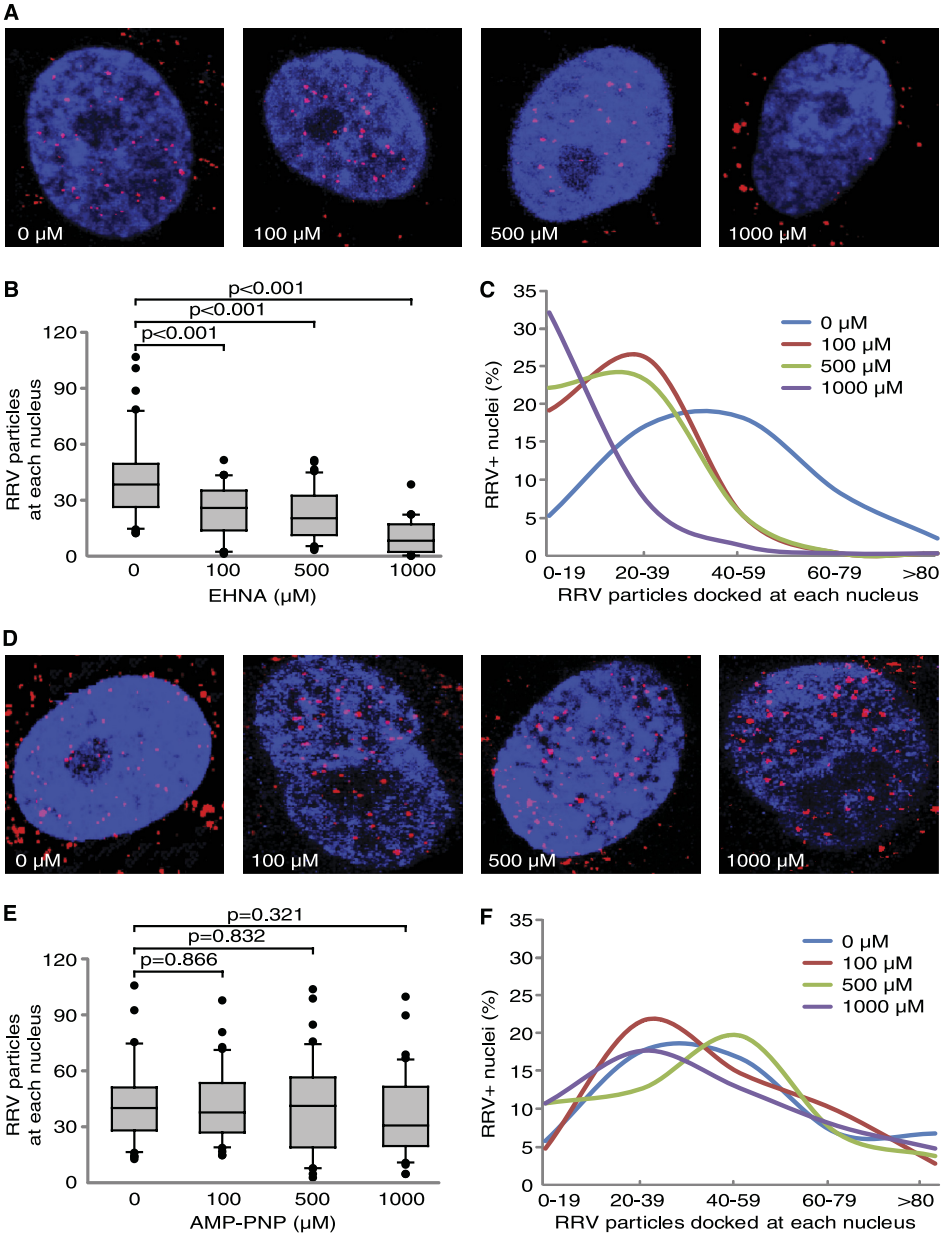

FIG 2 Inhibition of dynein but not kinesin blocks RRV trafficking. (A and D) RFs were pretreated with dynein inhibitor EHNA (A) or kinesin inhibitor AMP-PNP (D) for 1 h prior to infection with RRV for 4 h and stained for RRV particles (red) and nuclei (blue). (B and E) Quantification of the total numbers of RRV particles docked at each nucleus following treatment with EHNA (B) or AMP-PNP (E). (C and F) Distribution of nuclei with different numbers of RRV particles following treatment with EHNA (C) or AMP-PNP (F). Experiments and data analyses were carried out as described in the legend to Fig. 1.

hibitor of dynein ATPase, to disrupt the function of cytoplasmic dynein (37). EHNA strongly inhibited RRV intracellular trafficking (Fig. 2A). The numbers of RRV particles docked at nuclei were significantly reduced in a dose-dependent fashion in EHNA-treated cells (Fig. 2B and C). In contrast, inhibition of kinesin with 5-adenylyl-imidodiphosphate (AMP-PNP) (Sigma) had no effect on RRV trafficking (Fig. 2D to F). These results suggest that RRV trafficking in RFs is kinesin independent. Both EHNA and AMP-

on RRV trafficking. RFs were treated with nocodazole (B) or paclitaxel (F) for 1 h and inoculated with RRV in the presence of inhibitors, fixed at 4 h postinfection (hpi), and stained for RRV particles (red) and nuclei (blue). (C and G) Quantification of the total numbers of RRV particles docked at each nucleus following treatment with nocodazole (C) or paclitaxel (G). Images were acquired for 6 to 10 fields per coverslip to allow counting of 50 nuclei. The *t* test, analysis of variance, and/or Mann-Whitney tests were performed using SigmaPlot 11.0 (Systat Software, Inc., San Jose, CA) with $P < 0.05$ considered significant. The numbers of RRV particles docking on a nucleus upon drug or control treatment are presented in box and whisker plots showing the median values (middle dark lines in the boxes) and the upper 75% and lower 25% quartiles (top and bottom box lines of the open boxes). The top and bottom short lines showed the ranges of the data, and outliers were represented as black dots. All experiments were performed in triplicates. (D and H) Distribution of nuclei with different numbers of RRV particles following treatment with nocodazole (D) or paclitaxel (H).

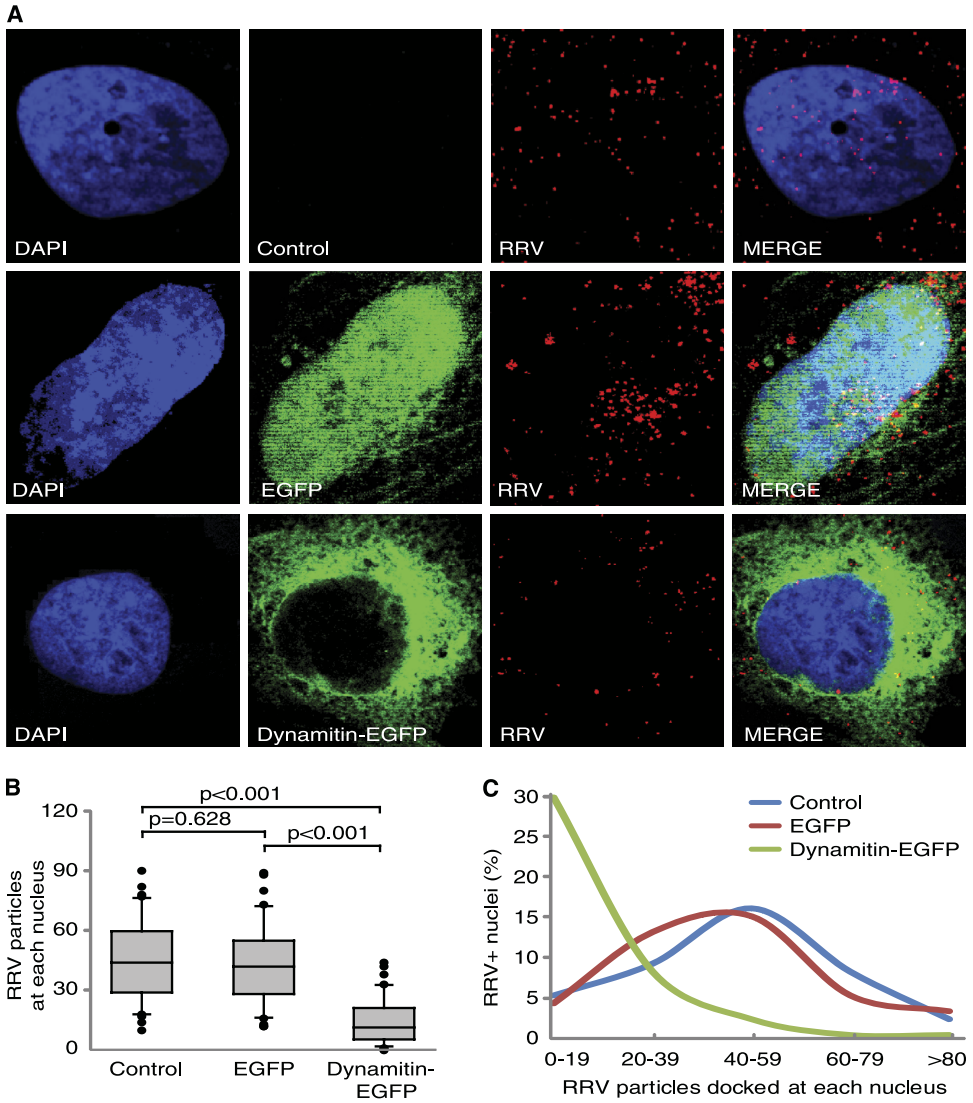

FIG 3 RRV trafficking is mediated by dynein. (A) Overexpression of dynamitin-GFP but not the enhanced green fluorescent protein (EGFP) vector efficiently inhibited RRV trafficking in RFs. Cells were transiently transfected with a dynamitin-GFP construct or a vector control. At 36 h posttransfection, cells were inoculated with RRV for 4 h and stained for RRV particles with a rabbit anti-RFP antibody. (B) Quantification of the total numbers of RRV particles docked at each nucleus. Images were acquired for 6 to 10 fields per coverslip to allow counting of 50 nuclei. Data were analyzed as described in the legend to Fig. 1. (C) Distribution of nuclei with different numbers of RRV particles.

PNP had minimal toxicity as fewer than 2% of the cells were PI positive following the treatments (data not shown).

The interaction between the cytoplasmic dynein complex and cargo is often mediated by a receptor protein complex called "dynactin." Dynactin consists of several subunits, including p150^{Glued}, Arp1, capping protein, p62, Arp11, and dynamitin (40). Overexpression of dynamitin leads to dissociation of the subunits from the dynein-dynactin complex, resulting in disruption of dynein motor function (4). We used a dynamitin-green fluorescent protein (GFP) expression plasmid to confirm the role of the dynein-dynactin complex in RRV transport. Compared to untransfected cells, cells transfected with the vector control had no change in the numbers of RRV particles docked at nuclei. In contrast, cells transfected with dynamitin-GFP had significantly lower numbers of RRV particles docked at the nuclei (Fig. 3), indicating that intact dynein-dynactin complex is essential for RRV trafficking. Compared to vector-transfected cells, cells transfected with dynamitin-GFP had fewer cells having a clear and single MTOC; however, the presence of a focus MTOC did not appear to affect RRV infection and trafficking (data not shown).

Our results so far indicate that RRV trafficking in RFs is microtubule and dynein dependent. To confirm these observations, we determined the association of RRV particles with microtubules. RFs infected with RRV-RFP were stained for RRV particles and β-tubulin. Z-stacks were acquired and used to generate three-dimensional (3D) projection XY overview images and the corresponding cross-sectional XZ images. 3D analysis indicated that more than 80% of incoming RRV particles were colocalized with microtubules (Fig. 4A; see Movie S1 in the supplemental material). A magnified 3D projection image of the individual RRV particles further illustrated their association with microtubules (insets in Fig. 4A).

Next, we determined the association of RRV particles with dy-

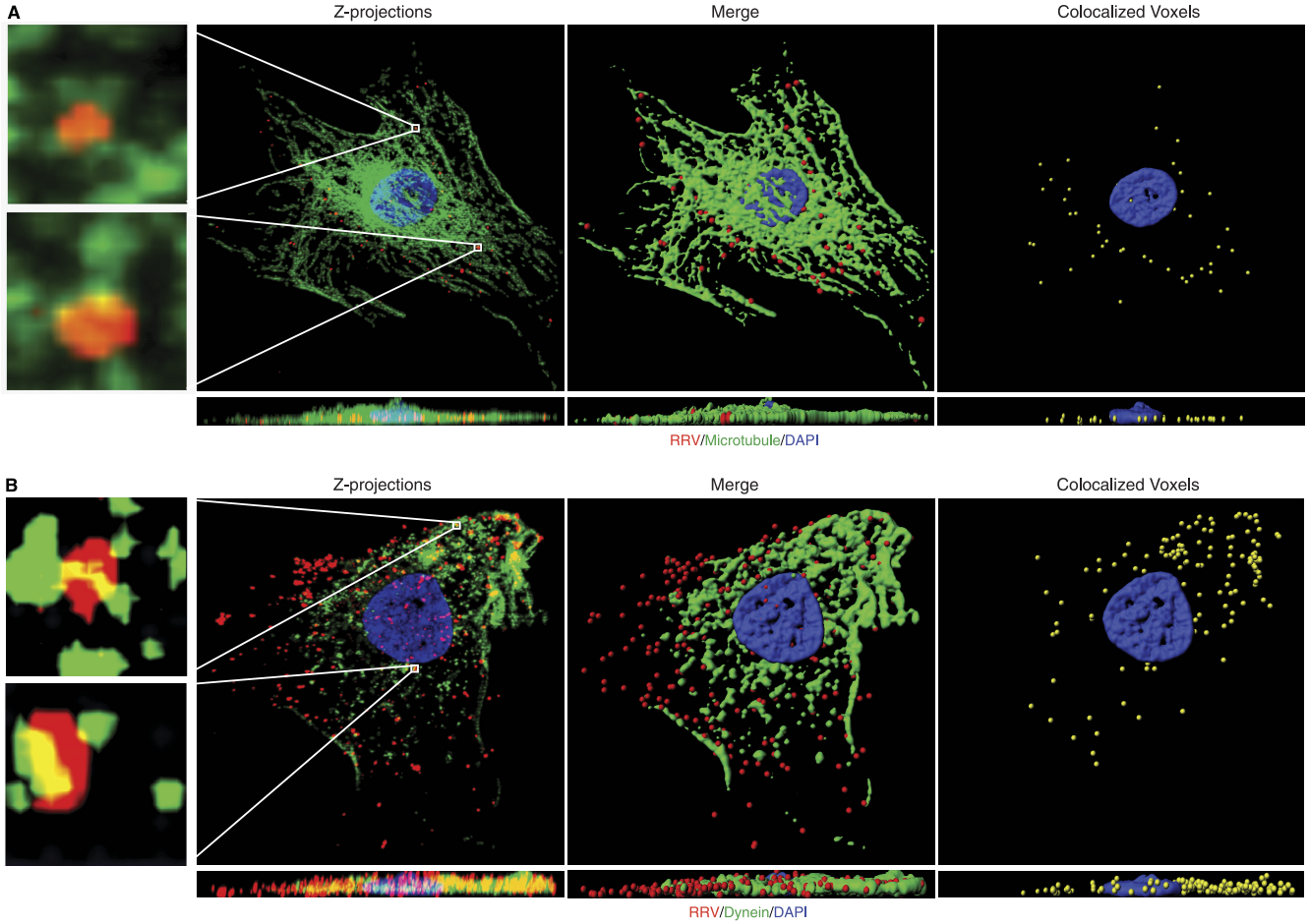

FIG 4 Colocalization of RRV particles with microtubules and the dynein motor proteins. (A) Colocalization of RRV particles with microtubules. RFs infected with RRV at a multiplicity of infection (MOI) of 50 were fixed at 1 h postinfection (hpi) and stained for RRV particles (red), microtubule cytoskeletons (green), and nuclei (blue). Z-stack images were acquired by Olympus FV1000 scanning confocal microscopy. Both the Z-projection and the XZ section showed colocalization of RRV particles with microtubules (yellow; left panel). Regions delineated by rectangle inserts were shown at higher magnifications in adjacent panels. 3D contoured images (middle and right panels) were generated with AutoQuant deconvolution (Media Cybernetics, Inc., Bethesda, MD) software and Imaris 3D image analysis software (Bitplane, Zurich, Switzerland). Corresponding XZ sections were visualized by rotating on the *x* axis to observe the location of virus particles. Movie S1 in the supplemental material corresponds to Fig. 4A. (B) Colocalization of RRV particles with dynein. RFs were infected with RRV for 1 h and stained for RRV particles and dynein heavy chain. Images were processed for 3D colocalization analysis. Regions delineated by rectangles are shown at higher magnifications in adjacent panels. Movie S2 in the supplemental material corresponds to Fig. 4B.

nein. By staining both RRV particles and dynein heavy chain, we observed colocalization of RRV particles with dynein (Fig. 4B; see Movie S2 in the supplemental material). These results further confirm that intact microtubule networks and microtubule dynamics are required for RRV trafficking to nuclei.

Our results show that RRV trafficking is regulated by both microtubules and dynein. Two models can explain these results. In the first model, low pH triggers the release of nucleocapsids from endosomes to cytosol. The nucleocapsids bind to microtubules through interactions with the dynein-dynactin complexes to hitch a ride along the microtubules. In the second model, virion-containing endosomes are transported along microtubules to the perinuclear regions before releasing the nucleocapsids. If the first model were correct, it would be important to identify the components of interactions between the dynein-dynactin complexes and virions. Almost all subunits in the dynein-dynactin complexes have the potential to directly bind to cargos, with the exception of dynein heavy chain (15). For example, herpes simplex virus 1 nucleocapsids bind to microtubules through the interaction between VP26 and dynein light chains RP3 and Tctex1 (12). In the second model, the interactions of the virions with microtubules and associated motor proteins are not required. Instead, the virion-containing endosomes directly bind to and travel along microtubules under the control of molecular motors such as dynein or kinesin prior to the release of nucleocapsids into the cytoplasm at low pH (3, 26, 31). In this case, examination of the colocalization of virions with intracellular endosomal markers and microtubules in real-time could define the precise stage and time and space of their association and release during virus entry.

Actin cytoskeletons are often involved in the trafficking of viruses. The involvement of either actin or microtubule cytoskeletons in KSHV trafficking is cell type dependent (16, 32, 39). In primary human umbilical vein endothelial cells, KSHV trafficking requires actin dynamics (16). However, in human fibroblasts, KSHV trafficking is mediated by microtubules (32). While we

have shown that microtubules mediate RRV trafficking in RFs, it is possible that actin cytoskeletons are also involved in this process. Further investigations are required to determine the role of actin cytoskeletons in RRV trafficking.

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