Volume 304, number 1, 12–14 FEBS 11111 June 1992

HSP70-related 65 kDa protein of beet yellows closterovirus is a microtubule-binding protein

Alexander V. Karasev[a,*], Anna S. Kashina[b], Vladimir I. Gelfand[b] and Valerian V. Dolja[c]

[a]*Institute of Microbiology, Academy of Sciences of Russia, Moscow 177811, Russia,* [b]*Institute of Protein Research, Academy of Sciences of Russia, Puschino, Moscow Region 142292, Russia* and [c]*A.N. Belozersky Laboratory, Moscow State University, Moscow 119899, Russia*

Received 26 March 1992

Beet yellows virus (BYV) genome encodes a 65 kDa protein homologous to the HSP70 family of cellular heat-shock proteins (Agranovsky, A.A., Boyko, V.P., Karasev, A.V., Koonin, E.V. and Dolja, V.V. (1991) J. Mol. Biol. 217, 603–610). The respective gene was cloned and expressed in vitro yielding a product of the expected size (p65). This product was found to bind to the purified microtubules with a binding constant of 4×10^{-7} M. The binding of p65 was stimulated if ATP presented in the translation mixture was hydrolyzed by apyrase. Removal of the short C-terminal domains of α- and β-tubulin by subtilisin digestion abolished the binding, demonstrating its specificity. The possible role of p65 association with microtubules in the movement of virus within and/or between plant cells is proposed.

Microtubule-associated protein; Plant virus; Cell-to-cell movement

1. INTRODUCTION

Beet yellows virus (BYV) is a type member of the closterovirus group combining filamentous plant viruses with large messenger-sense RNA genomes [1]. BYV is the only virus sequenced to date which has been found to code for the relatives of cellular heat-shock proteins [2,3]. The BYV 65 kDa protein encoded by open reading frame (ORF) 3 is a homologue of the HSP70 protein family, while the 64 kDa protein encoded by ORF 4 is a remote relative of the HSP90 family. Although the functions adopted by the virus to these proteins are unknown it was suggested that they could participate in a virus cell-to-cell movement by a molecular chaperone-like mechanism [2–4]. This crucial process for plant virus multiplication and spread is mediated by virus-encoded movement proteins, and is thought to include trafficking of viral nucleoproteins through the modified plasmodesmata [5–7]. The mechanism of the physical movement of virus-specific nucleoproteins is completely unknown. On the other hand, it is clear that most kinds of translocations of relatively large particles within the cell is mediated by the system of microtubules (MTs). Moreover, in several animal systems it was demonstrated that the addressed translocation of some mRNAs is an active process connected with the MTs [8–9]. At least two groups of microtubule-associated proteins (MAPs) are involved in the intracellular motility: 'structural' MAPs (MAP1, MAP2, τ, etc.) and translocators (kinesin, dynein, dynamin) (see [10] for review). Some of the HSP70s are also found in tight association with MTs both in vitro and in vivo [11–12]. Thus it seemed reasonable to address potential MT-associated properties of the BYV 65 kDa protein, a homologue of cell HSP70 molecular chaperones. In the present work we have demonstrated that the BYV 65 kDa protein does indeed possess MT-binding properties in vitro. We suggest that these properties are linked with the process of BYV cell-to-cell movement.

Abbreviations: BYV, beet yellows virus; PVX, potato virus X; HSP70, 70 kDa heat-shock proteins; HSP90, 90 kDa heat-shock proteins; MAP, microtubule-associated protein; MT, microtubule; ORF, open reading frame.

**Present address:* Department of Plant Pathology, University of California, Riverside, CA 92521-0122, USA.

Correspondence address: V.V. Dolja, Department of Biology, Texas A & M University, Texas 77843-3258, USA. Fax: (1) (409) 845-2891.

2. MATERIALS AND METHODS

Tubulin purification from bovine brain and MT polymerization, as well as subtilisin treatment of MTs, was performed as described [13]. Purified hsc70 protein from rabbit reticulocytes was a gift from Dr. O.N. Denisenko. Plasmid pBY65 contained the 65 kDa protein gene inserted in a pTZ19 transcription vector downstream of the T7 bacteriophage RNA polymerase promoter. Plasmid pA103 contained the capsid protein gene of potato virus X (PVX) under the control of the same promoter [14] (provided by Dr. S.Yu. Morozov). Transcription of both plasmids linearized with *Eco*RI or *Sal*I, and translation of the resulting uncapped transcripts in rabbit reticulocyte lysates, were as described [14]. The translation mixture (75 μl) contained 80–100 μg/ml of transcript and 60 μCi of L-[^{35}S]methionine. After 60 min incubation at 30°C, puromycin was added to a final concentration of

 Published by Elsevier Science Publishers B.V.

0.3 mM, and, after another 10 min, RNase A was added to release synthesized products from polysomes.

For the co-sedimentation tests translation mixtures were diluted two-fold and centrifuged at 100,000 × *g* for 40 min to remove non-specific aggregates. The supernatants were supplemented with taxol (20 mM) and mixed with normal or subtilisin-treated MTs. After 20 min of incubation at room temperature the mixture was pelleted through 4 M glycerol in buffer A [13], containing 5 mM taxol, at 190,000 × *g* for 40 min. Pellets were dissolved and analysed by electrophoresis on the 8–20% polyacrylamide gels. For the determination of the binding constant increasing quantities of MTs were mixed with equal amounts of labelled 65 kDa protein. After incubation one half of the resulting pellet was withdrawn and the trichloroacetic acid-insoluble label was counted; the remaining half was analyzed by electrophoresis. For apyrase experiments the translation mixture was incubated with 0.1 mg of apyrase (Sigma, grade VI) for 30 min at 37°C prior to addition of MTs. This amount was found sufficient to completely hydrolyze 10 mM ATP in the sample.

3. RESULTS AND DISCUSSION

After translation of the pBY65 plasmid transcripts in rabbit reticulocyte lysates a major labelled protein of the expected size became visible (Fig. 1A, lane 2). This protein was unequivocally identified as the 65 kDa product (p65) of BYV ORF 3 by hybrid-arrested translation (not shown). When pretreated translation mixture was incubated with taxol-stabilized MTs labelled p65 was found to co-sediment with MTs (Fig. 1A, lane 4). In the control experiments, in vitro-labelled capsid protein of PVX or heat-shock cognate protein 70 (hsc70) purified from rabbit reticulocytes did not bind to MTs (Fig. 1B and C, respectively). Titration of the constant amount of labelled p65 with increasing amounts of MTs showed that the concentration of tubulin sufficient for binding 50% of the maximal p65 amount, was approx. 4.1×10^{-7} M (calculated per dimer of tubulin; data not shown). The corresponding value for the binding of known MT-associated protein kinesin in a separate experiment was found to be 2.7×10^{-7} M. Similar values gave rough estimates of these constants for MAP2 and τ-binding (A.S.K., V.I. Rodionov and V.I.G., unpublished). Thus we concluded that p65 possessed MT-binding properties similar to those of several genuine MAPs. One of the differences in binding to MTs between 'structural' MAPs and translocators is that these two groups of proteins interact with different parts of the tubulin molecule [13]. After treatment of MTs with subtilisin, which removes the C-terminal fragments of tubulin subunits of about 4 kDa without affecting MT morphology, the modified MTs are completely unable to bind MAPs and still bind translocators normally [13]. This provides a test allowing to distinguish between MAP- and translocator-type binding. When translation mixture containing p65 was incubated with subtilisin-treated MTs its binding was drastically decreased (cf. Fig. 2, lanes 3 and 4). The residual binding could be the result of incomplete subtilisin cleavage of MTs which was undetectable by Coomassie staining. It can be concluded that p65, like MAP1, MAP2 and

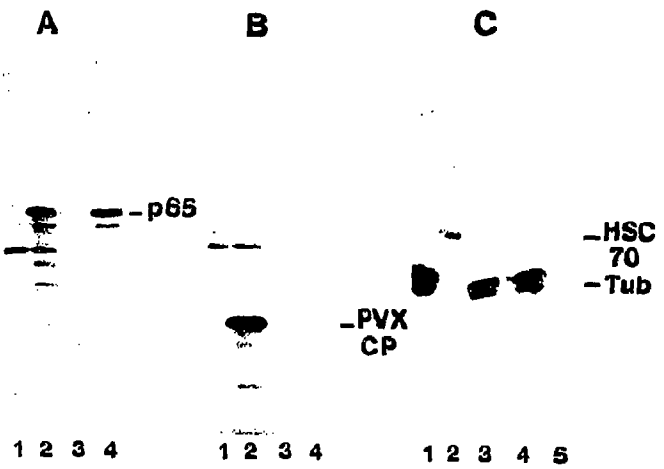

Fig. 1. Analysis of ^{32}P-labelled (A,B) or Coomassie-stained (C) proteins, co-sedimented with microtubules (MTs) on SDS-containing 8–20% polyacrylamide gels. (A and B) Products synthesized in rabbit reticulocyte lysates. (A) Lane 1, no RNA added; lane 2, translation of the RNA transcripts of the linearized pBY65 plasmid; lane 3, same as in lane 2 but sedimented without MTs; lane 4, same as in lane 2 but co-sedimented with 10 μg of MTs. (B) Lane 1, no RNA added; lane 2, translation of the RNA transcripts of the linearized pA103 plasmid; lane 3, same as in lane 2 but sedimented without MTs; lane 4, same as in lane 2 but co-sedimented with 10 μg of MTs. (C) Lane 1, 5 μg of MTs; lane 2, 0.5 μg of hsc70 protein; lane 3, 5 μg of MTs sedimented alone; lane 4, proteins co-sedimented upon incubation of 0.5 μg of hsc70 protein with 5 μg of MTs; lane 5, 0.5 μg of hsc70 protein sedimented alone.

τ, specifically interacts with C-terminal domains of α- and/or β-tubulin subunits.

It has been demonstrated that the binding properties of the proteins involved in the MT-dependent movement are modulated by ATP [10]. The same is true for numerous interactions involving HSP70 molecular chaperones, which are able to bind and hydrolyze ATP

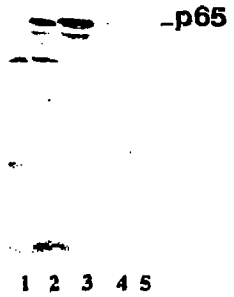

Fig. 2. Analysis of the co-sedimentation of the in vitro translated, ^{35}S-labelled BYV p65 with the subtilisin-treated MTs. Lanes 1 and 2, same as in Fig. 1A; lane 3, products of the pBY65 transcripts translation co-sedimented with 10 μg of MTs; lane 4, the same products co-sedimented with 10 μg of subtilisin-treated MTs; lane 5, the same products sedimented without MTs.

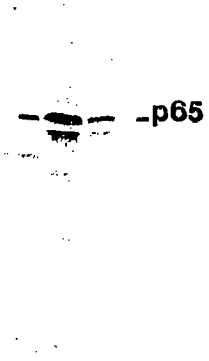

Fig. 3. Analysis of the co-sedimentation of the ^{35}S-labelled BYV p65 with the MTs after apyrase treatment of the translation mixture. Lanes 1 and 2, same as in Fig. 1; lane 3, products of the pBY65 transcripts translation co-sedimented with 10 μg of MTs after the apyrase treatment of the translation mixture; lane 4, the same products co-sedimented with 10 μg of MTs; lane 5, the same products sedimented without MTs.

[15]. To check the influence of ATP presence on the interaction between p65 and MTs we incubated translation mixtures containing 1 mM ATP with apyrase. As can be seen from Fig. 3, lanes 3 and 4, ATP hydrolysis strongly enhanced binding of p65 to MTs. Hence, p65 interaction with MTs seems to resemble, in this respect, the interaction of the proteins from HSP70 family to their various protein targets including denatured and nascent polypeptides, clathrin-coated vesicles, etc. [15].

There is some evidence that translocators alone are not sufficient for the MT-associated movement of particles within the cell and MAPs are the likely candidates to anchor the particles on MTs [16]. The involvement in MT-based motility has been suggested also for the β-internexin, a member of the HSP70 family exhibiting MAP-like properties [12]. A similar role could be proposed for the BYV p65, which might attach to the virus-specific ribonucleoproteins and to MTs mediating the translocation of ribonucleoproteins towards and/or through the plasmodesmata to the neighboring cell. Other viruses not coding for HSP70-related proteins possibly recruit for this purpose their own movement proteins or cellular molecular chaperones (cf. [3]). The specific need to adopt a HSP70 homologue might for BYV be connected with the mechanistic problem of movement of its relatively large RNA genome.

Acknowledgements: We are grateful to Dr. O.N. Denisenko for kind gifts of purified hsc70 and rabbit reticulocyte lysates, and to Dr. F.F. Severin for phosphocellulose-purified tubulin preparation. We thank Prof. J.G. Atabekov and Dr. J.C. Carrington for constant interest and support. Thanks are also due to Drs. A.A. Agranovsky, N.A. Lunina, A.R. Mushegian and E.V. Koonin for useful discussions and help at different stages of our work.

REFERENCES

[1] Bar-Joseph, M. and Murant, A.F. (1982) Closterovirus group, CMI/AAB Descr. of plant viruses, No. 260.
[2] Agranovsky, A.A., Boyko, V.P., Karasev, A.V., Koonin, E.V. and Dolja, V.V. (1991) J. Mol. Biol. 217, 603–610.
[3] Koonin, E.V., Mushegian, A.R., Ryabov, E.V. and Dolja, V.V. (1991) J. Gen. Virol. 72, 2895–2903.
[4] Agranovsky, A.A., Boyko, V.P., Karasev, A.V., Lunina, N.A., Koonin, E.V. and Dolja, V.V. (1991) J. Gen. Virol. 72, 15–23.
[5] Atabekov, J.G. and Dorokhov, Yu.L. (1984) Adv. Virus Res. 29, 313–364.
[6] Hull, R. (1989) Annu. Rev. Phytopathol. 27, 213–240.
[7] Wolf, S., Deom, C.M., Beachy, R.N. and Lucas, W.J. (1989) Science 246, 377–379.
[8] Davis, L., Banker, G.A. and Steward, O. (1987) Nature 330, 477–479.
[9] Yisraeli, J.K., Sokol, S. and Melton, D.A. (1990) Development 108, 289–298.
[10] Wiche, G., Oberkanins, C. and Himmler, A. (1991) Int. Rev. Cytol. 124, 217–273.
[11] Weller, N.K. (1988) Biol. Cell 63, 307–317.
[12] Green, L.A.D. and Liem, R.K.H. (1989) J. Biol. Chem. 264, 15210–15215.
[13] Rodionov, V.I., Gyoeva, F.K., Kashina, A.S., Kuznetsov, S.A. and Gelfand, V.I. (1990) J. Biol. Chem. 265, 5702–5707.
[14] Morozov, S.Yu., Miroshnichenko, N.A., Zelenina, D.A., Fedorkin, O.N., Solovijev, A.G., Lukasheva, L.I. and Atabekov, J.G. (1990) Biochimie 72, 677–684.
[15] Rothman, J.E. (1989) Cell 59, 591–601.
[16] Severin, F.F., Shanina, N.A., Kuznetsov, S.A. and Gelfand, V.I. (1991) FEBS Lett. 282, 65–68.