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Functional Analyses and Identification of Two Arginine Residues Essential to the ATP-Utilizing Activity of the Triple Gene Block Protein 1 of Bamboo Mosaic Potexvirus

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The TGBp1 of bamboo mosaic potexvirus (BaMV) is encoded by the first overlapping gene of the triple-gene-block (TGB), whose products are thought to play roles in virus movement between plant cells. This protein forms cytoplasmic inclusions associated with virus particles in the BaMV-infected tissues. It has been proposed that the inclusion is one of the active forms of TGBp1. To prove this idea, we purified the TGBp1 inclusions from both the BaMV-infected *Chenopodium quinoa* and *Escherichia coli* cells overexpressing this protein to test some of their biochemical activities. We found that the TGBp1 inclusions isolated from the infected plant leaves, but not from *E. coli,* possess the NTP-binding and NTPase activities. However, they lack the RNA-binding activity possessed by the soluble TGBp1. These results indicate that the TGBp1 proteins in the BaMV-infected tissues assume two different functional forms. Mutational analyses and competition experiments show that the two arginine residues, Arg-16 and Arg-21, essential to RNA binding, are also required for the ATP-utilizing activity of the soluble TGBp1. This indicates that a same-structure motif is required for the two functions of the soluble TGBp1. The location of the two arginine residues outside the seven conserved motifs of the NTP-utilizing superfamily I RNA helicases, to which TGBp1 belongs, suggests that an extra-structure motif, besides the seven conserved ones, is required for the NTP-utilizing activity of the TGBp1 protein of BaMV.

INTRODUCTION

It has been firmly established that the transport of plant viruses from cell to cell and over long distances requires the assistance of virus-encoded movement proteins (MPs) (Bleykasten *et al.,* 1996; Donald *et al.,* 1997; Lee *et al.,* 1994; Leisner *et al.,* 1993; Lucas and Gilbertson, 1994; Lucas, 1995; Meshi *et al.,* 1989; Schaad and Carrington, 1996; Simon *et al.,* 1992; Weber *et al.,* 1993). Two mechanisms for intercellular transport of plant viruses were previously reported (Carrington *et al.,* 1996). In the TMV-like mechanisms, the viral MPs possess multiple functions. They bind to single-stranded (ss) RNA nonspecifically and shape the viral nucleic acids into a transferable form (Citovsky *et al.,* 1990, 1992; Osman *et al.,* 1992; Schoumacher *et al.,* 1992; Giesman-Cookmeyer and Lommel, 1993; Li and Palukaitis, 1996). Besides, they interact with cytoskeleton upon which the viral movement complexes are transported to plasmodesmata (Carrington *et al.,* 1996; Heinlein *et al.,* 1995; McLean *et al.,* 1995) and increase the size-exclusion limit of plasmodesmata (Wolf *et al.,* 1989). In the tubule-based mechanisms, the viral MPs are involved in the formation of tubules through cell walls and/or plasmodesmata (Perbal *et al.,* 1993; Storms *et al.,* 1995; van Lent *et al.,* 1990; Wieczorek and Sanfacon, 1993). It is speculated that virions assembled in the cytoplasm are escorted to the tubular structure through the interaction with MP, and then transported through tubules via specific MP–capsid protein complexes (Wellink and van Kammen, 1989).

The three proteins encoded by the triple-gene-block (TGB) of certain viruses are also required for systemic spread of virus through plants (Angel *et al.,* 1996; Beck *et al.,* 1991; Bleykasten *et al.,* 1996; Donald *et al.,* 1997; Gilmer *et al.,* 1992; Herzog *et al.,* 1998; Petty *et al.,* 1990). However, they seem to be structurally and functionally different from the movement proteins encoded by viruses using the above-mentioned two transport mechanisms. The TGBp1 proteins encoded by the ORF2 of foxtail mosaic potexvirus (FMV), potato virus X (PVX), and bamboo mosaic potexvirus (BaMV) are associated predominantly with cytoplasmic inclusions (Chang *et al.,* 1997; Davies *et al.,* 1993; Rouleau *et al.,* 1994) and no such proteins are detected in plasmodesmata or in tubular structures. It was previously shown that the soluble form of TGBp1 binds ssRNA cooperatively, has an ATPase activity, and contains highly conserved helicase-like sequence motifs (Donald *et al.,* 1995; Rouleau *et al.,* 1994; Wung *et al.,* 1999). Recently, it was also reported that the RNA-binding activity of TGBp1 may serve to form a ribonucleoprotein complex analogous to that formed by the

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TMV-like MPs (Lough *et al.,* 1998). It remained unclear whether the TGBp1 inclusions have any function or whether they are just a waste of TGBP1. The proximity of virus aggregates to the cytoplasmic inclusions formed by the TGBp1 homologs in the PVX-, FMV-, and BaMV-infected tissues (Chang *et al.,* 1997; Davies *et al.,* 1993; Rouleau *et al.,* 1994) raises the possibility that the inclusions are active by themselves as reported for the insoluble P1 protein of cauliflower mosaic virus (CaMV) (Thomas and Maule, 1995) and the 66-kDa cytoplasmic inclusions of tamarillo mosaic potyvirus (TamMV) (Eagles *et al.,* 1994). Alternatively, they could be the source of TGBp1 from which the active TGBp1 is continuously released as a result of chemical equilibration.

We were able to isolate the TGBp1 inclusions from both the BaMV-infected tissues and *E. coli* cells and analyze their RNA-binding, NTP-binding, and NTPase activities. We found that the cytoplasmic TGBp1 inclusion, like the soluble TGBp1, is also an active form of TGBp1. We also found that the two arginine residues, Arg-16 and Arg-21, are essential to both the RNA-binding and ATP-utilizing activities of the soluble TGBp1, indicating that a same-structure motif is required for both functions of the soluble TGBp1. On the basis of our results and the proposed conserved motifs for the NTP-utilizing superfamily I RNA helicase, we propose that an extra-structure motif, besides the seven known ones, is important for the NTP-utilizing activity of the TGBp1 protein of BaMV.

RESULTS

Multiple functions of the TGBp1 protein

Although the TGBp1 protein of BaMV partitioned mainly in the cell wall and insoluble P30 fractions of the BaMV-infected tissue homogenate, some TGBp1 remained soluble in S30 of the homogenate (Chang *et al.,* 1997). The soluble TGBp1 of BaMV possesses the RNA-binding activity (Wung *et al.,* 1999), although it is not known whether it possesses the ATP-binding and ATPase activities as well. To answer this question, we first purified the TGBp1 inclusions from the BaMV-infected tissues and from the *E. coli* cells which overexpressed TGBp1 through organic solvent extraction, differential centrifugation, and sucrose density gradient centrifugation (see Materials and Methods). The highest purification fold was obtained at the step of sucrose density gradient centrifugation. The distribution of TGBp1 inclusions in the gradient is shown in Fig. 1. The TGBp1 inclusions isolated from the BaMV-infected tissues were fractionated mainly at 60 to 80% of sucrose (Fig. 1A), while those from *E. coli* were mainly at 60 to 70% (Fig. 1B). The homogeneity of TGBp1was above 90% for that isolated from the BaMV-infected tissues and about 60% for that isolated from *E. coli,* as examined by densitometric counting of the band densities of TGBp1 on the SDS–polyacrylamide gel.

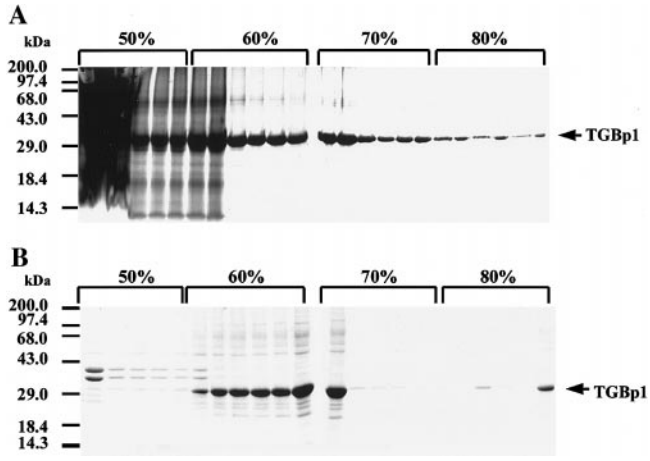

FIG. 1. Homogeneities of the TGBp1 inclusions isolated from the BaMV-infected *C. quinoa* and *E. coli* cells after sucrose density gradient centrifugation. Methods for the preparation of gradient sample are described under Materials and Methods. The 24 fractions collected from the sucrose gradient were electrophoresed through a 12% SDS–polyacrylamide gel. (A) Distribution of the TGBp1 inclusions extracted from *C. quinoa* in the sucrose gradient. (B) Distribution of the TGBp1 inclusions extracted from *E. coli* in the sucrose gradient. The positions at which the protein markers migrate are shown in the left margin. The arrowhead indicates the position at which the TGBp1 protein migrates on the SDS–polyacrylamide gel.

Then, we prepared protein samples containing the soluble TGBp1 by denaturation and refolding of the cytoplasmic inclusions isolated from both the BaMV-infected tissues and *E. coli* cells to test their ATP-binding and ATPase activities (see Materials and Methods). Comparison of the above-mentioned activities between these two samples would allow us to clarify whether there is any difference in TGBp1 of the plant and *E. coli* origins. No significant difference in these two activities was found between the two refolded TGBp1 samples (data not shown). Shown in Fig. 2 are the two activities of the refolded TGBp1 prepared from the BaMV-infected tissues. We found that they were present mostly in the supernatant fraction (Figs. 2A and 2B, all lanes for S) of the refolded TGBp1 sample (all lanes for T), suggesting that the active TGBp1 was mainly in the soluble form. The lack of the two activities in the pellet fraction (all lanes for P) probably resulted from the incorrect folding of TGBp1. Thus, the soluble TGBp1 protein possesses the ATP-utilizing activity besides the capability of RNA binding. Another noteworthy observation is that the soluble TGBp1 is able to bind ATP in the absence of Mg^{+2} (Fig. 2A, lane T*); however, the hydrolysis of ATP by TGBp1 is Mg^{+2}-dependent (Fig. 2B, lanes 5* for T and S).

The RNA-binding activity of TGBp1 resides in the soluble but not inclusion form of TGBp1

Since TGBp1 is mainly present in the form of cytoplasmic inclusions in the plant tissues infected with potexviruses and since virus particles are always observed in

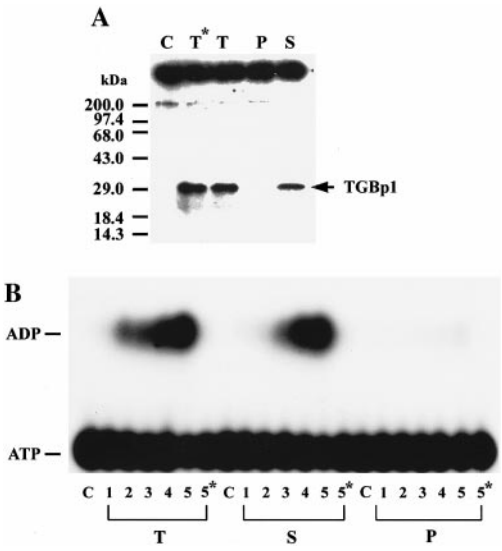

FIG. 2. ATP-binding and ATPase activities of the refolded TGBp1. Methods for preparation of the refolded TGBp1 (T) as well as the soluble (S) and pellet (P) fractions of the refolded TGBp1 are the same as those described under Materials and Methods. (A) ATP-binding activity of the refolded TGBp1. The refolded (66 pmol), soluble (10 pmol), and pellet (56 pmol) TGBp1 were mixed with 0.7 pmol of [α-^{32}P]ATP, respectively, and UV-crosslinked. (B) ATPase activity of the refolded TGBp1. 1, 2, 3, 4, and 5 represent 15, 30, 60, 120, and 240 pmol of the total refolded TGBp1 protein (T), respectively. Each of the soluble fractions (S) contains 15%, while each of the pellet fractions (P) contains 85% of the total refolded TGBp1 protein. Each of the samples was mixed with 0.17 pmol of [α-^{32}P]ATP. The ATPase activity was analyzed as described under Materials and Methods. The positions at which the protein markers migrate are shown in the left margin. The arrowhead indicates the position at which TGBp1 migrates on the SDS–polyacrylamide gel. C, [α-^{32}P]ATP only; *, ATP-binding reaction in the absence of Mg^{+2}.

the neighborhood of the inclusions, we wondered that the inclusions were able to bind RNA. To test this possibility, the TGBp1 inclusions isolated from both the BaMV-infected tissues and *E. coli* cells were tested for their RNA-binding activities. The results are shown in Fig. 3. Different from the refolded TGBp1 samples containing soluble TGBp1 (Fig. 3, lanes 3 and 4), the TGBp1 inclusions isolated from both the BaMV-infected tissues (Fig. 3, lane 1) and *E. coli* cells (Fig. 3, lane 2) were unable to bind RNA since no ^{32}P-labeled TGBp1 protein was detectable at the corresponding position. The additional bands observed with the *E. coli* TGBp1 inclusions (Fig. 3, lane 2) may arise from photochemical crosslinking of the ^{32}P-labeled RNA with certain contaminant proteins in the impure TGBp1 sample (Fig. 1). Thus, it seems that the RNA-binding activity of TGBp1 is restricted to the soluble TGBp1. Protein bands with molecular masses larger than that of TGBp1 and containing the RNA-binding activity were also detected in the refolded TGBp1 samples (Fig. 3, lanes 3 and 4) and *E. coli* inclusions (Fig. 3, lane 2). We suspected that they were multimeric TGBp1 proteins generated during the photochemical crosslinking.

The TGBp1 inclusions isolated from the BaMV-infected *C. quinoa* possess the NTP-binding and NTPase activities

Does the lack of RNA-binding activity of the TGBp1 inclusions mean that they also lack other biochemical activities? To answer this question, we analyzed whether the TGBp1 inclusions contained the NTP-binding and NTPase activities. As shown in Fig. 4A, ATP-, UTP-, CTP-, and GTP-binding activities were detected for the TGBp1 inclusions isolated from the BaMV-infected tissues (Fig. 4, lanes A, U, C, and G for 1). However, no such activity was found for the TGBp1 inclusions isolated from the *E. coli* cells (Fig. 4, lanes A, U, C, and G for 2) and for the proteins isolated in parallel from the healthy plant (Fig. 4, lanes A, U, C, and G for H).

Results for the analyses of the NTPase activity of TGBp1 inclusions are shown in Fig. 4B. Consistent with those found for the NTP-binding activity, the sample of TGBp1 inclusions isolated from the BaMV-infected tissues was able to hydrolyze ATP, UTP, CTP, and GTP (Fig. 4B, lane 1 for A, U, C, and G). The NTPase activity should not come from the trace amount of soluble TGBp1 possibly present in the sample of TGBp1 inclusions since no RNA-binding activity was found in the same TGBp1 sample (Fig. 3, lane 1). It is also not possible from a contaminated NTPase because no NTP-hydrolyzing activity was found for the protein sample isolated in parallel from the healthy plant (Fig. 4A, all lanes for H). Significant ATP-hydrolyzing activity was also observed for the TGBp1 inclusion sample isolated from *E. coli* (Fig. 4B, lane 2 for A). This activity was probably obtained by a contaminated ATPase in the partially purified *E. coli* TGBp1 inclusions, since the inclusion sample was rather impure (Fig. 1) and no ATP-binding activity was detected in the sample (Fig. 4A, lane A for 2). However, we still could not rule out the possibility that the *E. coli* TGBp1 inclusions did possess ATPase activity.

The preceding results indicate that the TGBp1 proteins synthesized in the BaMV-infected tissues are able to fold

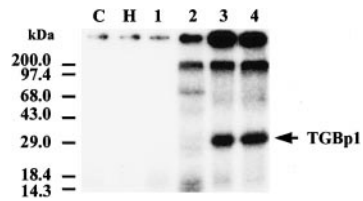

FIG. 3. RNA-binding activity of the TGBp1 inclusions isolated from the BaMV-infected *C. quinoa* and *E. coli* cells. The TGBp1 protein (4 μg) was incubated with 10 ng of the ^{32}P-labeled RNA transcript (220 bases) of the 3′ noncoding region of BaMV, and photochemically crosslinked as described previously (Wung *et al.,* 1999). C, RNA only; H, protein sample prepared in parallel from the healthy *C. quinoa*; 1, the TGBp1 inclusions isolated from the BaMV-infected *C. quinoa*; 2, the TGBp1 inclusions isolated from *E. coli* BL21(DE3)/pJP1, which overexpresses TGBp1; 3, the refolded TGBp1 prepared from the BaMV-infected tissues; 4, the refolded TGBp1 prepared from *E. coli* cells.

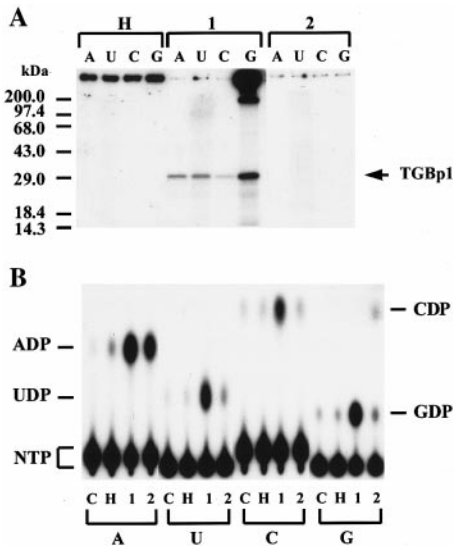

FIG. 4. NTP-binding and NTPase activities of the TGBp1 inclusions isolated from the BaMV-infected *C. quinoa* and *E. coli* cells. (A) NTP-binding activity of the TGBp1 inclusions. (B) NTPase activity of the TGBp1 inclusions. Methods for the assays of the NTP-binding and NTPase activities of TGBp1 are described under Materials and Methods. The positions at which the protein markers migrate are indicated in the left margin; the position at which TGBp1 migrates on the SDS–polyacrylamide gel is indicated by an arrow in (A). H, the protein isolated in parallel from the healthy *C. quinoa*. 1 and 2 indicate the TGBp1 inclusions isolated from the BaMV-infected *C. quinoa* and *E. coli,* respectively. A, U, C, and G are ATP, UTP, CTP, and GTP, respectively. Lanes C in (B) indicate NTP only, either ATP, UTP, CTP, or GTP. The positions at which NTP and NDP migrate on the polyethyleneimine plate are indicated in the right and left margins of (B).

and stack into an insoluble but functional form. A similar property was also found for the 66-kDa protein of tamarillo mosaic potyvirus (TamMV); its cytoplasmic inclusions contain the RNA-binding, NTPase, and RNA-helicase activities (Eagles *et al.,* 1994).

Mapping of the ATP-utilizing domain of TGBp1

Seven mutant TGBp1 proteins (M1 to M7) with amino acid deletions spanning from 3 to 24, 25 to 63, 64 to 96, 97 to 128, 129 to 164, 165 to 200, or 201 to 253, respectively, have been constructed to map the RNA-binding domains of TGBp1. With the aid of the seven purified mutant TGBp1 proteins overexpressed in *E. coli,* we found that the N-terminal 3 to 24 amino acids are essential to the RNA-binding activity of TGBp1 (Wung *et al.,* 1999). In the present study, the seven mutant TGBp1 proteins were further adopted to map the domains essential to the ATP-binding and ATPase activities of TGBp1. As shown in Fig. 5, each of the seven mutant TGBp1 proteins had a decreased ATP-binding activity compared with that of the wild-type counterpart, and only the activities for M5 and M6 were within the detection limit; they were about 8 and 12% of that of the wild-type, respectively (Fig. 5A, lanes 5 and 6). The decreased ATP-binding activity of the mutant TGBp1 also reflected on its ATPase activity. Most of the ATP-hydrolyzing activity was lost in the mutant TGBp1 (Fig. 5B). About 19% of the wild-type ATPase activity was possessed by M2 with amino acids 25 to 63 being deleted (Fig. 5B, lane 2), and less than 3% of the wild-type ATPase activity was possessed by the rest of mutant TGBp1 proteins. The destruction of the ATP-binding and ATPase activities by each individual deletion indicates that the structural integrity of TGBp1 is quite important to its ATP-utilizing activity.

Arg-16 and Arg-21 are essential to both the ATP-binding and the ATPase activities of TGBp1

It was previously reported that the mutant TGBp1 protein containing either the Arg-16-Ala or the Arg-21-Ala substitution loses most of the RNA-binding activity of TGBp1 (Wung *et al.,* 1999). In this study, we analyzed whether these two substitutions also affected the ATP-binding and ATPase activities of TGBp1 overexpressed in *E. coli.* We found that the ATP-binding activity of TGBp1 containing Arg-11-Ala was similar to that of the wild-type TGBp1 (Fig. 6A). However, the ATP-binding activity of TGBp1 containing either Arg-16-Ala or Arg-21-Ala was about 18 or 14%, respectively, of that of the wild-type

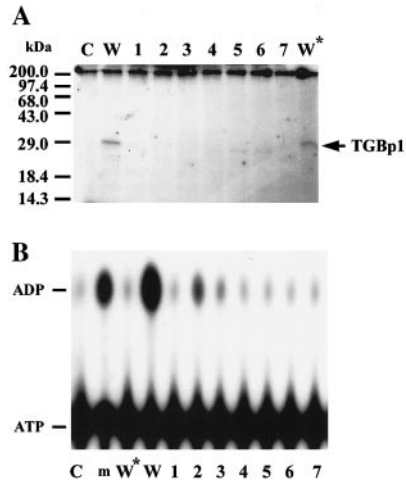

FIG. 5. Mapping of the domains required for ATP-binding and ATPase activities of TGBp1. (A) and (B) indicate the ATP-binding and ATPase activities of the wild-type and deletion mutant TGBp1, respectively. The proteins of the wild-type and mutant TGBp1 are obtained through the gel purification, denaturation, and refolding process as described previously (Wung *et al.,* 1999). Methods for the assay of ATP-binding and ATPase activities of TGBp1 are described under Materials and Methods. For ATP binding, 5 pmol of the gel-purified and refolded TGBp1 was mixed with 0.7 pmol of [α-^{32}P]ATP. For ATPase activity, 20 nmol of TGBp1 was mixed with 0.17 pmol of [α-^{32}P]ATP. C, [α-^{32}P]ATP only; m, ATPase activity obtained with 2 pmol of myosin; W, wild-type TGBp1; W*, ATP-binding and ATPase activities in the absence of Mg^{+2}; 1–7, activities of the seven deletion mutant proteins of TGBp1. The seven mutant TGBp1 proteins possess amino acid deletions spanning from 3 to 24, 25 to 63, 64 to 96, 97 to 128, 129 to 164, 165 to 200, and 201 to 253, respectively (Wung *et al.,* 1999).

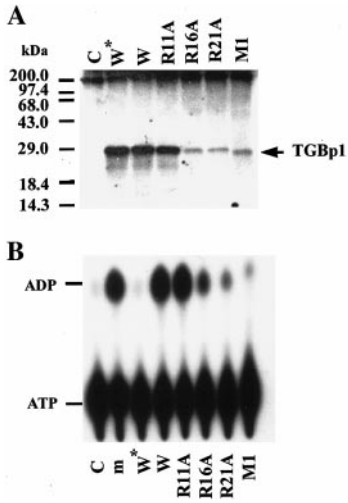

FIG. 6. Identification of amino acid residues involved in the ATP-binding and ATPase activities of TGBp1. (A) and (B) are the ATP-binding and ATPase activities of TGBp1 containing single Arg-to-Ala substitution, respectively. Methods for the assay of ATP-binding and ATPase activities of TGBp1 are described under Material and Methods. For ATP-binding assay, 15 pmol of TGBp1 was mixed with 0.7 pmol of [α-^{32}P]ATP; for ATPase activity assay, 50 pmol of TGBp1 was mixed with 0.17 mol [α-^{32}P]ATP. C, [α-^{32}P]ATP only; W, wild-type TGBp1; W*, wild-type TGBp1 in the absence of Mg^{+2}; m, 2 pmol of myosin; R11A, R16A, and R21A, the three Arg-to-Ala substitutions; M1, the mutant TGBp1 with amino acids 3–24 of the wild-type TGBp1 being deleted.

TGBp1 (Fig. 6A). The reduced ATP-binding activity of the mutant TGBp1 reflected on its ATPase activity. About 28 and 11% of the wild-type ATPase activity were detected for the TGBp1 proteins containing Arg-16-Ala and Arg-21-Ala, respectively (Fig. 6B). These activities were higher than that (less than 3% of the wild-type) of M1, which contains a 21-amino-acid deletion in the N-terminal region of TGBp1 (Fig. 6B). The above-noted results indicate either that Arg-16 and Arg-21 are essential to the ATP-binding and ATPase activities, besides the RNA-binding activity, of the soluble TGBp1 or that each of the two Arg-to-Ala substitutions would alter the overall conformation of TGBp1 and render it inactive to hydrolyze ATP. To clarify this point, competition between the RNA-binding activity and ATP-utilizing activity of TGBp1 was assayed (Fig. 7). Our data showed that the ATP-binding activity of TGBp1 was almost abolished when the molar ratio of RNA to ATP was raised to about 50:1 (Fig. 7A).

A similar trend was observed for the ATPase activity of TGBp1. Only about 20% of the ATPase activity of TGBp1 remained while 50-fold molar excess of competitor RNA was present in the reaction mixture (Fig. 7B). The effect of increasing ATP concentrations on the RNA-binding activity of TGBp1 was also examined; however, the competition effect was observed only when the molar concentration of ATP was at least 10^4-fold in excess of that of RNA (Fig. 7C). The requirement of a high ATP concentration to decrease the RNA-binding activity of TGBp1 may indicate the presence of a low binding constant for the complex of ATP and soluble TGBp1 and/or the existence of multiple TGBp1 proteins on the tested RNA molecule. In any case, our results manifest the existence of a competition between the RNA-binding and ATP-utilizing activities of TGBp1, albeit with a preference for RNA binding. Therefore, we conclude that a same-structure motif encompassing Arg-16 and Arg-21 is important to both the RNA-binding and the ATP-hydrolyzing activities of TGBp1. Since the two arginine residues are not located within the seven conserved motifs of the NTP-utilizing RNA helicases in superfamily I (Fig. 8) (Kadare and Haenni, 1997), we propose that an extra- and essential-structure motif for the NTP-utilizing activity is present in the TGBp1 protein of BaMV.

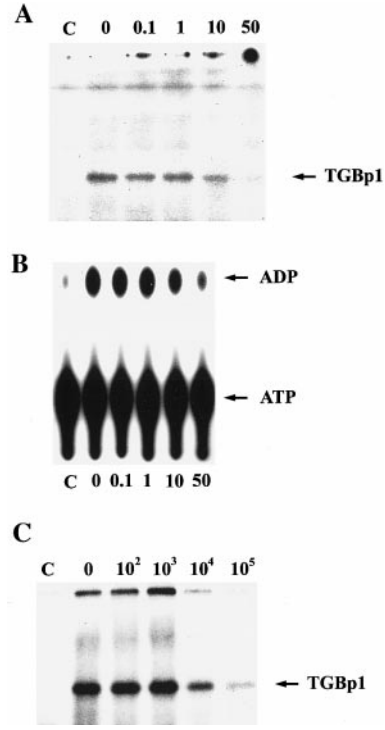

FIG. 7. Competition between the RNA-binding and ATP-utilizing activities of the soluble TGBp1. (A) and (B) Effects of increasing RNA concentrations on the ATP-binding and ATPase activities of the soluble TGBp1, respectively. Methods for the analyses of ATP-binding (A) and ATPase (B) activities of the soluble TGBp1 are the same as those mentioned in Materials and Methods, except that 20 pmol of the soluble TGBp1 was incubated with increasing amounts of unlabeled competitor RNA for 15 min at 4°C before mixing with 0.7 pmol of [α-P^{32}]ATP. The molar ratios of competitor RNA to ATP are shown above (A) and below (B). Lane C, [α-^{32}P]ATP only; lane 0, without the addition of competitor RNA. (C) Effect of increasing ATP concentrations on the RNA-binding activity of the soluble TGBp1. The method to assay the effect of ATP concentration on the RNA-binding activity of TGBp1 is the same as that described for RNA binding (see Materials and Methods), except that 40 pmol of the soluble TGBp1 was incubated with increasing amounts of unlabeled competitor ATP for 15 min at 4°C before mixing with 0.7 pmol of ^{32}P-labeled RNA. The molar ratios of competitor ATP to RNA are indicated above (C). Lane C, ^{32}P-labeled RNA only; lane 0, without the addition of competitor ATP.

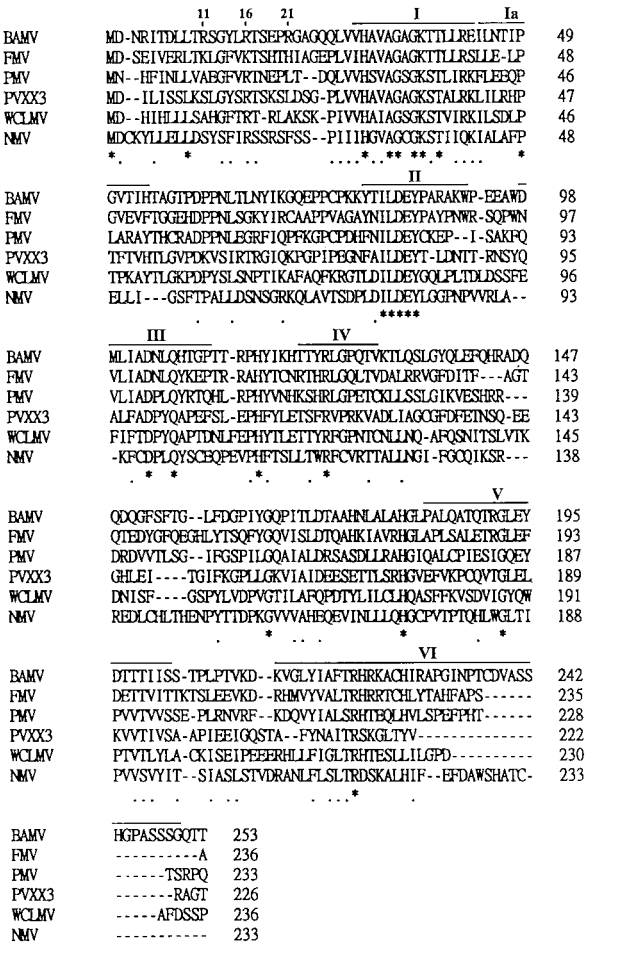

FIG. 8. Alignment of the amino acid sequences of the TGBp1 homologs of potexviruses. The TGBp1 homologs of potexviruses belong to the RNA helicase of superfamily I (Kadare and Haenni, 1997). The seven conserved motifs of RNA helicase are indicated by I, Ia, and II to VI. Motifs I and II are proven to be involved in NTP binding. The three mutated arginine residues, Arg-11, Arg-16, and Arg-21, are indicated above the amino acid sequence of TGBp1 of BaMV. Amino acid residues that are identical among the TGBp1 homologs are indicated by asterisks; those that are homologous are indicated by dots. BaMV, bamboo mosaic virus; FMV, foxtail mosaic virus; PMV, papaya mosaic virus; PVXX3, potato virus X; WCIMV, white clover mosaic virus; NMV, narcissus mosaic virus.

DISCUSSION

We have isolated the TGBp1 inclusions from both BaMV-infected tissues and *E. coli* cells and have analyzed their RNA-binding, NTP-binding, and NTPase activities. Our data reveal that the TGBp1 inclusions formed in the BaMV-infected tissues, but not in *E. coli,* possess the NTP-binding and NTPase activities; however, the soluble TGBp1 contains an extra RNA-binding activity besides the above-mentioned two functions. Our data also indicate that Arg-16 and Arg-21, which are critical to both RNA binding and ATP hydrolysis, are located outside the seven conserved motifs of the superfamily I RNA helicases (Kadare and Haenni, 1997). These results suggest that the structure motifs required for the NTPase activity of a NTP-utilizing RNA helicase in superfamily I are not restricted to the seven known conserved ones. Arginine residues with similar RNA-binding and ATPase functions have not yet been identified in the TGBp1 homologs of other known potexviruses.

Our results show that the refolded and soluble TGBp1 derived from both *E. coli* and plant inclusions are biologically active and behave similarly (Fig. 2). However, only the TGBp1 inclusions isolated from plant are biochemically active (Figs. 3 and 4). It is possible that the inactive *E. coli* TGBp1 inclusions result from the off-pathway aggregation of the overexpressed TGBp1 (Wetzel, 1994). The mechanism leading to the formation of functional TGBp1 inclusions is interesting but unclear. It is probable that either a host factor or a viral component (RNA or protein) is responsible for the assembly of the functional TGBp1 inclusions in plants, although no such evidence has been obtained yet.

The presence of two functional forms of TGBp1 is novel. It has been reported that microinjection of the soluble TGBp1, capsid protein, and viral RNA would enable movement of the infectious transcripts from cell to cell (Lough *et al.,* 1998). However, this could not rule out the possibility that the TGBp1 inclusions still have certain functions such as increasing the efficiency of virus movement. The proximity of virus particles to the TGBp1 inclusions as observed for potexviruses (Chang *et al.,* 1997; Davies *et al.,* 1993; Rouleau *et al.,* 1994) supports this idea. It is probable that the viral RNAs are modified in the vicinity of the TGBp1 inclusions through their NTPase activity. The energy released by NTP hydrolysis may assist unwinding of the secondary structure of viral RNA, since TGBp1 contains the conserved motifs for the NTP-utilizing RNA helicase (Fig. 8). Unfortunately, our efforts on testing this hypothesis were unsuccessful (data not shown). It could be that TGBp1 exhibits the helicase activity only when it is complexed with other viral and/or cellular proteins as suggested for superfamily I RNA helicase (Kadare *et al.,* 1996; Kadare and Haenni, 1997; Rikkone *et al.,* 1994). It is also possible that the inclusions are the active sources of TGBp1, which continuously release soluble TGBp1 through chemical equilibration. The soluble TGBp1 then functions to bind the nearby viral RNA and helps it to transport to the plasmodesmata as proposed for the TMV-like transport mechanism (Carrington *et al.,* 1996; Heinlein *et al.,* 1995; McLean *et al.,* 1995).

It was previously reported that N-terminal deletion of the superfamily I RNA helicase motifs I, Ia, and II would result in the loss of the ATPase activity of the TGBp1 homolog of PVX (Morozov *et al.,* 1999). Our identification of two essential arginine residues outside the seven conserved motifs of the superfamily I RNA helicase is intriguing. As revealed from the X-ray crystallographic data, the conserved motif I is directly involved in binding of the β and γ phosphates of the NTP, while the con-

served motif II serves to chelate the Mg^{+2} of the Mg^{+2}–NTP complex (Kadare *et al.,* 1997). The decrease in the ATPase activity of TGBp1 by replacement of the two positively charged arginines with alanines supports the supposition that the two arginines are involved at certain step(s) for the function of ATPase. It seems that they serve at least as enhancers for NTP binding, since both the two Arg-to-Ala substitutions decrease the ATP-binding activity of the TGBp1 protein of BaMV (Fig. 6A). Thus, binding of ATP to the BaMV TGBp1 in the absence of Mg^{+2} is probably attributed to the existence of Arg-16 and Arg-21. The lack of ATP-utilizing activity in the absence of Mg^{+2} for the TGBp1 homologs of FMV and PVX (Rouleau *et al.,* 1994; Kalinina *et al.,* 1996), which do not contain both the two essential arginines at parallel positions (Fig. 8), may support this idea.

The effect of R11A on the biochemical properties of TGBp1 is different from those of R16A and R21A. The R11A affects only the RNA-binding activity (Wung *et al.,* 1999), while R16A and R21A affect both the RNA-binding and ATP-utilizing activities of TGBp1 (Fig. 6) (Wung *et al.,* 1999). Since each of the three Arg-to-Ala substitutions destroys most of the RNA-binding activity (Wung *et al.,* 1999), we favor the notion that the functioning of the RNA-binding activity requires a certain structure(s) encompassing the three arginine residues of TGBp1, rather than just the particular amino acids. The absence of α-helix or β-structure preceding the conserved motif I of the superfamily I RNA helicase, to which the TGBp1 protein belongs (Chou and Fasman, 1974) (Fig. 8), may indicate that a loop or bulge structure in this region is critical to the RNA-binding activity of TGBp1. The mechanism responsible for the differential effect of R11A on RNA binding and ATP utilization of TGBp1 remains unclear. A possible explanation is that the ATP-utilizing activity, different from that for the RNA-binding activity, requires a proper orientation of only Arg-16 and Arg-21 of TGBp1. The involvement of Arg-16 and Arg-21 in both RNA binding and ATP utilization makes the functional states of the soluble TGBp1 more complicated than expected. It is unlikely that the two arginine residues play the RNA-binding and ATP-utilizing functions simultaneously, because (1) the ATP-utilizing activity of TGBp1 is RNA-independent (Figs. 4, 5, and 6), (2) the RNA-binding activity of TGBp1 does not require ATP (Fig. 3), and, more important, (3) there is a competition between these two activities (Fig. 7). Therefore, the two functions may be carried out by two different sets of soluble TGBp1, with each participating in only one of the specific functions.

MATERIALS AND METHODS

Purification of the TGBp1 inclusions from the BaMV-infected *C. quinoa* and *E. coli* cells

The method for the purification of TGBp1 inclusions is derived from a previous report (Eagles *et al.,* 1994). Basically, the three steps of organic solvent clarification, differential centrifugation, and sucrose density gradient are involved. In detail, 50 g of the BaMV-infected *C. quinoa* was ground first with liquid nitrogen then with 200 ml of buffer A (0.333 M potassium phosphate, pH 7.5, 0.17% [w/v] sodium sulfite, 17% [v/v] chloroform, 17% [v/v] carbon tetrachloride, 0.53 M urea, 1 mM DTT, and 1 mM PMSF), before homogenization with a polytron for 15 min and centrifugation at 1020 *g* for 5 min. The supernatant (S1) thus obtained was further centrifuged at 13,000 *g* for 15 min to collect the pellet (P2). The P2 pellet was subsequently homogenized with 20 ml of buffer B (50 mM potassium phosphate, pH 8.2, 0.1% [v/v] 2-mercaptoethanol, 1 mM DTT, and 1 mM PMSF). The homogenate was centrifuged at 27,000 *g* for 15 min and the pellet (P3) was extracted three times with 0.4× buffer B. The extract thus obtained was centrifuged at 500 *g* for 5 min to harvest the supernatant (S4), which was then spun at 27,000 *g* to get the protein pellet (P5). The P5 pellet was suspended with 1 ml of buffer C (20 mM Tris–HCl, pH 8.2) and the suspension was layered on a 50–80% sucrose step gradient (37.2 ml), centrifuged at 70,000 *g*, and fractionated with a fractionation collector (1.5 ml/tube). After SDS–PAGE, the fractions with high homogeneities of TGBp1 were collected and diluted four times with buffer C. The diluted protein sample was centrifuged at 27,000 *g* for 15 min to pellet the TGBp1 inclusions (F), which were then stored in buffer C at 4°C.

Methods for the purification of TGBp1 inclusions from *E. coli* were similar to those used for the purification of protein inclusions from the BaMV-infected tissues, except the preparation of *E. coli* BL21(DE3)/pJP1 culture overproducing TGBp1 (Chang *et al.,* 1997).

Isolation of TGBp1 from SDS–polyacrylamide gel and refolding of the TGBp1 protein

The TGBp1 inclusions isolated from the BaMV-infected *C. quinoa* or from the *E. coli* cells were run on an SDS–polyacrylamide gel, stained with 0.25 M ice-cold KCl solution, excised from the gel, and electroeluted in TAE buffer (40 mM Tris–HCl, pH 8.6, 2 mM EDTA) with an Isco eluter. The recovered TGBp1 was precipitated with 4 volumes of −20°C cold acetone. The pelleted TGBp1 was washed four times with a solution containing 80% (v/v) acetone and 20% (v/v) buffer A (50 mM Tris–HCl, pH 8.0, 0.1 mM EDTA, 0.15 M NaCl, 1 mM DTT, and 1 mM PMSF) to remove residual SDS as much as possible. The TGBp1 pellet was denatured with buffer L (10 mM Tris–HCl, pH 8.0, 0.2 M NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, and 10% glycerol) supplemented with 6 M urea. Refolding of TGBp1 (66 pmol) was then performed by dialysis against buffer L containing 50 mM NaCl for 1 h using a microdialysis apparatus (BRL/Life Technologies, Gaithersburg, MD). The refolded TGBp1 (T) was separated into the soluble (S) and pellet (P) fractions by

centrifugation at 100 *g* for 1 min. The TGBp1 pellet was suspended with buffer L containing 50 mM NaCl to the same volume of the soluble fraction. This protocol results in pelleting of about 85% of the refolded TGBp1, leaving 15% of the refolded TGBp1 in solution. The TGBp1 protein prepared by this protocol was highly pure (>99% in homogeneity) as examined by densitometric counting.

Preparation of ^{32}P-labeled RNA template and photochemical crosslinking of TGBp1 and RNA

Methods for the preparation of RNA template *in vitro* and for the photochemical crosslinking of TGBp1 and RNA are the same as those reported previously (Wung *et al.,* 1999). The ^{32}P-labeled RNA template is the 3′-end noncoding sequence of BaMV and is 220 nucleotides in length. The radioactivity of the RNA is about 5×10^7 cpm/μg RNA as measured by a scintillation counter.

Assay of the NTP-binding activity of TGBp1

A 2-μCi sample of either [α-^{32}P]ATP, -UTP, -CTP, or -GTP (3000 Ci/mmol; Amersham Life Science, Arlington Heights, IL) was mixed with 1 μg of TGBp1 in a buffer containing 10 mM Tris–HCl, pH 8.0, 50 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, and 10% glycerol. The final volume of the binding mixture was 10 μl. The mixture was spotted on a piece of parafilm and incubated on ice for 15 min before irradiation for 8 min in a Stratalinker (Stratagene, La Jolla, CA) at 8 cm from the light source (0.78 J/cm^2). After UV crosslinking, the sample was boiled for 5 min with an equal volume of sample buffer (0.125 M Tris–HCl, pH 6.8, 4% SDS, 20% glycerol, and 0.002% bromphenol blue) and electrophoresed through a 12.5% SDS–polyacrylamide gel. The gel was stained with Coomassie brilliant blue, dried, and autoradiographed after the electrophoresis. The NTP-binding activity of TGBp1 was determined by densitometric counting of the band density of TGBp1 with [α-^{32}P]NTP crosslinked to it.

Assay of the NTPase activity of TGBp1

TGBp1 (2 μg) was mixed with 0.5 μCi of either [α-^{32}P]ATP, -UTP, -CTP, or -GTP (3000 Ci/mmol; Amersham Life Science) in a buffer containing 20 mM Tris–HCl, pH 8.0; 150 mM NaCl; 5 mM KCl; 2.5 mM $MgCl_2$; 1 mM DTT; 1 mM PMSF; 100 μM ATP, UTP, CTP, or GTP; and 10% glycerol. The mixture (10 μl) was incubated at 37°C for 1 h and the NTP hydrolysis reaction was inhibited by adding EDTA to the mixture at a final concentration of 20 mM. About 0.5 μl of the reaction mixture was then spotted on a polyethyleneimine–cellulose plate (BDH) for an ascending thin-layer chromatography. The developer used for the separation of NTP and NDP was a solution containing 0.5 M LiCl and 0.5 M formic acid. After chromatography, the plate was dried and autoradiographed. The NTPase activity was determined by densitometric counting of the spot of [α-^{32}P]NDP thus detected.

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