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Protein Kinase Activity Associated with the Extracellular and Occluded Forms of the Baculovirus *Autographa californica* Nuclear Polyhedrosis Virus

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Protein kinase activity is associated with both the extracellular and the occluded forms of *Autographa californica* nuclear polyhedrosis virus, a baculovirus. Serine and threonine are the predominant amino acids phosphorylated by the kinase activity associated with both viral forms; no phosphotyrosine was detected. The addition of calcium, cAMP, or cGMP has no apparent effect on the amount of phosphorylation or the substrates phosphorylated.

The enveloped, rod-shaped nucleocapsids of baculoviruses contain large, double-stranded, circular DNA molecules which replicate in the nuclei of invertebrate host cells (16, 18). Nuclear polyhedrosis viruses have two infectious forms. The extracellular nonoccluded viruses (NOV) bud from the plasma membrane and are responsible for systemic infection of the host. Occluded viruses (OV) are produced in the nucleus; each is composed primarily of a crystalline protein matrix in which enveloped nucleocapsids are embedded. OV are involved in the primary infection of the host organism. Protein kinases are associated with virions of a variety of viruses (1, 2, 4, 6, 12–15, 17), have regulatory roles in cellular metabolism (7), and have been identified as key enzymes in viral oncogenesis (3, 10). Protein kinases are often associated with membranes. In this study, both NOV and OV forms of the baculovirus *Autographa californica* nuclear polyhedrosis virus were analyzed for the presence of protein kinase since the membranes of the two forms differ in origin.

The presence of protein kinase activity in preparations of virions purified from OV was detected by gel electrophoresis of ^{32}P-labeled viral proteins after the incubation of Nonidet P-40 (NP-40)-disrupted virions with [γ-^{32}P]ATP. Two proteins of approximately 15 and 17 kilodaltons were the primary targets for the protein kinase activity of occluded virions (Fig. 1A). Two proteins in the 30- to 34-kilodalton region were phosphorylated; the major occlusion matrix protein, polyhedrin, migrates in this region of the gel. The labeling of a 42- and a 54-kilodalton protein is more clearly observed in Fig. 1B, in which the 15-kilodalton protein was run off the bottom of the gel and the 17-kilodalton protein was overexposed.

Treatment of the virions with 0.1% NP-40 before the assay stimulated incorporation of ^{32}P into acid-precipitable material approximately fourfold. Treatment of the virions with 1% NP-40 inhibited the reaction. No significant differences in the relative activity of the protein kinase or in the ^{32}P-labeled protein electrophoretic pattern were observed upon the addition of calcium chloride, cAMP, or cGMP (Table 1).

Bovine serum albumin, phosvitin, and protamine sulfate were tested for their ability to serve as substrates for the kinase activity. The addition of phosvitin stimulated incorporation of ^{32}P label into acid-precipitable protein 15-fold (Table 1). Gel analysis indicated that phosvitin served as a substrate for the kinase activity. No stimulation of ^{32}P incorporation was observed with bovine serum albumin or protamine sulfate. In the course of these experiments, we attempted to monitor kinase activity by a procedure involving phosphocellulose paper (12, 19). Using this assay, we observed that phosvitin inhibited the reaction—a result which was clearly erroneous as determined by trichloroacetic acid precipitation followed by Cerenkov counting or by gel electrophoretic observations. We ascribe the erroneous result to the inability of phosvitin to bind (or remain bound) to the phosphocellulose paper under the assay conditions employed and therefore suggest caution in using such as assay technique.

Protein kinase activity was also detected in preparations of NOV isolated from the media of infected monolayers of either *Trichoplusia ni* (TN-360) or *Spodoptera frugiperda* (IPLB-SF-21) cells. The NOV and mock-infected controls were prepared by collecting media, clearing cell debris by centrifugation at 10,000 × *g* for 20 min, pelleting the virus by centrifugation at 90,000 ×

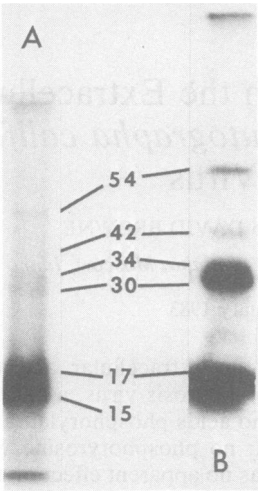

FIG. 1. Autoradiograph of ^{32}P-labeled virus proteins phosphorylated by protein kinase associated with virions of OV. Molecular sizes of proteins in kilodaltons are shown by the numbers. OV of *Autographa californica* nuclear polyhedrosis virus L-1 (9) were purified (11) from infected larvae of *T. ni*. After equilibrium buoyant density centrifugation in a linear 40 to 66% (wt/wt) sucrose gradient, the OV were disrupted with 0.05 M sodium carbonate. The released virions were purified by equilibrium centrifugation on linear 20 to 60% (wt/wt) sucrose gradients. The virion bands (9) were collected, diluted, pelleted by centrifugation, suspended in 0.01 M Tris (pH 7.6), and stored in liquid N_2 until used. Virions were removed from liquid N_2 and quickly thawed. An equal volume of cold disruption buffer containing 0.10 M potassium phosphate (pH 7.2), 20 mM 2-mercaptoethanol, 2 mM EDTA, and 0.2% NP-40 was added. The concentration of virion proteins was maintained at 1 mg/ml. Virions were allowed to disrupt overnight at 5°C in the disruption buffer. Protein kinase assays were carried out in 50-μl reaction mixtures containing 20 mM Tris (pH 8.0), 10 mM $MgCl_2$, 2 mM dithiothreitol, and 50 μM ATP, including 5 μCi of [γ-^{32}P]ATP per reaction. Final protein concentrations were 200 μg/ml. Reactions were incubated 1 h at 32°C and terminated by the addition of an equal volume of cold 20% trichloroacetic acid. The samples were placed at 5°C overnight and then were centrifuged in an Eppendorf at 12,800 × *g* for 5 min. The pellets were washed three times with 1 ml of cold 5% trichloroacetic acid, washed twice with 1 ml of acetone, and air dried. The pellets were suspended in Laemmli sample buffer, heated to 100°C for 1 min, and electrophoresed on sodium dodecyl sulfate-polyacrylamide gels as previously described (8). Electrophoresis was carried out for a shorter time for A than for B. Gels were fixed, stained, dried, and autoradiographed.

g for 1 h, suspending the virus, and centrifuging it on 20 to 66% (wt/wt) linear sucrose gradients at 90,000 × *g* for 2 h. The virus band or equivalent region of the sucrose gradient was collected, diluted, pelleted by centrifugation, suspended in a small volume of 0.01 M Tris (pH 7.6), and stored in liquid N_2 until use. Under the conditions used to disrupt the virions and assay the kinase activity (Fig. 1, legend; Table 1), approximately 10-fold more acid-insoluble ^{32}P cpm were observed with NOV (e.g., 70,000 cpm in the standard reaction; Table 1) than with OV. Very low levels of phosphorylation were observed in mock-infected controls, even when phosvitin was added as an exogenous substrate. Phosvitin stimulated the NOV reaction, but the addition of calcium chloride, cAMP, or cGMP had no effect on the level of phosphorylation or the type of protein phosphorylated (data not shown). The predominant endogenous proteins labeled by NOV from *S. frugiperda* cells were 98, 70, 60, 54, 37, 28 and 15 kilodaltons.

Since we do not know whether the kinase activity of the virions is of host origin or viral, the importance of the difference in the level of kinase activities in NOV and OV virion preparations is difficult to assess. If the kinase activity is a contaminating host enzyme inserted, for instance, in the membranes of the virions, then the difference in levels may only reflect the difference in origins of the two proteins, one being derived from the nucleus and the other from host plasma membrane. Similarly, a difference in the ^{32}P-labeled protein profile as observed in NOV and OV virion assays may only reflect differences in membrane composition. It must be remembered that the substrates for the in vitro reaction may not be those observed in vivo, and protein phosphorylation in vitro may only reflect

TABLE 1. Influence of reaction conditions on OV-associated protein kinase activity

Reaction conditions[a]	Acid-insoluble ^{32}P (cpm)
Standard	7,300
Standard + 1.0 mM $CaCl_2$	7,010
Standard + 0.5 mM cAMP	7,160
Standard + 0.3 mM cGMP	7,360
Standard + 0.1 mg of phosvitin per ml	95,500
Standard + 0.1 mg of bovine serum albumin per ml	7,150
Standard + 0.1 mg of protamine sulfate per ml	7,180

[a] Standard reaction mixture was 20 mM Tris (pH 8.0), 10 mM $MgCl_2$, 2 mM dithiothreitol, 50 μM ATP (including 5 μCi of [γ-^{32}P]ATP), and 200 μg of protein per ml from NP-40-disrupted OV virions. The reactions were incubated at 32°C and stopped by the addition of trichloroacetic acid as described in the legend to Fig. 1. Acid-soluble radioactivity was removed by trichloroacetic acid and acetone washing (Fig. 1).

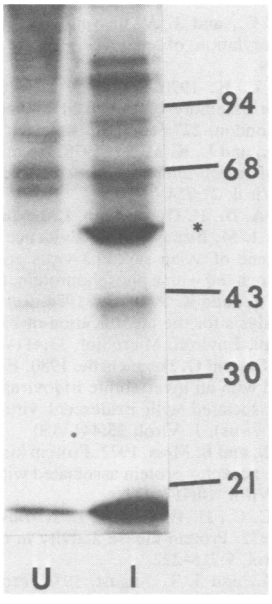

FIG. 2. Comparison of endogenous proteins phosphorylated by protein kinases in infected and uninfected cell extracts. Infected and uninfected cells were pelleted by centrifugation, disrupted with NP-40, and assayed for protein kinase as described in the legend to Fig. 1. Autoradiograms of phosphorylated proteins separated on sodium dodecyl sulfate-polyacrylamide gels reveal a number of protein substrates in whole cell extracts. The star indicates a 54-kilodalton phosphoprotein which is phosphorylated in infected cell extracts (I) but not in uninfected cell extracts (U). The numbers to the right show the molecular sizes of the standard proteins in kilodaltons.

the availability of specific amino acid substrates for the kinase. The fact that phosvitin serves as an excellent substrate illustrates this point.

A comparison of proteins labeled in vitro by protein kinase present in infected and uninfected *S. frugiperda* cells is shown in Fig. 2. The most noticeable difference in the ^{32}P-labeled protein profiles is the intensive labeling of a protein of approximately 54 kilodaltons in the infected cell extract. To investigate this phenomenon further, the kinases of the infected cell extract were denatured by incubating the extract of 75°C for 15 min, and the heat-denatured, infected cell extract was then used as a substrate for the kinases of infected cell extracts and of control uninfected cell extracts. The 54-kilodalton protein was labeled intensively by the uninfected cell extract as well as by the infected cell extract, indicating that the 54-kilodalton protein may simply be a virus-induced protein which is an active substrate for a cellular protein kinase. A protein of 54 kilodaltons was also labeled in purified NOV extracts.

To determine the target amino acids of the protein kinase activity present in the virion preparations, the proteins radioactively phosphorylated by kinase activity of NOV from *S. frugiperda* cells and OV from *T. ni* larvae were partially hydrolyzed, and the products were analyzed by electrophoresis (Fig. 3). The ^{32}P label was found to be distributed primarily between phosphoserine and phosphothreonine. There was no indication of phosphotyrosine with preparations from either NOV or OV viri-

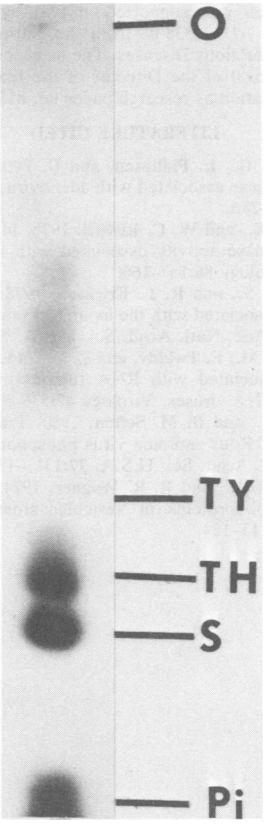

FIG. 3. Determination of the amino targets of the protein kinase activity associated with NOV. In vitro ^{32}P-labeled phosphoproteins of NOV were hydrolyzed with acid (5). The hydrolysates were dissolved in a mixture containing 20 μg each of phosphoserine, phosphothreonine, and phosphotyrosine, and the products were analyzed by electrophoresis on a 56-cm strip of Whatman 3MM paper at pH 3.6 for 75 min at 2 V in glacial acetic acid-pyridine-water (12:1:337, vol/vol). The strips were treated with 0.1% ninhydrin in acetone and dried for 5 min at 80°C. The positions of the marker amino acids were denoted on the strip, and the strip was then autoradiographed for detection of ^{32}P-labeled amino acids. The origin is shown by O. The positions of the phosphoserine, phosphothreonine, and phosphotyrosine markers are shown by S, TH, and TY, respectively. The position of Pi is also shown.

ons that had been disrupted by NP-40. Spots close to the origin of electrophoresis are apparently incompletely hydrolyzed phosphopeptides (5); partial hydrolysis was utilized to avoid breakdown of phosphorylated amino acids, particularly phosphotyrosine (5). Therefore, the virion-associated kinase activities are not closely related to the kinases involved in animal tumor virus transformation.

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