JOURNAL OF VIROLOGY, Feb. 1972, p. 216-222

Vol. 9, No. 2
Printed in U.S.A.

Protein Kinase Activity in Equine Herpesvirus

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Received for publication 15 November 1971

A protein kinase which is intimately associated with equine herpesvirus (equine abortion virus) was found by using adenosine triphosphate-γ-^{32}P as a phosphate donor and virus protein as an acceptor. Consistent demonstration of the activity requires prior removal of phosphohydrolase. The kinase activity requires Mg^{2+}, is not stimulated by cyclic adenosine monophosphate, but is enhanced by added protamine or arginine-rich histone. The labeled product is resistant to ribonuclease, deoxyribonuclease, and chloroform-methanol but is sensitive to Pronase. Other tests suggest that serine and threonine residues are the acceptor sites. In the in vitro reaction, the incorporation represents an average of approximately 4,500 phosphate residues per virion, and all 17 virus protein bands resolved by polyacrylamide gel electrophoresis appear to be labeled.

While this manuscript was being prepared, a paper by Strand and August (11), describing the presence of protein kinase activity in certain enveloped viruses, was published. Their contribution was the first published report of this nature. In addition, this important paper has reviewed, with appropriate references, the significance of the phosphorylation of proteins. The authors described the enzyme activity in avian myeloblastosis, Rauscher leukemia, and vesicular stomatitis viruses, but none was detected in poliovirus or adenovirus, which are reputed not to be enclosed by a membranous envelope. Subsequently, high levels of protein kinase activity were found in frog virus 3, a large deoxyribonucleic acid (DNA) virus replicating in the cytoplasm (J. T. August, *personal communication*).

This laboratory has long investigated a member of the herpes group, equine abortion (herpes) virus (EAV), which has the advantage (to the investigator) of replicating in the Syrian hamster with the production of a rapidly lethal hepatitis and pronounced viremia (10). The plasma of infected animals contains approximately 10^{10} particles per ml; thus, the virus is relatively easy to purify (1, 2, 4). It seemed reasonable that EAV, which buds through the nuclear membrane (5), might contain adenosine triphosphatase or related enzymes (6). As expected, EAV was shown to contain phosphohydrolase activity (C. C. Randall, J. Szabocsik, and H. W. Rogers, Jr., *unpublished data*). It was assumed that the presence of this enzyme would materially effect the demonstration of kinase activity. This proved to be the case and explained the erratic or negative results of our earlier attempts. Fortunately, the competing hydrolase activity was in material loosely adherent to the envelope, so that it could be dislodged from the virus particles without loss of infectivity or apparent morphological alteration by a slight modification of the purification procedure (1). The characteristics of the extraneous hydrolase will be described elsewhere. The present paper describes endogenous protein kinase activity and substrate protein in preparations of purified EAV.

MATERIALS AND METHODS

Virus. The strain of EAV, its propagation in animals, purification from viremic hamsters, and quantitation have been described previously (1, 2, 4, 10). Viruses may be purified by differential centrifugation, followed by banding either in sucrose or tartrate gradients. The resulting preparations are free from contaminating material as judged by electron microscopy (2). The virus prepared by banding in sucrose gradients was found to have significant phosphohydrolase activity (9). Further treatment of the virus by suspending it in 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride and 0.001 M ethylenediaminetetraacetic acid (EDTA) buffer, *p*H 7.4, followed by layering in 20 to 42% potassium tartrate and centrifuging in a Spinco SW 25.1 rotor for 2 hr at 5 C at 20,000 rev/min, caused release of a top component containing most of the hydrolase activity. The virus, stripped of hydrolase activity, banded about halfway down the tube and was collected by side puncture, dialyzed overnight against Tris-EDTA buffer, and concentrated by pelleting at 20,000 rev/min for 0.5 hr in a Spinco no. 30 rotor. The pooled virus pellets ("tartrate purified") were sonically treated and suspended in distilled water to an optical density of 1.0 at 260 nm, approximately

equivalent to 7×10^{10} particles or 222 μg of protein per ml (2; *unpublished data*). By the criteria of electron microscopy, the virions were not morphologically different from the crude virus obtained directly from the blood. Samples of 1 ml were stored at −20 C. Prior to use, preparations were thawed and suspended by brief sonic treatment, and appropriate samples were used as indicated in the individual experiments.

Reagents. Nuclease-free Pronase B (Calbiochem) was incubated at 37 C for 2 hr. Salmon sperm protamine sulfate and arginine-rich calf thymus histone (Sigma Chemical Co.) were dissolved in distilled water. A solution of pancreatic ribonuclease A, five times crystallized (Calbiochem), was heated for 3 min at 100 C prior to use. Deoxyribonuclease I was obtained from Worthington Biochemical Corp. Alkaline phosphatase was purchased from Calbiochem. Succinic acid was obtained from Eastman Organic Chemicals, and hydroxylamine hydrochloride was purchased from Fisher Scientific Co. Anhydrous caffeine was from Calbiochem, and cyclic adenosine 3′,5′ monophosphate (AMP) was obtained from Sigma Chemical Co. Nonidet P-40 (NP-40) is a product of Shell Chemical Co. Venom phosphodiesterase was obtained from Sigma Chemical Co. All isotopes were obtained from New England Nuclear Corp.

Protein kinase assay. A typical reaction mixture is shown in Table 1. The amount of adenosine triphosphate (ATP)-γ-^{32}P (New England Nuclear Corp.) is given in the individual experiments along with other specific conditions. After incubation for the indicated interval, 25 μliters of the reaction mixture was placed on Whatman 3MM filter paper discs, which were then processed by the method of Bollum (3). At the end of the incubation period, the contents of each tube were mixed with 200 to 400 μg of bovine serum albumin, and the mixture was precipitated with an equal volume of 10% trichloroacetic acid (final dilution 5%). After brief storage at 4 C, each tube was centrifuged at low speed for 10 min at 4 C, the precipitate was washed three to four times with cold 5% trichloroacetic acid, and the supernatant fluid was discarded. The washed pellet was dissolved in 0.2 ml of 0.2 N NaOH, and a sample was counted in a scintillation counter (Packard Instrument Co., Inc.).

TABLE 1. *Complete reaction mixture*[a]

Component	Concn (stock)	Vol (μliters)
ATP-γ-^{32}P	12.2 pmoles/μliter	10
Tris-hydrochloride, *p*H 8.5	0.5 M	30
$MgCl_2$	0.1 M	20
$MnCl_2$	0.01 M	30
Dithiothreitol	0.1 M	20
Nonidet P-40	15%	10
Purified EAV[b]	222 μg of protein/ml	50
Water		130

[a] Initial specific activity = 20.5 Ci/mmole. Samples of isotope were counted on day of experiment to determine specific activity in calculating incorporation.

[b] Tartrate purified virus.

Gel electrophoresis of phosphorylated proteins. ^{32}P-phosphorylated equine abortion virus was dissociated by treatment with 2% sodium dodecyl sulfate (SDS), 1% 2-mercaptoethanol (ME), and 0.5 M urea as described by Abodeely et al. (2). After dissociation and treatment in 0.1 M sodium phosphate buffer (*p*H 7.2) for 1 hr at 37 C, the samples were heated to 100 C for 1 min before overnight dialysis against 0.1% SDS, 1% 2-ME, 0.5 M urea, and 10% glycerol in 0.1 M phosphate buffer, *p*H 7.2. The samples, containing 100 to 150 μg of protein in less than 100 μliters, were then mixed with 10 μliters of 0.02% bromophenol blue (BPB) and layered over gels consisting of 10% Eastman acrylamide (for electrophoresis), 0.26% *N,N′*-methylenebisacrylamide, 0.04% *N,N,N′,N′*-tetramethylenediamine, and 0.1% SDS in 0.1 M phosphate buffer, *p*H 7.2. The gels were electrophoresed at 3 ma per gel for 12 hr at room temperature. The electrode buffer was 0.1 M sodium phosphate, *p*H 7.2, containing 0.1% SDS. Electrophoresis was continued until the tracking dye was 1 cm from the end of the gel, when the dye front was marked with India ink by using a 27-gauge needle. The gels were sliced into 1-mm segments, hydrolyzed in 0.2 ml of 30% hydrogen peroxide, and counted in Aquasol (New England Nuclear Corp.) in a liquid scintillation counter (Packard Instrument Co., Inc.). When the gels were stained for protein, samples were fixed overnight in 10% trichloroacetic acid, stained 6 to 8 hr in 0.05% Coomassie Blue in 7% acetic acid, and then destained in 7% acetic acid.

RESULTS

Conditions for viral enzyme activity. The incubation of appropriately prepared virions in the complete reaction mixture (Table 1) in the presence of ATP-γ-^{32}P resulted in optimal incorporation of the label into acid-insoluble material (Table 2). Caffeine inhibited the reaction about 25% and was omitted from further assays. Various omissions showed that full activity was not obtained in the absence of NP-40, dithiothreitol, or Mg^{2+}. Since early experiments indicated that Mn^{2+}, in addition to Mg^{2+}, might enhance the reaction, Mn^{2+} was included in all relevant reactions. As shown in Table 2, Mn^{2+} may partially replace Mg^{2+}, whereas Co^{2+} and Ca^{2+} cannot. It is significant that the sucrose gradient-purified virus gave only 15% of the incorporation seen with tartrate-banded virus. The competing phosphohydrolase activity (C. C. Randall et al., *unpublished data*) appears to be responsible. The *p*H range was not thoroughly investigated, although preliminary experiments suggested the optimum was about 8.5. About two-thirds of the maximal activity was obtained at *p*H 7.2. Cyclic AMP at the concentrations used (25, 100, and 200 pmoles) did not stimulate incorporation of ^{32}P, which was contrary to the finding

TABLE 2. *Factors affecting incorporation*[a]

Additions	Amt/300 μliters of reaction mixture	^{32}P incorporated (pmoles/mg of EAV protein)
Complete[b]		2,238
Omit Nonidet P-40		1,742
*p*H 7.2		1,502
Omit $MgCl_2$		786
Omit $MnCl_2$		2,237
Omit $MnCl_2$, $MgCl_2$		48
Omit virus		8
Complete[c]		969
Substitutions for tartrate-purified virus:		
Sucrose-purified virus	50 μliters (11.1 μg of EAV protein)	149
Virus-free serum[d]	50 μliters	21
Water (omit virus)	50 μliters	5
Complete[e]		1,068
Omit dithiothreitol		639
Cyclic AMP	100 pmoles	933
Cyclic AMP	25 pmoles	900
Omit virus		5

[a] After 1 hr of incubation at 37 C, 25-μliter samples were processed by the disc method and counted.

[b] Complete reaction mixture as described in Table 1. Specific activity (SA) = 0.04 pmole/10^3 counts/min.

[c] Complete reaction mixture as described in Table 1, except that each 300-μliter sample contained 5 μliters of 11.7 μM adenosine triphosphate (ATP), and $MnCl_2$ was omitted. SA = 0.037 pmole/10^3 counts/min.

[d] Obtained by centrifugation.

[e] Complete reaction mixture as in (c). SA = 0.050 pmole/10^3 counts/min.

for several other enveloped viruses (11). Most protein kinases are stimulated by cyclic AMP (7, 12).

Association of enzyme activity with virions. The dependence of the reaction on the amount of added virus is shown in Fig. 1. At high levels of virus concentration, the incorporation appears to be limited by the amount of ATP-γ-^{32}P. The relationship between the protein kinase activity and the virions banded in tartrate gradients appears well established and indicates that the enzyme protein is intimately associated only with the virus and is not soluble. Serum from infected animals from which the virus had been pelleted and various supernatant fluids above the pelleted virus contained no significant activity.

Kinetics of reaction and temperature optimum. That the rate of reaction was quite rapid can be seen from Fig. 2; no significant incorporation was seen with an incubation period longer than 1 hr. No activity was seen with the virus heated at 80 C for 10 min.

The capacity to incorporate ATP-γ-^{32}P into an acid-insoluble product was determined at several temperatures as shown in Fig. 3, which indicates that the kinase activity was greatest at 37 C. Con-

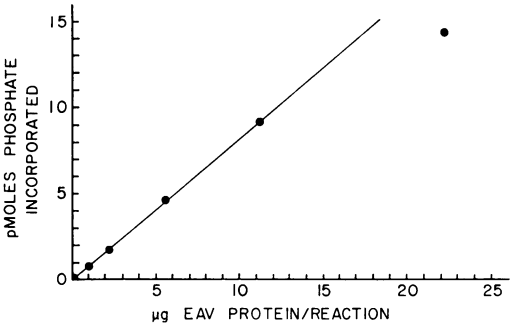

FIG. 1. *Incorporation of ^{32}P as a function of purified virus. Reaction mixture as in Table 1, except that each 300-μliter sample contained 5 μliters of adenosine triphosphate (ATP), 12.2 pmoles/μliter, and virus as indicated. After 1 hr at 37 C, 25-μliter samples were processed by the disc method. Specific activity of ATP-γ-^{32}P = 0.056 pmoles/10^3 counts/min.*

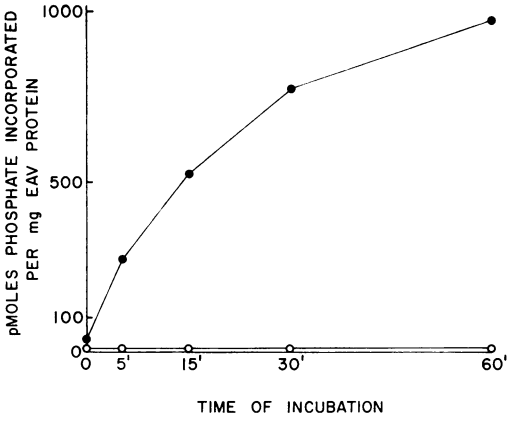

FIG. 2. *Kinetics of incorporation. Closed circles, complete reaction mixture as described in Table 1 with incubation at 37 C, except that each 300-μliter sample contained 5 μliters of 11.7 μM adenosine triphosphate (ATP). Open circles, same, except that the virus was omitted. At the times indicated, 25-μliter samples were processed as described. Specific activity of ATP-γ-^{32}P = 0.057 pmoles/10^3 counts/min.*

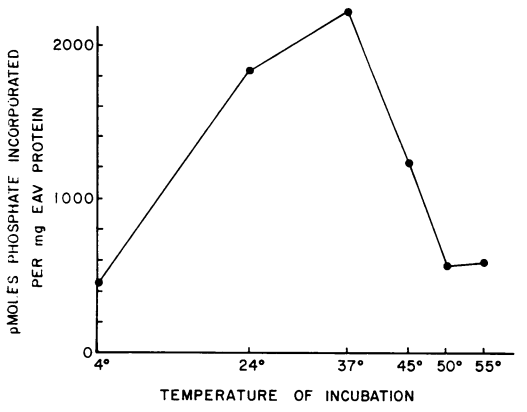

FIG. 3. *Incorporation of ^{32}P as a function of temperature. Reaction mixture as in Table 1, except that each 300-μliter sample contained 10 μliters of adenosine triphosphate (ATP), 2 pmoles/μliter. After 1 hr of incubation at the indicated temperature, 25-μliter samples were processed by the disc method. Specific activity of ATP-γ-^{32}P = 0.228 pmoles/10^3 counts/min.*

siderable enzyme activity was also demonstrated at 24 and 45 C.

Phosphate donors. Preliminary experiments showed that neither ATP-α-^{32}P nor guanosine 5′-triphosphate-(GTP)-α-^{32}P were phosphate donors. GTP-γ-^{32}P, substituted for ATP-γ-^{32}P in the reaction, gave significant acid-insoluble incorporation, but not as much as ATP-γ-^{32}P. A mixture of uridine 5′-triphosphate (UTP), cytidine 5′-triphosphate (CTP), and GTP at a final concentration of 1.6×10^{-3} M completely blocked the reaction.

Effect of other proteins on the endogenous viral enzyme. From Table 3, it is evident that incorporation of acid-insoluble ^{32}P is stimulated when certain proteins (protamine and arginine-rich histone) are exposed to the endogenous enzyme of the virus. It is presumed that the proteins in question are acting as phosphate acceptors.

Characterization of the reaction product. The data presented here, based on the incorporation of ^{32}P from ATP-γ-^{32}P into acid-insoluble material intimately associated with virus, indicate that virions of EAV contain endogenous kinase as well as substrate activity. The nature of the acid-insoluble radioactive product is shown in Tables 4 and 5. After treatment of separate samples of virus with chloroform-methanol (2:1) and hot trichloroacetic acid, approximately 90 to 95% of the label remained acid insoluble. The label was also stable after exposure to nucleases. These findings showed that there was little or no incorporation into lipids or nucleic acid. Pronase released approximately 87% of the label, indicating the protein nature of the acid-insoluble product. The solubilization of the label by alkaline phosphatase, stability in succinic acid and succinic acid-hydroxylamine, and sensitivity to hot NaOH all suggest that the phosphorylated amino acids are most likely serine and threonine, as discussed by Strand and August (11).

TABLE 3. *Effect of phosphate acceptor proteins*[a]

Additions	Amt/300 μliters of reaction mixture	^{32}P incorporated (pmoles/mg of EAV protein)
Complete[b]		859
Protamine	7 μg	1,519
Histone, arginine-rich	7 μg	1,083
Omit virus		16

[a] After 1 hr of incubation at 37 C, 25-μliter samples were processed by the disc method and counted.

[b] Complete reaction mixture as in Table 1, except that each 300-μliter sample contained 5 μliters of 11.7 μM adenosine triphosphate. Specific activity = 0.052 pmole/10^3 counts/min.

TABLE 4. *Characteristics of reaction product: enzymatic studies*[a]

Treatment	Amt/200 μliters of reaction mixture	^{32}P incorporated[b]
Control		1,801
Deoxyribonuclease[c]	15 μg	1,899
Ribonuclease[c]	15 μg	1,779
Pronase[d]	15 μg	239
Control		593
Alkaline phosphatase[c]	200 μg	113

[a] Although these experiments were done in batches from which 0.2-ml samples were boiled for 3 min after 1 hr of incubation at 37 C, the proportions of the ingredients in the reaction mixtures are the same as in Table 1, except that in the first part (nucleases and Pronase) each 300-μliter volume of reaction mixture contained 20 μliters of adenosine triphosphate (ATP), 12.2 pmoles/ml. Specific activity = 0.031 pmoles/10^3 counts/min. In the second batch (alkaline phosphatase), each 300-μliter volume contained 5 μliters of ATP, 11.7 pmoles/ml. Specific activity = 0.052 pmoles/10^3 counts/min.

[b] Picomoles per milligram of EAV protein; remaining acid insoluble.

[c] Incubated for 1 hr at 37 C, precipitated, washed, and counted. The ribonuclease was boiled for 3 min before it was added to the 200-μliter sample of reaction mixture.

[d] Incubated for 2 hr at 37 C, precipitated, washed, and counted.

TABLE 5. *Characteristics of product: chemical studies*[a]

Treatment	^{32}P incorporated,[b] pmoles per mg of EAV protein remaining acid insoluble
Control	986
Hot trichloroacetic acid[c]	875
Succinic acid[d]	1,045
Succinic acid-hydroxylamine[d]	1,160
Hot NaOH[e]	3

[a] Although this experiment was a batch reaction from which 0.2-ml samples were precipitated and washed prior to treatment, the proportions of ingredients in the reaction mixture are the same as in Table 1, except that each 300-μliter volume contained 6.25 μliters of 11.7 μM adenosine triphosphate. Specific activity = 0.041 pmole/10^3 counts/min. The incubation period was 1 hr at 37 C.

[b] Picomoles per milligram of EAV protein remaining acid insoluble.

[c] A pellet from a 0.2-ml portion of the reaction mixture was suspended in 0.2 ml of 10% trichloroacetic acid, extracted for 20 min at 90 C, sedimented, washed in trichloroacetic acid, and counted.

[d] Pellets from 0.2-ml samples of the reaction mixture were suspended in 1 M succinic acid (*p*H 5.5) or in 1 M succinic acid in 1 M hydroxylamine (*p*H 5.5), heated for 60 min at 37 C, precipitated with bovine serum albumin (BSA) and trichloroacetic acid, washed, and counted.

[e] A pellet from a 0.2-ml sample of reaction mixture was dissolved in 0.2 ml of 1 N NaOH, heated for 15 min at 100 C, precipitated with 0.1 ml of BSA (200 μg) and 0.2 ml of 50% trichloroacetic acid, washed, and counted.

Gel electrophoresis of protein kinase reaction mixture. Electrophoresis of the phosphorylated EAV protein revealed at least seventeen ^{32}P-labeled peaks (Fig. 4). These correspond in distribution to those reported by Abodeely et al. (2) and include species ranging from 11,000 to 100,000 daltons. At least three of the higher molecular weight peaks corresponded to the glycolipoprotein bands of the envelope (2). It therefore appears that most, if not all, of the major structural proteins of EAV are phosphorylated by the protein kinase. Experiments to locate the enzyme activity within the virion are in progress.

Protein kinase activity in other viruses. The endogenous phosphorylating ability of other viruses also was considered. In preliminary experiments with fowlpox virus (FPV) and vaccinia virus (VV), only the latter showed any activity, about 5% of that seen with EAV. It was later found that the VV used was contaminated with mycoplasma, and further studies were not done. In any case, both these viruses are cell-associated, are assembled in the cytoplasm, and apparently do not escape from the cell in significant numbers by budding. The great majority of particles, therefore, would not be expected to have an associated plasma membrane or enzymes from this source.

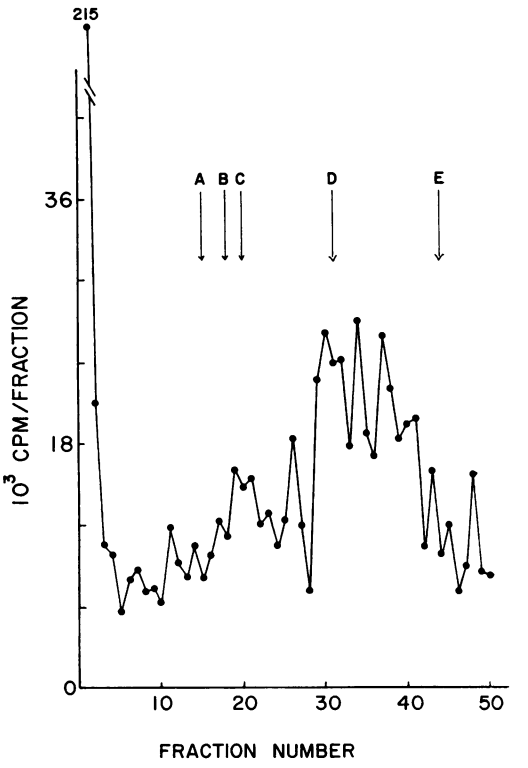

FIG. 4. *Polyacrylamide gel electropherograms of phosphorylated EAV proteins. Purified EAV equivalent to 480 μg of protein was incubated in an eightfold reaction mixture (2.4 ml) minus Nonidet P-40, and containing 10 nmoles of ATP-γ-^{32}P (Specific activity = 0.039 pmoles/10^3 counts/min) for 1 hr at 37 C. At the end of incubation, 0.2 ml of the mixture was precipitated, washed, and counted. The remainder was divided into three 0.7-ml fractions and precipitated and washed with trichloroacetic acid. These samples were then washed once with 3 ml of acetone and solubilized in 50 μliters of sodium dodecyl sulfate-urea-2-mercaptoethanol. The samples were mixed with 10 μliters of 0.02% bromophenol blue in 20% glycerol and electrophoresed as described by Abodeely et al. (2). The relative positions of molecular weight marker proteins (Schwarz/Mann) from companion gels are indicated by arrows (A, bovine serum albumin, 67,000 daltons; B, gamma globulin, heavy chain, 50,000 daltons; C, ovalbumin, 45,000 daltons; D, chymotrypsinogen, 22,500 daltons; and E, cytochrome C, 12,400 daltons).*

This may, however, be an oversimplification, as there may be, in addition to the mechanism of release, other more subtle differences between viruses to explain differences in kinase activity.

DISCUSSION

The results presented here show that equine herpesvirus contains an endogenous kinase which, in the presence of ATP-γ-^{32}P, phosphorylates at least 17 substrate polypeptides intrinsic to the virus. These represent most of the structural proteins and the envelope glycolipoproteins (2). The localization of the enzyme is probably amenable to fractionation techniques (2). If it is an integral part of the envelope (which is quite difficult to remove), it is hard to explain the phosphorylation of viral proteins internal to this structure unless there is a mechanism to transfer the phosphate. The in vitro phosphorylation of other proteins which stimulate incorporation of ^{32}P poses another problem. This may be a surface phenomenon, or the virus may be penetrated by these substrates.

The role of the phosphohydrolase is not clear, but it is of interest that frog virus 3, which has high protein kinase activity (T. August, *personal communication*), also contains a phosphohydrolase (13) which specifically converts ATP to adenosine diphosphate. Perhaps the hydrolase facilitates the transfer of phosphorus. On the other hand, it is possible that it serves to prevent phosphorylation of the virion proteins during the sojourn of the mature virion through the variety of environments it encounters between maturation, entry, and uncoating in its subsequent host cell. As a third possibility, it may be simply fortuitous.

The role of the protein kinase also is not clear, but it is possible that, in the normal growth cycle, phosphorylation may occur after infection of the host cell but before uncoating. The evidence for this is that the virus used here is mature, having been released and freed in the plasma. Presumably the phosphohydrolase would prevent phosphorylation of the virion proteins until it is removed at the time of initiation of the subsequent growth cycle. In any case, the maximum amount of in vitro phosphorylation observed represents only about 4,500 amino acid residues per virion (from 2,500 pmoles of phosphate incorporated per mg of EAV protein, and 3.17 fg of protein per EAV virion; *unpublished data*). If, as appears likely, it is confined to threonine and serine, it would represent only a small fraction of the more than 10^6 such residues in each virion (O'Callaghan et al., *in press*). Whether or not such a small number could be important in initiating the growth cycle remains to be determined. Alternatively, a substantial number of the threonine and serine residues may have been phosphorylated during maturation.

Although it is not easy to identify the origin of the protein kinase, it is well known that herpesviruses, including EAV, are assembled in the nucleus and must have intimate contact with nuclear chromatin. Since it has been shown that phosphoprotein kinases may be associated with chromatin (12), this source should not be overlooked. Whether or not the enzyme is coded for by EAV, it is apparently an integral part of the virion and probably serves an important function in some stage of replication.

We have not been able to demonstrate stimulation of protein kinase by cyclic AMP. It has been established, however, that the prominent action of cyclic AMP-dependent protein kinases is the ability to phosphorylate histone (8). The failure to respond to cyclic AMP may indicate that it is already bound or that the viral kinase may lack a regulatory subunit (11). Other studies have reported a lack of stimulation of protein kinase by cyclic-AMP (7, 12).

The nature of the product was studied further with venom diesterase (2×10^{-2} units/300 μliters of reaction mixture, 2 hr at 37 C, *p*H 8.4), but these experiments gave anomalous results. In each case, almost two-thirds of the radioactivity was rendered acid soluble. This suggests that some of the incorporated phosphate may be involved in a phosphodiesterase bond, linking the protein to molecules which are small enough to be acid soluble. Studies are presently underway to identify these products.

Preliminary experiments in this laboratory indicate that there may be fundamental differences in the behavior of ATP and GTP as phosphate donors, which might indicate an important control mechanism. The biological function exemplified by the phosphorylation of viral proteins remains obscure and poses many challenging problems.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grants AI-08421, AI-02032, and 5-K3-AI-07021 (G.A.G.) and training grant AI-69 from the National Institute of Allergy and Infectious Diseases. Howell W. Rogers is a postdoctoral trainee (AI-69).

We acknowledge with thanks the excellent technical assistance of DeSha Montgomery and Judy Prather.

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