Actin Cytoskeleton Is Involved in Targeting of a Viral Hsp70 Homolog to the Cell Periphery

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The cell-to-cell movement of plant viruses involves translocation of virus particles or nucleoproteins to and through the plasmodesmata (PDs). As we have shown previously, the movement of the *Beet yellows virus* requires the concerted action of five viral proteins including a homolog of cellular ~70-kDa heat shock proteins (Hsp70h). Hsp70h is an integral component of the virus particles and is also found in PDs of the infected cells. Here we investigate subcellular distribution of Hsp70h using transient expression of Hsp70h fused to three spectrally distinct fluorescent proteins. We found that fluorophore-tagged Hsp70h forms motile granules that are associated with actin microfilaments, but not with microtubules. In addition, immobile granules were observed at the cell periphery. A pairwise appearance of these granules at the opposite sides of cell walls and their colocalization with the movement protein of *Tobacco mosaic virus* indicated an association of Hsp70h with PDs. Treatment with various cytoskeleton-specific drugs revealed that the intact actomyosin motility system is required for trafficking of Hsp70h in cytosol and its targeting to PDs. In contrast, none of the drugs interfered with the PD localization of *Tobacco mosaic virus* movement protein. Collectively, these findings suggest that Hsp70h is translocated and anchored to PDs in association with the actin cytoskeleton.

In addition to plasma membranes, plant cells are surrounded by cell walls that define tissue and organ architecture and provide protection from the environment and pathogens. Even though cell walls are complex and dynamic structures whose functions are regulated by developmental and environmental cues (62), they are also a barrier for efficient intercellular communications. To facilitate such communications, plants possess plasmodesmata (PDs), organelles that contain cytoplasmic microchannels lined with the derivatives of plasma and endoplasmic reticulum membranes (19, 31, 44). Early research suggested that molecules of less than ~1 kDa can freely diffuse between cells via PDs, while diffusion of larger molecules is generally restricted. Recent discoveries, however, brought about a conceptual shift in understanding of PD function.

It was found that certain proteins termed non-cell-autonomous proteins, or NCAPs, can traffic to and through PDs either by default or using an active transport pathway (31, 40, 65). Moreover, some of the NCAPs potentiate intercellular transport of mRNAs, therefore contributing to programs of cell differentiation and plant development (25, 26, 30). It is also believed that PDs provide a conduit for transport of RNA silencing signals (4, 21, 37, 67). Despite the rapidly growing appreciation of the PDs' importance for intercellular communications, little is known about the molecular composition of PDs or mechanisms involved in delivery of macromolecules to and through the PDs. Although several candidate PD proteins were identified (16, 19, 28, 64), it remains to be determined which of these proteins are structural components of the PDs

and which are parts of the PD targeting pathway. For instance, it appears that NCAPs associate with PDs only transiently, such that they cannot be considered PD-residential proteins (31).

Infection cycles of plant viruses include a phase of cell-to-cell movement via PDs. Typically, this process is potentiated by dedicated movement proteins (MPs) that are able to modify PDs and aid translocation of virions or viral nucleoproteins through these organelles (8, 11, 29). Despite their structural and mechanistic diversity, many MPs have been reported to associate with PDs. Because of this, plant viruses and their MPs provide useful tools for probing PD functions. One of the most acclaimed models is the *Tobacco mosaic virus* (TMV) MP, which is found in PDs of infected cells (45, 63). Furthermore, the TMV MP is autonomously targeted to PDs upon transient or transgenic expression in a free form or as a fusion with the green fluorescent protein (GFP) (12, 55). The ability of TMV MP-GFP to accumulate in PDs provides a reliable *in vivo* PD marker. In addition to residing in PDs, TMV MP binds viral RNA (10) and associates with cytoskeletal elements and the endoplasmic reticulum (ER) (20, 33, 35). However, the exact mechanism of its delivery to PDs remains a matter of debate (6, 17).

Other advanced models of PD biology are MPs of the viruses in the families *Comoviridae* and *Nepoviridae*. These MPs restructure PDs by assembling tubules through which icosahedral virions are transported from cell to cell. Recent work has demonstrated that targeting of these MPs to PDs and tubule assembly involve a complex interplay of secretory and cytoskeletal trafficking pathways (28, 52). Movement of many rod-shaped and filamentous viruses is enabled by three proteins encoded by conserved triple gene block (TGB) (38). Interestingly, intracellular trafficking of TGB2 MP was found to involve the endocytic pathway and actin cytoskeleton (18).

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The cell-to-cell movement of the *Beet yellows virus* (BYV) (14) requires concerted action of five proteins (1, 49). One of those is a dedicated MP characterized as a type III transmembrane protein that is specifically targeted to the ER (50). Four other proteins are major and minor capsid proteins (CP and CPm, respectively), a 64-kDa protein that harbors a CP-like domain, and a homolog of cellular molecular chaperones from a family of ~70-kDa heat shock proteins (Hsp70h) (2, 41). Strikingly, each of these four proteins is an integral component of the ~1,400-nm-long filamentous virions with CP coating ~97% of the 15.5 kb RNA genome. The remaining three proteins, CPm, p64, and Hsp70h, assemble a ~100-nm-long tail that encapsidates a 5'-terminal, ~600-nucleotide (nt)-long RNA segment (47). An additional, 20-kDa, tail protein is not required for the cell-to-cell movement, but is essential for the viral transport through the phloem (53). Extensive mutation analysis demonstrated that assembly of the tailed virions is a prerequisite for BYV movement (2, 41). Using immunogold electron microscopy, we found that in addition to virions, Hsp70h also accumulates in PDs of BYV-infected cells (36).

In this work, we demonstrate that Hsp70h is autonomously targeted to PD-rich areas at the cell periphery. We also reveal that Hsp70h associates with the actin microfilaments and traffics in a cytoplasm with a mean speed of ~1 μ m/s. Using various inhibitors of cytoskeletal functions, we show that both the intracellular trafficking and PD localization of Hsp70h require a functional actomyosin motility system.

MATERIALS AND METHODS

Binary vectors for expression of Hsp70h variants and fluorescent reporters.

Generation of the Hsp70h-GFP expression cassette in a mini binary vector pCB302 (66) was described earlier (46, 53). To obtain Hsp70h-yellow fluorescent protein (YFP) and Hsp70h-mRFP cassettes, the GFP open reading frame (ORF) in the Hsp70h-GFP cassette was replaced with the YFP ORF (39) or the mRFP1 ORF (7) using AvrII or XbaI sites, respectively. A similar approach was used to engineer GFP fusions of the BYV minor capsid protein (CPm; GenBank accession no. AAF14304) and *Arabidopsis thaliana* Hsc70-3 (GenBank accession no. AY096676; a cDNA clone was obtained from the Arabidopsis Biological Resource Center, Ohio State University, Columbus, OH). Binary vectors for expression of the wild-type Hsp70h or GFP fused to the bacterial β -glucuronidase (GFP-GUS) were described previously (53).

To obtain fluorescent markers of the actin cytoskeleton, we engineered binary vectors for expression of the actin-binding domain of the mouse talin (27) fused to GFP (GFP-talin) or DsRed (DsRed-talin) (34). The TMV MP ORF was PCR-amplified using pTMV-208 as a template, a full-length cDNA clone of TMV (a gift from W. O. Dawson, University of Florida), and fused to GFP ORF by replacing Hsp70h ORF in a Hsp70h-GFP cassette. For visualization of microtubules, we used the transgenic *Nicotiana benthamiana* line CB13 provided by Karl Oparka (17).

A plasma membrane marker was engineered by fusing GFP ORF with a nucleotide sequence encoding a C-terminal hypervariable region of a maize ROP7 GTPase (22). A Golgi-specific fluorescent reporter in which a transmembrane domain and cytoplasmic tail of the rat A-2,6-sialyltransferase were fused with GFP was provided by C. Hawes (58).

Transient protein expression in plants and immunoblot analysis. The binary vectors used in this work were transformed to *Agrobacterium tumefaciens* strain C58 GV2260 by electroporation. The lower leaf surfaces of young (six- to seven-leaf stage) *N. benthamiana* plants were infiltrated with a syringe lacking a needle. The infiltrated bacteria (optical density at 600 nm [OD₆₀₀] of 0.2 to 0.5) were suspended in a buffer containing 10 mM MES-KOH (pH 5.85), 10 mM MgCl₂, and 150 mM 4'-hydroxy-3',5'-dimethoxyacetophenone (TCI). Plants were kept for 18 to 22 h in a growth chamber at 24°C prior to analyses.

For immunoblot analysis, samples that contained ~15 μ g of protein extracted from infiltrated leaves were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore). The GFP fusions were detected using mono-

clonal anti-GFP antibody (Roche) and horseradish peroxidase-conjugated secondary antibody (Bio-Rad) in a 1:5,000 dilution. The Hsp70h and its fusion derivatives were also detected using Hsp70h-specific antibody as described previously (48).

Immunofluorescence labeling and confocal laser scanning microscopy. For immunofluorescence detection, patches of leaves that transiently expressed Hsp70h were gently abraded with carborundum using a soft paintbrush and treated with 0.1% pectolyase Y-23 (Kikkoman Co., Tokyo, Japan) and 0.2% driselase (Sigma) for 10 min. After that, patches were washed twice by PBS-TB, pH 7.4 with 1 mg/ml of bovine serum albumin (BSA; Sigma) and 0.5% of Triton X-100 (ICN), and fixed for 30 min with formaldehyde in MTSB-T buffer [25 mM piperazine-*N,N'*-bis(z-ethanesulfonic acid), pH 6.9, 2 mM EGTA, 1 mM MgSO₄] (61) with 0.5% of Triton X-100. Additional fixation of patches was in 100% methanol and 100% acetone at -20°C followed by brief washing in MTSB-T. Patches were then blocked in PBS-TB with 0.8% Triton X-100 and 1% BSA for 30 min and incubated with primary rabbit polyclonal anti-Hsp70h antibody (48) followed by goat anti-rabbit secondary antibody fused to Alexa488 (Molecular Probes), each at a 1:100 dilution. After three washes in PBS-TB buffer, patches were mounted in CrystalMount (Biomedex). For propidium iodide staining, leaf segments were incubated in an aqueous solution of 10 μ g/ml propidium iodide (MP Biomedical, Germany) for 30 min.

Confocal laser scanning microscopy was done using a Zeiss LSM 510 META (Zeiss, Germany) microscope fitted with the following configurations of excitation and emission filters, respectively: 488 nm and 508 nm for Alexa 488 and GFP, 513 nm and 527 nm for YFP, 558 nm and 583 nm for DsRed and mRFP, and 530 nm and 620 nm for propidium iodide. The software package provided by the manufacturer was used for 3D reconstructions and image processing.

Drug treatments and time-lapse experiments. Dimethyl sulfoxide (DMSO) stock solutions containing latrunculin B (2 mM, Calbiochem), cytochalasin D (20 mM, ICN), oryzalin (20 mM, ChemService), phalloidin (5 mM, Calbiochem), and taxol (10 mM, Sigma) were used for drug treatment experiments. Water solutions of 2,3-butanedione monoxime (BDM; Sigma) were prepared fresh for each experiment. The drug treatments were done using incubation of leaf disks in water solutions of drugs for 24 h in small Petri dishes in a growth chamber. Equivalent concentrations of DMSO were used as controls; optimal concentrations of drugs resulting in desired effects were determined in preliminary experiments.

For subcellular localization experiments involving fluorophore-tagged Hsp70h and TMV MP variants, young *N. benthamiana* leaves were infiltrated with corresponding drugs 24 h prior to agrobacterial infiltration. The infiltrated bacterial suspension was also supplemented with the same concentrations of drugs.

For time-lapse experiments, 25 consecutive images were taken at 3.96-s intervals. Individual Hsp70h-GFP granules or Golgi stacks were followed from frame to frame, and the distance between their positions in consecutive frames was measured using LSM5 Image Browser software (Zeiss, Germany). A mean translocation speed was determined using at least 30 individual measurements. Stops in the granule or Golgi movements related to "stop-and-go" translocation manner (43) were also factored into speed measurements. For time-lapse experiments involving drugs, leaves were infiltrated by bacterial suspensions and propagated for 20 to 36 h prior to treatments with drugs or an equivalent amount of DMSO as a control. Mobility measurements were started at 1 h after drug applications.

RESULTS

Localization of Hsp70h to cell periphery. To investigate the subcellular distribution of Hsp70h, we employed transient expression of the fluorophore-tagged Hsp70h variants in plants of a BYV experimental host, *N. benthamiana* (14). Leaves were infiltrated with *Agrobacterium* strains containing plasmids capable of expressing relevant fusion proteins. A ~65-kDa Hsp70h was fused to GFP (Hsp70h-GFP), YFP, or monomeric red fluorescent protein (Hsp70h-mRFP), and its localization in live cells of the leaf epidermis was compared to localization of other fusion proteins. In accord with our previous observations (53), Hsp70h-GFP was present in small granules at the cell periphery (Fig. 1A). In contrast, GFP fusion with a ~68-kDa, bacterial, β -glucuronidase was uniformly distributed throughout the cortical cytoplasm and trans-vacuolar cytoplasmic

FIG. 1. Expression of fluorophore-tagged proteins in epidermal cells of *N. benthamiana* leaves at ~20 h after infiltration with *Agrobacterium*. (A) BYV Hsp70h-GFP forms discrete granules at the cell periphery. (B) GFP-GUS is distributed throughout the cortical cytoplasm and transvacuolar cytoplasmic strands. (C) and (D) Similar cytoplasmic localization of the CPm-GFP and *A. thaliana* Hsc70-3-GFP, respectively. Red spots in panels A through D represent chloroplast autofluorescence. (E) High-magnification image of paired Hsp70h-GFP granules at the peripheries of adjacent cells. (F) Cell wall stained with propidium iodide (red) is present between pairs of the peripheral Hsp70h-GFP granules. (G) Cells at the borders of the infiltrated regions contain unpaired peripheral granules of Hsp70h-GFP. (H and I) Paired granules formed by Hsp70h-YFP and Hsp70h-mRFP, respectively. (J) Coexpression of Hsp70h-mRFP and plasma-membrane-targeted GFP indicates close association of red granules with the plasma membrane. (K) Expression of a nontagged, wild-type, BYV Hsp70h visualized by immunocytochemical staining with Hsp70h-specific antibodies and Alexa 488-conjugated secondary antibody reveals formation of the peripheral granules. (L) TMV MP-GFP also forms paired granules in the adjacent cells. (M) Coexpression of Hsp70h-mRFP with TMV MP-GFP reveals colocalization of the two fluorophore-tagged viral proteins (yellow and orange granules marked by white arrows). (N and N') Consecutive images taken at 3.96-s intervals show translocation of a cytoplasmic Hsp70h-GFP granule (marked by an arrow) relative to immobile cell wall. (O and O') Consecutive images showing movement of a pair of Golgi stacks (marked by an arrow). (P) Coexpression of Hsp70h-mRFP and GFP-talin demonstrates colocalization of red granules with green actin MFs (yellow and orange). (Q) Coexpression of Hsp70h-mRFP and GFP-talin reveals tight association of red granules with green MFs upon image processing using the VisArt program. (R) Coexpression of Hsp70h-GFP and dsRed-talin reveals tight association of green granules with red MFs upon three-dimensional image reconstruction using the VisArt program. (S) Expression of Hsp70h-mRFP in GFP-tubulin transgenic plants shows no significant association of tagged Hsp70h with green microtubules. Bars: 10 μ m in panels A through D, M through P, and S; 1 μ m in panels E through L, Q, and R.

strands (Fig. 1B). Similar cytoplasmic distributions were observed for GFP fused to a 24-kDa minor capsid protein of BYV (Fig. 1C) or to *Arabidopsis thaliana* Hsc70-3 (Fig. 1D). The latter result indicated that peripheral localization is not a generic property of Hsp70s but is specific to viral Hsp70h. Immunoblot analysis using GFP-specific (Fig. 2A, lanes 2 through 5) or Hsp70h-specific (Fig. 2B, lanes 1 through 3) antisera confirmed that the fusion proteins of expected sizes were produced in the agroinfiltrated leaves. Moreover, this analysis indicated that the accumulation levels of transiently-expressed, fluorophore-tagged Hsp70h variants (Fig. 2B, 1

through 3) were similar to that of the wild-type Hsp70h produced in BYV-infected plants (Fig. 2B, lane 4).

In higher magnification images of a single focal plane through the middle of two adjacent cells, many of the Hsp70h-GFP granules appeared as pairs of similarly sized bodies apparently separated by the cell wall (Fig. 1E). Cell-wall-specific staining with propidium iodide confirmed this assumption (Fig. 1F). Interestingly, cells at the periphery of *Agrobacterium*-infiltrated areas showed unpaired fluorescent granules (Fig. 1G), suggesting that Hsp70h-GFP is unable to autonomously translocate to the neighboring cells that do not express this

FIG. 2. Detection of transiently expressed, fluorophore-tagged proteins using immunoblot analysis and anti-GFP (A and C) or anti-Hsp70h (B) antibodies. (A) Lane 1, mock-infiltrated plants; lane 2, GFP-GUS; lane 3, Hsc70-3-YFP; lane 4, Hsp70h-GFP; lane 5, Hsp70h-YFP. (B) Lane 1, Hsp70h-GFP; lane 2, Hsp70h-YFP; lane 3, Hsp70h-mRFP; lane 4, protein extract from BYV-infected plants. (C) Accumulation of transiently expressed Hsp70h-GFP (lanes 2 through 4) and TMV MP-GFP (lanes 6 through 8) in leaves treated with drugs. Lanes 1 and 5, mock-infiltrated plants; lane 2, Hsp70h-GFP in the presence of DMSO (control); lane 3, Hsp70h-GFP in the presence of 2 μ M latrunculin B; lane 4, Hsp70h-GFP in the presence of 20 μ M oryzalin; lane 6, TMV MP-GFP in the presence of DMSO (control); lane 7, TMV MP-GFP in the presence of 2 μ M latrunculin B; lane 8, TMV MP-GFP in the presence of 20 μ M oryzalin. Arrows indicate positions of the protein size markers.

protein. Paired granules were also observed upon expression of Hsp70h-YFP and Hsp70h-mRFP (Fig. 1H and I, respectively). Coexpression of Hsp70h-mRFP with the plasma membrane marker generated by fusing GFP with a fragment of maize ROP7 GTPase (22) revealed tight association of the granules with the membrane (Fig. 1J).

To determine if the peripheral localization of the Hsp70h fusions is due to Hsp70h moiety rather than to incidental properties of the fusion products, a wild-type, tag-free Hsp70h was expressed in plants. Immunofluorescence detection using Hsp70h-specific antibodies and Alexa 488-conjugated secondary antibodies resulted in localization patterns very similar to those observed using fluorophore-tagged Hsp70h except for smaller sizes of the granules (Fig. 1K). Taken together with the detection of Hsp70h in the PDs of BYV-infected cells (36), the appearance of the discrete, opposing Hsp70h granules in the adjacent cells and their tight association with the plasma membrane and cell wall suggested that Hsp70h has an intrinsic ability to accumulate in the PD-rich areas.

To further address possible PD localization of Hsp70h, we used TMV MP-GFP, which is a well-characterized PD-residential protein. Accordingly, we observed typical punctate ac-

FIG. 3. Mean translocation speeds of cytoplasmic Hsp70h-GFP granules and GFP-labeled Golgi stacks in the presence of drugs. Control, upon infiltration with bacterial cultures, leaf disks were placed in DMSO solution for 1 h. LatB, incubation with 25 μ M latrunculin B. Oryzalin, incubation with 200 μ M oryzalin. BDM, incubation with 200 mM BDM. Standard deviations are shown as black bars.

cumulation of TMV MP-GFP following its transient expression in the leaf epidermal cells (Fig. 1L). Results of coexpression of the MP-GFP PD reporter with Hsp70h-mRFP are shown in Fig. 1M, where the majority of the fluorescent granules appeared yellow or orange in merged images (Fig. 1M, arrows), indicating colocalization of the two fusion proteins. Some small granules retained their green or red color, but they were located in close proximity to each other (Fig. 1M). However, a few TMV MP-GFP granules were distributed singly and were not accompanied by Hsp70h-mRFP (Fig. 1M). Collectively, these results indicated that the viral Hsp70h, but not its plant homolog Hsc70-3, is specifically and autonomously targeted to the PD-rich areas at the cell periphery.

Motile granules of fluorophore-tagged Hsp70h are associated with actin microfilaments. In addition to immobile, peripheral granules, mobile granules of Hsp70h-GFP and Hsp70h-YFP were detected in the cytosol (Fig. 1N and N'). Time-lapse experiments demonstrated that these granules trafficked in transvacuolar cytoplasmic strands and in cortical cytoplasm with a mean speed of $\sim 1 \mu$ m/s (Fig. 3, Control). It is believed that the predominant manner of physical translocation within plant cells involves actomyosin motility system with the Golgi trafficking being the best studied example (24, 43, 60). To compare trafficking of Hsp70h-GFP granules with that of Golgi, we used Golgi-specific GFP reporter derived from a rat sialyltransferase (58). The observed speed of Golgi trafficking was in close agreement with previous measurements (43) and was similar to that of Hsp70h (Fig. 1O and O' and 3, Control). The movies showing cytoplasmic trafficking of Hsp70h-GFP and GFP-labeled Golgi are available upon request. These results were suggestive of a possible involvement of the actin cytoskeleton in trafficking of Hsp70h in cytoplasm.

To determine if cytoplasmic granules of Hsp70h are indeed

FIG. 4. Effects of various drug treatments on the integrity of cytoskeleton and subcellular localization of Hsp70h-GFP or TMV MP-GFP. (A) Intact microtubules labeled by transgenically expressed GFP-tubulin in epidermal *N. benthamiana* cells in the presence of DMSO. (B and C) Treatments with 20 μ M oryzalin or trifluralin, respectively, result in disassembly of microtubules. (D) Treatment with 10 μ M taxol stabilizes microtubules. (E) Intact microfilaments labeled with GFP-talin in the presence of DMSO. (F and G) Treatments with 2 μ M latrunculin B or 20 μ M cytochalasin D, respectively, result in disassembly of actin microfilaments. (H) Treatment with 2 μ M phalloidin stabilizes microfilaments. Oval-shaped pairs of guard cells are seen in panels A through D, F, and G. Red and orange spots correspond to autofluorescent chloroplasts. (I) to (X) High magnification images of the cell periphery in epidermal cells that express Hsp70h-GFP (I through P) or TMV MP-GFP (Q through X) following treatments with various drugs as indicated between the rows of panels. Triflur., trifluralin; Lat B, latrunculin B; Cyt D, cytochalasin D; Phalloid., phalloidin. Drug concentrations are the same as in panels A through H and Y. Treatments with latrunculin B (M), cytochalasin D (N), and BDM (P) prevent formation of the peripheral granules of Hsp70h-GFP, whereas the remaining drugs do not affect granule formation. None of the tested drugs prevented formation of the peripheral granules of TMV MP-GFP (Q through X). (Y) Treatment with 50 mM BDM does not affect microfilaments. Bars: 10 μ m in panels A through H and Y; 1 μ m in panels I through X.

associated with actin microfilaments (MFs), we used coexpression of Hsp70h-mRFP and GFP-talin that is a specific marker of MFs (27). Stacks of images representing consecutive optical cross sections through the cortical cytoplasm at the top parts of the epidermal cells were projected into a single plane to facilitate detection of GFP-labeled MFs and cytoplasmic Hsp70h-mRFP granules. As seen in Fig. 1P, most of the Hsp70h-mRFP granules appear orange or yellow rather than red due to their

coalignment with the green MFs. Virtually no granules were observed in the areas devoid of MFs. The computer-enhanced, 3D analysis of relative localization of Hsp70h-mRFP granules and MFs using the VisArt program (Zeiss, Germany) confirmed association of the granules with the green MFs (Fig. 1Q). Similar results were obtained in a reciprocal experiment: green granules of Hsp70h-GFP were tightly associated with red MFs labeled with dsRed-talin (Fig. 1R).

To determine if Hsp70h can also associate with the microtubules, we expressed Hsp70h-mRFP in *N. benthamiana* plants in which microtubular cytoskeleton was visualized via transgenic expression of GFP-tubulin (17). As shown in Fig. 1S, no reliable co-localization of Hsp70h-mRFP with microtubules was observed.

The pattern and speed of the trafficking of fluorophore-tagged Hsp70h in cytoplasm, as well as its specific association with MFs but not microtubules, suggested that the actomyosin motility system is involved in intracellular translocation of Hsp70h.

Trafficking and localization of Hsp70h require actomyosin motility system. A pharmacological approach was used to determine the functional significance of microtubules and actin MFs in Hsp70h trafficking and peripheral localization. Oryzalin and trifluralin were used to disassemble microtubules in plants, while taxol was used to stabilize microtubular cytoskeleton (32). Analogously, cytochalasin D and lantrunculin B treatments were used to dissociate MFs, whereas phalloidin was used to stabilize MFs and prevent their normal dynamics (13, 58). In addition, an inhibitor of the myosin-type ATPases, BDM that was reported to interfere with the myosin function (43) was used to inhibit actomyosin motility without disrupting MFs.

In preliminary experiments, a range of drug concentrations was tested (not shown), and concentrations that exerted desired effects were determined. As illustrated in Fig. 4, applications of microtubule- or MF-depolymerizing drugs resulted in virtual disappearance of corresponding types of fibrils (Fig. 4B and C and F and G, respectively). As expected, treatments with taxol and phalloidin enhanced the appearance of the microtubules (Fig. 4D) and MFs (Fig. 4H), respectively, while BDM did not affect the integrity of MFs (Fig. 4Y).

The drugs were used to treat leaf tissue that transiently expressed Hsp70h-GFP (Fig. 4I through P). None of the microtubule-specific drugs had a detectable effect on the formation of peripheral fluorescent granules by Hsp70h-GFP (Fig. 4J through L). Strikingly, both lantrunculin B and cytochalasin D, which dissociated MFs, also abolished granule formation as evidenced by the diffuse green fluorescence seen in the cortical cytoplasm (Fig. 4M and N). In contrast, stabilization of MFs following treatment with phalloidin resulted in a very clear appearance of fluorescent Hsp70h-GFP granules at the opposite sides of the cell wall (Fig. 4O). Finally, BDM treatment also inhibited granule formation (Fig. 4P).

Dramatically different results were obtained using drug treatments of the tissues that expressed TMV MP-GFP: none of the drugs was capable of preventing formation of the peripheral fluorescent granules (Fig. 4Q through X). It should be emphasized that the localization patterns presented in Fig. 4 were observed for $\geq 95\%$ of the screened cells ($n \geq 200$). The immunoblot analyses demonstrated that treatments with the cytoskeleton-specific drugs did not affect either the accumulation levels or the integrity of the Hsp70h-GFP (Fig. 2C, lanes 2 through 4) or TMV MP-GFP (Fig. 2C, lanes 6 through 8).

A subset of cytoskeleton-specific drugs described above was used to compare the requirements for cytosol trafficking of Hsp70h-GFP and Golgi stacks. As seen in Fig. 3, the microtubule-destabilizing drug oryzalin had no significant effects on the trafficking of Hsp70h-GFP granules or GFP-tagged Golgi.

In contrast, both latrunculin B and BDM interfered with trafficking.

Collectively, these data strongly suggest that both active translocation of Hsp70h in the cytosol, and its accumulation in the PD-rich areas, involve the actomyosin motility system but not the microtubules.

DISCUSSION

The actin cytoskeleton has one of the central roles in the physiology of eukaryotic cells and is also involved in the virus-host interactions required for trafficking of viral proteins and particles within and between cells (51). In plants, the actomyosin motility system functions in cytoplasmic streaming that results in rapid movement of different organelles, including Golgi stacks and peroxisomes (60). Actin MFs were also implicated in vesicular trafficking and cell plate formation (5, 24). Furthermore, several studies suggested that actin and myosin may participate in PD biogenesis and function (13, 54, 64). The data presented in this paper provide experimental evidence for the role of MFs in protein transport to PDs.

Our previous work using electron microscopy demonstrated that Hsp70h is present in PDs of BYV-infected cells (36). Here we show that Hsp70h localizes to PDs of noninfected cells in the absence of other viral proteins. This conclusion is based on a characteristic appearance of paired Hsp70h-containing granules at opposite sides of cell walls, and colocalization of these granules with an established PD marker, TMV MP-GFP. A very similar pattern of peripheral localization, as well as colocalization with TMV MP-GFP, was observed for a recently discovered cellular PD-residential protein, AtRGP2 (57). Because the resolution of the light microscope is not sufficient to detect individual PDs, we assume that the observed granules of the nontagged or fluorophore-tagged Hsp70h or TMV MP accumulate in the PD-rich areas or pit fields.

In addition to immobile granules of Hsp70h tightly associated with the plasma membrane and cell wall, time-lapse confocal microscopy revealed granules that moved with a mean speed of $\sim 1 \mu\text{m/s}$. We found that these cytoplasmic granules were associated with MFs, but not with microtubules. To identify mechanisms responsible for granule transport, we used seven drugs that affected either microtubular or actomyosin motility system. These experiments demonstrated that drugs that either disassembled MFs or inhibited myosins without affecting MF integrity completely abolished motility of the Hsp70h granules. Moreover, treatment with these drugs prevented formation of the peripheral immobile granules and resulted in a diffuse localization of Hsp70h in the cortical cytoplasm.

The negative effects of the MF- and myosin-specific drugs on both motility and localization of the Hsp70h do not necessarily imply that the former is required for the latter. However, the most parsimonious hypothesis states that trafficking of Hsp70h in association with MFs is the pathway for transporting this viral protein to the PD. Tight association of the cytoplasmic Hsp70h granules with MFs is in accord with this hypothesis. Furthermore, this association strongly suggests that Hsp70h translocation involves specific interaction with the actomyosin motility system rather than passive trafficking due to cytoplasmic streaming.

In accord with previous research, microtubule-specific drugs had only a moderate negative effect on cytoplasmic streaming (43). These drugs did not significantly inhibit trafficking, or altered partitioning of the Hsp70h to the cell periphery. These results indicated that the intact microtubular cytoskeleton is not required for Hsp70h localization to pit fields.

Interestingly, none of the tested inhibitors was able to markedly affect PD targeting of the GFP-tagged TMV MP. These unexpected observations could be due to higher tolerance of this process to drug treatments. An alternative explanation implies a cytoskeleton-independent mechanism of TMV MP targeting and therefore suggests that more than one mechanism could be used by viral proteins to reach PDs. The ability of TMV MP to interact with the cell-wall-associated pectin methylesterase could be a part of such alternative mechanism (9). It should also be emphasized that our experiments with TMV MP indicate that the drug treatments do not disrupt PDs that remain competent for TMV MP docking.

It seems likely that trafficking of the viral proteins, genomes, and particles within and between cells relies on preexisting pathways of intercellular communication used by host NCAPs (31). It will be interesting to see if the actin cytoskeleton is involved in the targeting of NCAPs to PDs. Recent work showed that the non-cell-autonomous behavior of certain plant Hsc70s is mediated by a specific amino acid sequence motif (3). In the case of another NCAP, transcription factor KN1, such behavior relies on a C-terminal homeodomain (25). None of these signals is present in BYV Hsp70h. Our previous work showed that the GFP fusions of either the N-terminal ATPase domain or C-terminal domain of Hsp70h localized to peripheral punctate bodies (53). These results suggested that Hsp70h may possess redundant signals for peripheral targeting.

The pathways of MP translocation upon transient expression do not necessarily reproduce events of the viral infection that may involve association of MPs with other viral proteins, genomes, or virions. Indeed, it has been demonstrated that TMV MP colocalizes and traffics with viral replication complexes (23, 33). The TGB MPs are involved in multiple interactions between themselves (18, 38), while tubule-forming MPs enclose virions into the tubes formed during infection (29). In addition to accumulation in PDs, BYV Hsp70h is incorporated to virion tails that function in virus transport (2, 36, 42, 47, 53).

We have also tested effects of cytoskeletal inhibitors on the BYV replication and cell-to-cell movement. It was revealed that while intact microtubules are dispensable for both processes, MF disassembly by drugs dramatically reduces viral RNA accumulation and abolishes BYV movement from cell to cell (A. I. Prokhnevsky and V. V. Dolja, unpublished data). Therefore, demonstration of a possible direct function of MFs in BYV movement that might be overshadowed in these experiments by MF requirement for replication needs to be addressed using an alternative approach.

Investigation of the targeting pathways of individual viral proteins described in this and other recent papers (18, 23, 28) continues to provide important insight into molecular mechanisms of virus interactions with the plant cells. In addition to findings with NCAP trafficking in plants, one of the herpesvirus tegument proteins exhibits NCAP-like behavior in being able to translocate viral mRNAs between animal cells (15, 59). Moreover, cultured animal cells can interconnect via nanotub-

ules that facilitate intercellular vesicular trafficking in an actomyosin-dependent manner (56). It appears that direct intercellular communications involving actin cytoskeleton may become a common theme in plant and animal cell biology and virology.

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