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Interaction between HSP70 Homolog and Filamentous Virions of the Beet Yellows Virus

Alberto J. Napuli,* Bryce W. Falk,† and Valerian V. Dolja*,‡,[1]

*Department of Botany and Plant Pathology and ‡Center for Gene Research and Biotechnology, Oregon State University, Corvallis, Oregon 97331; and †Department of Plant Pathology, University of California, Davis, California 95616

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An HSP70 homolog (HSP70h), encoded by the *Closterovirus* Beet yellows virus (BYV), functions in viral movement from cell to cell. A previous study revealed that in infected cells, HSP70h colocalizes with the masses of BYV filamentous virions. Here we demonstrate that HSP70h forms a physical complex with BYV virions. This conclusion is based on both the comigration of HSP70h with BYV virions in sucrose density gradients and the coimmunoprecipitation of the HSP70h and BYV capsid protein using anti-HSP70h serum. The HSP70h–virion complex is stable at high concentrations of sodium chloride; its dissociation using sodium dodecyl sulfate, lithium chloride, or alkaline pH was accompanied by virion disassembly. However, the complex formation does not involve covalent bonds between HSP70h and virion components. Each BYV virion contains approximately 10 molecules of HSP70h. The possible role of HSP70h interaction with the virions in cell-to-cell movement of BYV is discussed.

INTRODUCTION

Molecular chaperones from the family of ~70-kDa heat shock proteins (HSP70s) are conserved through all kingdoms of unicellular and multicellular organisms. In eukaryotic cells, the specialized members of the HSP70 family are present in all cell compartments (Boorstein *et al.,* 1994). Some of the HSP70s are stress-inducible and play important roles in cell survival under heat shock and other stress conditions. Other HSP70s are constitutively expressed and are involved in protein folding in the cytoplasm, protein import into the endoplasmic reticulum, mitochondria, chloroplasts, or trafficking of the receptors and coated vesicles, etc. (Bukau and Horwich, 1998; Pilon and Schekman, 1999).

Although viruses do not normally encode HSP70s, there are multiple examples of viruses relying on cellular HSP70s at different phases of their life cycles. For several animal viruses, the interaction with HSP70s appears to be involved in virion assembly that may take place in cytoplasm, nucleus, or endoplasmic reticulum, depending on the requirements of different viruses (Macejak and Sarnow, 1992; Cripe *et al.,* 1995; Xu *et al.,* 1998; Liberman *et al.,* 1999). The induction of a host HSP70 gene by diverse plant viruses suggested that the corresponding protein may provide an important function in the infected cell, although it is not clear who is a beneficiary, the virus or the host (Escaler *et al.,* 2000).

The *Closteroviridae* is a family of positive-strand RNA viruses (closteroviruses) that possess ~15- to 20-kb genomes (Bar-Joseph *et al.,* 1979; Dolja *et al.,* 1994b; Martelli *et al.,* 1999). These genomes are encapsidated into filamentous virions that are assembled from major and minor capsid proteins (CP and CPm, respectively) (Agranovsky *et al.,* 1995; Tian *et al.,* 1999). Although closteroviruses are remarkably divergent in their genetic organization, all of them possess a gene encoding an HSP70 homolog (HSP70h) (Agranovsky *et al.,* 1991; Fazeli and Rezaian, 2000). Viruses of no other family described so far are reported to harbor an HSP70 gene.

Functional characterization of several closteroviruses revealed that HSP70h is not required for genome amplification (Klaassen *et al.,* 1996; Peremyslov *et al.,* 1998; Satyanarayana *et al.,* 1999). The Beet yellows virus (BYV) gene coding for HSP70h is expressed early in infection and to relatively low levels (Hagiwara *et al.,* 1999). We demonstrated recently that HSP70h is essential for BYV movement from cell to cell (Peremyslov *et al.,* 1999). Similarly to movement proteins of many other plant viruses (Carrington *et al.,* 1996; McLean *et al.,* 1997; Lazarowitz and Beachy, 1999), BYV HSP70h was observed in association with the plasmodesmata (Medina *et al.,* 1999). It was also found that in the cytoplasm of BYV-infected cells, HSP70h colocalizes with the virion masses, suggesting that it may physically interact with the virions (Medina *et al.,* 1999). This possibility was supported by the finding of HSP70h in the partially purified virions of the Lettuce infectious yellows virus (LIYV), a member of the genus *Crinivirus* in the family *Closteroviradae* (Tian *et al.,* 1999). In addition to HSP70h, a 6-kDa protein, a 64-kDa protein, CP, and CPm are required to potentiate the movement of BYV (Alzhanova *et al.,* 2000). These results suggested that BYV translocation may in-

[1] To whom correspondence and reprint requests should be addressed. Fax: (541) 737-3573. E-mail: doljav@bcc.orst. edu.

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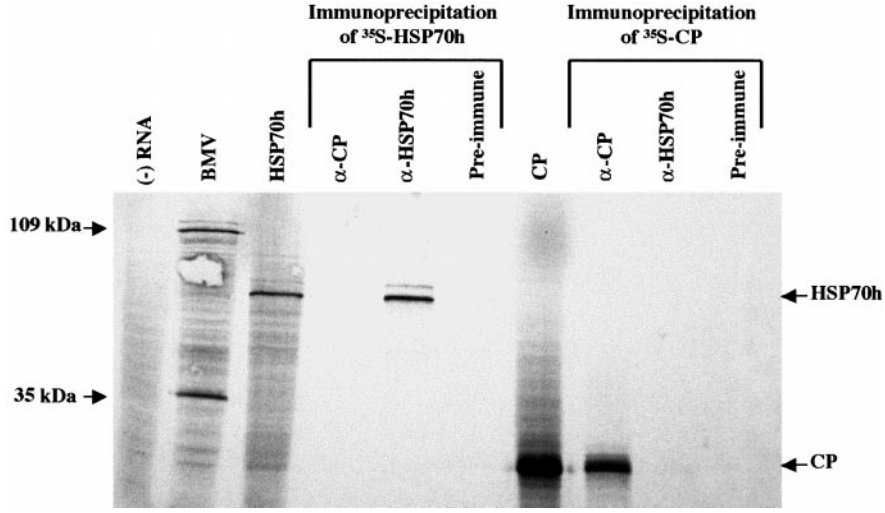

FIG. 1. Immunoprecipitation of the ^{35}S-labeled HSP70h and capsid protein using anti-HSP70h (α-HSP70h), anti-capsid protein (α-CP), or preimmune sera. The products of *in vitro* translation reactions programmed with no RNA (negative control, (−)RNA lane), Brome mosaic virus RNA (positive control, BMV lane), HSP70h mRNA (HSP70h lane), and capsid protein mRNA (CP lane) were separated by SDS–PAGE, blotted onto a nitrocellulose membrane, and subjected to autoradiography. The bracketed sets of three lanes show the analysis of the immunoprecipitation products using BYV ^{35}S-HSP70h and ^{35}S-capsid protein and antisera as indicated. The arrows mark the positions of corresponding BMV proteins (left) or BYV proteins (right).

volve virion formation, as well as interaction between HSP70h and other components of the viral movement machinery.

We report here that the virions isolated from BYV-infected plants are physically associated with HSP70h. Formation of these HSP70h–virion complexes does not involve covalent bonds with any of the virion components. However, our attempts to dissociate complexes using various treatments resulted in at least partial virion disassembly. A hypothetical model of BYV translocation involving formation of the HSP70h–virion complexes is proposed.

RESULTS

Characterization of the anti-HSP70h and anti-CP sera

In this work, we have utilized sera raised against BYV HSP70h and CP that were previously used for serologically specific electron microscopy (Medina *et al.,* 1999) and immunoblot analysis (Dolja *et al.,* 1998), respectively. The former antiserum was derived using the recombinant C-terminal fragment of the HSP70h superexpressed in *E. coli,* whereas the latter was raised against purified BYV virions. We felt it necessary to further characterize these antisera, since their performance in immunoprecipitation experiments was of critical importance for testing possible association of HSP70h with virions.

To this end, we generated ^{35}S-cysteine-labeled HSP70h and CP using *in vitro* translation of corresponding mRNAs in the wheat germ extracts. Each mRNA directed formation of the labeled product of expected mol wt, that is ~65 kDa for the HSP70h and ~22 kDa for CP (Fig. 1, lanes HSP70h and CP, respectively). These labeled products were immunoprecipitated using the homologous, heterologous, and preimmune sera. As seen in Fig. 1, each antiserum specifically immunoprecipitated the homologous antigen, whereas preimmune sera did not precipitate any. These results confirmed the utility of anti-HSP70h and anti-CP sera for immunoprecipitation experiments. It should be noted that, since anti-CP serum was raised against purified BYV virions, it may also recognize CPm and other proteins potentially present in virions. Accordingly, we used this antiserum only as a positive control for immunoprecipitating virions that contain ~95% of the CP (Agranovsky *et al.,* 1995).

HSP70h comigrates with the virions in sucrose density gradient

To examine the possible association between HSP70h and virions, we employed centrifugation in sucrose density gradients and analysis by SDS–PAGE and transmission electron microscopy (TEM). The SDS–PAGE analysis showed that the virions purified from BYV-infected plants migrated in gradients to form a broad zone with peak in fractions 11–14 (Fig. 2B). TEM analysis revealed that the gradient fractions with the majority of BYV CP also contained apparently intact filamentous particles of the size and morphology characteristic of BYV (Fig. 2C) (Bar-Joseph and Hull, 1974). The presence of the HSP70h in gradient fractions was tested using immunoblot analysis and an HSP70h-specific antiserum. As is obvious from comparison between Figs. 2A and 2B, the distribution of the antiserum-reactive protein among the gradient fractions was very similar to that of BYV CP and virions.

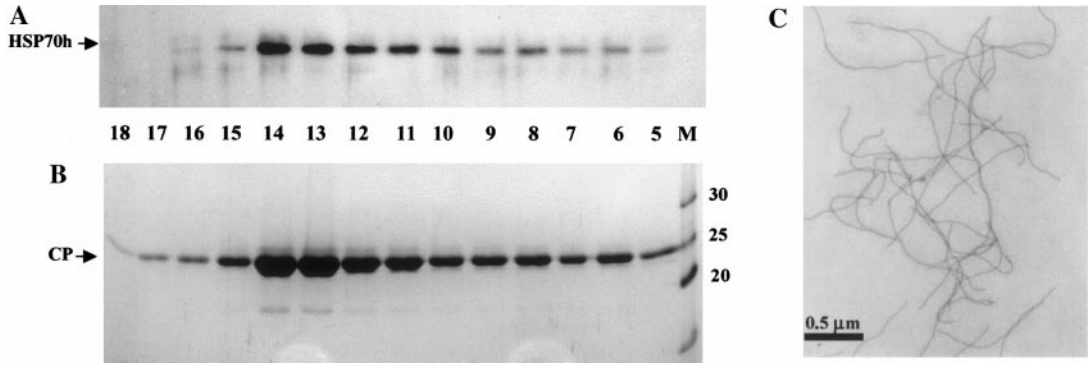

FIG. 2. Comigration of the HSP70h and virions in sucrose density gradient. (A) Immunoblot analysis of the gradient fractions (numbering from the bottom up) using anti-HSP70h serum; position of the ^{35}S-labeled HSP70h generated via *in vitro* translation is marked by the arrow. (B) Same gradient fractions separated by PAGE and stained with Coomassie brilliant blue. The position of the BYV CP is marked by the arrow; the numbers at the right indicate mol wt of the protein markers (M). (C) Transmission electron micrograph of the BYV virions isolated from peak fractions of the gradient.

Moreover, the mobility of the detected protein in SDS–polyacrylamide gels was identical to the mobility of ^{35}S-cysteine-labeled HSP70h derived *in vitro* (Fig. 2A). Taken together, these results not only demonstrated that the HSP70h comigrates with BYV virions in sucrose density gradients but also suggested that there is a physical association between the two.

Coimmunoprecipitation of the HSP70h and virions

Since it can not be excluded that the comigration of the HSP70h and BYV virions in sucrose density gradients was coincidental, we used an independent experimental approach to provide further evidence for the existence of the HSP70h–virion complexes. The BYV virions isolated from the peak fractions of the gradient were incubated with either anti-CP or anti-HSP70h serum, and the resulting antibody–antigen complexes were precipitated with the aid of protein A–Sepharose. As expected, the following analysis of the products immunoprecipitated using anti-CP serum revealed the presence of BYV CP (positive control; Fig. 3, lane 5). Conspicuously, the comparable amount of the CP was immunoprecipitated using anti-HSP70h serum (Fig. 3, lane 6). A series of control reactions demonstrated that the appearance of the CP in this latter immunoprecipitated material was strictly dependent on the presence of HSP70h-specific serum (Fig. 3, lanes 1–4 and 7). The coimmunoprecipitation of the BYV HSP70h and CP from gradient fractions containing intact virions (Fig. 2C) by anti-HSP70h serum demonstrates the physical association of HSP70h with the virions. The identical result was obtained using the virions which were not fractionated in gradient, indicating that the HSP70h–virion complexes were formed prior to, rather than during, the gradient centrifugation (data not shown). Collectively, the results of sucrose density gradient centrifugation and coimmunoprecipitation experiments provided strong evidence for the existence of HSP70h–virion complexes in the BYV-infected plants.

Stability and composition of the HSP70h–virion complexes

The fact that the antiserum-reactive protein detected in virion preparations possessed the mol wt expected for monomeric HSP70h (Fig. 1A) suggested that HSP70h is not covalently linked to the virion components. However, if the putative covalent bonds were formed by disulfide bridges, they could dissociate during the sample boiling in the presence of β-mercaptoethanol prior to its electrophoretic analysis. To test this possibility, the parallel samples of the virions were treated by 2% SDS in or without the presence of 100 mM DTT and analyzed using

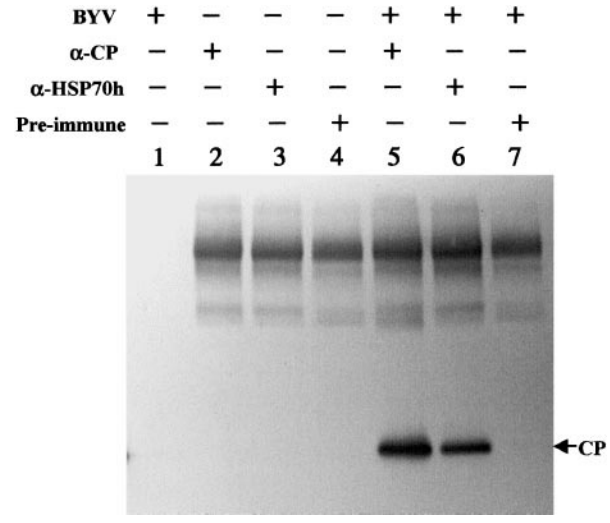

FIG. 3. Coimmunoprecipitation of the HSP70h and BYV virions derived from the peak fractions of sucrose density gradients. The products of immunoprecipitation reactions were analyzed using immunoblotting and anti-CP serum. The principal components of the reactions corresponding to each lane are shown in the table above the photograph. Arrow marks the position of the BYV CP. The bands in the upper portion of the membrane correspond to rabbit antibodies present in immunoprecipitated material and recognized by the goat anti-rabbit serum conjugated to alkaline phosphatase.

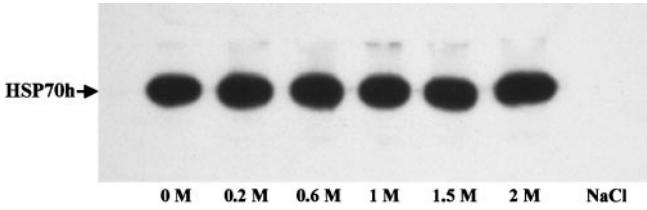

FIG. 4. Stability of the HSP70h–virion complexes in the presence of NaCl. The BYV virions incubated in the presence of indicated concentrations of NaCl were precipitated by ultracentrifugation and analyzed using immunoblotting and anti-HSP70h serum.

SDS–PAGE without the boiling in β-mercaptoethanol-containing buffer. The SDS treatment alone should result in complete dissociation of the virions (see below) without disrupting disulfide bridges, whereas combined treatment with SDS and DTT should disrupt both. Comparative immunoblot analysis of these parallel samples using antiserum to HSP70h revealed identical bands corresponding to monomeric HSP70h (data not shown). These results allowed us to exclude the involvement of covalent bonds in the formation of the HSP70h–virion complex.

To characterize the complex stability under conditions of different ionic strength, we incubated the virions in the presence of various concentrations of NaCl. After the incubation, the virions were precipitated by ultracentrifugation and analyzed using immunoblotting and anti-HSP70h serum. The salt-induced dissociation of the HSP70h–virion complex was expected to result in decreasing amounts of HSP70h detected in the precipitated virions. As shown in Fig. 4, the complex remained stable under the concentrations of NaCl up to 2 M.

In the next series of experiments we used LiCl, which is capable of dissociating protein–protein and protein–nucleic acid complexes including virions (Goodman, 1975). The treatment of the virions with increasing concentrations of LiCl resulted in a gradual decrease in the amount of virions precipitated by ultracentrifugation (Fig. 5B), indicating virion destabilization. The immunoblot analysis demonstrated that the amount of the HSP70h present in treated virions was roughly proportional to the amount of precipitated virions (compare Figs. 5A and 5B). Similar results were obtained when the virions were exposed to various pH. The decrease in the HSP70h amount detected in virions after incubation under alkaline (pH 11) or acidic (pH 4) conditions was approximately proportional to the amount of precipitated virions (data not shown). These results suggested that the HSP70h–virion complexes are relatively stable; their dissociation by either LiCl or extreme pH was accompanied by virion disassembly.

We have also examined the stability of the HSP70h–virion complex using SDS treatments. It was found that the treatment with 2% SDS results in virtually instantaneous virion disassembly, whereas this process proceeds much slower in the presence of 0.5% SDS (data not shown). The time-course experiment shown in Fig. 6 revealed the concomitant dissociation of the complex and the virions. However, it appears that the virion stability in the presence of 0.5% SDS is somewhat higher than that of the HSP70h–virion complexes. Indeed, the amount of the virions precipitated after 5-min treatment with 0.5% SDS is comparable to that at time zero, whereas the amount of HSP70h associated with the virions at this time point is much lower than at the beginning of treatment (compare Figs. 6A and 6B). These

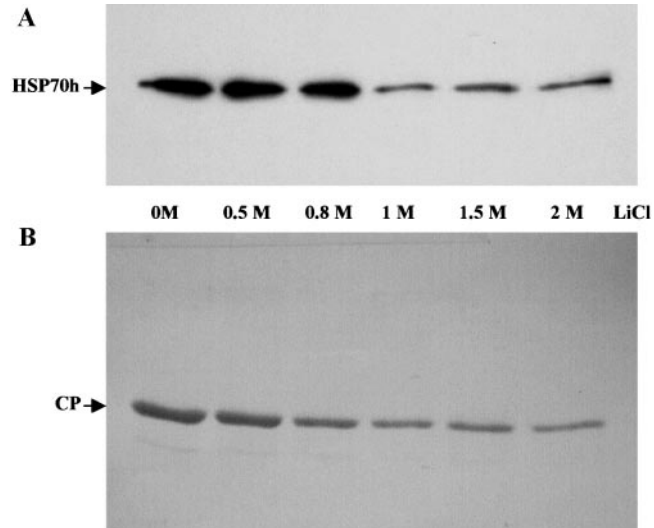

FIG. 5. Stability of the HSP70h–virion complexes in the presence of LiCl. The virions incubated in the presence of indicated concentrations of LiCl were precipitated by ultracentrifugation and analyzed using immunoblotting and anti-HSP70h serum (A) or SDS–PAGE and Coomassie staining (B).

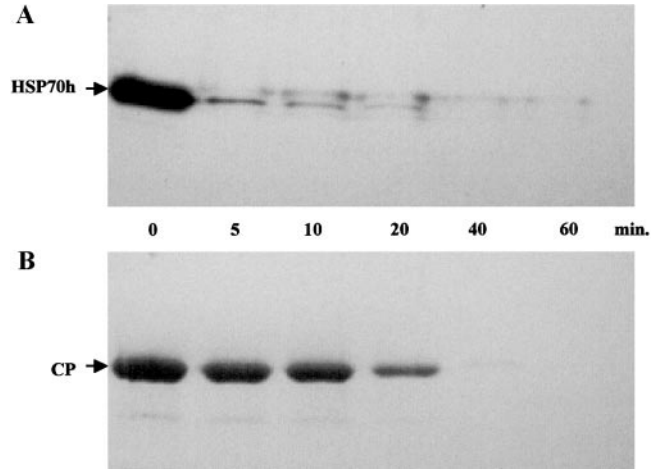

FIG. 6. Time-course analysis of the disassembly of the HSP70h–virion complexes in the presence of 0.5% SDS. The virions were incubated in the presence of SDS for the indicated periods of time, precipitated by ultracentrifugation, and analyzed using immunoblotting and anti-HSP70h serum (A) or SDS–PAGE and Coomassie staining (B).

results indicate that the stability of the HSP70h–virion complexes and the virions are different under the used experimental conditions.

To determine the number of HSP70h molecules per virion, we separated virion proteins using SDS–PAGE. The approximate amount of HSP70h present in the sample was determined by comparing to standardized dilutions of a marker protein, bovine serum albumin (data not shown). Given that the intact BYV virion contains ~3300 molecules of the CP (Bar-Joseph and Hull, 1974), we estimated that each virion is bound on average to ~10 molecules of HSP70h.

DISCUSSION

Using two experimental approaches, the sedimentation in sucrose density gradients and the immunoprecipitation, we revealed that the virions isolated from BYV-infected plants contain one of the viral movement proteins, HSP70h. The HSP70h–virion complexes withstood the treatment by high concentrations of sodium chloride, whereas utilization of other dissociating agents resulted in concomitant disassembly of the complexes and virions. Our data indicate that the complexes are maintained by strong nonionic interactions that do not involve covalent bonds between HSP70h and other components of the virion. Finding the HSP70h associated with virions of BYV (genus *Closterovirus*) and LIYV (genus *Crinivirus*) (Tian *et al.,* 1999) suggests that this may be a feature shared by viruses in family *Closteroviridae.*

What is the role played by the HSP70h molecules that are attached to or present in the virions? Although HSP70h may be an integral virion component, it seems unlikely that its role is only structural or protective. Indeed, the RNA genomes of all rod-shaped and filamentous plant viruses, except for closteroviruses, are encapsidated into and protected by a single type of capsid protein. The major distinction of closteroviruses from other elongated plant viruses is the heterodimeric morphology of the virions with the minor capsid protein that forms a short tail at one end of the virion (Agranovsky *et al.,* 1995; Tian *et al.,* 1999). It is possible that HSP70h aids in the assembly of the tailed BYV virions. On the other hand, high stability of the HSP70h–virion complexes is suggestive of the HSP70h involvement in the phases of virus life cycle that follow encapsidation.

At least three of those additional functions are feasible with the existing data. First, HSP70h may be required for the virion disassembly at the onset of the infection process. Second, finding of the HSP70h in the insect-transmissible virion preparations of LIYV is compatible with its role in vector transmission (Tian *et al.,* 1999). Third, HSP70h may mediate the intercellular translocation of the virions. This latter possibility is supported by the requirement for both HSP70h activity and virion assembly to potentiate BYV cell-to-cell movement (Peremyslov *et al.,* 1999; Alzhanova *et al.,* 2000). Moreover, association of HSP70h with the virions and plasmodesmata (Medina *et al.,* 1999; this study) allowed us to propose that HSP70h functions via binding virions and aiding their translocation toward and through the plasmodesmata.

The requirements for cell-to-cell movement in distinct filamentous plant viruses reveal some intriguing analogies. Similar to BYV (genus *Closterovirus*), virions were implicated in the cell-to-cell movement of the members of the genera *Potexvirus* (Chapman *et al.,* 1992; Forster *et al.,* 1992; Santa Cruz *et al.,* 1998) and *Potyvirus* (Dolja *et al.,* 1994a; Rodriguez-Cerezo *et al.,* 1997). Movement machineries of each of these three virus genera include proteins possessing (putative) ATPase activity that may generate energy for virus translocation. Examples of such proteins are provided by the HSP70h of BYV (Peremyslov *et al.,* 1999), the 25-kDa protein of the Potato virus X (*Potexvirus*) (Morozov *et al.,* 1999), and the CI protein of the Tobacco etch virus (*Potyvirus*) (Carrington *et al.,* 1998). These analogies suggest that the filamentous viruses share some of the mechanisms of their cell-to-cell movement. It will be interesting to see whether the formation of complexes between virions and movement-associated ATPases takes place in *Potexvirus* and *Potyvirus* genera.

Although the gene encoding HSP70 homolog is found only in representatives of *Closteroviridae,* there is at least one example of unrelated virus that may utilize host HSP70 for the cell-to-cell movement. It was found recently that the movement protein of Tomato spotted wilt virus (genus *Tospovirus*) is capable of binding viral nucleocapsid protein and plant DnaJ-like proteins (Soellick *et al.,* 2000). Since the members of the DnaJ family function via interactions with HSP70s (Pilon and Schekman, 1999), it appears that the recruitment of these molecular chaperones in virion or nucleocapsid translocation may have evolved independently in very distinct families of plant viruses.

MATERIALS AND METHODS

Isolation, fractionation, and electron microscopy of the BYV virions

BYV virions were isolated from infected *Nicotiana benthamiana* plants 2 to 3 weeks postinoculation, following the protocol developed for LIYV (Klaassen *et al.,* 1994). Sucrose density gradient centrifugation of BYV virions was conducted as described (Bar-Joseph and Hull, 1974). In brief, 1 to 2 ml of the partially purified virions (~1 mg/ml) were loaded onto 10–40% sucrose density gradients and centrifuged at 25,000 rpm for 4 h using Beckman SW40 rotor at 4°C. The gradients were prepared by layering 10, 20, 30, and 40% sucrose solutions (5, 7, 8, and 8 ml, respectively) containing TE buffer (10 mM Tris and 1 mM EDTA; pH 7.6) in corresponding centrifuge tubes, and stored for 1 h at the room temper-

ature. Gradients were fractionated into 1-ml fractions by gravity flow.

TEM was conducted using the virions isolated from the peak gradient fractions. Formvar/carbon-coated grids were allowed to float on 25-μl drops of the 1:100 diluted virions for 10 min. The grids were rinsed three times in drops of the water and stained using 2% uranyl acetate for 10 min prior to viewing by TEM.

In vitro translation, immunoprecipitation, and immunoblot analysis

The ORFs coding for BYV HSP70h and CP were cloned into the pTL7SN plasmid downstream from an SP6 RNA polymerase promoter and a Tobacco etch virus leader region (Carrington and Freed, 1990) using *Nco*I and *Sma*I sites. These sites were introduced into the terminal regions of corresponding DNA fragments concomitantly with their generation via PCR. The *Sma*I-linearized plasmids and SP6 RNA polymerase were used to generate uncapped *in vitro* RNA transcripts as described (Peremyslov *et al.,* 1998). The resulting mRNAs were precipitated using LiCl, resuspended in water, and stored at −80°C. *In vitro* translations of CP and HSP70h transcripts in the wheat germ extracts (Promega, Madison, WI) were conducted in 100-μl reaction mixtures according to the manufacturer's protocol. The translation products were labeled using L-[^{35}S]cysteine (Amersham Pharmacia Biotech, Piscataway, NJ). The Brome mosaic virus RNA and water were used as positive and negative controls, respectively.

The immunoprecipitation assays were conducted as described by Cripe *et al.* (1995) with some modifications. For the labeled translation products, the 200-μl reaction mixtures contained 180 μl of the IP buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 1% Triton X-100), 20 μl of the products, and 1 μl of rabbit antisera against CP (Dolja *et al.,* 1998) or HSP70h (Medina *et al.,* 1999). Immunoprecipitations of the virions were conducted in 1-ml reaction mixtures containing 900 μl of IP buffer, 100 μl of BYV virions derived from the peak fractions of the gradients, and 1 μl of the rabbit antiserum against CP or HSP70h. The preimmune sera were used as negative controls. The reactions with primary antibodies were incubated at 4°C for 16 h with gentle mixing, followed by addition of 100 μl of 20% protein A–Sepharose CL-4B (Amersham Pharmacia Biotech, Piscataway, NJ). After 4-h incubation at 4°C, the beads were loaded into 1-ml microspin columns and washed five times with the IP buffer and five times with the TE buffer. Proteins were eluted by boiling the beads in protein dissociation buffer, and the immunoblot analysis was performed as described (Dolja *et al.,* 1993) using anti-BYV serum at 1:1000 dilution.

The immunoblot analysis of HSP70h was conducted using a chemiluminescent assay kit ECL Western Blotting System (Amersham Pharmacia Biotech, Piscataway, NJ) and anti-HSP70h serum at 1:2000 dilution. Secondary goat anti-rabbit antibodies conjugated to horseradish peroxide were used at 1:4000 dilution.

Examination of the stability and composition of the HSP70h–virion complexes

Preparations of BYV virions were used to determine the stability of the HSP70h–virion complexes in the presence of varying concentrations of NaCl, LiCl, and SDS, as well as under alkaline or acidic conditions. For NaCl experiments, 100 μl of the virions (~1 mg/ml) were incubated on ice for 30 min in 0, 0.2, 0.6, 1, 1.5, and 2 M solutions of NaCl in 10 mM Tris–HCl, pH 7.4 (final volume of the mixture was 450 μl). The reaction mixtures were layered onto 150 μl of 20% sucrose cushion in 1.5-ml tubes and centrifuged at 100,000 *g* in the Beckman TLA 100.3 rotor at 4°C for 2 h to pellet virions. The pellets were resuspended in 50 μl of 2× protein dissociation buffer, boiled for 10 min, and analyzed using SDS–PAGE or immunoblotting.

The LiCl experiments were conducted in general accord with the method described by Goodman (1975) for the isolation of Potato virus X CP subunits by LiCl dissociation. To determine the effects of LiCl on the HSP70h–virion complexes, reaction mixtures containing 100 μl of the BYV virions were incubated on ice for 1 h in 0, 0.5, 0.8, 1, 1.5, and 2 M concentrations of LiCl (final volume of reactions was 600 μl). The virions were centrifuged and analyzed as described earlier.

The SDS experiments followed the method described by Mundry *et al.* (1991) for disassembly of Tobacco mosaic virus. A 150-μl sample of the virions was mixed with 420 μl of the buffer containing 50 mM Tris–HCl, pH 7.4, 200 mM NaCl, and 2% Triton X-100. The 100-μl aliquot of the reaction mixture was transferred immediately into 900 μl of the protein dissociation buffer to represent a zero time point. After that, 10% SDS was added to a final concentration of 0.5%. The 100-μl aliquots of the reaction mixture were quenched in 900 μl of the same buffer at 5, 10, 20, 40, and 60 min after addition of SDS. The samples were centrifuged and analyzed as described earlier.

The pH experiments were conducted using the modified protocol of Pelcher and Halasa (1979) for alkaline degradation of virions of Tobacco mosaic virus. The BYV virions were dialyzed against 10 mM EDTA, pH 7.4, for 16 h to standardize the pH of the starting material. Alkaline buffer solution was prepared using 20 mM sodium tetraborate at pH 11. Acidic buffer solution was prepared using 20 mM sodium citrate at pH 4.5. To examine the stability of the HSP70h–virion complexes at varying pH conditions, the reaction mixtures containing 50 μl of the virions were mixed with 1 ml of corresponding buffer solution. The reactions were incubated for 1 h on ice, then centrifuged and analyzed as described.

To determine the amount of HSP70h present in virions, the concentration of the total protein in virion preparation was measured using Bradford protein assay (BioRad Laboratories, Hercules, CA). The sample containing 10 μg of the total protein was separated using 12.5% SDS–PAGE; the samples containing 0.1, 0.2, 0.5, 1, and 1.5 μg of the bovine serum albumin were used as standards. After staining the gels with Coomassie brilliant blue, the approximate concentration of the HSP70h was determined by comparing the intensity of its band with that of the standards. The molar ratio of the CP to HSP70 was determined using the known mol wt of each protein and the Avogadro number. The approximate number of HSP70h molecules per virion was calculated assuming that each BYV virion contains ~3300 molecules of the CP (Bar-Joseph and Hull, 1974).

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