STRUCTURAL POLYPEPTIDES OF FROG VIRUS 3, PHOSPHORYLATED PROTEINS

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Received 20 February 1980

1. Introduction

Many DNA and RNA viruses contain phosphorylated structural proteins. This seems to be a general rule when a protein kinase is associated with the virus particle, although it has not been established that the viral enzyme itself is responsible for the phosphorylation. Frog virus 3 (FV 3) contains a >20-fold higher specific activity of protein kinase than several other animal viruses and this enzyme has been purified and characterized as virus specified [1,2]. In an in vitro system the purified FV 3-associated protein kinase will phosphorylate virion polypeptides [3]. Thus it is very likely that some of the structural proteins of the mature virions are phosphorylated.

Here we report the identification of these polypeptides and their internal localization. In addition we show that some of the viral proteins phosphorylated in vivo are still susceptible to an in vitro phosphorylation by the virion protein kinase.

2. Materials and methods

2.1. *Growth and purification of FV 3 virus*

Propagation and radioactive labeling of FV 3 were carried out on BHK cells. For preparation of amino acid-labeled virus, the growth medium (Eagle's minimum essential medium – MEM Eurobio – supplemented with 5% dialyzed calf serum) was replaced after 3 h infection with a similar medium modified to contain 1/10th of the normal level of amino acids and 2 μCi/ml [^{14}C]protein hydrolysate (spec. act. 45 mCi/matom carbon – CEA). To label the virus with [^{32}P]orthophosphate, a phosphate-free MEM medium containing 10 μCi carrier free ^{32}P/ml was used (Amersham). After 36 h the virus was collected and purified as in [4] with the following modifications. The viral suspension obtained after cell lysis and elimination of cellular debris was centrifuged through a sucrose cushion (36%, w/w, in 10 mM Tris–HCl, pH 8.5) for 4 h at 90 000 × g. The resuspended virus pellet was submitted to a velocity sedimentation on linear sucrose gradients (10–40%, w/w, in Tris buffer) at 25 000 × g for 45 min. The virus band was collected and subjected to isopycnic centrifugation on a 35–65%, w/w, sucrose gradient (90 000 × g, 16 h).

One major and a slightly slower sedimenting minor band of virus were located below the middle of the tube. The major faster sedimenting band was harvested, pelleted by centrifugation (100 000 × g, 45 min) and suspended by brief ultrasonic vibration, in 10 mM Tris–HCl (pH 8.5). This purified preparation will be refered to as 'dense virus particle'. Alternatively, unlabeled FV 3 was produced on chorioallantoic membranes of 9 day-old embryonated eggs. After inoculation and incubation at 29°C for 8 days, membranes were collected, diluted with 3 vol. 10 mM Tris–HCl (pH 8.5) buffer and homogenized. The virus was submitted to the complete cycle of purification above except that, as proposed by G. Obert, a 43% sucrose cushion was used instead of 36% sucrose.

2.2. *SDS–polyacrylamide gel electrophoresis*

The samples were submitted to electrophoresis on slab gels [5] using a discontinuous system with regard to pH value [6] and a resolving gel composed of a linear gradient, 10% or 12.5% in acrylamide, as indicated in the legends. Virus suspensions were diluted with an equal volume of dissociating buffer A consisting of 50 mM Tris–HCl (pH 6.8), 2% SDS, 2% 2-mercaptoethanol, 15% glycerol 0.004% bromophenol blue, heated at 100°C for 2 min and electrophoresed at 15 mA/slab gel (30–50 μg proteins/slot).

The gels were stained with Coomassie brilliant blue, destained, dried and exposed to Kodirex films.

2.3. *In vitro phosphorylation of FV 3 polypeptides by the endogenous protein kinase*

The standard reaction was as follows: purified FV 3 (100 μg proteins) was disrupted in 25 mM Tris–HCl (pH 8) buffer, 10 mM DTT, 0.5% NP40 (Nonidet P40) at 30°C for 30 min and proteins phosphorylated by addition of 3 μCi [γ-^{32}P]ATP (spec. act. 15 Ci/mol) 10 mM $MgCl_2$ in 200 μl final vol. Samples were reincubated at 30°C and, at different times, reactions were terminated by addition of 10% trichloroacetic acid, 20 mM Na-pyrophosphate and treated as in [3].

3. Results

3.1. *Polypeptide composition of the virions*

Analysis of FV 3 structural polypeptides by polyacrylamide gel electrophoresis using a discontinuous buffer system revealed the presence of high molecular weight polypeptides (from 90 000 to 140 000) (fig.1) that were not detected in phosphate containing polyacrylamide gels run at pH 7.2 [7,8]. The polypeptide patterns were compared in the case of:

(i) Virus suspensions after velocity or isopycnic centrifugation;

(ii) Viruses produced in different cells.

The main difference in the polypeptide composition of the virus obtained after velocity banding (fig.1.1) and the virus obtained after a subsequent sucrose isopycnic centrifugation (main dense peak,

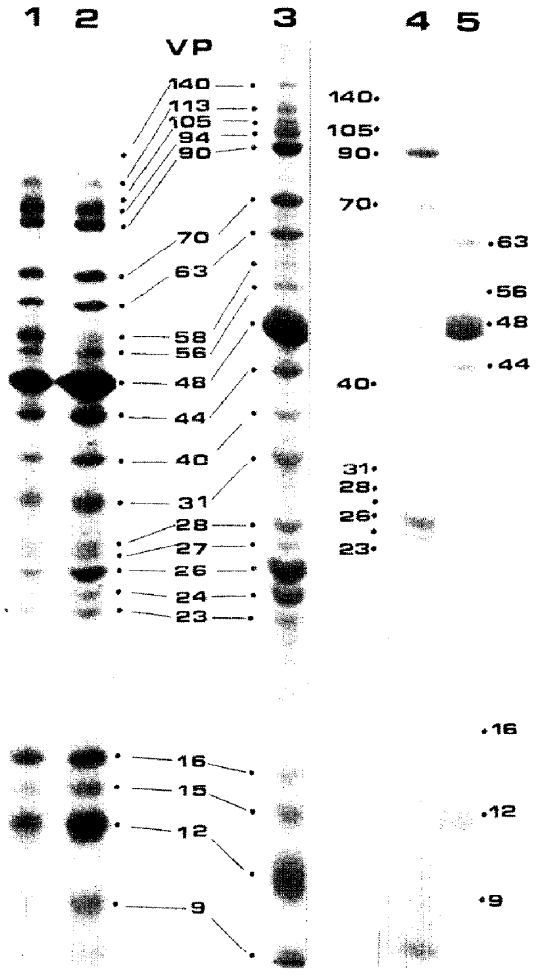

Fig.1. Analysis of FV 3 structural polypeptides by SDS–polyacrylamide gel electrophoresis using a 8–15% linear gradient in acrylamide. Autoradiogram of [^{14}C]polypeptides associated with FV 3 at different steps of virus purification. Virus after sedimentation velocity banding (slot 1), dense virus particles obtained after an additional isopycnic centrifugation (slot 2), Coomassie blue staining of FV 3 grown on chorioallantoic membranes of embryonated eggs (dense particle, slot 3). Analysis of FV 3 core polypeptides (slot 4) and external polypeptides (slot 5) on 12.5% acrylamide gel–Coomassie blue staining. The samples were prepared as follows: virus was disrupted by an incubation in 25 mM Tris–HCl (pH 8), 10 mM dithiothreitol, 0.5% NP40 at 30°C for 1 h. This suspension was sedimented on a sucrose gradient (35–65%, w/w), 4 h, 90 000 × *g*. The visible core band, located below the middle of the gradient, was collected with a syringe and the particles sedimented whereas the release polypeptides were trichloroacetic acid precipitated from the upper third of the gradient, both fractions being redissolved in the dissociating buffer A. The intermediate fraction of the gradient was not analyzed, it may contain polypeptides apparently missing in the core and solubilized fractions. Estimation of polypeptide molecular weights: the migration distance being proportional to the logarithm of the molecular weight, the value of standard proteins were used to derive a curve by the method of least squares to calculate the molecular weight (× 10^{-3}) of viral polypeptides (*E. coli* RNA polymerase, subunits β β' 165, 155, α 39; phosphorylase *b*, 94; bovine serum albumin, 68; catalase, 60; glutamate dehydrogenase, 53; ovalbumin 45; DNase I, 31; chymotrypsinogen 25.7; trypsin inhibitor from soybean 21.5; RNase 13.7; cytochrome *c* 11.7). For more accurate determinations polypeptides of mol. wt < 21 × 10^3 were estimated on 12.5% acrylamide gels and those of higher molecular weight on 10% acrylamide gels. VP, viral polypeptide, mol. wt × 10^{-3}.

fig.1.2) concerns the polypeptide of 58 000 mol. wt which was present in a much smaller amount in the dense virus particles. FV 3 is known to bud at the plasma membrane where it acquires an envelope, no release of unenveloped particles has been described [9–11]. When the purification was performed using the virus of the extracellular medium alone, collected before cell lysis, the majority of the particles were found after the isopycnic centrifugation in the lighter band (not shown); this band was enriched in 58 000 mol. wt polypeptide (not shown) and must contain enveloped particles. Therefore 58 000 mol. wt polypeptide is very likely a component of the virus envelope. Analysis of enveloped particles has been recently developed [12].

For the dense particles, used in all the following experiments, the profiles obtained after autoradiography (virus produced in BHK cells, fig.1.2) or Coomassie blue staining (virus grown on chorioallantoic membranes of embryonated eggs, fig. 1.3) were remarkably similar. 22 predominant size classes of polypeptides were identified and named according to their app. mol. wt over 140 000–9000. Although this pattern has been obtained with four different virus preparations, depending mostly on the conditions of analysis, some variations may be observed in the regions 113 000–105 000 and 27 000–23 000 mol. wt. Treatment of the virus with 0.5% NP40, 10 mM dithiothreitol led to the formation of a core particle [13] (fig.1.4) and solubilized 7 polypeptides (fig.1.5) presumably external components.

3.2. *Identification of the structural phosphoproteins*

Examination of ^{32}P-labeled virions revealed numerous components of different electrophoretic mobilities containing ^{32}P (fig.2A1,4). The ^{32}P label remained associated with the viral polypeptides when disrupted virus was incubated before electrophoresis with DNase, or when proteins were extracted by phenol and treated with chloroform:methanol (1:1). To eliminate any nucleic acid fragments the gels were also immersed after electrophoretic separation in hot trichloroacetic acid according to [14]. The resulting autoradiograph was compared to the one obtained with an untreated gel (fig.2A4,5); the principal difference is the reduced amount of phosphate in the 15 000 mol. wt region after trichloroacetic acid treatment. The main phosphorylated species were found to migrate with app. mol. wt between 113 000 and 90 000, 40 000, 23 000, and slightly <48 000.

3.3. *In vitro phosphorylation by the endogenous protein kinase*

The virion phosphoproteins were compared with the in vitro reaction product of the virus-associated protein kinase to determine if the same molecules could still be phosphorylated. When the in vitro reaction was carried out under the optimal conditions after dissociation of the particle by NP40 [1] 3 zones corresponding to products of mol. wt >70 000, between 30 000 and 20 000 and 16 000–15 000 were heavily phosphorylated (fig.2B2). This results in a diffuse distribution of the polypeptides in the gel, principally of mol. wt ($\times 10^{-3}$): 94, 90, 70, 40, 28, 27, 26, 24, 16, 15 (fig.2B1). Although the phosphorylation reaction was accompanied by a slight release of amino acids in an acid-soluble form (2–4% of ^{14}C-labeled amino acid labeled virions; data not shown), this hydrolysis was not sufficient to explain the extensive modification in the apparent molecular weight of certain polypeptides.

This modification was not observed after an incubation in the absence of ATP or when the phosphorylation reaction was limited by omitting Mg^{2+} or substituting it by Mn^{2+}. In the presence of Mn^{2+}, the addition of phosphate may be antagonized by the activity of the endogenous phosphoprotein phosphatase [1]. Fig.2B3 shows that addition of Mn^{2+} led to the formation of distinct phosphorylated species, some of them comigrating with polypeptides revealed by Coomassie blue staining. A faint phosphorylation was observed even without any addition of Mg^{2+} or Mn^{2+}, only the 90 000 and 105 000 mol. wt polypeptides being labeled; this phosphorylation was abolished in the presence of EDTA (data not shown).

3.4. *Localization of protein kinase and phosphoproteins in the virions*

The protein kinase activity associated with the solubilized external polypeptides or the core particle was measured in vitro. The results reported in table 1 indicate that the totality of the enzyme activity remained associated with the core particle.

To determine if the in vitro hyperphosphorylation may lead to a dissociation of the nucleoprotein complex, the virus was phosphorylated under optimal conditions in the presence of NP40, subviral particles and soluble proteins isolated thereafter and subsequently analysed by SDS–polyacrylamide gel electrophoresis. The solubilized phosphorylated polypeptides were of ~16 000 and 12 000 mol. wt

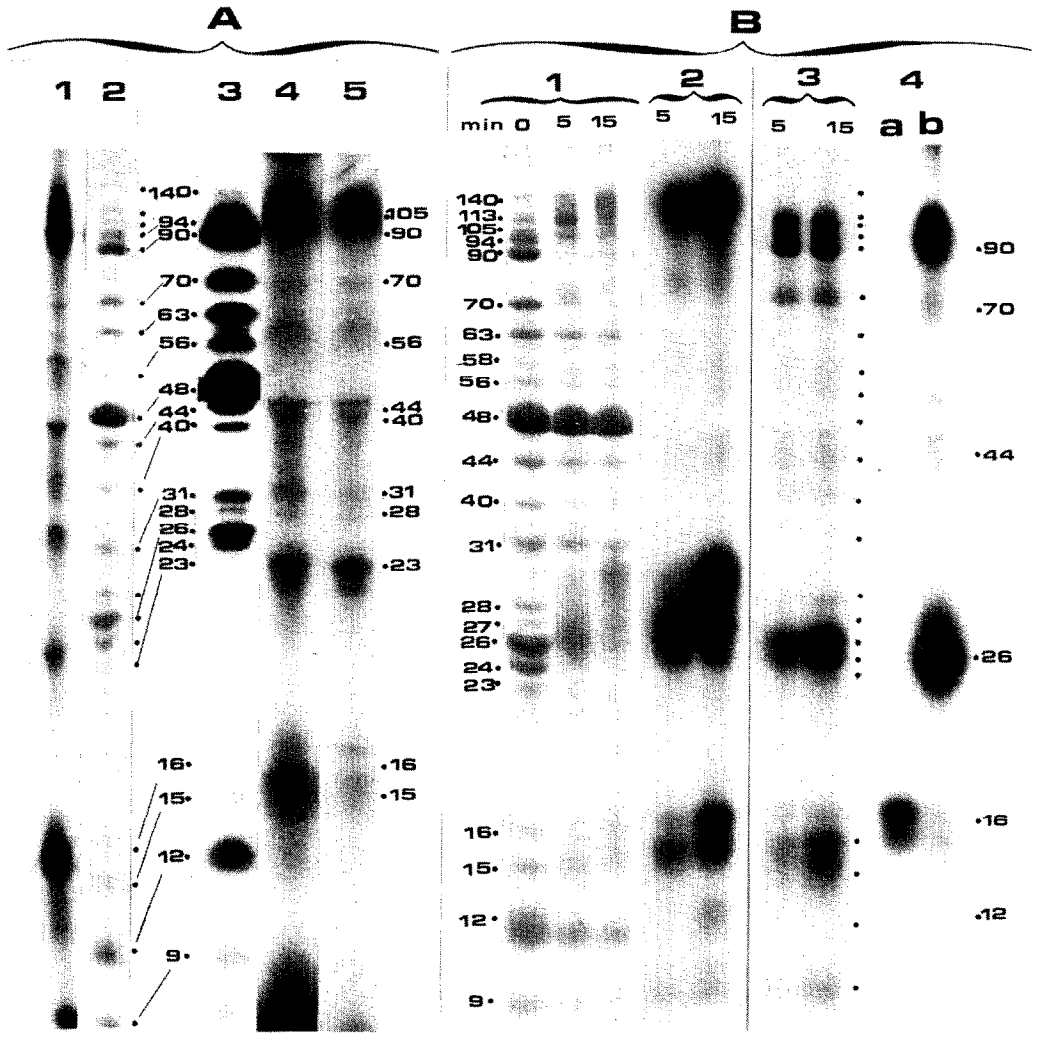

Fig.2. Electrophoretic separation of FV 3 polypeptides phosphorylated in vivo and in vitro. (A) Autoradiogram of [^{32}P]FV 3 labeled in vivo and purified as in section 2 (slot 1) analyzed on 10% acrylamide gel, Coomassie blue staining of the same sample (slot 2). ^{14}C-labeled amino acid labeled FV 3 (slot 3), [^{32}P]FV 3 labeled in vivo (slot 4) and after trichloroacetic acid treatment of the gel (slot 5) as in [14], analysis on 12.5% acrylamide gel (different virus preparation than slot 1). (B) In vitro phosphorylation of FV 3 polypeptides by the endogenous protein kinase. Virus samples were electrophoresed on 10% acrylamide gel. Coomassie blue staining of FV 3 polypeptides after a 0, 5 or 15 min phosphorylation in the standard conditions (slots 1), autoradiogram of the same gel (slots 2). Autoradiogram of proteins phosphorylated in the presence of 10 mM $MnCl_2$ (instead of 10 mM $MgCl_2$) (slots 3). The points localize the bands revealed by Coomassie blue staining. Localization of the in vitro phosphorylated proteins: at the end of a 15 min incubation of the virus in the standard conditions, the reaction mixture was sedimented (1 h, 80 000 × *g*) and the supernatant (solubilized proteins, slot 5a) and the pellet (core particles, slot 5b) electrophoresed separately.

Table 1
Localization of protein kinase in the virions

Fraction	Protein kinase[a] ^{32}P incorp. (pmol/15 min)
Complete virus	5440
Cores	4311
Solubilized proteins	174

[a] Protein kinase was assayed in the standard conditions in section 2, using 170 μg protein for the complete virus and cores or soluble proteins prepared from the same amounts of virus. In all cases 100 μg casein were added as phosphate acceptor

(fig.2B4a), polypeptides which were already liberated from the particle by NP40 treatment alone (fig.1.5). Thus the phosphorylation process did not result in a release of phosphoproteins from the particulate fraction (fig.2B4b). The polypeptide composition of this phosphorylated subviral particle and the core particle in fig.1.4 should be very similar but it cannot be rigorously established since phosphorylation was accompanied by a modification of polypeptide electrophoretic mobility (analog to fig.2B1).

4. Discussion

Regarding FV 3 polypeptide composition, our results correlate well with those in [3]. The slightly different polypeptide patterns obtained by others [15,16] mainly in the region 23 000–27 000 mol. wt might be due to the lower amounts of protein they loaded on the gels. We did not detect the groups of 5 polypeptides between 23 000 and 27 000 mol. wt with dilute virus suspension of high specific activity (2 μg protein/slot). Thus these 5 polypeptides could be selectively lost during the dissociation procedure and/or the electrophoresis when these steps were carried out with dilute virus suspensions, either because they may be bound to DNA and be difficult to release quantitatively or because due to their chemical composition they were preferentially damaged. Additional proof that these polypeptides are structural polypeptides and not contaminants comes from their internal location in the particle (fig.1.4).

Many FV 3 structural polypeptides are susceptible to phosphorylation in vivo as well as in vitro. The predominantly in vitro phosphorylated polypeptides were species of app. mol. wt 113 000–90 000 and 26 000, 24 000. Whereas, in vivo, no phosphorylation of polypeptides 26 000–24 000 was detected, polypeptides 113 000–90 000 were the most heavily phosphorylated molecules. However no additional information is available to explain this different behaviour. Anyhow, these phosphoproteins are components of the cores while 48 000, an external protein, was never found phosphorylated neither in vivo nor in vitro. A similar situation was found for adenovirus since polypeptides internally located are phosphorylated whereas the external proteins of the capsid are not substrates for the virion protein kinase [17]. The degree of phosphorylation may determine whether or not a protein becomes associated with the nucleocapsid [18]. In this respect it would be interesting to know whether the phosphorylation occurs during the maturation of the FV 3 particle or takes place in the cell prior to assembly. The large number of ^{32}P-labeled polypeptides found reflect a relatively non-specific phosphorylation process but the hyperphosphorylation observed in vitro suggests that not all of the available sites on viral proteins are phosphorylated in vivo therefore the in vivo reaction must be in fact well controlled.

Acknowledgements

We are grateful to R. Drillien for his assistance in the preparation of the manuscript and to F. Lecerf for photographic work. This work was supported by grants from INSERM (CRL no. 78 5 122-1) and from the DRET.

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