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The beet yellows closterovirus p65 homologue of HSP70 chaperones has ATPase activity associated with its conserved N-terminal domain but does not interact with unfolded protein chains

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The positive-strand RNA genome of beet yellows closterovirus (BYV) encodes a 65 kDa protein (p65) related to the HSP70 family of cell chaperones. The full-sized BYV p65, and N- and C-terminal fragments, with $(His)_6$ tails, were overexpressed in bacteria and purified by metal-chelate chromatography. Using a polyclonal antiserum raised against the C-terminal fragment of p65, evidence was obtained for expression of the viral protein *in planta*. Purified recombinant p65 and its N-terminal 40 kDa fragment exhibited Mg^{2+}-dependent ATPase activity *in vitro*. However, unlike its cellular HSP70 homologues, p65 was unable to bind to denatured protein and its ATPase activity was not stimulated by synthetic peptides which are known to stimulate HSP70 ATPases. Hence, the BYV p65, although being a chaperone-type ATPase, may have a distinct substrate specificity and function in BYV-infected cells.

Introduction

The family *Closteroviridae*, of which beet yellows virus (BYV) is the most studied member, contains several filamentous positive-strand RNA viruses that are semi-persistently transmitted by insect vectors (Agranovsky, 1996; Bar-Joseph *et al.*, 1979). Among plant positive-strand RNA viruses they have the largest genomes (up to 19 kb) containing peculiar gene arrays that are likely to have resulted from duplication of intrinsic viral sequences and insertion of foreign genes via RNA recombination (reviewed in Agranovsky, 1996; Dolja *et al.*, 1994). A hallmark of closteroviruses is the putative 65 kDa protein (p65) evidently related to the family of 70 kDa heat shock proteins (HSP70s) (Agranovsky *et al.*, 1991; Pappu *et al.*, 1994; Klaassen *et al.*, 1995). It has been hypothesized that the p65 gene arose from recombination between the genome of an ancestor of closteroviruses and a cellular HSP70 mRNA (Agranovsky *et al.*, 1991). So far, p65 has not been detected in infected plants, and its role in closterovirus infection remains unknown.

In cells, HSP70s act as 'molecular chaperones' and mediate a variety of functions connected with stress responses, protein folding, oligomerization and intracellular transport (Craig *et al.*, 1993; Gething & Sambrook, 1992; Rothman, 1989). Cell chaperones apparently function by transiently interacting with their protein targets in an ATP-dependent manner, a property that has been modelled *in vitro* using oligopeptides as targets (Gragerov & Gottesmann, 1994; Flynn *et al.*, 1989). Using proteolytic fragments of the bovine heat-shock cognate protein (HSC70), ATPase- and peptide-binding activities have been assigned to the N-terminal (44 kDa) and C-terminal (29 kDa) parts of the protein, respectively (Chappell *et al.*, 1987). It has been proposed that ATP hydrolysis by HSP70 leads to a conformational change in its ATPase domain which is then imposed on the protein target (Flaherty *et al.*, 1990). Cellular HSP70 sequences are highly conserved, mostly in their N-terminal part, with the C-terminal part being more variable (reviewed in Craig *et al.*, 1993). The N-terminal domains of the 65 kDa proteins of BYV, citrus tristeza virus (CTV), and lettuce infectious yellows virus (LIYV) show high levels of similarity to the HSP70 ATPases, whereas their C-terminal portions, being moderately similar to each other, are distinct from the equivalent domains in the cellular HSP70s (Agranovsky *et al.*, 1991; Klaassen *et al.*, 1995; Pappu *et al.*, 1994).

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In this work, we show that purified BYV p65 exhibited *in vitro* ATPase activity, mapped to its N-terminal fragment, but was unable to bind to unfolded poly- and oligopeptides in an ATP-dependent fashion. Using polyclonal antibodies to the C-terminal fragment of p65, the protein was detected in BYV-infected plants and found in the subcellular fractions of cytoplasm, membranes and cell walls. A preliminary report on the ATPase activity of BYV p65 has been published (Nikiforova *et al.*, 1995).

Methods

■ **Expression plasmids.** The BYV p65 gene sequence from p65Nco-pGEM plasmid (Fig. 1; Nikiforova *et al.*, 1995) was cloned into pQE vectors (Diagen) under the control of a bacteriophage T5 promoter and a ribosome-binding site. To obtain pQE-N6H-p65, the complete 1·8 kb insert was excised with *Bam*HI and *Sal*I and ligated into the similarly cleaved pQE-9. The inserts for pQE-p65-C6H and pQE-N6H-52K were obtained by PCR on a p65Nco-pGEM template with positive-sense primer oligo65(+) (5′ dCGGATCCCCTCGTGCCATGGTTG 3′) and either of two negative-sense primers, oligo65(-)Bgl or oligo65 (-)Sal (5′ dGC<u>AGATCT</u>GACATCAAGTTTTTCCACTC 3′ or 5′ dGA<u>GTCGAC</u>GTTCGACGCTCGAAACG 3′, *Bgl*II and *Sal*I sites underlined). The 1·8 and 1·5 kb amplification products were cloned between the *Nco*I and *Bgl*II sites of pQE-60 or the *Bam*HI and *Sal*I sites of pQE-9, to yield pQE-p65-C6H and pQE-N6H-52K, respectively. Plasmids pQE-N6H-40K and pQE-N6H-35K were constructed by cloning the 1·1 kb *Bam*HI-*Xho*I and 0·95 kb *Bam*HI-*Sca*I fragments of pQE-N6H-p65, respectively, between the *Bam*HI and *Sal*I or *Bam*HI and *Sma*I sites of pQE-30. Plasmid pQE-N6H-13K contained the 0·35 kb *Sau*3a-*Sal*I fragment of p65Nco-pGEM cloned between the *Bam*HI and *Sal*I sites of pQE-11. The plasmids were used to transform *Escherichia coli* strains M15 or SG13009 (Diagen) containing the repressor pREP4 plasmid. Recombinant inserts were selected by restriction of the plasmid DNA isolated from progeny clones.

■ **Expression and purification of p65 and its truncated derivatives with His$_6$ tags.** The procedure was essentially as described by Hochuli *et al.* (1988). Briefly, transformed cells harbouring over-expressed proteins with a tag of six histidines (His$_6$) were harvested 2 h after IPTG-induction and lysed in buffer A (6 M guanidine.HCl, 0·1 M NaH$_2$PO$_4$, 0·01 M Tris–HCl, pH 8·0). The clear lysate was applied to an Ni–NTA agarose column (Diagen) and eluted with buffer A followed by buffers B, C, D and E (8 M urea, 0·1 M NaH$_2$PO$_4$, 0·01 M Tris–HCl, pH 8·0, 6·4, 5·9 and 4·5, respectively). The pH 5·9 and 4·5 eluates containing pure recombinant protein were diluted 10-fold with a high-salt buffer (1 M NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol, 0·05% Tween 20, 50 mM HEPES–KOH pH 7·0) containing 4 M urea, and subjected to gradient dialysis against the same buffer without urea (Agranovsky *et al.*, 1994). Finally, the protein was dialysed against buffer G (0·2 M NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol, 0·05% Tween 20, 50 mM HEPES–KOH pH 7·0) and concentrated by incubating the dialysis bag on dry Sephadex G-250 (Pharmacia).

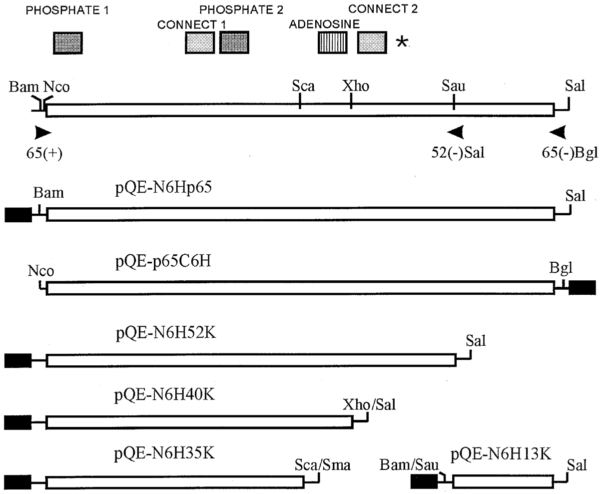

Fig. 1. Schematic representation of the expression cDNA clones used in this study. The restriction map of the BYV p65 gene cloned into p65Nco-pGEM, with encoded amino acid sequence motifs common in HSP70 ATPases (Bork *et al.*, 1992) is indicated above. The asterisk marks the position of the C terminus of the HSC70 44 kDa fragment retaining the ATPase activity (Chappell *et al.*, 1987). The arrowheads denote the positions of PCR primers. Restriction sites used for cloning manipulations are Bam, *Bam*HI; Bgl, *Bgl*II; Nco, *Nco*I; Sal, *Sal*I; Sau, *Sau*3a; Sca, *Sca*I; Xho, *Xho*I. BYV p65 expression inserts are shown as open boxes, and the vector sequence coding for His$_6$ tags as black boxes. Drawn approximately to scale.

For native p65 purification, the bacterial cell pellet was resuspended in sonication buffer (50 mM NaH$_2$PO$_4$ pH 7·0, 300 mM NaCl; 3 vols/g wet wt), followed by several cycles of sonication on ice (30 s burst at 200 W with 1 min cooling). The lysate was clarified by centrifugation at 14000 ***g*** for 20 min, mixed with Ni–NTA agarose, and gently stirred on ice for 1 h. The slurry was applied onto a column, and the resin was washed with sonication buffer and then with wash buffer (50 mM NaH$_2$PO$_4$, pH 6·0, 300 mM NaCl). Adsorbed proteins were eluted with a gradient of 20–250 mM imidazole in the wash buffer and peak fractions were dialysed and concentrated as described above for refolded proteins.

Soluble His$_6$-containing p22 and p24 BYV CPs were expressed and purified as described earlier (Agranovsky *et al.*, 1994). Preparations of the recombinant proteins were analysed by electrophoresis in SDS–polyacrylamide gels (Laemmli, 1970). The mid-range and high-range protein molecular mass markers (Promega) were used as standards. Protein concentration was determined using a BSA protein assay kit (Pierce).

■ **Production of antiserum and detection of the BYV p65 in virus-infected plants.** Polyclonal antiserum against a recombinant 13 kDa fragment (13K) of BYV p65 (As-p65C) was raised in rabbits by three intramuscular injections made at 2 week intervals. The mixture for each injection consisted of 0·3 mg soluble 13K in 1 ml PBS (10 mM NaH$_2$PO$_4$ buffer, pH 7·0, 0·85% NaCl) emulsified with 1 ml of Freunds' complete adjuvant. Blood was collected 14, 28, 42, 56 and 70 days after the last injection.

One gram of healthy or BYV-infected (3–4 weeks post-inoculation) *Tetragonia expansa* leaf tissue was ground in a mortar with 1·5 ml of ice-cold extraction buffer (100 mM Tris–HCl, pH 8·0, 11 mM KCl, 5 mM MgCl$_2$, 400 mM sucrose, 10% glycerol, 10 mM β-mercaptoethanol). Non-fractionated extracts were mixed with an equal volume of electrophoresis sample buffer (75 mM Tris–HCl, pH 6·1, 4·5% SDS, 9 M urea, 7·5% β-mercaptoethanol) and boiled for 5 min, followed by centrifugation at 14000 ***g*** for 10 min. Subcellular fractions of BYV-infected and healthy plants were prepared as described previously (Agranovsky *et al.*, 1994; Godefroy-Colburn *et al.*, 1986). Ten μl of each sample was applied onto a 15% polyacrylamide denaturing gel, and the proteins were separated by electrophoresis. For Western blotting, proteins were transferred from the gel onto nitrocellulose membranes, and the blots were treated with As-p65C diluted 1:500, followed by goat anti-rabbit IgG–alkaline phosphatase conjugate (Sigma). Detection was by treatment with naphthol as-mx phosphate and 5-chloro-2-toluene-diazonium chloride (Sigma).

■ ***In vitro* transcription and translation.** The *Nco*I–*Pst*I fragment from pGEM-p65 was cloned between the same restriction sites of pK3, an expression vector produced by inserting the *αβ* translational enhancer of potato virus X (Smirnyagina *et al*., 1991) and the *Nco*I site into pTZ18R (Pharmacia; O. Fedorkin & A. Solovyev, unpublished). The resulting pK3-p65 construct contained, under the control of a bacteriophage T7 promoter, the complete sequence of the BYV p65 gene in a context favourable for translation in eukaryotic cell-free systems. *Pst*I-linearized pK3-p65 was transcribed with T7 RNA polymerase (Promega). The uncapped transcripts were translated in rabbit reticulocyte lysates as described earlier (Boyko *et al*., 1992).

■ **ATPase assay.** ATPase activity in protein samples was assayed in 20 µl mixtures containing buffer G, 0·5 µg purified protein, 0·2 µM ATP, and 0·1 µCi $[\gamma\text{-}^{32}P]$ATP. Samples were supplemented with 5 mM Mg^{2+} and peptides (1 mM final concentration) as specified. Linear peptides NRLWLTG, NLLRLTG, NDLLLTG and SNGSLQCRIC were kindly provided by A. Gragerov (Institute of Cancer Research, Columbia University, New York, USA) and E. Maiss (Institute of Plant Pathology and Plant Protection, University of Hanover, Hanover, Germany), and the cyclic peptide, bacitracin, was from Serva. After incubation at 37 °C for 60 min, 1 µl was applied onto a cellulose thin-layer chromatography plate (Kodak), allowed to dry, and run in 1 M ammonium acetate, 70% ethanol. Samples of $[\gamma\text{-}^{32}P]$ATP incubated in buffer G with or without apyrase (Sigma) served as the ATP and Pi mobility markers, respectively. The spots were developed by autoradiography, excised and counted in a toluene scintillation cocktail to determine the percentage hydrolysis of ATP. The ATP chemical decay background (estimated at 0·2% for the sample of $[\gamma\text{-}^{32}P]$ATP incubated without any protein added) was subtracted from each value.

■ **Protein binding assay.** Ovalbumin (5 mg/ml solution in 100 mM Na_2CO_3) was heated at 100 °C for 5 min, NaCl was added to 500 mM and the pH adjusted to pH 9·0 with 1 M HCl. Denatured protein was linked to BrCN–agarose 4B (Pharmacia) as per the manufacturer's protocol. The sorbent (0·3 ml equilibrated in 100 mM KCl, 1 mM $MgCl_2$, 10 mM Tris–HCl, pH 7·6) was incubated for 30 min at room temperature with 10 µg of purified BYV p65, or with nonfractionated rabbit reticulocyte lysate containing the $[^{35}S]$methionine-labelled p65 resulting from *in vitro* translation of T7 transcripts of pK3-p65. Ovalbumin–Sepharose was then transferred to a column, washed with 10 column vols of equilibration buffer and eluted with 1 mM ATP. Protein, in 10 µl aliquots taken from 0·3 ml column fractions, was analysed on denaturing protein gels and developed by immunoblotting with As-p65C (for purified p65), or by Coomassie staining and autoradiography (for $[^{35}S]$methionine-labelled protein). A preparation of monoclonal antibodies (MAbs) to mammalian 70 and 72 kDa heat shock proteins (kindly made available by W. J. Welch, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, USA) was used to detect endogenous rabbit reticulocyte HSP70s on sister immunoblots (Milarski *et al*., 1989).

Results

Bacterial overexpression and purification of BYV p65 and its truncated forms

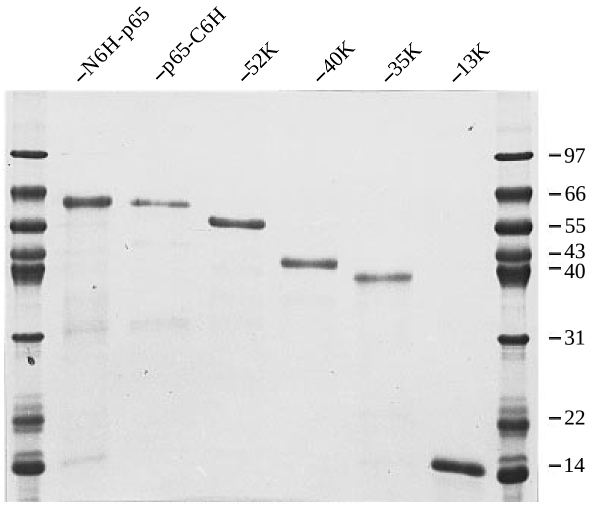

Fig. 2. SDS–PAGE separation (8–20% polyacrylamide gradient gel) of the recombinant proteins expressed in *E. coli* strains M15 and SG13009 and purified by affinity chromatography on Ni–NTA agarose. N6H-p65 and p65-C6H are full-sized BYV p65 having N- or C-terminal His$_6$ tags (isolated under native conditions); 52K, 40K and 35K are N-terminal fragments of p65, and 13K is a C-terminal fragment of p65 (isolated under denaturing conditions). Lanes to the left and the right contain molecular mass standards (kDa; mid-range protein markers, Promega).

We produced full-sized, recombinant BYV p65 as two variants: N6H-p65 and p65-C6H, having an N- or C-terminal His$_6$ tag, respectively. p65 fragments representing the N-terminal 317, 363 and 482 amino acids (35K, 40K and 52K, respectively), or the C-terminal 116 amino acids (13K), had N-terminal His$_6$tags. The proteins were expressed in IPTG-induced *E. coli* cells bearing plasmids pQE-p65-C6H, pQE-N6H-p65, pQE-N6H-52K, pQE-N6H-40K, pQE-N6H-35K and pQE-N6H-13K (Fig. 1). Expression levels of both versions of the full-sized p65 were much lower than any of its fragments (ca. 2% compared with 20–35% of total bacterial protein, respectively). This may reflect, for example, a lower stability of the full-sized protein compared to its fragments. The proteins purified by affinity chromatography on Ni–NTA agarose had apparent molecular masses very close to the expected values (Fig. 2).

Refolding of denatured p65 and fragments thereof presented a problem, as the proteins readily precipitated upon direct dialysis of the 8 M urea eluates against urea-free buffers. The soluble protein preparations were obtained by gradient dialysis against a buffer containing a non-ionic detergent, EDTA, *β*-mercaptoethanol and high salt. Alternatively, soluble p65 was purified from *E. coli* under non-denaturing conditions, using sonication to disrupt the bacterial cells and imidazole-containing buffers for Ni-NTA agarose chromatography. Attempts to produce the 52K, 40K and 35K fragments using this method were unsuccessful, possibly because of their higher expression leading to accumulation of protein aggregates that resisted sonication.

Expression of p65 in infected plants

So far, the 65 kDa proteins of closteroviruses have not been detected *in vivo* and, although their conservation within the

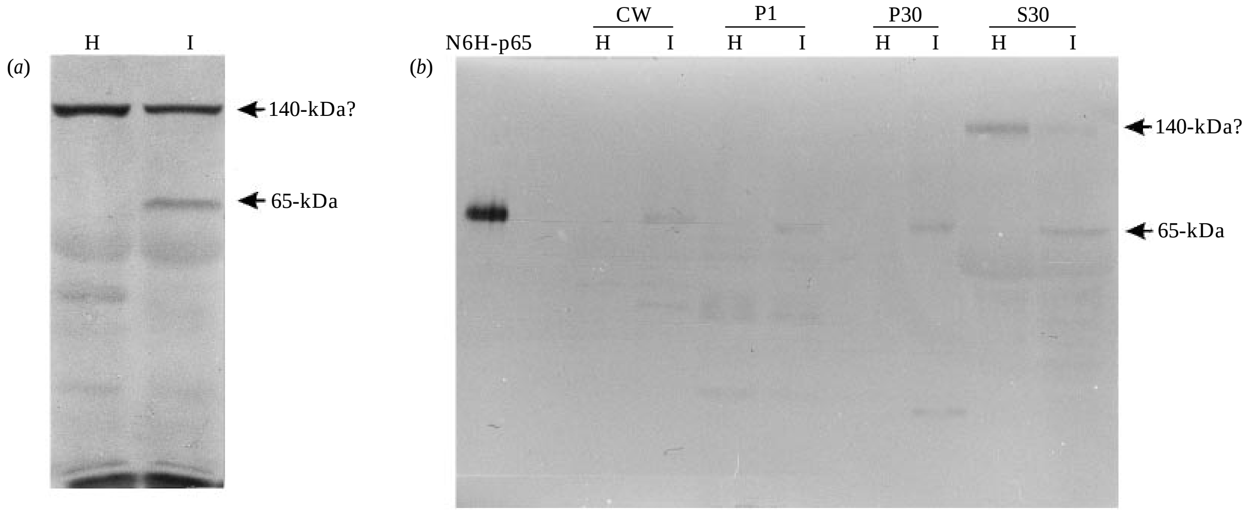

Fig. 3. (*a*) Western blot of the total protein extracted from healthy (lane H) and BYV-infected (lane I) *Tetragonia expansa* plants. Protein extracts were separated on a linear (15% polyacrylamide) SDS gel, transferred onto nitrocellulose, and the blot was developed with As-p65C. Arrows indicate the major bands corresponding to proteins of 65 kDa and 140 kDa, as determined by comparison with the molecular mass standards (high-range protein markers, Promega) in an adjacent lane. (*b*) Western blot of subcellular fractions from BYV-infected (I) and healthy (H) *T. expansa* leaves. Lane N6H-p65, the recombinant BYV p65 (4 ng of the protein isolated in denaturing conditions); lane CW, cell wall debris; lane P1, 1000 ***g*** pellet; lane P30, 30000 ***g*** pellet; lane S30, 30000 ***g*** supernatant.

closterovirus group suggests functionality, it cannot be formally excluded that the respective ORFs are pseudogenes. To detect BYV p65 in infected plants, we produced a polyclonal antiserum specific to its unique C-terminal part. This 13 kDa fragment of p65 was purified from *E. coli* (Fig. 2) and used as antigen for rabbit immunization. The polyclonal antiserum (As-p65C) reacted specifically with the cognate antigen (not shown) and with full-sized recombinant p65 on Western blots (Fig. 3 *b*).

Proteins extracted from healthy and BYV-infected *T. expansa* plants were separated by SDS–PAGE, blotted onto nitrocellulose, and probed with As-p65C. On parallel blots, pre-immune serum or the second antibody alone showed a fairly low background reaction with plant proteins and did not produce bands corresponding to any proteins specific to the virus-infected plant tissue (not shown). The only prominent band detected by As-p65C in the 'infected' lane, but absent from the 'healthy' lane of the unfractionated extracts, corresponded to a protein with an apparent molecular mass of 65 kDa (Fig. 3*a*), thus showing that p65 is indeed expressed upon BYV infection in plants. The absence of cross-reaction of As-p65C with endogenous plant HSP70s (Fig. 3*a*, *b*) indicates that the p65 C-terminal domain shares no common epitopes with these proteins. The 65 kDa band had roughly equal intensity in lanes corresponding to subcellular fractions S30 (cytoplasm), P30 (membranes), P1 (nuclei and large organelles) and CW (cell walls) (Fig. 3*b*).

In addition, a prominent band corresponding to an ~ 140 kDa protein was revealed by As-p65C in both 'infected' and 'healthy' lanes of unfractionated extracts (Fig. 3*a*). Unlike the 65 kDa band, the 140 kDa band was detected only in the cytoplasmic fraction of BYV-infected and healthy *T. expansa* cells (Fig. 3*b*). It could be noted that the 140 kDa band was more prominent in the 'healthy' lanes compared to the 'infected' ones (Fig. 3*a*, *b*). Further experiments will be needed to elucidate whether this band was artefactual, or reflected the presence of an abundant host protein serologically related to the BYV p65.

Purified p65 has ATPase activity associated with its N-terminal domain

ATPase activity in protein samples was determined *in vitro* by the release of inorganic phosphate from [γ-^{32}P]ATP. The data in Fig. 4(*a*) clearly show that the full-sized BYV p65 has a magnesium-stimulated ATPase activity, which is typical of the HSP70 chaperones (Flynn *et al*., 1989). There was an apparent difference in the ATPase activity of the p65 preparations produced by different methods. The activities of N6H-p65 and p65-C6H purified under native conditions were higher than those of the respective refolded proteins (Fig. 4*a*), possibly because of incomplete restoration of the proper p65 conformation after gradient dialysis. Besides the ATPase activity of the 'native' and 'refolded' preparations of the N6H-p65 was lower compared to that of the respective p65-C6H preparations, indicating that the vector-derived sequence placed at the N terminus, instead of at the C terminus, impaired either the folding or the activity of the recombinant protein. In general, the 'native' purification protocol consistently yielded lower amounts of p65, having a higher specific ATPase activity, compared to the 'refolding' protocol.

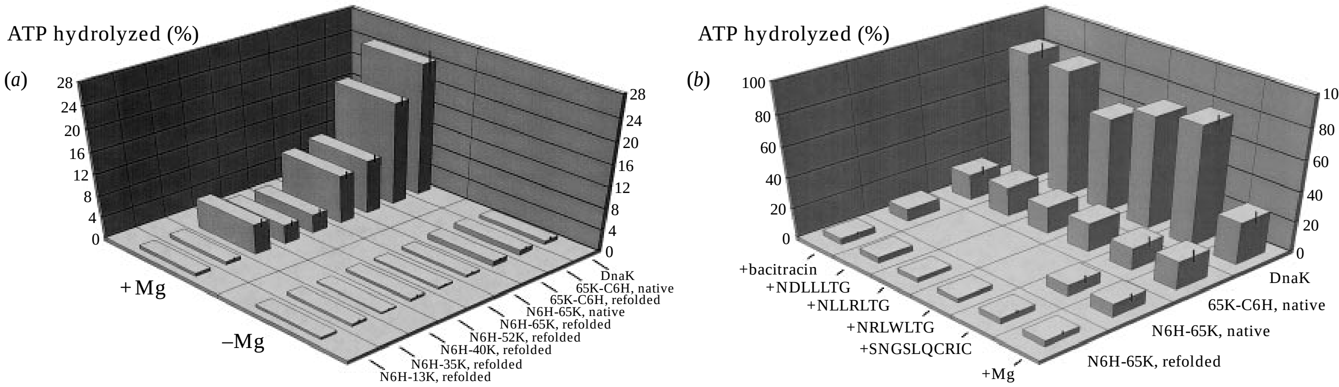

Fig. 4. ATPase activity of BYV p65. Column heights correspond to the percentage of [γ-^{32}P]ATP hydrolysed in the sample (means from at least two independent experiments). Error bars shown are standard deviations whenever data from three independent experiments were available. (*a*) Magnesium-dependent ATPase activity in preparations of p65 and its fragments. DnaK, a bacterial HSP70, was used as a positive control. (*b*) Activation of p65 and DnaK ATPases by peptides. All samples were incubated in the presence of 5 mM $MgCl_2$. Peptides SNGSLQCRIC (Flynn *et al*., 1989), NRLWLTG, NLLRLTG, NDLLLTG (Gragerov & Gottesman, 1994) and bacitracin were added as indicated.

The ATPase activity was retained in the N-terminal 52K and 40K fragments of p65, but not in its N-terminal 35K fragment (Fig. 4*a*), thus confirming the predictions for the ATPase domain in the N-terminal part of the protein (Agranovsky *et al*., 1991). The C-terminal 13K fragment of the p65 and the p22 and p24 BYV capsid proteins did not display the magnesium-dependent ATPase activity (Fig. 4*a* and data not shown). Likewise, no ATPase activity was observed in the following *E. coli* M15-derived preparations obtained under 'native' conditions: (i) histidinylated rat prothymosin alpha; (ii) histidinylated S7 ribosomal protein of *Thermus aquaticus* (kindly provided by A. Vartapetyan and A. Kopylov, Belozersky Institute); and (iii) imidazole eluate from the column loaded with the lysate of cells carrying pQE-30 without the p65 gene insert (data not shown). Taken together, these data rule out the possibility that the observed ATPase activity was due to minor amounts of contaminating bacterial proteins that might be present in the p65 preparations.

p65 does not interact with peptides and denatured protein

The ability of cellular HSP70s to interact, in an ATP-dependent fashion, with peptides and unfolded proteins has been demonstrated for DnaK, a multifunctional chaperone encoded by a single *hsp*70 gene of *E. coli* (Gragerov & Gottesman, 1994; Skowyra *et al*., 1990), and for several products of the eukaryotic *hsp*70 multigene family, including constitutive chaperones HSC70 and BiP (Beckmann *et al*., 1990; Flynn *et al*., 1989). Although HSP70s bind some amino acid sequences better than others (Flynn *et al*., 1989; Gragerov & Gottesman, 1994), these interactions may be considered sequence-nonspecific with respect to (i) a broad range of proteins that individual chaperones can recognize in cells, and (ii) their ability to interact *in vitro* with synthetic peptides chosen at random.

It has been reported that [^{35}S]methionine-labelled BYV p65, expressed in a cell-free system, cosediments with externally added bovine brain microtubules (MTs), and that the amount of pelleted p65 increases after ATP hydrolysis by apyrase (Karasev *et al*., 1992). Association of HSP70 with MTs had been well-documented in biochemical and genetic studies (Green & Liem, 1989; Gupta, 1990), and it was asserted that the closterovirus protein binds to its protein target in much the same way as cellular HSP70s (Karasev *et al*., 1992). However, it remained an open question as to whether p65 is able to interact with a broader range of protein substrates in a chaperone mode.

We compared stimulation of the ATPase activity of the purified BYV p65 and DnaK by synthetic linear peptides NRLWLTG, NLLRLTG, NDLLLTG (efficient substrates for DnaK, BiP and HSC70; Gragerov & Gottesman, 1994) and SNGSLQCRIC (which, among several peptides tested, had been found to stimulate the ATPase activity of BiP most effectively; Flynn *et al*., 1989). All these peptides clearly stimulated the ATPase of DnaK as a positive control (Fig. 4 *b*). Interestingly, we found that the DnaK ATPase was also stimulated by a cyclic peptide (Fig. 4 *b*), thus suggesting that an extended conformation of a protein chain may not be necessary for chaperone binding, at least *in vitro*. In contrast to the DnaK ATPase, the activity of p65 did not depend on the presence of any peptide tested (Fig. 4 *b*).

To investigate further whether BYV p65 is able to bind unfolded protein chains, we used chromatography on denatured protein–Sepharose, a sorbent that selectively retains HSP60 and HSP70 (Sherman & Goldberg, 1992; O. Denisenko, unpublished data). In our hands, chromatography of rabbit reticulocyte lysates (programmed by the T7 transcripts of expression clone pK3-p65) on ovalbumin–Sepharose was quite specific, as it allowed retention and subsequent elution with 1 mM ATP of an endogenous 70 kDa protein(s) identified as

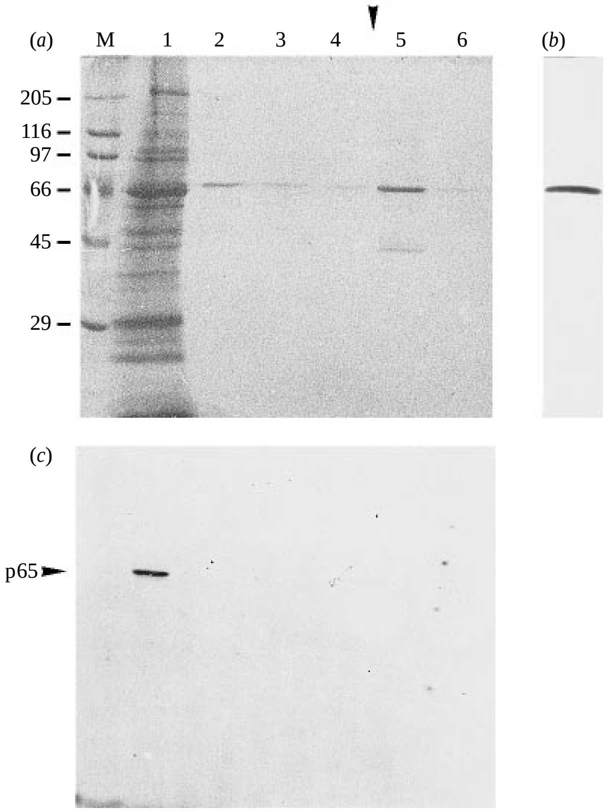

Fig. 5. Protein binding assay. Rabbit reticulocyte lysates programmed with a T7 transcript of the expression clone pK3-p65 were fractionated on a denatured ovalbumin–Sepharose column, and the fractions were analysed by SDS–PAGE (8–20% polyacrylamide gradient). (*a*) Coomassie staining of the gel loaded with specified column fractions; arrow indicates the onset of elution with 1 mM ATP. Lane M, molecular mass standards (kDa). (*b*) Western blot of lane 5 from a parallel gel developed with MAbs to mammalian HSP70/72. (*c*) Detection of the [^{35}S]methionine-labelled p65 by autoradiography of the dried gel shown in Fig. 3 (*a*).

an HSP70 with MAbs to mammalian HSP70/72 (Fig. 5*a*, *b*). The [^{35}S]methionine-labelled p65, synthesized in and cochromatographed with the same lysate sample, failed to bind under these conditions (Fig. 5*c*). The purified recombinant N6H-p65 was also found to be incapable of binding to denatured ovalbumin (data not shown).

Discussion

The p65 homologue of HSP70s encoded by the RNA genomes of closteroviruses consists of an N-terminal domain whose sequence and structural fold appear to be very similar to that of the cell chaperones, and a C-terminal part which is clearly distinct from the HSP70 peptide-binding domain (Agranovsky *et al.*, 1991; Bork *et al.*, 1992; Klaassen *et al.*, 1995; Rippmann *et al.*, 1991; F. Rippmann, personal communication). This implies that the p65 gene, since its capture from the host genome, has undergone uneven evolutionary pressure, or that its N- and C-terminal domains have different origins. In accord with this, we show here that the purified BYV p65 possesses the *in vitro* magnesium-dependent ATPase activity associated with its N-terminal portion, but is unable to interact with unfolded protein chains in an ATP-dependent manner, thus suggesting a function different from those of classical 'polypeptide chain binding proteins' (Rothman, 1989). Despite the apparent inability of p65 to bind peptides, it is still possible that its interactions with a target (e.g. microtubules) might be driven by a conformational shift transmitted from the N-terminal ATPase 'muscle' to the substrate-bound C-terminal domain, in a mode postulated for orthodox chaperones (Flaherty *et al.*, 1990). Conservation of a glutamic residue in the closterovirus p65 proteins (Glu-181 in BYV p65) circumstantially supports this assumption. As demonstrated for DnaK, and proposed for other family members, an equivalent glutamic residue in HSP70s (Glu-171 in DnaK or Glu-175 in HSC70) is required for coupling ATPase activity with substrate binding and release, which is crucial for chaperone action (Buchberger *et al.*, 1994).

In earlier work, Chappell *et al.* (1987) have shown that the ATPase activity of an HSP70 is associated with its N-terminal 44 kDa fragment obtained by chymotrypsin digestion. We supplemented their analysis by producing several 3′-terminal deletions in the p65 gene cloned into an expression plasmid. The N-terminal 40 kDa fragment of p65 was still capable of hydrolysing ATP, thus indicating that a part of the conserved interface in the ATPase structure (CONNECT 2; Fig. 1) is not essential for the enzymatic activity. The ATPase activity dropped when a deletion (in the 35 kDa fragment) intruded further into the conservative domains.

In this work we show for the first time that a closterovirus p65 is expressed upon virus infection *in vivo*. The observed distribution of p65 among the subcellular fractions of cytoplasm, membranes, nuclei and cell walls of BYV-infected *T. expansa* plants (Fig. 3*b*) provides no firm clue to the function of the protein, specifically in view of its suggested involvement in cell-to-cell and long-distance transport of virus infection (Agranovsky *et al.*, 1991; Dolja *et al.*, 1994). The movement proteins of tobamo-, ilar-, diantho- and trichoviruses, whose subcellular distribution has been assessed by crude fractionation, have been found predominantly in the membrane and cell wall fractions (Deom *et al.*, 1990; Godefroy-Colburn *et al.*, 1986; Osman & Buck, 1991; Sato *et al.*, 1995). On the other hand, a significant portion of the triple gene block-encoded hordeivirus 58K and potexvirus 26K movement proteins is in the cytoplasmic fraction (Donald *et al.*, 1993; Rouleau *et al.*, 1994).

What substrate(s), then, might p65 interact with in the cell? It has been demonstrated that BYV p65 synthesized *in vitro* cosediments with externally added bovine brain MTs in a mode similar to that of structural microtubule-associated proteins (Karasev *et al.*, 1992). This might indicate that the postulated involvement of p65 in cell-to-cell movement of virus infection would operate *via* specific interactions with the plant cell cytoskeleton and translocation machinery (Dolja *et al.*, 1994; Karasev *et al.*, 1992). It is noteworthy that the 58K and 26K movement proteins of hordeiviruses and potexviruses encompass the NTPase domain conserved in putative DNA and RNA helicases (Gorbalenya *et al.*, 1988) and do possess magnesium-stimulated ATPase activity *in vitro* (Rouleau *et al.*, 1994; A. O. Jackson, personal communication). Although these proteins are not related to the closterovirus p65 ATPase, the remarkable similarity of their enzymatic activities might indicate a common energy-dependent function.

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