Evidence for a Protein Kinase Activity Associated with Purified Particles of Cauliflower Mosaic Virus

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(Accepted 30 May 1986)

SUMMARY

A cyclic nucleotide-independent protein kinase (PK) activity has been found to be associated with purified particles of cauliflower mosaic virus. The main acceptors of phosphorylation were proteins with mol. wt. of 42000 (the capsid protein), 58000 (which may be the capsid protein precursor) and 110000 (of unknown function). Acid hydrolysis and phosphoamino acid analysis of nucleocapsid proteins phosphorylated in vitro showed that the PK catalyses the transfer of phosphate to both serine and threonine residues. Activation of the PK made the DNA more accessible to DNase I, suggesting that a modification of the structure of the capsid had occurred.

INTRODUCTION

Cauliflower mosaic virus (CaMV) is the type virus of the caulimovirus group. CaMV particles consist of an icosahedral capsid containing a double-stranded circular relaxed DNA.

From the sequence of the DNA (Frank et al., 1980; Gardner et al., 1981; Balazs et al., 1982) six major open reading frames (ORFs) have been deduced. The assignments of four ORFs are now established. ORF II encodes the aphid transmission factor (Howarth et al., 1981; Armour et al., 1983; Woolston et al., 1983; Givord et al., 1984). The protein encoded by gene IV is the coat protein precursor (Frank et al., 1980). ORF V is assigned to the viral reverse transcriptase (Toh et al., 1983; Volovitch et al., 1983; Ziegler et al., 1985; Laquel et al., 1986) and ORF VI encodes the major protein of the cytoplasmic inclusion bodies in which viral particles are stored (Odell & Howell, 1980; Al Ani et al., 1980; Covey & Hull, 1981; Xiong et al., 1982).

Because ORFs I and III remain unaccounted for, we have investigated the possibility that enzyme activities might be associated with CaMV particles. We have previously shown that a DNA polymerase activity co-sediments with purified particles (Ménissier et al., 1984). This enzyme is probably the reverse transcriptase described by Laquel et al. (1986). We have also looked for a protein kinase (PK) activity associated with CaMV particles for two reasons. First, preliminary studies by Hahn & Shepherd (1980, 1982) indicated that the coat protein (mol. wt. 42000; 42K) of CaMV and its precursor (58K) are phosphorylated in vivo. Second, a PK activity has been found associated with many viruses and notably retroviruses and hepatitis B virus, which replicate in a manner very similar to that postulated for CaMV; these three types of virus have been classified under the new group name ‘retroid’ virus (Pfeiffer & Hohn, 1983).

Al Ani et al. (1979b) demonstrated that CaMV coat protein is a single protein that can be both degraded and aggregated. The molecular mechanism of the proteolysis (or maturation) of either the 58K precursor nor the 42K coat protein is known and we suggest in this paper that it is probably related to the phosphorylation of this protein.

This study also presents evidence that a PK is associated with CaMV particles. We have characterized the kinase activity with respect to cyclic nucleotide and cation requirements, exogenous and endogenous protein acceptors and phosphoamino acid targets within the acceptor proteins. Phosphorylation of the 42K protein results in an increased accessibility of the viral DNA to DNase.
METHODS

Virus preparation. CaMV (Cabb-S) was propagated in turnip (*Brassica rapa* L. cv Just Right) and particles were isolated as described by Hull et al. (1976). Virus preparations were further purified by isopycnic centrifugation as described by Al Ani et al. (1979a) and stored at 4 °C.

Standard PK reaction mixture. The standard in vitro PK reaction mixture consisted of 15 μg of purified virus, 20 mM-Tris–HCl pH 7-4, 10 mM-MnCl₂, 0.8 mM-dithiothreitol, 0.25 mM-EDTA and 2 μCi [γ-32P]ATP (3000 Ci/mmol, Amersham) in a final volume of 100 μl. The standard incubation was for 1 h at 37 °C.

Activity was assayed as the amount of radioactivity in aliquots spotted Whatman 3MM filter paper squares that was insoluble in 10% TCA.

Polyacrylamide gel electrophoresis. Proteins were subjected to electrophoresis in 10% SDS–polyacrylamide slab gels using the discontinuous buffer system of Laemmli (1970). Samples (25 μl) of the kinase reaction mixture were mixed with 25 μl 125 mM-Tris–HCl pH 6.8, containing 4% SDS, 4% 2-mercaptoethanol, 30% glycerol, and boiled for 4 min. Electrophoresis was carried out at 30 mA. Gels were stained with Coomassie Brilliant Blue, destained, dried, and radioactive proteins were detected by autoradiography.

Phosphoamino acid analysis. Fifteen μg of CaMV particles phosphorylated in vitro was precipitated from 10% TCA and the pellet was washed three times with cold acetone, dried, dissolved in 6 M-HCl and sealed in a capillary tube. After incubation at 105 °C for 3 h, HCl was evaporated and the pellet was resuspended in 5 μl H₂O containing 1 μg each of O-phospho-L-tyrosine, O-phospho-DL-threonine and O-phospho-L-serine (Sigma). The sample was then spotted on a cellulose layer (DC-Plastikfolien Cellulose, Merck). After two-dimensional ascending chromatography in pyridine/n-butanol/acetic acid/H₂O (100/150/30/120, by vol.), the dried plate was sprayed with ninhydrin and autoradiographed.

DNase susceptibility of the phosphorylated virus. Samples of 5 μg purified virus were incubated in the standard kinase reaction mixture containing 1 mM-ATP instead of [γ-32P]ATP. A control sample was incubated in the same mixture but without ATP. After 1 h at 37 °C, 1 μg DNase I (Boehringer) was added and the samples were kept at 37 °C for 30 min. To detect the effect of DNase I, the DNA was nick-translated by the addition of 0.2 mM each of dATP, dGTP and dTTP and 1 μCi [α-32P]dCTP (3000 Ci/mmol, Amersham) and 1 unit Kornberg DNA polymerase I (Boehringer). The mixture was incubated for 30 min at 37 °C. Proteins were then digested with proteinase K (1 μg/ml) (Sigma) for 2 h at 37 °C in the presence of 1% SDS and 0.5 mM-NaCl and extracted by shaking with 1 vol. phenol saturated with 50 mM-Tris–HCl pH 8. DNA was purified by two successive ethanol precipitations from 1 M-ammonium acetate and 0.2% SDS. DNA samples were analysed on a 1% agarose gel in a buffer containing 36 mM-Tris–HCl pH 7.9, 30 mM-Na₂HPO₄, 1 mM-EDTA. After electrophoresis at 30 V for 16 h, the agarose gel was dried and autoradiographed.

RESULTS

Conditions for kinase activity

When purified CaMV virions were incubated in the kinase reaction mixture containing [γ-32P]ATP, incorporation of 32P into acid-precipitable material increased with time as shown in Fig. 1. When the viral preparation was treated for 5 min at 65 °C before assay, no incorporation was detected. Assays with CaMV isolates Cabb-S, D/H (Baláz et al., 1982) and SA2 (Givord et al., 1984) all yielded similar results.

We tested the kinase activity at pH 7.4 and 37 °C. It required the presence of a divalent cation. Three cations (Mg²⁺, Mn²⁺ and Ca²⁺) were added to the reaction mixture in concentrations ranging from 0-1 to 20 mM. Manganese was the preferred cation; optimum incorporation was obtained at a concentration of 10 mM. Addition of calcium and magnesium instead of manganese resulted in lower levels of 32P incorporation (20% and 56% of the standard reaction value, respectively; see Table 1).

PKs can be broadly categorized by the response elicited when cyclic nucleotides are added to the reaction mixture in vitro. The CaMV-associated PK was cAMP-independent since no enhancement was obtained in the presence of cAMP at concentrations ranging from 1 μM to 1 mM (Table 1).

Pyrophosphate (PPi) is a known inhibitor of phosphatase (Khandelwal, 1977). In our conditions, when 2 μM-sodium pyrophosphate was included in the reaction mixture the incorporation of 32P was dramatically inhibited (Table 1).
Fig. 1. Time course of the protein kinase phosphorylation reaction. Fifteen µg purified CaMV particles was incubated in 100 µl standard reaction mixture in the presence of 2 µCi [γ-32P]ATP (■) and as a control 15 µg of a CaMV preparation which had been heated at 65 °C for 5 min was incubated in the same conditions (●).

Table 1. Influence of reaction conditions on kinase activity

<table>
<thead>
<tr>
<th>Reaction conditions*</th>
<th>% of standard†</th>
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<tbody>
<tr>
<td>Standard</td>
<td>100</td>
</tr>
<tr>
<td>Standard – MnCl₂</td>
<td>2.8</td>
</tr>
<tr>
<td>(Standard – MnCl₂) + 5 mM-MgCl₂</td>
<td>56</td>
</tr>
<tr>
<td>(Standard – MnCl₂) + 5 mM-CaCl₂</td>
<td>20</td>
</tr>
<tr>
<td>Standard + 20 mM-EDTA</td>
<td>16</td>
</tr>
<tr>
<td>Standard + 10 µM-cAMP</td>
<td>104</td>
</tr>
<tr>
<td>Standard + 2 µM-PP₁</td>
<td>2.9</td>
</tr>
<tr>
<td>Standard using heated virion‡ (or without virion)</td>
<td>0</td>
</tr>
</tbody>
</table>

* Standard reaction conditions are described in Methods. They correspond to 10 mM-MnCl₂. Standard – MnCl₂ corresponds to the standard mixtures, but without MnCl₂. CaMV (5 µg) was used as the enzyme source in all cases and no exogenous acceptor proteins were added. This table corresponds to the results obtained in one experiment. However, all tests have been performed at least twice, yielding very similar results.

† Standard kinase reaction was taken as 100% and percentages of other reactions are relative to the standard.

‡ Virus was heated for 5 min at 65 °C before the reaction.

Acceptor proteins

The reaction of the CaMV-associated PK was stopped after 1 h by the addition of 1 vol. sample buffer and the proteins were analysed on polyacrylamide gels. The Coomassie Brilliant Blue-stained gel (Fig. 2, lane 2) shows a typical pattern of the viral proteins in which the major polypeptide was 37K although this protein is considered to be a degradation product of the 42K protein. The higher mol. wt. species (84K) was shown by Al Ani et al. (1979b) to consist of aggregates of various partially degraded proteins. Autoradiography (Fig. 2, lane 3) revealed that the major phosphorylated protein was the 42K component that was present in relatively small amounts. A 58K component was also labelled as was a high mol. wt. protein of about 110K whose function and origin remain to be determined. The 37K protein was not phosphorylated in vitro under our conditions although in some experiments a 39K protein and what we assume...
Fig. 2. Electrophoretic identification of endogenous viral proteins phosphorylated in vitro by the CaMV-associated kinase. Fifteen µg phosphorylated CaMV particles was loaded on a 10% SDS-polyacrylamide gel. Lane 1, molecular weight markers; numbers on the left are their mol. wt. × 10^{-3}. Lane 2, Coomassie Brilliant Blue-stained pattern of CaMV proteins. In this preparation no 58K precursor could be seen. Lane 3, autoradiograph of lane 2 exposed for 2 h. Lane 4, autoradiograph of the products of attempted phosphorylation of a heated virus sample; exposure for 16 h.

were radioactive degradation products ranging from 30K to 15K were weakly labelled. We interpret the phosphorylated radioactive material remaining at the top of the revolving gel or just entering it, as undissociated nucleocapsid.

When virus was heated at 65 °C for 5 min, the PK activity was totally lost since no radioactive protein could be detected even upon long exposures (Fig. 2, lane 4). It is well known that many kinases are substrate-specific (Mangeat et al., 1978). CaMV PK phosphorylated casein and histones H2A and H2B in addition to the 42K protein but did not phosphorylate protamines (data not shown).

Determination of the phosphorylated amino acids

A two-dimensional separation of acid-hydrolysed phosphorylated products revealed predominantly phosphoserine (P-Ser) and small amounts of phosphothreonine (P-Thr) (Fig. 3). No radioactivity corresponding to phosphotyrosine (P-Tyr) could be detected. The dark trail seen in Fig. 3 was probably caused by inorganic 32P because it was also found when hydrolysed [γ-32P]ATP was analysed on a cellulose plate under the same conditions.

DNase susceptibility of virus particles phosphorylated in vitro

Native CaMV particles are unaffected by DNase I but after in vitro phosphorylation of the capsid, viral DNA became partially accessible to DNase I. To enhance the visualization of the action of the DNase I, the nicked DNA was labelled with DNA polymerase I in the presence of
Fig. 3. Detection by autoradiography of the labelled phosphoamino acids present in an HCl hydrolysate of CaMV protein. Dotted circles show the position of ninhydrin-stained phosphoamino acid markers: O-phospho-L-tyrosine (P-tyr), O-phospho-DL-threonine (P-thr) and O-phospho-L-serine (P-ser). The dark line at the left of the figure is traces of $^{32}$PO$_4$.

Fig. 4. DNase sensitivity of the virus phosphorylated in vitro. DNA from 5 µg native (lane 1) or phosphorylated (lane 2) CaMV particles was incubated with DNase I (10 µg/ml) and then was labelled in the presence of [$\alpha$-$^{32}$P]dCTP and 1 unit Kornberg polymerase I. Purified viral DNA was analysed on 1% agarose gel and detected by autoradiography. C, Circular form of the DNA; L, linear form of the DNA (Ménissier et al., 1983).

radioactive precursor ([$\alpha$-$^{32}$P]dCTP) prior to purification and analysed by electrophoresis in 1% agarose. The results are presented in Fig. 4. In a control sample of native virus (lane 1) the labelling of the DNA was slight and could correspond to the endogenous DNA polymerase reaction (Ménissier et al., 1984) whereas DNA from virus phosphorylated in vitro (lane 2) was much more radioactive, showing that it had been partially nicked by DNase I.

**DISCUSSION**

The PK activity present in purified virions is tightly associated with the capsid structure. We think that it is not simply a fortuitous contaminant which became absorbed to the surface of the virions during purification for the following reasons. (i) The last step of virus purification was in a CsCl gradient and it is known that in a high salt concentration contaminant proteins should be released (Hull & Shepherd, 1976). (ii) We have checked that a crude extract of healthy plant in the standard reaction mixture is not able to phosphorylate added heated virus although some cellular proteins are labelled (data not shown). No PK activity was detected in the pellets of a mock virus preparation from healthy plants, even in the presence of acceptor viral proteins from heated virions. (iii) Since the $^{32}$P phosphogroup is resistant to the action of alkaline
phosphatase, the acceptor site of the PK is probably not located at the surface of the capsid protein, but rather in the interior of the viral particle. Neutron small-angle scattering experiments have shown that the protein shell of CaMV particles, although very stable, consists of loosely packed protein subunits (J. Kruse, P. Timmins & J. Witz, personal communication). This unusual architecture may explain why exogeneous proteins can also reach the active site of the PK.

The CaMV PK seems to be specific with respect to protein acceptors. We found that both the 42K coat protein and a 58K protein, assumed to be its precursor, were phosphorylated in vitro. But in virions the 42K species is more actively labelled than the 58K one, whereas Hahn & Shepherd (1982) found that in vivo the specific radioactivity of the 58K was always higher than that of the 42K protein. This observation suggests that phosphorylation in vivo takes place at least in part before encapsidation. Perhaps the 58K protein possesses phosphorylation sites at its termini and these are excised during maturation; both extremities are unusually rich in serine and threonine residues (Franck et al., 1980).

It is interesting to note that the 37K protein is never phosphorylated either in vitro or in vivo (Hahn & Shepherd, 1982). Consequently, the site(s) of phosphorylation of the 42K protein is located within 50 amino acids of the N or C terminus, perhaps at both extremities. Furthermore, the 37K protein is more stable towards proteolysis than the 58K or the 42K proteins since it is the major protein observed in CaMV obtained by more drastic purification procedures. Consequently, a relationship could exist between the phosphorylation of the CaMV structural proteins and their maturation. Phosphorylation could also emphasize the accessibility of the extremities of both the 42K and 58K proteins.

The role of virion-associated kinase in the replication cycle remains unknown but it is now established that phosphorylation (as well as dephosphorylation) of enzymes and proteins can play a significant part in the control of a wide variety of cellular or viral functions. A striking example of the role of a protein kinase in a granulosis virus is presented by Wilson & Consigli (1985b). These authors have shown that upon phosphorylation the DNA-binding capacity of a basic viral core protein is reduced and viral DNA is released. In CaMV, phosphorylation of the 42K protein also induces structural modification of the virions since the DNA becomes more accessible to DNase I. We have estimated that the observed phosphorylation corresponds to the incorporation of about one phosphate group per 100 virions. This rate is only ten times lower than that observed by Wilson & Consigli (1985a). Such a low level of phosphorylation could be explained in two ways. First, the methods used to purify CaMV are drastic and could partially inhibit the protein kinase. It is indeed possible that only some particles possess an active PK. Second, polyacrylamide gel analysis of many CaMV preparations indicates that in our hands the 37K protein species is present in much larger amounts than any other degradation or maturation product of the 58K precursor. The 42K protein is present in small amounts only.

It is not yet known if the virion-associated PK described in this paper is also responsible for the phosphorylation in vivo observed by Hahn & Shepherd (1980, 1982) in the maturation process of CaMV particles. Indeed, it is possible that phosphorylation of the 58K and 42K proteins plays a role in their processing by cellular or viral proteases. Experiments are in progress to determine the phosphorylation site(s) on the viral capsid protein and its precursor in an attempt to test this idea.

We are grateful to Professor L. Hirth for helpful discussions. We wish to thank Dr A. Mazen for useful comments. Dr J. Witz is to be thanked for useful advice and critical reading of the manuscript. The collaboration of G. Daney de Marcillac is gratefully acknowledged.

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