VIROLOGY 219, 274–278 (1996)
ARTICLE NO. 0246

w metadata, citation and similar papers at core.ac.uk
brought to you
provided by Elsevier - Publis

Expression of the 69K Movement Protein of Turnip Yellow Mosaic Virus in Insect Cells

KARIN SÉRON,*[,1] LILIA BERNASCONI,† BERNARD ALLET,† and ANNE-LISE HAENNI*

*Institut Jacques Monod, 2, place Jussieu—Tour 43, 75251 Paris Cedex 05, France; and †Glaxo Institute for Molecular Biology S.A., 14, chemin des Aulx—Case Postale 674, 1228 Plan-les-Ouates/Geneva, Switzerland

Received October 23, 1995; accepted February 21, 1996

The nonstructural 69-kilodalton (K) protein of turnip yellow mosaic virus is necessary for systemic spread of the virus within the plant. To examine the behavior of the 69K protein *in vivo,* antibodies were raised against the carboxy-terminal region of this protein. The full-length 69K protein was also expressed in insect cells using a recombinant baculovirus. Studies on the posttranslational modifications of the 69K protein in insect cells revealed that the protein is phosphorylated but not glycosylated. Further experiments of subcellular fractionation and indirect immunolocalization in insect cells showed that the 69K protein is localized in the cytoplasm and/or in the plasma membrane. © 1996 Academic Press, Inc.

Turnip yellow mosaic tymovirus (TYMV) possesses a monopartite single-stranded RNA genome of positive polarity (*1*) which directs the expression of two overlapping nonstructural proteins. The longer 206-kilodalton (K) protein undergoes autocatalytic cleavage (*2*) and is necessary for RNA replication (*3*). The shorter 69K protein is involved in systemic spread of the virus within the plant and possibly also in cell-to-cell movement of TYMV (*4*).

The movement proteins of many different plant viruses have been widely studied (reviewed in *5–7*). They are involved in the process which allows the virus to move from cell to cell via plasmodesmata. Their mechanisms of action are, however, still hypothetical. To date, the best studied movement protein is the 30K protein of tobacco mosaic tobamovirus (TMV). The TMV model proposes that the 30K protein, which is a single-stranded nucleic acid binding protein (*8*), forms a complex with TMV RNA and targets it to plasmodesmata where it can traffic from the infected cell to the neighboring noninfected cell. The 30K protein is localized in the plasmodesmata of infected plants (*9*) and is phosphorylated by a cell wall-associated protein kinase (*10*); this may lead to regulation of the association of the protein with plasmodesmata. The second most studied virus from the point of view of movement is cowpea mosaic comovirus. As opposed to TMV, it moves as intact virus particles within tubules that are produced by its movement protein (*11, 12*).

Computer analyses of the amino acid sequences of a number of plant viruses have led to the identification of conserved motifs within many movement proteins. These motifs possess similarities with the heat shock protein HSP90 (*13*). Surprisingly, the TYMV 69K protein contains none of these motifs and may therefore belong to another family of movement proteins (*14*). It is longer than the other known movement proteins, and because it is very proline-rich (19.4%) it could have a secondary and/or tertiary structure similar to that of proline-rich filamentous proteins such as type I collagen.

To better characterize the 69K protein, it was expressed in a baculovirus system. This approach was adopted to circumvent the fact that the 69K protein is produced in extremely low amounts in TYMV-infected plants and that we were unable to express it in *Escherichia coli.* Polyclonal antibodies were raised against the carboxy-terminal part of the 69K protein. The full-length 69K protein expressed in baculovirus was then characterized and localized in infected insect cells.

A construct corresponding to the 73 carboxy-terminal amino acids of the 69K protein linked downstream of, and in phase with, the glutathione S-transferase (GST) of pGEX-3X (Pharmacia) was produced starting with F34, a TYMV cDNA subclone (*15*), using standard protocols (Fig. 1). The fusion protein GST-C69 was expressed in isopropyl β-D-thiogalactopyranoside-induced *E. coli* DH5α cells and purified by affinity chromatography using glutathione resin. The purified GST-C69 was injected into rabbits (six subcutaneous injections each of 20 μg, at 3-week intervals) and the IgG fraction was purified from the crude serum (*16*). The antibodies specifically immunoprecipitated the 69K protein synthesized in a reticulocyte lysate programmed with TYMV RNA and also detected the 69K

[1] To whom correspondence and reprint requests should be addressed. Fax: (33 1) 44 27 35 80.

0042-6822/96 $18.00
Copyright © 1996 by Academic Press, Inc.
All rights of reproduction in any form reserved.

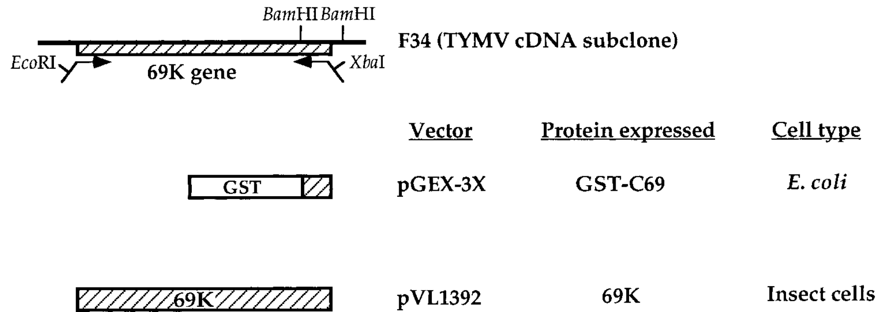

FIG. 1. Schematic representation of the TYMV 69K protein expressed in *E. coli* and insect cells. The open reading frame of the 69K protein (hatched box) appears on the TYMV cDNA (black bar). Bent arrows correspond to primers with appropriate restriction sites used for PCR amplification. The 73 carboxy-terminal amino acids of the 69K protein were expressed as fusion protein GST-C69 with GST (open box) in *E. coli* to produce antibodies. The full-length 69K protein was expressed as recombinant AcNPV in insect (Sf9 or Tn5) cells for posttranslational modification and immunolocalization studies.

protein by Western blots of proteins extracted from TYMV-infected rapeseed leaves (not shown).

Attempts to express the entire 69K protein in *E. coli* were unsuccessful and may have been due to codon usage in bacteria. Indeed, the 69K protein is very proline-rich and the proline is encoded by its four possible codons. However, the CCC codon is seldom used in bacteria (*17*). This codon is abundant (25% of the proline codons) in the 69K protein gene and could possibly lead to stalling or blocking of protein synthesis in *E. coli,* especially when three consecutive CCC codons occur (*18*) as in the nucleotide positions 947 to 955 [amino acids 254 to 256 of the 69K protein sequence, numbering according to (*1*)]. Expression of the C-terminal part of the 69K protein was easily accomplished, possibly because it corresponded to a small part (~12%) of the protein, was less rich in proline (~10%), and did not contain contiguous proline codons. In contrast the codon usage of insect (*Drosophila*) allows the coding of all the proline codons (*17*).

The entire 69K protein was expressed in insect cells using a recombinant *Autographa californica* nuclear polyhedrosis virus (recombinant AcNPV). Recombinant AcNPV was generated by cotransfection of *Spodoptera frugiperda* (Sf9) lepidopteran cells with the pVL1392-69K recombinant clone [obtained from the 69K protein gene amplified by PCR using primers containing *Eco*RI and *Xba*I restriction sites (Fig. 1) introduced into similarly digested pVL1392 (PharMingen, San Diego, CA)] and BaculoGold baculovirus DNA. The virus stock was titrated and used to infect Sf9 or *Trichoplusia ni* (Tn5) cells at a multiplicity of infection of 1. Since the results obtained with either cell type were essentially the same, Sf9 and Tn5 cells were used interchangeably.

In crude extracts the yield of 69K protein expressed in insect cells was low. The protein could not be detected by Coomassie blue staining of SDS–polyacrylamide gels containing protein extracts of infected Sf9 (Fig. 2A, lane 1) or Tn5 cells (not shown), but it was very easily detected 42 hr postinfection by Western blot using the anti-69K serum (Fig. 2B, lane 1), appearing as a discrete set of bands migrating at ~80K. In addition, a set of bands of ~40K was always visible in the total protein extracts and could correspond to degradation products of the 69K protein.

To determine whether the aberrant migration pattern could be due to posttranslational modifications of the 69K protein, the following experiments were performed.

Western blot analyses of the 69K protein expressed from recombinant AcNPV in the presence of tunicamycin (4 μg/ml) produced no detectable shift in migration of this protein (not shown). Consequently, the 69K protein is not glycosylated in insect cells as expected from the analysis of its amino acid sequence, which did not demonstrate the presence of potential glycosylation sites.

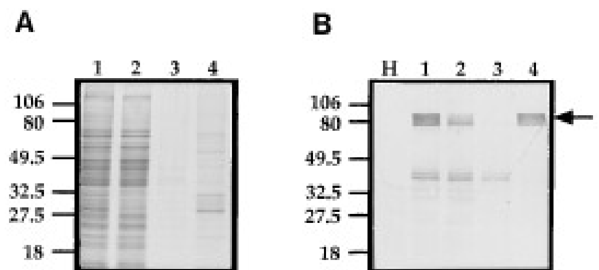

FIG. 2. Fractionation of Sf9 cells infected by the recombinant AcNPV expressing the 69K protein and analysis by 10% SDS–PAGE. Cells (3×10^6) were fractionated into cytoplasmic extract, nuclear extract, and pellet fraction (*19*). The latter contains membranous material and insoluble proteins. Each fraction was resuspended in 3 ml of buffer independent of protein concentration. Aliquots (20 μl) of each fraction were stained with Coomassie blue (A) or treated for Western blot analysis using purified anti-69K serum and a horseradish peroxidase-linked second antibody (B). The chromogenic substrate 3,3′-diaminobenzidine-tetrahydrochloride (Sigma) of horseradish peroxidase was used for detection. Total infected (lane 1) Sf9 cell extracts, cytoplasmic extract (lane 2), nuclear extract (lane 3), and pellet fraction (lane 4). H in (B) refers to total healthy insect cell extracts. The numbers to the left indicate (in K) the position of prestained protein markers (Bio-Rad) loaded onto an adjacent lane of the same gel; the arrowhead to the right indicates the position of the 69K protein.

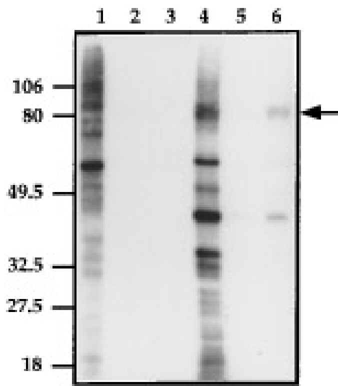

FIG. 3. Phosphorylation of the 69K protein in insect cell extracts. The phosphorylation assay was performed essentially as described (*20*) on 2×10^4 healthy (lanes 1–3) or infected (lanes 4–6) Tn5 cells harvested and resuspended in 10 μl of phosphate-buffered saline (PBS; 138 m*M* NaCl, 8.1 m*M* Na_2HPO_4–$7H_2O$, 1.2 m*M* KH_2PO_4, and 2.7 m*M* KCl, pH 7.4). The reaction (50 μl) was performed for 15 min at 30° in 50 m*M* Tris–HCl, pH 7.5, 10 m*M* $MgCl_2$, 0.1 m*M* ATP, and 1 μCi (37 kBq) of [γ-^{32}P]ATP (167 TBq/mmol; ICN). Samples (30 μl) were immunoprecipitated with either preimmune serum (lanes 2 and 5) or crude anti-69K serum (lanes 3 and 6). The remaining 20 μl, from healthy (lane 1) or infected (lane 4) cells, as well as the immunoprecipitated samples were analyzed by 10% SDS–PAGE and transferred onto a nitrocellulose membrane. The membrane was exposed to an X-ray film with an intensifying screen at −80° and submitted to autoradiography. Other notations are as in Fig. 2.

On the other hand, computer analyses of the amino acid sequence of the 69K protein demonstrated several potential phosphorylation sites. Phosphorylation of the 69K protein was tested using extracts obtained from Tn5 cells infected with the recombinant AcNPV and incubated with [γ-^{32}P]ATP. A band was detected (Fig. 3, lane 4) at about 80K that was specifically immunoprecipitated with the anti-69K serum (lane 6). No band corresponding to the 69K protein was detected in noninfected cell extracts (lane 1) or was immunoprecipitated from healthy cell extracts (lane 3) using the anti-69K serum or from healthy (lane 2) or infected (lane 5) cell extracts using the preimmune serum.

Hence the 69K protein is phosphorylated. This could be either by autophosphorylation or by a protein kinase present in the cell extract. The unusual migration of the 69K protein at the position of 80K could thus be due at least in part to phosphorylation. The faster migrating protein (~40K) which is phosphorylated in infected cell extracts (lane 4) and which is absent in healthy cell extracts (lane 1) could correspond to a degradation product of the 69K protein or to a baculovirus protein since it is detected by the anti-69K (lane 6) and very faintly by the preimmune (lane 5) sera.

The apparent size (~80K) of the 69K protein produced in infected insect cells (Fig. 2) and in TYMV-infected leaves was identical (not shown). It is possible that the same posttranslational modifications such as phosphorylation could occur in both eukaryotic systems. The fact that Western blot analyses revealed a discrete set of bands at ~80K (Fig. 2B, lane 1) could result from different phosphorylation states. The 30K movement protein of TMV was first demonstrated to be phosphorylated when expressed in insect cells (*21*). Later it was shown that in transgenic tobacco plants expressing the 30K protein it is phosphorylated by a cell wall-associated protein kinase (*10*). These two results demonstrate that experiments performed with insect cells can be a good preamble to the study of proteins that are expressed to very low levels in infected plants.

Sf9 cells expressing the 69K protein were fractionated to yield a cytoplasmic extract, a nuclear extract, and a pellet that were analyzed by SDS–polyacrylamide gel electrophoresis and stained with Coomassie blue (Fig. 2A). The vast majority of the cellular proteins was present in the cytoplasmic extract (lane 2). A series of bands corresponding to the 69K protein and detected by Western blot using anti-69K serum (Fig. 2B) was present in the cytoplasm (lane 2) and the pellet (lane 4). Thus, the pellet is very highly enriched in 69K protein. Whereas the 69K protein in the pellet fraction was intact, it was partly degraded in the cytoplasmic fraction. The fact that the 69K protein present in the pellet fraction appears insoluble or tightly associated with membranes may explain why it remains intact. This correlates with the conclusions reached (*4;* our unpublished results) concerning the difficulties encountered in attempting to extract the 69K protein from infected tissues. Strong denaturing conditions such as 2 *M* urea together with 1% SDS and 0.6% β-mercaptoethanol were required to solubilize the protein.

Confocal microscopy and indirect immunofluorescent staining of fixed Sf9 cells infected by recombinant AcNPV expressing the 69K protein were used to more precisely localize the 69K protein. As detected by anti-69K serum (Fig. 4B), the 69K protein was located outside the nucleus, either in the cytoplasm which is completely compressed by the nucleus or more likely in the plasma membrane which would be compatible with the cell fractionation experiments. No staining was detected in healthy cells (Fig. 4A). The cytoplasm in the insects cells is so tenuous that it was very difficult to discriminate between cytoplasm and plasma membrane, even when staining was superimposed to the phase contrast (Fig. 4C).

In the case of squash leaf curl geminivirus a correlation was established between insect and plant cells concerning the subcellular localization of the viral BR1 and BL1 proteins. These proteins have the same subcellular localization in both systems (*19*). BR1 was located in the nucleus of insect cells infected with a recombinant baculovirus expressing this protein and of pumpkin cells infected with squash leaf curl geminivirus, whereas BL1 was localized in the plasma membrane in both cell types

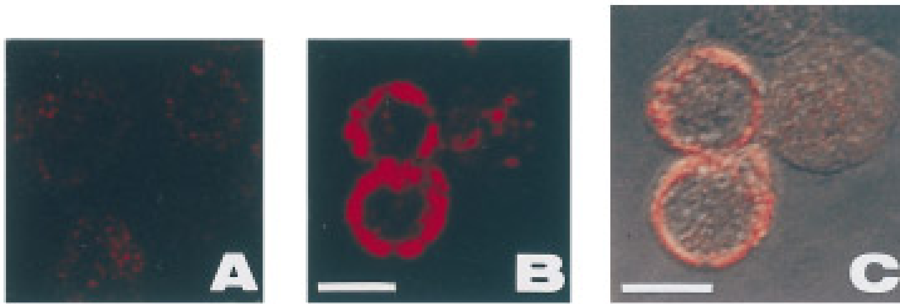

FIG. 4. Indirect immunofluorescent staining of the 69K protein in baculovirus-infected Sf9 cells. Cells (1.5×10^6) were seeded in slideflasks (Nunc) and infected with the recombinant AcNPV stock. After 42 hr, the cells were washed with PBS and fixed in 95% ethanol at −20° for 5 min. Staining was performed as follows: the cells were washed with PBS, incubated for 2 hr at room temperature with purified anti-69K serum diluted 1/250 in PBS containing 2% goat serum, washed with PBS, incubated with Texas red-labeled goat anti-rabbit secondary antibody (Vector laboratories, Burlingame, CA) for 1 hr, washed with PBS, and mounted in Vectashield (Vector Laboratories). The slides were observed with a confocal laser-scan microscope (Zeiss LSM 410 inverted). (A) Healthy Sf9 cells and (B) Sf9 cells infected with recombinant AcNPV expressing the 69K protein. (C) is (B) superimposed to phase contrast to precisely locate the staining. Bars in (B) and (C), 20 μm. The magnification in (A) and (B) is the same.

(*19, 22*). Thus, we presume that the cytoplasm/plasma membrane localization of the TYMV 69K protein in insect cells will correlate with its localization in TYMV-infected plant cells. It is also possible that depending on its phosphorylation state, the 69K protein may occupy different cell compartments. Nevertheless, the exact localization of the protein, whether in the cytoplasm or the plasma membrane, remains unclear.

It was shown recently that TYMV does not need the coat protein to move from cell to cell (*23*). Furthermore, all attempts to detect tubule formation in TYMV-infected cowpea protoplasts or TYMV-infected rapeseed plants were unsuccessful (K. Séron, D. Kasteel, J. van Lent, J. Wellink, unpublished results), which may suggest that TYMV may move from cell to cell as a ribonucleoprotein as does TMV. These observations together with the study of the 69K protein reported here favor the TMV model for two other reasons. First, the 69K protein is located in the cytoplasm and/or the plasma membrane of the insect cells. This localization is consistent with a TMV-like mechanism in which the movement protein must first bind the viral RNA in the cytoplasm and then target it to the plasmodesmata. In insect cells that are devoid of plasmodesmata, the movement protein would remain in the cytoplasm or in the plasma membrane, which in plants forms part of the plasmodesmata. All attempts to locate the protein in sections of infected leaves were unsuccessful. Second, the 69K protein is phosphorylated as is the 30K protein of TMV. Cell-to-cell movement is certainly highly regulated, and the fact that the 69K protein is phosphorylated may result from this regulation. In the case of TMV it was postulated that phosphorylation of the protein regulates the association of the 30K protein with plasmodesmatal proteins or the association of the movement protein with its RNA and subsequent release of the viral material into the neighboring healthy cell.

Despite the lack of sequence homology between the TYMV movement protein and the movement proteins of other viruses known to date, a common mechanism of action may nevertheless prevail. Comparable conclusions have recently been drawn concerning the movement proteins of other viruses (*24, 25*). However, the TYMV 69K protein might still be very different in structure from other movement proteins because of its unusually high proline content. When sufficient amounts of 69K protein will become available, it will be important to determine whether the protein binds nucleic acids as does the TMV 30K protein, and to precisely localize the protein in infected leaves. Essential information on the 69K protein and insight into the mechanism of TYMV cell-to-cell movement have been provided and facilitated by the expression of the 69K protein in insect cells.

ACKNOWLEDGMENTS

The authors are very grateful to J.-M. Camadro for his help with the rabbits and to F. Bernardi and G. Mazzei for constructive discussions. K.S. is the recipient of a fellowship from the "Ministère de la Recherche et de l'Enseignement Supérieur." The Institut Jacques Monod is an "Institut Mixte, CNRS—Université Paris 7."

REFERENCES

1. Morch, M.-D., Boyer, J.-C., and Haenni, A.-L., *Nucleic Acids Res.* **16,** 6157–6173 (1988).

2. Morch, M.-D., Drugeon, G., Szafranski, P., and Haenni, A.-L., *J. Virol.* **63,** 5153–5158 (1989).

3. Weiland, J. J., and Dreher, T. W., *Proc. Natl. Acad. Sci. USA* **90,** 6095–6099 (1993).

4. Bozarth, C. S., Weiland, J. J., and Dreher, T. W., *Virology* **187,** 124–130 (1992).

5. Hull, R., *Semin. Virol.* **2,** 89–95 (1991).

6. Deom, C. M., Lapidot, M., and Beachy, R. N., *Cell* **69,** 221–224 (1992).

7. Maule, A. J., *Trends Microbiol.* **2,** 305–306 (1994).

8. Citovsky, V., Knorr, D., Schuster, G., and Zambryski, P., *Cell* **60,** 637–647 (1990).

9. Tomenius, K., Clapham, D., and Meshi, T., *Virology* **160,** 363–371 (1987).
10. Citovsky, V., McLean, G. B., Zupan, J. R., and Zambryski, P., *Genes Dev.* **7,** 904–910 (1993).
11. van Lent, J., Wellink, J., and Goldbach, R., *J. Gen. Virol.* **71,** 219–223 (1990).
12. Kasteel, D., Wellink, J., Verver, J., van Lent, J., and Goldbach, R., *J. Gen. Virol.* **74,** 1721–1724 (1993).
13. Koonin, E. V., Mushegian, A. R., Ryabov, E. V., and Dolja, V. V., *J. Gen. Virol.* **72,** 2895–2903 (1991).
14. Mushegian, A. R., and Koonin, E. V., *Arch. Virol.* **133,** 239–257 (1993).
15. Boyer, J.-C., Drugeon, G., Séron, K., Morch-Devignes, M.-D., Agnès, F., and Haenni, A.-L., *Res. Virol.* **144,** 339–348 (1993).
16. Harlow, E., and Lane, D., "Antibodies: A Laboratory Manual." Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1988.
17. Zhang, S., Zubay, G., and Goldman, E., *Gene* **105,** 61–72 (1991).
18. Zhang, S., Goldman, E., and Zubay, G., *J. Theor. Biol.* **170,** 339–354 (1994).
19. Pascal, E., Sanderfoot, A. A., Ward, B. M., Medville, R., Turgeon, R., and Lazarowitz, S. G., *Plant Cell* **6,** 995–1006 (1994).
20. Mazzei, G. J., Schmid, E. M., Knowles, J. K. C., Payton, M. A., and Maundrell, K. G., *J. Biol. Chem.* **268,** 7401–7406 (1993).
21. Atkins, D., Roberts, K., Hull, R., Prehaud, C., and Bishop, D. H. L., *J. Gen. Virol.* **72,** 2831–2835 (1991).
22. Pascal, E., Goodlove, P. E., Wu, L. C., and Lazarowitz, S. G., *Plant Cell* **5,** 795–807 (1993).
23. Bransom, K. L., Weiland, J. J., Tsai, C.-H., and Dreher, T. W., *Virology* **206,** 403–412 (1995).
24. Thomas, C. L., and Maule, A. J., *Plant Cell* **7,** 561–572 (1995).
25. Giesman-Cookmeyer, D., Silver, S., Vaewhongs, A. A., Lommel, S. A., and Deom, C. M., *Virology* **213,** 38–45 (1995).