Induction of a novel protein kinase in pupae of the silkworm *Bombyx mori* after infection with nuclear polyhedrosis virus

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Protein kinases induced by *Bombyx mori* nuclear polyhedrosis virus in pupae of the silkworm *B. mori* were examined by activity gel analysis using phosvitin as a protein substrate. The method involved PAGE of the soluble fraction from pupae under native conditions and in the presence of SDS, followed by in situ renaturation of proteins and recovery of protein kinase activity in the intact gel. A novel protein kinase able to phosphorylate phosvitin was detected in the infected pupae from 2 days post-infection. This enzyme was not present in uninfected silkworms at any stage of the pupal period. The novel kinase activity was found by SDS–PAGE to be associated with a single polypeptide with an apparent *M*<sub>r</sub> of 50K. However, on electrophoresis under native conditions its activity was associated with a set of polypeptides with similar but not identical electrophoretic mobilities. Microheterogeneity of the catalytically active polypeptides suggests that the virus-induced protein kinase undergoes post-translational modification during the course of infection.

Protein phosphorylation plays a key role in the regulation of cellular metabolism and viral oncogenesis. Although the function of protein kinases in the reproduction of viruses is less clear, specific protein kinases have been found associated with virions of many RNA and DNA viruses of mammals (Hatanaka *et al.*, 1972; Rubenstein *et al.*, 1972; Roux & Kolakofsky, 1974; Tan & Sokol, 1974; Blair & Russell, 1978; Lemaster & Roizman, 1980; Stevely *et al.*, 1985; Britt & Auger, 1986). The protein kinases induced in human cells by herpes simplex virus (HSV) and cytomegalovirus are encoded by the viral genomes, but that of HSV-1 is not essential for growth in cell culture (Purves *et al.*, 1987b; Frame *et al.*, 1987; Somogyi *et al.*, 1990). The pseudorabies virus protein kinase has been purified to homogeneity from infected hamster fibroblasts (Purves *et al.*, 1987a).

Although virus-specific phosphoproteins appear in insect cells after inoculation of baculoviruses (Maruniak & Summers, 1981; Miller *et al.*, 1983; Kelly & Lescott, 1984; Zemskov *et al.*, 1991), little is known about baculoviral protein kinases. Protein kinase activity has been detected in virions of *Autographa californica* nuclear polyhedrosis virus (Miller *et al.*, 1983) and in virions of *Plodia interpunctella* granulosis virus (PiGV) (Wilson & Consigli, 1985a). Incubation of PiGV with ATP results in the release of DNA from the nucleocapsid, suggesting that the endogenous protein kinase activity participates in the uncoating event (Wilson & Consigli, 1985b). Whether the kinase activity associated with the nucleocapsids of baculoviruses is of viral or host origin remains unknown.

*Bombyx mori* nuclear polyhedrosis virus (BmNPV) belongs to the subfamily Eubaculovirinae of the family Baculoviridae (Francki *et al.*, 1991). Its genome consists of a circular dsDNA of 130 kbp (Maeda & Majima, 1990). During infection of silkworm pupae, BmNPV induces synthesis of virus-specific phosphoproteins, including two structural components of the virions, a 35K polypeptide (pp35) and a basic DNA-binding protein (ppB) (Zemskov *et al.*, 1991). The protein kinase activity in extracts from infected silkworms shows a prominent specificity for viral phosphoproteins, suggesting that the virus-specific protein kinase(s) are induced in the course of BmNPV infection. In the present study we tried to detect viral protein kinase in extracts prepared from silkworm pupae at different times after infection with BmNPV. Protein kinases were detected after electrophoresis of the extracts in polyacrylamide gels by using phosvitin as the kinase substrate. A novel 50K protein kinase appeared in the pupae 2 days post-inoculation, and it could be demonstrated in the infected pupae up to the final stages of infection.

BmNPV was injected into silkworm pupae on the 3rd day of the pupal period as described earlier (Onodera *et al.*, 1965; Mikhailov *et al.*, 1986). The pupae were homogenized in buffer (5 ml/pupa) containing 20 mM-Tris–HCl pH 7.5, 1 mM-EDTA, 0.5 mM-EGTA, 5 mM-2-mercaptoethanol, 1 mM-PMSF. The homogenates were...
centrifuged at 3000 g for 15 min and then at 105000 g for 1 h. The final supernatant was used in all experiments.

Extracts from isolated nuclei were prepared in buffer containing 2 M-NaCl as described earlier (Zemskov et al., 1991). One or two frozen pupae were homogenized in a Dounce homogenizer in 10 ml 0.3 M-sucrose in buffer A (20 mM-Tris–HCl pH 7.5, 5 mM-MgCl₂, 1 mM-PMSF, 1 µM-leupeptin, 1 µg/ml pepstatin). The homogenate was diluted with 10 ml 1-M-sucrose in buffer A, and then centrifuged at 50000 g for 1 h. The nuclear pellet was suspended in 7 to 8 ml of 0.3 M-sucrose in buffer A, and nuclei were collected by centrifugation at 1000 g for 10 min and resuspended in the same buffer without MgCl₂. Extraction buffer (1 ml; 3 M-NaCl, 10 mM-Tris–HCl pH 7.5, 0.2 mM-DTT, 1 mM-PMSF, 1 µM-leupeptin, 1 µg/ml pepstatin) was added to 0.5 ml of nuclear suspension and incubation at 0 °C was continued for 1 h with stirring. The supernatant obtained after centrifugation at 5000 g for 30 min was used as the soluble fraction of nuclei.

A modification of the method termed activity gel analysis was used for detection of protein kinase catalytic polypeptides. Extracts were electrophoresed in polyacrylamide gels under native conditions and in the presence of SDS. The native electrophoresis was performed using 5 to 30% gradient polyacrylamide slab gels (14 × 13 × 0.08 cm) prepared in electrode buffer, 0.04 M-Tris–boric acid pH 9.0. After loading of the samples (300 µg of protein each), electrophoresis was at a constant 400 V for 21 h in a cold box (4 °C). Discontinuous SDS-polyacrylamide slab gels (14 × 13 × 0.08 cm) were run using the system described by Laemmli (1970). The separating gel contained 10% acrylamide. SDS and 2-mercaptoethanol were added to the samples (150 µg of protein each) to a final concentration of 1% just prior to sample application. Electrophoresis was performed overnight in a cold box (4 °C) at a constant current of 10 mA/gel. To remove SDS, the gel was rinsed in 500 ml 0.04 M-Tris–HCl pH 8.0 twice for 1 h each time at room temperature, then overnight at 4 °C, and finally for 1 h at room temperature. For detection of protein kinase activity the gel was covered with a sheet of Whatman 3MM paper soaked in buffer containing 0.1 M-Tris–HCl pH 8.0, 3.75 mg/ml phosvitin, 10 mM-MgCl₂, 5 mM-DTT, 15 to 20 µCi/ml [γ-³²P]ATP (2000 Ci/mmol). After incubation at 32 °C for 40 min (native electrophoresis) or for 4 h (denaturing electrophoresis) the paper was discarded. The gel was washed in 10% TCA with 1% sodium pyrophosphate, stained with Coomassie blue, dried and autoradiographed.

A new protein able to phosphorylate phosvitin was detected in the BmNPV-infected pupae after electrophoresis of extracts in native polyacrylamide gels (Fig. 1a, lanes 1 to 4). Visualization of the radioactive band depended on the presence of an exogenous protein substrate (phosvitin). The level of non-specific adsorption of [γ-³²P]ATP and autophosphorylation of proteins in the gels was negligible (Fig. 1a, lanes 5 to 8). The virus-induced protein kinase appeared in the pupae 2...
days post-infection and could be detected up to 7 days, i.e. until the terminal stages of the infection cycle (Fig. 1b). From 3 days post-infection the protein kinase activity was associated with both the original band and a set of polypeptides of higher electrophoretic mobility. The observed microheterogeneity of the catalytically active polypeptides suggests that the virus-induced protein kinase undergoes post-translational modification during the course of infection.

The virus-induced protein kinase was also detected after electrophoresis of the extracts under denaturing conditions in the presence of SDS (Fig. 2). In this case detection of the protein kinase activity also was dependent on the addition of phosphitin (Fig. 2a, lanes 2 and 6). Heat treatment of the extract (3 min at 100 °C) before electrophoresis resulted in complete enzyme inactivation (Fig. 2a, lane 4). The induced protein kinase has an apparent Mr of 50K, and the time course of enzyme induction was the same as that determined by native electrophoresis (compare Fig. 2b and Fig. 1b). However, in the presence of SDS the overall protein kinase activity was associated with a single polypeptide band which showed no mobility shift during the course of infection. Since the same extracts were used for both the native and denaturing electrophoresis, it is unlikely that the multiple forms of the protein kinase observed under the native conditions were produced by proteolysis or glycosylation of the original polypeptide during infection. It is more likely that the high mobility forms are a result of extensive phosphorylation of the original form. Thus, post-translational modification of the virus-induced protein kinase during the course of infection may involve the phosphorylation of the catalytically active polypeptide.

Although an active protein kinase capable of phosphitin phosphorylation is present in host cells (Fig. 1), it was not detected in the extracts after electrophoresis in the presence of SDS (Fig. 2). This result may be due to low activity of the soluble host cell enzymes in pupae or their incomplete renaturation in the gels after removal of SDS. The detection of host cell protein kinases in some subcellular fractions was more successful. A host cell protein kinase utilizing phosphitin was identified in respectively, but the samples were heated to 100 °C for 3 min prior to electrophoresis; lanes 5 to 8, as lanes 1 to 4, respectively, but recovery of the protein kinase activity in the absence of phosphitin. (b) Lane 1, uninfected pupae on the 7th day of the pupal period; lane 2, BmNPV-infected pupae 4 days post-infection; lanes 3 and 4, as lanes 1 and 2.

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Fig. 2. Identification of protein kinases after electrophoresis of extracts in 10% polyacrylamide gels in the presence of SDS. (a) Lane 1, uninfected pupae on the 7th day of the pupal period; lane 2, BmNPV-infected pupae 4 days post-infection; lanes 3 and 4, as lanes 1 and 2.
nuclear extracts made using 2 M-NaCl (Fig. 2c), and was associated with a 40K polypeptide. This activity was detected in uninfected pupae and in infected pupae at early stages of BmNPV infection, but completely disappeared upon induction of the 50K protein kinase. This result shows that the activity of host cell protein kinases may change dramatically after virus infection. In nuclei from infected pupae the host cell 40K protein kinase appeared to be completely replaced by the virus-induced 50K protein kinase.

We do not know whether the 50K protein kinase is encoded by the viral genome, but the absence of similar activity in uninfected silkworm pupae strongly suggests a viral origin. The other important question concerns the relationship between the novel protein kinase and the kinase activity detected in baculovirus nucleocapsids (Miller et al., 1983; Wilson & Consigli, 1985a, b). The virus-induced protein kinase may be sequestered during maturation of virions and involved in release of DNA from the nucleocapsid during the next infection cycle.

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References


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