In Vivo and In Vitro Phosphorylation of Murine Mammary Tumour Virus Proteins

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SUMMARY

A comparative study of in vitro and in vivo phosphorylation of murine mammary tumour virus, a type B RNA virus, is reported. The protein kinase activity associated with murine mammary tumour virus catalysed the in vitro phosphorylation of endogenous virus polypeptides. This kinase activity required a divalent metal cation, a non-ionic detergent, and was stimulated in the presence of dithiothreitol. Exogenous cyclic AMP was not required. The ^3P-labelled products of the in vitro reaction were completely sensitive to pronase digestion and the phosphate was attached mainly by phosphomonoester linkage to serine residues. As determined by SDS-polyacrylamide gel electrophoresis, heterogeneous labelling of major and minor virus polypeptides was observed under in vitro conditions.

In contrast, the in vivo labelling of type B virus produced by a continuous cell line (MuMT-73), established from pooled mammary adenocarcinomas of Balb/cfC3H mice, demonstrated specific phosphoproteins associated with murine mammary tumour virus. The major phosphorylated proteins were found to have mol. wt. of 18000 and 12000 (p18 and p12) after isolation by molecular sieving chromatography and analysis by gel electrophoresis.

INTRODUCTION

Strand & August (1970) initially reported protein kinase activities associated with Rauscher leukaemia virus (RLV), avian myeloblastosis virus (AMV), and vesicular stomatitis virus (VSV); however, these enzymic activities were absent from preparations of polio- and adenoviruses. In addition, protein kinase activities have been demonstrated to be associated with diverse RNA and DNA viruses, including Sendai (Roux & Kolakofsky, 1974), vaccinia (Paoletti & Moss, 1972; Rosemond & Moss, 1973), frog virus 3 (Roux & Kolakofsky, 1974), equine herpesvirus (Randall et al. 1972) and RNA tumour viruses isolated from diverse hosts (Hatanaka et al. 1972). In most instances, the origin of these kinase activities, whether virus or cellular, has not been ascertained; however, Silberstein & August (1976) demonstrated that frog virus 3 protein kinase was virus-coded. With regard to the latter, Collett & Erikson (1978) recently reported that the src gene product of avian sarcoma virus was a protein kinase; the relationship of this enzymic activity to phosphoproteins or protein kinase activities associated with RNA tumour viruses, however, remains to be elucidated.

Possible functional roles for protein kinase activities have been investigated by com-
parative studies of in vitro and in vivo phosphorylation of virus proteins. For example, both in vivo and in vitro phosphorylation of VSV yields mainly one phosphoprotein, i.e. NS (Moyer & Summers, 1974). In contrast, vaccinia contains a low mol. wt., core-associated phosphoprotein after in vivo labelling and no major structural proteins were phosphorylated under in vitro conditions (Rosemond & Moss, 1973). That an associated virus protein kinase is not a requisite for phosphorylation of virus structural proteins is amply demonstrated by three viruses which lack this enzymic activity. For example, Sindbis contained predominantly E2 as the phosphoprotein (Waite et al. 1974) and all of the major structural proteins of SV40 (Tan & Sokol, 1972) and adenovirus (Russell et al. 1972) are phosphorylated.

Comparative studies of mammalian type C viruses by Pal et al. (1975) demonstrated that viruses of mouse, rat and feline origins contained a predominant 12000 mol. wt. phosphoprotein (pp12). In addition, rat viruses contained a 10000 mol. wt. phosphoprotein (pp10). In contrast, endogenous viruses of primate origin (baboon endogenous virus and RD-114) possessed a phosphorylated protein with a mol. wt. of 15000 (pp15).

Type B viruses (MuMTV) have been less intensively investigated. From a number of assayed RNA tumour viruses, Hatanaka et al. (1972) observed the highest level of virus-associated protein kinase activity for MuMTV; however, the extent and specificity of the in vitro phosphorylation was not monitored and in vivo phosphoprotein(s) associated with MuMTV was not investigated. The purpose of this study was the investigation of in vitro and in vivo phosphorylation of MuMTV proteins.

METHODS

Chemicals and radiochemicals. Adenosine 3', 5' cyclic monophosphoric acid, calf thymus histone (arginine-rich), O-phospho-L-serine, dl-O-phosphothreonine, salmon protamine sulphate and alkaline phosphatase (E. coli) were purchased from Sigma Chemical Company, St Louis, Missouri, U.S.A. DNase I (electrophoretically pure and further treated to remove RNase by iodoacetate treatment (Zimmerman & Sandeen, 1966), pancreatic RNase A (previously boiled for 10 min. to remove DNase) and phospholipase C were obtained from Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A. Pronase and Nonidet P40 (NP40) were products of Miles-Seravac Laboratories, Kankakee, Illinois, U.S.A. and International Shell Chemical Co., London, U.K. respectively. γ-32P-ATP (sp. act. 12.6 Ci/mmol) was obtained from Amersham/Searle (Arlington Heights, Illinois, U.S.A.). Carrier-free 32P-phosphate (500 m Ci/mmol) and a mixture of 3H-labelled amino acids (1.3 to 88.5 Ci/mmol) were purchased from New England Nuclear Boston, Mass., U.S.A.).

In vivo labelling of MuMTV phosphoproteins. A continuous cell line (MuMT-73), established from spontaneous Balb/cfC3H mammary adenocarcinomas, was employed as a source of radiolabelled MuMTV (Sarkar et al. 1977). These cells were maintained in Hanks' Eagle's minimum essential medium, supplemented with 15% foetal calf serum and insulin (10 μg/ml). For the labelling of virus proteins, five confluent flasks (75 cm², Falcon Plastics) were each washed twice with phosphate-free Hanks’ Eagle's medium and re-fed with phosphate-free medium containing 10% foetal calf serum, insulin (10 μg/ml), hydrocortisone (10 μg/ml), carrier-free 32P-phosphate (70 μCi/ml) and 3H-labelled amino acid mixture (10 μCi/ml). After 24 h, the culture fluids were pooled and radiolabelled virus was purified by sedimentation and isopycnic centrifugation as previously described (Sarkar et al. 1977).

Identification of in vivo labelled MuMTV phosphoproteins. Radiolabelled virus pellets were re-suspended in 0.01 M-tris-HCl, pH 7.8, containing 10 mM-EDTA, 0.2% (v/v) β-mercaptoethanol, 1% (w/v) SDS and 4.5 mg unlabelled RIII-MuMTV (purified from
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RIII milk) in a total vol. of 1.0 ml. Virions were disrupted by heating at 50°C for 30 min and allowed to cool to room temperature. Sucrose was then added to a final concentration of 10% (w/v) and the preparation was layered on a Sephadex G-200 column (1:5 x 90 cm) equilibrated with 0.01 M-tris-HCl, pH 7.8, containing 1 mM-EDTA and 1% SDS (Dion et al. 1977). One ml fractions were collected at a flow rate of 8 ml/h and a 200 µl sample of each fraction was counted after the addition of Hydromix (Yorktown Research, Hackensack, New Jersey, U.S.A.). Identification of MuMTV phosphoproteins was accomplished by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), as described below.

In vitro protein kinase assay. MuMTV was purified from RIII mouse milk as described previously (Dion & Moore, 1973) and the virus pellet was resuspended in 0.01 M-tris-HCl, pH 8.3, at a concentration of 1 mg virus protein/ml. Each assay contained 3 µg virus protein, 25.0 mM-tris-HCl, pH 8.6, 1.5 mM-Mg²⁺, 1.0 mM-dithiothreitol (DTT), 0.05% NP40 and 0.1 nmol γ³²P-ATP in a final vol. of 40 µl, as described by Hatanaka et al. (1972). Unless otherwise specified, incubation proceeded for 20 min at 22°C. The reactions were terminated by placing the samples on ice and by the addition of 100 µg bovine serum albumin (BSA) and 2.5 ml 5% trichloroacetic acid (TCA). After 10 min the acid-insoluble γ³²P was collected on Millipore filters and counted in 2.5-bis-(2-(5-tert-butyl-benzoxazolyl)-thiophene (BBOT)-toluene (3:2 g/l) in a Packard liquid scintillation spectrometer. γ³²P counts obtained after a 0 min incubation of a complete reaction mixture were subtracted as background.

Polyacrylamide gel electrophoresis (PAGE). TCA precipitates were washed with 1 ml 5% TCA and re-centrifuged. After draining, the precipitates were resuspended in 50 µl of 0.01 M-sodium phosphate, pH 7.8, 1% SDS, 1% β-mercaptoethanol (Sarkar & Dion, 1975) and incubated to 60°C for 30 min. After cooling, glycerol, containing bromphenol blue as tracking dye, was added to a final concentration of 10%. Electrophoretic analyses were performed on 7.5% polyacrylamide gels at 3 mA/gel for 30 min, followed by 3 to 3.5 h at 7 mA/gel. Following electrophoresis, the gels were frozen, sectioned at 1.2 mm intervals and each section was dissolved in 100 µl 30% H₂O₂ overnight at 60°C. After cooling, samples were counted in Aquasol (New England Nuclear, Boston, Mass., U.S.A.). Unlabelled standards containing approx. 50 µg virus protein/gel were electrophoresed simultaneously and stained with Coomassie blue R-250.

Phosphate acceptor: enzyme and chemical digestions. A tenfold standard in vitro reaction mixture was incubated at 22°C for 20 min and the reaction was terminated by boiling for 3 min. Samples (40 µl) containing 10 µg DNase I, RNase A, pronase or phospholipase C were incubated at 37°C for 60 min. These enzyme digestions were stopped by adding 100 µg BSA and 2.5 ml 5% TCA. Half of each digestion mixture was filtered as above to determine acid-insoluble γ³²P. The other half was prepared for electrophoretic analysis on 7.5% gels as described above. Alkaline phosphatase and various chemical digestions were performed according to Strand & August (1971) on γ³²P-labelled MuMTV proteins prepared as above.

Phosphate acceptor: analysis for phosphoserine and phosphothreonine. TCA-insoluble precipitates were washed with 1 ml 5% TCA, re-centrifuged and the pellets were re-suspended in 0.01 M-tris-HCl, pH 8.3. For partial acid hydrolysis, an equal vol. of 12 N-HCl was added and the sample was incubated at 110°C for 30 min in a sealed ampoule. After cooling, the samples were evaporated to dryness in vacuo, re-suspended in water and applied to a 10 x 20 cm cellulose thin layer chromatography plate (Avicel, Analtech, Newark, Delaware). Phosphoserine and phosphothreonine (1 µmol of each) were applied as standards. Thin layer electrophoresis was performed at 500 V for 180 min. in 2:5% (w/v) formic acid, 7:8% (w/v) acetic acid, pH 1.9. The standards were developed by spraying with ninhydrin. To locate γ³²P-labelled compounds, 5 mm strips were scraped from the plate and counted in 10 ml BBOT-toluene scintillation fluid.
Table 1. Requirements for protein kinase activity

<table>
<thead>
<tr>
<th>Reaction mixture*</th>
<th>(32p) incorporation (ct/min)</th>
<th>% of complete</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>21,578</td>
<td>100.0</td>
</tr>
<tr>
<td>Omit (\text{MgCl}_2)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Omit DTT</td>
<td>14,677</td>
<td>68.0</td>
</tr>
<tr>
<td>Omit NP40</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Omit (\text{MgCl}_2), Add Mn ((\text{CH}_3\text{COO})_2)</td>
<td>15,531</td>
<td>72.3</td>
</tr>
<tr>
<td>Add NaF</td>
<td>1,399</td>
<td>6.5</td>
</tr>
<tr>
<td>Add cyclic AMP</td>
<td>20,989</td>
<td>97.3</td>
</tr>
<tr>
<td>Add protamine</td>
<td>20,616</td>
<td>95.5</td>
</tr>
</tbody>
</table>

* The complete reaction mixture is described in the Methods. The substitution of magnesium chloride was equimolar, i.e. 1.5 mM. Where indicated, NaF, cAMP or protamine were added to final concentrations of 50 mM, 1 \(\mu\)M and 75 \(\mu\)g/ml, respectively.

RESULTS

Requirements for in vitro protein kinase activity

In determining the requirements for the protein kinase activity of MuMTV, the standard in vitro reaction mixture described in the Methods was employed. As shown in Table 1, the optimal incorporation of \(\gamma-32p\)-ATP was dependent upon the presence of a non-ionic detergent, a divalent metal cation and a reducing agent, dithiothreitol. In separate experiments (not shown), the optimal \(\text{Mg}^{2+}\) concentration was found to be 2.5 mM; however, the results to be described were performed in the presence of 1.5 mM-Mg\(^{2+}\) in order to be consistent with the conditions employed by Hatanaka et al. (1972). Incorporation in the presence of 2.5 mM-Mg\(^{2+}\) was approx. 25\% greater than at 1.5 mM. Equimolar Mn\(^{2+}\) partially replaced Mg\(^{2+}\) in fulfilling the divalent cation requirement. Fluoride, which was reported by Strand & August (1971) to stimulate the in vitro phosphorylation of Rauscher leukaemia virus proteins, strongly inhibited the phosphorylation of MuMTV proteins for all concentrations in the range of 2.5 to 56.2 mM. In addition, Table 1 demonstrates that the addition of cAMP or protamine had little effect on the incorporation of \(32p\). Subsequent studies (not shown) with arginine-rich histone resulted in a twofold stimulation of incorporation when added at a final concentration of 3 \(\mu\)g/ml. Finally, the incorporation of \(32p\) catalysed by the endogenous protein kinase of MuMTV was linear for approx. 20 min at 22°C. The latter observation corroborates the finding of Hatanaka et al. (1972).

The nature of in vitro phosphate acceptors

The nature of endogenous phosphate acceptors of MuMTV was assayed by enzymic and chemical digestions which are described in the Methods. As shown in Table 2 (experiment 1), the digestion of in vitro labelled MuMTV with DNase or RNase did not appreciably solubilize the label; however, digestion with pronase rendered the label completely acid soluble. In view of the latter result, the partial solubilization of \(32p\) label with phospholipase C is probably due to contaminating proteases. Further data regarding phosphate transfer to protein and the nature of the phosphate-protein linkage are given in experiment 2 (Table 2). Indicative of phosphate monoester linkage to serine and/or threonine residues, \(32p\) label was solubilized by digestion with alkaline phosphatase or by NaOH hydrolysis. Treatment of \(32p\)-labelled MuMTV proteins with succinic acid plus hydroxylamine resulted in only a slight reduction in acid precipitability, precluding the possibility of large amounts of phosphohistidine or the presence of phosphorylated adenosine triphosphatase.

In order to determine the amino acid acceptor for phosphate transfer from \(\gamma-32p\)-ATP,
Table 2. Nature of the in vitro $^{32}$P-acceptor

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$^{32}$P (ct/min)</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control*</td>
<td>11713</td>
<td>(100.0)</td>
</tr>
<tr>
<td>DNase</td>
<td>10051</td>
<td>85.8</td>
</tr>
<tr>
<td>RNase</td>
<td>14183</td>
<td>121.1</td>
</tr>
<tr>
<td>Pronase</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Phospholipase C</td>
<td>7857</td>
<td>67.1</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>13476</td>
<td>(100.0)</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>7116</td>
<td>52.8</td>
</tr>
<tr>
<td>Succinic acid</td>
<td>11676</td>
<td>86.7</td>
</tr>
<tr>
<td>Succinic acid plus</td>
<td>11331</td>
<td>84.1</td>
</tr>
<tr>
<td>hydroxylamine</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NaOH</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* A 20-fold reaction mixture was prepared as described in the Methods. Digestions with DNase, RNase, pronase or phospholipase C (experiment 1) were performed at a final concentration of 250 μg enzyme/ml. Chemical and enzymic digestions (experiment 2) were performed according to Strand & August (1971).

Fig. 1. Thin layer electrophoretic analysis of partial acid hydrolysate of MuMTV labelled with $^{32}$P by the in vitro virus protein kinase. Conditions of incubation and electrophoretic analysis are given in the Methods. P-SER, phosphoserine; P-THREO, phosphothreonine; Pi, inorganic phosphate.

partial acid hydrolysates of $^{32}$P-labelled MuMTV were analysed by thin layer electrophoresis. Fig. 1 illustrates the distribution of $^{32}$P label as a function of electrophoretic mobility at pH 11.9. Under these conditions, $^{32}$P label is associated with inorganic phosphate (Pi), presumably incompletely hydrolysed phosphopeptides and with phosphoserine as located by ninhydrin staining of standards electrophoresed simultaneously. A smaller amount of labelling was associated with phosphothreonine; however, the proximity of the latter to incompletely hydrolysed phosphopeptides precludes obtaining a ratio of $^{32}$P labelling of phosphoserine to phosphothreonine. Nevertheless, it is apparent that serine is a more frequent acceptor of phosphate than is threonine.
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Fig. 2. Co-electrophoresis of $^{32}$P- and $^{125}$I-labelled MuMTV. $^{32}$P labelling of MuMTV was performed by in vitro phosphorylation utilizing the endogenous protein kinase as described in the Methods. The chloramine T procedure of Greenwood et al. (1963) was employed for $^{125}$I labelling. Protein designations correspond to the nomenclature proposed by August et al. (1974), i.e. mol. wt. $\times 10^{-3}$. ● $^{125}$I label; ▲ $^{32}$P label. Arrows designate positions of casein mol. wt. markers prepared by the technique of Enami & Nandi (1977) from virus-free C57Bl mouse milk. A, pp44; B, pp26; C, pp22. Additional external mol. wt. markers included bovine serum albumin (68000), ovalbumin (45000), $\beta$-lactoglobulin (17500) and cytochrome c (12500).

Identification of in vitro phosphorylated MuMTV proteins

A number of major and minor proteins have been reported for detergent-disrupted MuMTV when analysed by SDS-PAGE (Dion & Moore, 1977). As mentioned previously, $^{32}$P-phosphate incorporation proceeds linearly for 20 min at 22°C and these results were corroborated by SDS-PAGE analyses of samples taken at various time intervals. By way of illustration, a sample of in vitro, $^{32}$P-labelled MuMTV (20 min at 22°C) was analysed by SDS-PAGE to determine which MuMTV proteins were phosphorylated, and RIII-MuMTV labelled with $^{125}$I by the chloramine T procedure (Greenwood et al. 1963) was co-electrophoresed to ascertain known mol. wt. assignments. The results shown in Fig. 2 illustrate the heterogeneity of in vitro phosphorylation of MuMTV proteins. The major proteins associated with MuMTV, i.e. gp68, gp55, gp34, p28, p18, p14 and p12, may be phosphorylated, with the possible exception of p12, but it is also obvious that several minor proteins, especially with mol. wt. equal to or greater than 68000 were highly labelled. The phosphorylation of minor MuMTV proteins is also indicated by the lack of constancy of $^{32}$P to $^{125}$I labelling ratios. From these data, we conclude that in vitro phosphorylation of MuMTV proteins possesses little specificity. Finally, consistent with the results presented in Table 2, labelling profiles on SDS-PAGE analyses were maintained after digestion with DNase or RNase, but were completely abolished by prior treatment with pronase.
**Phosphoproteins of MuMTV**

Fig. 3. Molecular sieving chromatography of SDS-disrupted MuMTV proteins labelled *in vivo* in the presence of $^{32}$P-phosphate and $^{3}$H-amino acids. Experimental protocols of cell culture, labelling and chromatography are presented in the Methods. ——, $^{3}$H label; ——, $^{32}$P label. The void (Vo) and total volumes (Vt) were determined with dextran blue and phenol red, respectively. Mol. wt. markers for column calibration included bovine serum albumin (68000), ovalbumin (45000), soybean trypsin inhibitor (21 500) and lysozyme (14 400).

![Molecular sieving chromatography of SDS-disrupted MuMTV proteins](image)

Fig. 4. SDS-PAGE analysis of pooled fractions of low mol. wt., *in vivo* labelled MuMTV proteins obtained by Sephadex G-200 chromatography after SDS-disruption (Fig. 3). ——, $^{3}$H label; O—O, $^{32}$P label. (a) SDS-PAGE analysis of pooled fractions 117 to 138 (Fig. 3); (b) and (c), analyses of pooled fractions 117 to 127 and 128 to 138, respectively. Mol. wt. markers included those given in the legend to Fig. 2.

![SDS-PAGE analysis of pooled fractions](image)
In vivo phosphorylation of MuMTV proteins

MuMTV, labelled in the presence of $^{32}$P-phosphate and $^3$H-amino acids, was purified from a continuous cell line, MuMT-73, as described in the Methods. After SDS disruption and molecular sieving chromatography on Sephadex G-200, the labelling profile given in Fig. 3 was observed. Most of the $^{32}$P label was found to be associated with the lower mol. wt. proteins of MuMTV. Mol. wt. assignments given in Fig. 3 were established by SDS-PAGE analyses of individual fractions and correspond to previous results (Dion et al. 1977).

Corroboration and identification of MuMTV phosphoproteins was investigated by SDS-PAGE analyses as shown in Fig. 4. For these assays, fractions from the low mol. wt. peak were pooled and chromatographed on an anion exchange column to effect the removal of SDS, nucleic acids or unincorporated $^{32}$P label (Dion et al. 1977). Following concentration by in vacuo dialysis, SDS-PAGE analyses (Fig. 4) revealed that pI8, pI2 and possibly pI4 were phosphorylated under in vivo conditions. These latter data, therefore, are clearly distinct from results obtained by in vitro phosphorylation.

DISCUSSION

The in vitro protein kinase activity of RIII-MuMTV requires non-ionic detergent-disruption, a divalent metal cation and DTT for maximal activity and proceeds linearly for 20 min at 22°C (Table 1). These data and the lack of an appreciable effect with added cAMP have also been noted by Hatanaka et al. (1972). In contrast to the results of Strand & August (1971) for RLV, the addition of fluoride inhibits the incorporation of phosphate for in vitro protein kinase activity of MuMTV. Schrecker et al. (1972) reported that fluoride sometimes enhanced the endogenous RNA-directed DNA polymerase reaction of RNA tumour viruses. These authors proposed that this effect was perhaps due to the inhibition of phosphatase by fluoride. From the data reported here and the observation by Hatanaka et al. (1972) that both dATP and ATP can act as phosphate donors, it is possible that fluoride may also have a sparing effect on dATP for the virus DNA polymerase by virtue of protein kinase inhibition and may also contribute to the enhancement of virus RNA-directed DNA polymerase activity.

As shown in Table 2, the in vitro phosphorylation of MuMTV-associated proteins catalyses the transfer of phosphate from $\gamma$-$^{32}$P-ATP to proteins mainly via monoester linkage to serine (Fig. 1). The distribution of $^{32}$P label, as analysed by SDS-PAGE (Fig. 2) indicates that in vitro phosphorylation is heterogeneous. This lack of specificity for in vitro phosphorylation of MuMTV polypeptides is similar to the results obtained for RLV (Strand & August, 1971) and Rous sarcoma virus (Lai, 1976). In both instances, the phosphorylation of several minor virus protein components was also apparent. For example, of the ten RLV proteins found to be phosphorylated, only three were major components. Similarly, the in vitro phosphorylation of Rous sarcoma virus proteins resulted in $^{32}$P labelling of numerous proteins, only two of which coincided with virus polypeptides, namely p19 and p12. With regard to MuMTV, this milk-born virus is contaminated with small amounts of the caseins which are known to be phosphorylated and have mol. wt. of 44000, 26000 and 22000 (pp44, pp26 and pp22; Enami & Nandi, 1977). As shown in Fig. 2, casein mol. wt. markers coincide with three minor proteins of MuMTV which are highly phosphorylated in vitro. Tentatively, therefore, we conclude that these $^{32}$P-acceptor proteins represent casein contamination of MuMTV.

In contrast to the results of in vitro phosphorylation, the in vivo reaction appears to be specific as shown in Fig. 3 and 4. Initially, assays of in vivo phosphorylated proteins were
Attempted by direct SDS-PAGE analyses of MuMTV after disruption with NP40, nuclease digestion and lipid extraction with isoamyl alcohol; however, \(^{32}\)P label was routinely observed to be diffusely distributed between p18 and gp34. These results most probably reflect contamination with nucleic acid fragments and precluded the use of this technique. Similar results were reported by Lai (1976). Therefore, this technique was abandoned and the characterization of MuMTV phosphoproteins proceeded after molecular sieving chromatography (Fig. 3). Corroborating the protein nature of p18 and p12 (Fig. 4), pronase digestion completely eliminated the labelling profiles associated with these proteins (not shown). The nearly complete recovery of \(^{32}\)P label following anion exchange chromatography obviated nucleic acid contamination; however, a labelled, low mol. wt. contaminant was routinely observed in SDS-PAGE analyses which was not pronase-sensitive and probably represents a phosphorylated glycolipid which is stainable with periodic acid-Schiff reagent but not with Coomassie blue (Sarkar & Dion, 1975). In addition to the loss of \(^{32}\)P label due to radioactive decay, the resolution of this contaminant from p18 and p12 would explain the obvious decrease in the ratio of \(^{32}\)P to \(^{3}$$H$$ counts observed in Fig. 4 as compared to Fig. 3.

Finally, electrophoretic analyses of partial acid hydrolysates of each of these \(^{32}\)P-labelled proteins (p18 and p12) gave results similar to those illustrated in Fig. 1, i.e. phosphoserine was the predominant phosphorylated amino acid residue; smaller quantities of phosphothreonine were also found.

Because \textit{in vivo} phosphorylation was investigated with C3H-MuMTV in a Balb/c host (Balb/c/fC3H), it was of interest to determine whether this phenomenon was applicable to a milk-borne MuMTV from a different isogeneic mouse strain (RIII-MuMTV). As previously reported (Dion \textit{et al.} 1977), techniques were developed for the isolation of all of the major structural proteins of RIII-MuMTV and these have been extensively characterized (A. S. Dion \textit{et al.} manuscript submitted). Of the internal, non-glycosylated proteins of RIII-MuMTV, i.e. p28, p18, p14 and p12, only p28 was found to lack a detectable level of phosphorylated amino acids after partial acid hydrolysis. Furthermore, we presented chemical evidence (N-termini, tryptic peptide maps and quantitative amino acid analyses) that p14 probably represents a cleavage product of p18. From these data we conclude that MuMTV contains at least two major phosphoproteins (pp18 and pp12). Further studies of these internal virus polypeptides with regard to function and localization within the virus structure are presently in progress.

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\textit{Note added in proof.} Following submission of this manuscript, Sarkar \textit{et al.} (\textit{Virology} 91, 407-422, 1978) reported that MuMTV, purified from cell cultures of MuMT-73 as in the present study, contained a major, \textit{in vivo} phosphorylated species (pp23) which represented a minor protein of this virus. These seemingly disparate data may simply reflect difficulties in assigning mol. wt. designations according to the present nomenclature; however, a more probable explanation resides in techniques of analysis. For example, gradient slab gel electrophoresis following affinity chromatography could possibly select for a highly phosphorylated species of pp18, i.e. pp23, by virtue of increased hydrophilicity and decreased electrophoretic mobility effected by phosphorylation.
REFERENCES


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