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# CHARACTERIZATION AND PROPERTIES OF A PROTEIN KINASE ASSOCIATED WITH MURINE SARCOMA-LEUKEMIA VIRUS (MSV-MuLV). ENDOGENOUS PHOSPHORYLATION OF A UNIQUE p10 POLYPEPTIDE

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## SUMMARY

MSV-MuLV virions produced by the rat embryo fibroblast 78 $A_1$ cell line and extensively purified by treatment with trypsin (M. Rucheton, D. Blaas and Ph. Jeanteur, Biochimie, in press) were shown to contain an endogenous cyclic AMP independent protein kinase activity which is located within the viral core. Enzymatic properties were investigated and an apparent molecular weight of 40,000 daltons determined by gel filtration. It shows a marked preference for acidic exogenous substrates and especially α-casein. During endogenous reaction, only the viral protein p10 was phosphorylated.

## INTRODUCTION

The occurrence of a protein kinase activity independent of cyclic AMP seems to be a common feature of enveloped animal viruses, whether DNA or RNA-containing (for review, see ref. 1). Mention of such an activity was made in various retroviruses (2-5). The most extensive studies, however, have been carried out on the protein kinase from avian myeloblastosis virus (AMV) which has been significantly purified and well characterized (6,7).

We have previously reported that murine sarcoma-leukemia virus (Moloney MSV-MuLV) extensively purified by trypsin treatment (8) contained an endogenous protein kinase activity (3). The present paper further reports on the enzymological characterization of this activity, its localization in viral cores and the identification of a unique viral phosphate acceptor protein.

## MATERIALS AND METHODS

MSV-MuLV virus was produced by the chronically infected $78A_1$ rat cell line (9) and purified by polyethylen-glycol precipitation followed by trypsin treatment (8). The pellet of purified virus was resuspended at 10 mg/ml in buffer A (10 mM Na phosphate buffer pH 7, 100 mM NaCl, 1 mM EDTA).

Poly(rA)-oligo(dT) stimulated reverse transcriptase activity (RDDP) was determined as described previously (8).

Protein-kinase activity was determined in a final volume of 0.1 ml of a reaction-mixture containing 2.5 micromoles Tris-HCl pH 8, 1 micromole DTT, 1 micromole $MgCl_2$, 0.2% w/v Nonidet NP 40, 2 nanomoles $\gamma$-($^{32}$P) ATP (approximately 1000 cpm/picomole) and eventually 250 micrograms of α-casein (Merck) as exogenous phosphate acceptor when indicated. Incubation was carried out for 30 minutes at 37°C and stopped by addition of 1 ml of 10% trichloracetic acid containing 20 mM sodium pyrophosphate and 0.2 mg ATP. Acid precipitable radio-

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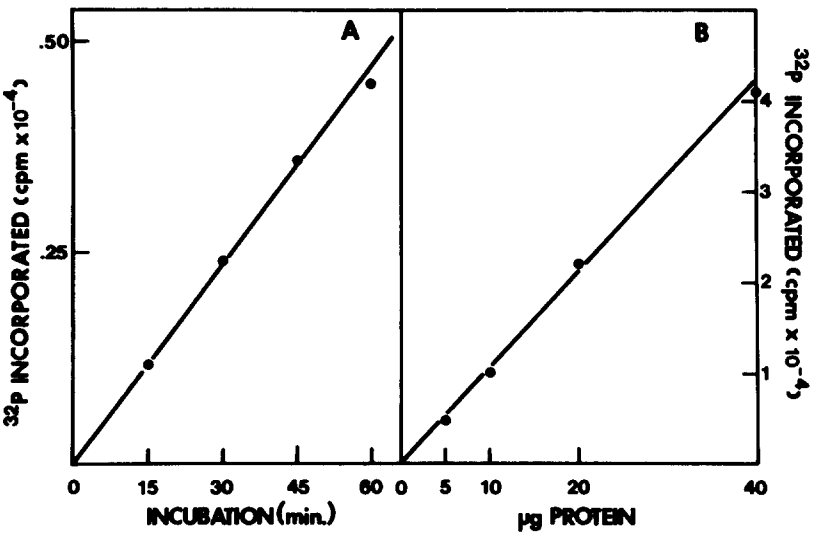


Figure 1 : Reaction kinetics versus time (A) and enzyme concentration (B).

A) A 0.6 ml standard endogenous reaction mixture (no α-casein added) containing 60 micrograms of viral protein was incubated at 37°C and 0.1 ml aliquots withdrawn at indicated times and processed as described in Materials and Methods.

B) Several 0.1 ml reaction mixtures containing various concentrations of viral protein were incubated under standard conditions in the presence of α-casein and processed as described in Materials and Methods.

activity was collected on fiberglass filter discs GF/C (Whatman) and counted in a Nuclear Chicago gas flow counter. γ-($^{32}$P)-ATP was prepared according to Glynn and Chappell (10) using carrier-free $H_3(^{32}P)O_4$ from CEA (Saclay, France).

Protein concentration was determined according to Bradford (11).

## RESULTS

### Identification of a protein kinase activity

When supplied with γ($^{32}$P)-ATP, purified virus disrupted with a non-ionic detergent incorporates ($^{32}$P) into acid-insoluble material in a linear manner for up to at least one hour under standard assay conditions (Figure 1 A). When α-casein is added as an exogenous substrate, incorporation remains linear versus enzyme concentration up to at least 40 micrograms viral proteins per standard assay (Figure 1 B). Controls reported in Table 1 established that ($^{32}$P) was covalently linked to viral proteins by a phosphoester bond, as confirmed by the presence of phosphoserin and phosphothreonin (respectively 66 and 34%) in mild acid hydrolysates (not shown). The low recovery of hot TCA

Table 1 : Evidence for ($^{32}P$) incorporation into phosphoprotein.

| Treatment | Remaining acid-insoluble radioactivity (% of control) |
|---|---|
| 1) TCA 0°C (control) | 100 |
| 2) TCA 90°C, 20 minutes | 20 |
| 3) Chloroform-ethanol (2:1), 1 hour, 25°C | 101 |
| 4) Micrococcal nuclease (125 unit/ml), 1 hour, 37°C | 101 |
| 5) Proteinase K (25 µg/ml in 0.375% SDS), 1 hour, 37°C | 2 |
| 6) NaOH (1N), 1 hour, 30°C | 17 |

Incubation was carried out under standard conditions in the presence of 0.1 mg/ml of viral protein. After 60 min. of incubation, reaction mixtures 1 to 3 were precipitated with 10% TCA, filtered on GF/C filters which were treated as indicated before counting. Reaction mixtures 4 to 6 were exposed to the indicated set of conditions before precipitation and collection on filters as above.

Table 2 : Efficiency of various exogenous proteins as phosphate acceptors.

| Protein | ($^{32}P$) incorporated (pmol/min/mg) |
|---|---|
| No (endogenous reaction) | 1.2 |
| α-Casein | 17.0 |
| Phosvitin | 9.1 |
| Histone IIa | 1.1 |
| Protamine sulfate | 2.1 |

Incubation was carried out under standard conditions in the presence of 0.25 mg/ml of various substrats and 0.1 mg/ml of viral proteins. Calf thymus histones (IIa), and phosvitin were from Sigma, protamine sulfate from NBC.

resistant material may be explained by the small size (9,100 daltons) of the viral polypeptide substrate (see below).

Enzymatic properties of protein kinase

The ability of various proteins to serve as exogenous phosphate acceptor for the viral protein kinase bas been investigated (Table 2). Basic proteins like histones and protamine had very low, if any, acceptor activity. Acidic proteins like phosvitin and especially α-casein were efficient acceptors, the latter one resulting in about 15-fold stimulation of the endogenous reaction. α-Casein was therefore selected as the standard substrate for investigating the requirements for optimal protein kinase activity.

Table 3 : Requirements for MSV-MuLV protein kinase reaction.

| Reaction components | ($^{32}$P) incorporated (% of complete reaction mixture)[a] |
|---|---|
| - Nonidet NP40 | 6 |
| - $MgCl_2$ | 7 |
| - DTT | 92 |
| + NaCl (0.6 M) | 15 |
| + Cyclic AMP (1 µM) | 94 |
| + Rabbit muscle protein kinase inhibitor (0.7 Unit/ml)[b] | 100 |
| + Hemin (150 µM) | 20 |

[a] Reaction mixture as described in "Materials and Methods" containing 0.25 mg α-casein and 10 µg of viral protein in 0.1 ml.

[b] Kindly supplied by Dr J. Demaille, 1 unit of inhibitory activity is defined as the amount of inhibiting a protein kinase incorporating 30 picomoles Pi/min. to an extent of 50% (12).

Requirements for optimum kinase activity and the influence of various effectors are summarized in Table 3. The presence of a non-ionic detergent (NP40) appeared to be a stringent condition for activity. An optimum pH between 7.5 and 8 was observed with Tris-HCl as a buffer with only a small decrease in activity up to pH 9. Omission of $Mg^{++}$ resulted in a nearly complete loss of activity with an optimum being reached at about 15 mM under otherwise standard assay conditions. However, α-casein substrate started to precipitate at this $Mg^{++}$ concentration. A standard concentration of 10 mM was therefore adopted. Addition of NaCl was progressively inhibitory and reached 85% for 0.6 M NaCl. When varying ATP concentrations, a maximum of activity was reached at about 40 µM and a $K_M$ value for ATP of 20 µM could be determined under standard assay conditions with α-casein as phosphate acceptor. Although insufficient to establish excess ATP conditions, a standard concentration of 20 µM was selected in order to maintain a manageable level of incorporated counts especially in the case of the endogenous reaction. Neither cyclic AMP nor an excess of the rabbit muscle inhibitor (12) which is diagnostic for the catalytic subunits of cyclic AMP regulated protein kinase had any significant effect on the reaction, whether endogenous or α-casein stimulated. Hemin, a known inhibitor of various protein kinases cAMP dependent (13) or independent (14,15), slightly inhibits

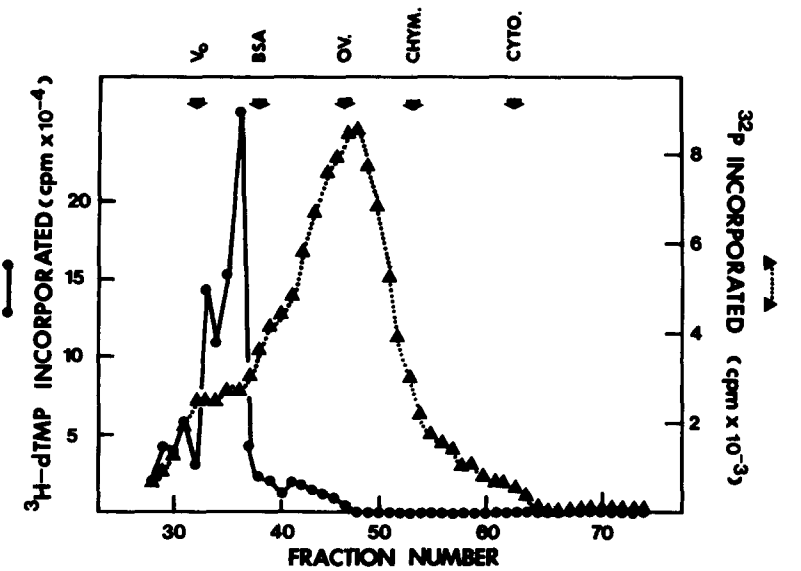


Figure 2 : Behavior of RDDP and protein kinase from detergent disrupted virus on Sephadex G-75.

4.3 mg of viral protein were incubated at 37°C for 5 min. in 0.5 ml disruption buffer (10 mM K phosphate buffer pH 8, 1 M KCl, 5 mM EDTA, 10 mM β-mercaptoethanol, 2% Triton X-100, 1 mM phenylmethylsulfonylfluoride) centrifuged at low speed to remove debris and layered onto a 1.2 x 75 cm Sephadex G-75 column equilibrated in buffer B. Elution was carried out by the same buffer under a head pressure of 28 cm. 1 ml fractions were collected and assayed for RDDP and protein kinase activities. The positions of marker proteins are indicated by arrows.

the phosphorylation of the endogenous substrate (not shown) and to a larger extent that of α-casein.

## Molecular weight

When purified virus was disrupted in the presence of 2% Triton X-100 and applied to a Sephadex G-75 column equilibrated with buffer B (10 mM K phosphate buffer pH 8, 150 mM KCl, 1 mM EDTA, 5 mM β-mercaptoethanol, 0.2% NP40° (Figure 2), protein kinase eluted at a position corresponding to an apparent molecular weight of about 40,000 with reference to known markers. In the same experiment, RDDP had an apparent molecular weight of 83,000 in good agreement with published values for Friend and Moloney MuLV (16,17). Significant amounts of kinase activity eluted closer to the void volume and overlapped the RDDP region indicating a tendency of the kinase to aggregate with other viral components. Similar results were obtained with Sephadex G-100 but both enzymes were abnormally retarded on Sephacryl S-200 suggesting an

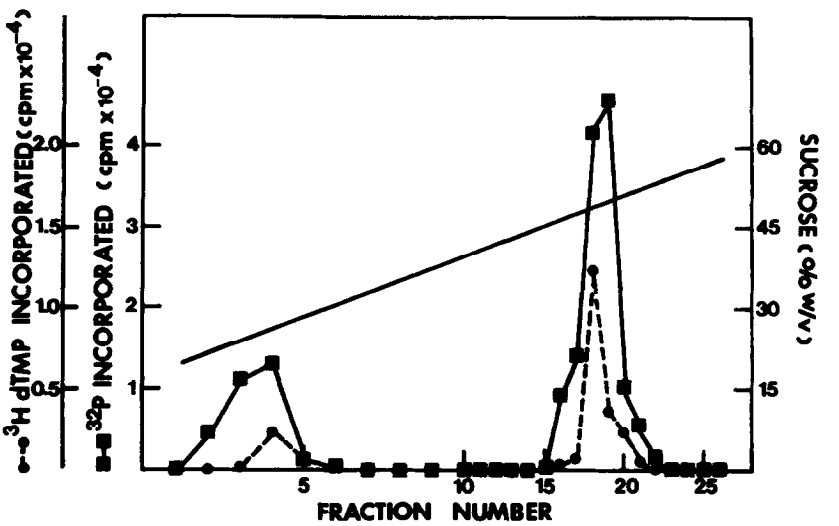


Figure 3 : Location of protein kinase in viral cores.

Viral cores were prepared by a modification of Stromberg's procedure (19). Two milligrams of viral protein were incubated at 37°C for 10 min. in buffer A containing 2 mM β-mercaptoethanol and 2% Sterox SL. The cooled suspension was then layered on top of a sucrose gradient (30-80% w/w in the above buffer without detergent) and centrifuged for 5 hrs at 50,000 rpm (IEC Rotor 498). 0.1 ml fractions were collected by a peristaltic pump and assayed for sucrose concentration (by means of refractive index), RDDP and protein kinase activity.

interaction with the matrix (18). We have so far been unable to perform this kind of experiment with protein kinase partially purified due to the very low recovery of activity.

## Localization of kinase activity within virus cores

Establishing that a protein belongs to the viral core can be regarded as strong evidence in support of its being a genuine constituent of the virion. Reverse transcriptase, unanimously recognized as a viral component, is indeed located within the viral core and can therefore serve as a reference.

Evidence that protein kinase is also located in the viral core already suggested by the fact that it withstood the trypsin digestion step during virus purification (8), is confirmed by the demonstration that viral cores purified from detergent treated virions by sucrose gradient centrifugation do contain the majority of both RDDP and protein kinase (Figure 3).

## Identification of the viral phosphate acceptor

When trypsin purified virus was incubated with ($^{32}$P)γ-ATP in the presence

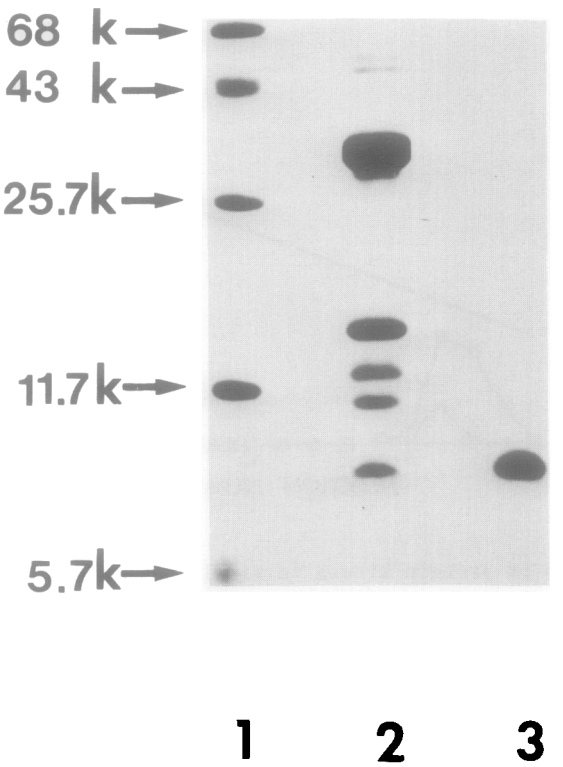


Figure 4 : Identification of the endogenous substrate by SDS acrylamide gel electrophoresis.

A 1 ml endogenous reaction mixture (no α-casein) containing 100 micrograms of viral protein was incubated, processed for electrophoresis, run and stained (track 2) along with reference marker proteins (track 1) according to Laemmli (20) except that the resolving gel contained 14.6% acrylamide and 0.4% bis-acrylamide. The autoradiogram of track 2 is shown in track 3. The following markers were used : bovine serum albumin (68 K), ovalbumin (43 K), chymotrypsinogen (25.7 K), cytochrome C (11.7 K) and insulin (5.7 K).

of NP40 (endogenous reaction) and viral proteins were subsequently analysed by gel electrophoresis, the autoradiograph of Figure 4 (Track 3) was produced. Only one labeled phosphoprotein band, comigrating with viral structural protein p10 could be detected after one day exposure time. A much longer exposure (3 days) led to the appearance of several minor, very weakly labeled bands (not shown), none of which corresponding to major structural proteins of the virus (Figure 4, Track 2).

## DISCUSSION

The present work establishes the occurrence of a protein kinase in MSV-MuLV as a genuine constituent of the virion by ruling out the possibility of

a cellular cytoplasmic enzyme being adventitiously wrapped with the virus during the budding process. Three lines of evidence are in support of this contention : 1) the protein kinase activity is not destroyed by the trypsin treatment used to purify the virus and which attacks only surface glycoproteins (21) ; 2) its activity is dependent on the presence of a non-ionic detergent as required to disrupt the virion ; 3) its remains associated with the viral core along with RDDP. A similar localization has been demonstrated for RD114 (5).

As a common feature with all other viral kinases described so far (1,2,6, 22) the MSV-MuLV enzyme is insensitive to cAMP. Furthermore, the possibility that it could be a free catalytic subunit of an otherwise cAMP dependent kinase is excluded by its insensitivity to the specific inhibitor from rabbit muscle (12). The enzyme shows a marked preference for acidic proteins like α-casein and phosvitin as exogenous substrates as does the AMV protein kinase (6). Its apparent molecular weight determined as about 40,000 by gel filtration is also close to the value measured for the AMV protein kinase (45,000) (6).

Endogenous phosphorylation results in the labeling of a unique polypeptide with the same apparent mobility as structural protein p10. This protein has been shown to occur in phosphorylated form as the second major phosphoprotein after p12 only in type C viruses which have been propagated in rat cells (5,23,24). This situation applies to the $78A_1$ cell line used in this work and which is derived from rat embryo fibroblasts chronically infected by the Moloney strain of MSV-MuLV (9). We have confirmed that both p10 and p12 were phosphorylated after in vivo labeling of $78A_1$ cells with ($^{32}P$) orthophosphate (results not shown). Our failure to phosphorylate p12 may be explained in different ways. Some specificity factor required for p12 phosphorylation may be lacking or inoperative in our trypsin purified viral preparation. Alternatively, p12 may not be phosphorylatable in vitro either because it can only be phosphorylated at some precursor level (22,25) or as a result of its being already maximally phosphorylated. This latter explanation does not fit well with the observation of multiple phosphorylated states of p12 (26) as the least phosphorylated species should have sites available for further phosphorylation in vitro. In any case, no labeling was detected in association with the RDDP band around 80,000 (16,17, Rucheton et al., unpublished results) in agreement with recent data in the AMV system (6).

Another question which remains open is whether the protein kinase described here and associated with the viral core is viral-coded or of cellular origin. Should it be virus specific, it would have to be encoded in one of the four genes recognized in RNA tumor viruses : gag, pol, env and src. (27). The product of avian src gene has recently been detected by two groups (28,29) by immunoprecipitation from extracts of RSV transformed cells and identified

as a protein kinase (30). The protein kinase associated with avian retroviruses and the src gene product appear to be clearly different both by their size, respectively 45,000 (6) and 60,000 (30), and the fact that the latter does not seem to be present in virions (30). No such identification has yet been made for the MSV-MuLV src gene and speculations as to its possible relevance to the present enzyme are therefore premature.

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