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# Phosphoproteins: Structural Components of Oncornaviruses

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Oncornaviruses, which contain a virion-associated protein kinase, were found to possess phosphoproteins as virion structural components. One major phosphoprotein common to strains of laboratory and wild mouse oncornaviruses and a strain of feline leukemia virus was shown to be a polypeptide of about 12,000 mol wt. In addition to this, the Kirsten strain of murine sarcoma virus contained a second major phosphoprotein of about 10,000 mol wt, and mouse erythroblastosis virus contained a second major phosphoprotein that was either identical to or comigrated with the virion glycoprotein of about 74,000 mol wt. The major phosphoprotein of RD-114 virus was found to be of about 16,000 mol wt. The major phosphoamino acid of the 12,000-mol wt polypeptide of the mouse erythroblastosis virus was identified as phosphoserine, and that of the 16,000-mol wt polypeptide of the RD-114 virus was identified as phosphothreonine.

Protein kinase activity has been demonstrated in a large number of enveloped RNA- and DNA-containing animal viruses. The RNA viruses known to contain this activity are oncornaviruses (9, 26), rhabdoviruses (24, 26), togaviruses (28), and probably other budding RNA viruses such as parainfluenza, influenza, and feline syncitium-forming viruses (9). All of the structural polypeptides of the togaviruses studied are present in the virion as phosphoproteins (28), and one or two of the rhabdovirus structural proteins are phosphoproteins (24), implicating association of the virion protein kinase with the intracellular phosphorylation of virion proteins. Although protein kinase has been detected in oncornaviruses, earlier attempts to detect phosphoproteins as structural components of two oncornaviruses were unsuccessful (24). We now describe the presence of specific phosphoproteins in mouse and feline type C oncornaviruses as structural components of these virions.

## MATERIALS AND METHODS

**Reagents.** All radiochemicals were obtained from New England Nuclear. Agarose, acrylamide, and bisacrylamide were purchased from Bio-Rad Laboratories; guanidine hydrochloride, Triton X-100, phosphoserine, and phosphothreonine were from Sigma Chemical Co.; sucrose (RNase free) and protein molecular weight markers were from Schwarz/Mann; phosphorylase *a* and pancreatic RNase were from Worthington Biochemicals Corp.; and Eagle minimum essential medium and fetal bovine serum were from Flow Laboratories. Sodium dodecyl sulfate (SDS), obtained from Matheson Scientific, Inc., was recrystallized prior to use.

**Cells and viruses.** The Rauscher strain of mouse leukemia virus (MuLV-R) was grown in an infected NIH Swiss mouse embryo cell culture. The Kirsten strain of mouse sarcoma virus (Ki-MSV) and the original mouse erythroblastosis virus (MEV) (11) that gave rise to Ki-MSV after in vivo passage through rats (10, 25) were grown in normal rat kidney cell lines. The tissue culture line used as a source of Ki-MSV released both Ki-MSV and MEV with 10- to 100-fold excess of transforming particles (4, 14). The Gardner-Arnstein strain of feline leukemia virus (FeLV-GA) was propagated in the human rhabdomyosarcoma cell line RD (12). RD-114 virus is an endogenous feline type C virus that was picked up by the RD cells after these cells were passed in the brain of a feline embryo (13). The wild mouse type C viruses WM-275 and WM-292 were grown in infected NIH Swiss mouse embryo cell cultures (5). WM-275 virus was derived from the central nervous system tissue of a paralyzed NIH Swiss mouse that had been inoculated with WM-1504E virus originating from an embryo wild mouse cell culture (6). WM-292 virus was isolated from the central nervous system of a naturally paralyzed wild mouse (5, 18).

cells were grown in 50% phosphate-free minimal essential medium containing 10% dialyzed fetal bovine serum in the presence of the $^3$H-labeled L-amino acid mixture (2 $\mu$Ci/ml) and carrier-free [$^{32}$P]phosphate (80 $\mu$Ci/ml). Two changes of the medium were made at 24-h intervals, and the virus was purified from the pooled culture fluids by the published procedure (19, 21).

**Separation of virion phosphoprotein.** Pellets of purified virions, simultaneously labeled with the [$^3$H]amino acid mixture and [$^{32}$P]phosphate, were disrupted in 0.2 ml of 0.1% Nonidet P-40 in 20 mM

Tris-hydrochloride (pH 7.4) for 1 h at 0 C. The disrupted virus preparation was then mixed with 0.2 ml of RNase solution (1 mg/ml; preheated at 100 C for 5 min in 20 mM sodium acetate, pH 5.0) and incubated at 37 C for 2 h. The solution was then brought to 8 M guanidine hydrochloride and 0.3% β-mercaptoethanol by the addition of solid guanidine hydrochloride and β-mercaptoethanol, heated at 56 C for 45 min, and applied to Bio Gel A-5m column containing 6 M guanidine hydrochloride for separation of virus polypeptides (16, 19).

**Polyacrylamide-gel electrophoresis of virion phosphoproteins.** Virion proteins, containing both $^{3}H$ (amino acids) and $^{32}P$ (phosphate) labels, isolated by guanidine-agarose chromatography were analyzed further by SDS-polyacrylamide gel electrophoresis according to the procedure of Summers et al. (27). Dialyzed and lyophilized materials from the pooled chromatographic peak fractions were dissolved in 0.01 M sodium phosphate (pH 7.2) containing 1% SDS and 0.1% β-mercaptoethanol by heating at 100 C for 2 min. Electrophoresis was carried out in 7.5% gels (0.5 by 10.5 cm) containing 0.1% SDS at 9 mA/gel for 4 h at room temperature. After electrophoresis, gels were cut into 1-mm slices as described (19). Two slices were taken as one fraction.

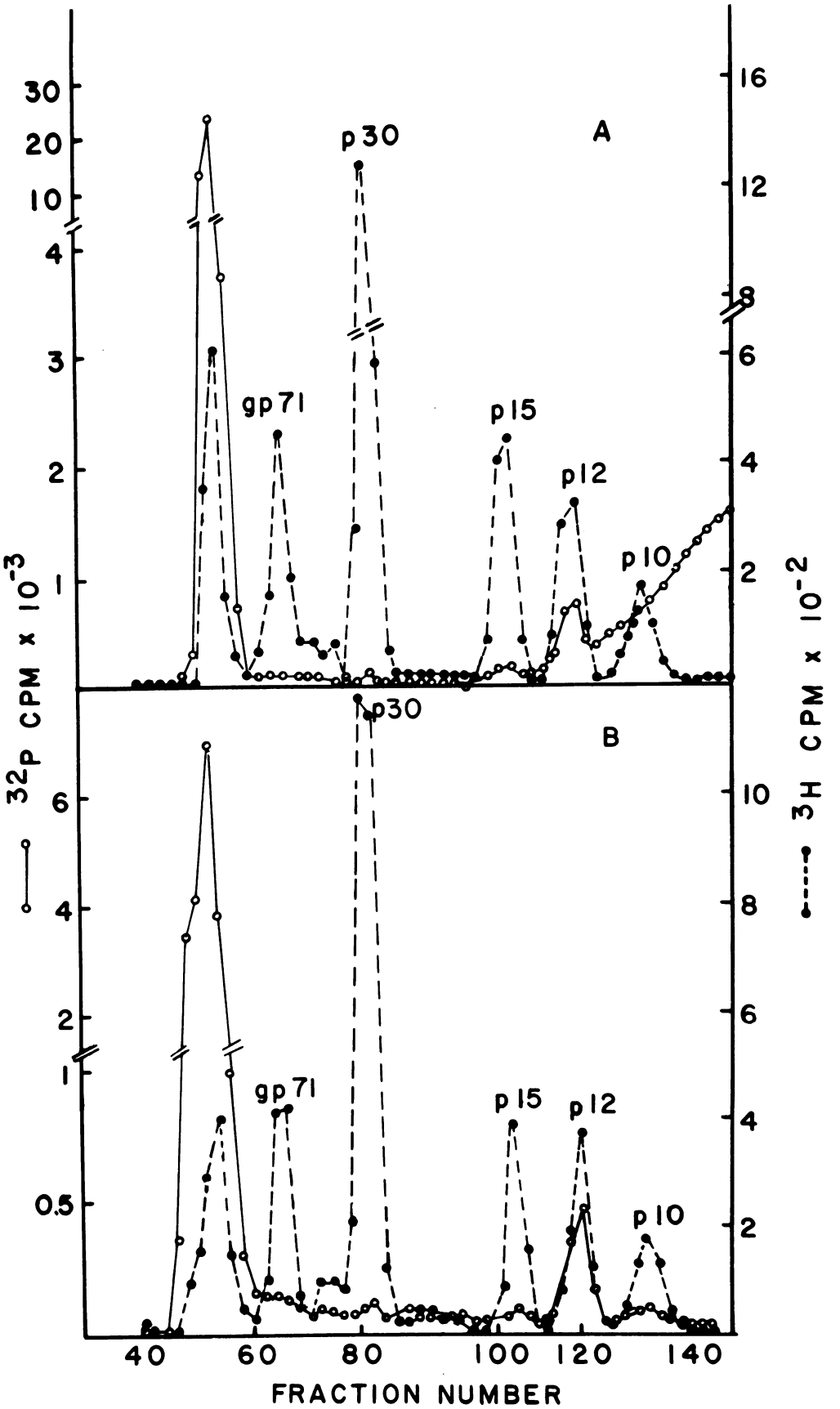
**Molecular weight determinations.** The molecular weights of the labeled proteins (10,000 to 16,000) were estimated from the results of guanidine-agarose chromatography (2). The values for higher-molecular-weight proteins (20,000 to 77,000) were estimated on the basis of their relative electrophoretic mobility in SDS-gels (2, 23). Standard proteins, phosphorylase *a*, bovine serum albumin, ovalbumin, chymotrypsinogen A, myoglobin, and cytochrome *c* were always included in identical gels as molecular weight markers. For staining, gels were fixed in 20% sulfosalicylic acid for 18 h and stained in Coomassie brilliant blue (15). Proteins and glycoproteins, respectively, were designated by "p" and "gp" placed before the number indicating the molecular weight in thousands (2). The glycoproteins were detected in separate experiments by guanidine-agarose chromatography of virions labeled with [$^{3}H$]glucosamine and $^{14}C$-labeled amino acids (19). The molecular weights of MuLV-R polypeptides were published previously (2).

**Paper electrophoresis of acid-hydrolyzed phosphoproteins.** To determine the nature of phosphoamino acids in the in vivo phosphorylated viral polypeptides, the phosphoprotein peaks, p12 of MEV and p16 of RD-114, from the guanidine-agarose column were dialyzed extensively against distilled water containing 0.1% β-mercaptoethanol, lyophilized, and then hydrolyzed in 1 ml of 6 N HCl at 110 C for 5 h (1). The hydrolysate was dried on a watch glass placed over a boiling-water bath and desiccated with NaOH beads in vacuo. The residue was then dissolved in a minimum volume of water containing 50 μg each of unlabeled phosphoserine and phosphothreonine and subjected to paper electrophoresis by the method of Allerton and Perlmann (1) with minor modifications. Electrophoresis was carried out in horizontal strips (4 by 23 cm) of Whatman no. 1 paper with a formic acid-acetic acid-water (30:90:880) mixture (pH 1.85) at 600 V for 6 h at 4 C. After electrophoresis the paper strips were stained with ninhydrin and cut into 3-mm strips for counting of radioactivity.

**Liquid scintillation counting.** Aliquots (0.1 ml) of the fractions from guanidine-agarose chromatography were mixed with 0.3 ml of water and 6 ml of scintillation fluids (8.0 g of butyl-PBD and 0.5 g of PBBO in 750 ml of toluene and 250 ml of Triton X-100) in small vials (Cal-Glass), thoroughly mixed to obtain a clear solution, and then counted in standard size carrier glass vials. Gel slices from electrophoresis experiments were counted in 6 ml of the above scintillation fluid after shaking with 0.4 ml of 2% periodic acid for 30 min at 60 C (22). Paper strips from the electrophoresis experiments were counted in 6 ml of a toluene scintillation fluid without Triton X-100. A Beckman LS-250 liquid scintillation counter with automatic quench correction was used for differential counting of $^{3}H$ and $^{32}P$.

## RESULTS

**Identification of the major structural phosphoproteins of mouse oncornaviruses.** When purified WM-275 virus labeled with [$^{3}H$]amino acids and [$^{32}P$]phosphate was dissociated by treatment with guanidine hydrochloride and separated into usual six major components (2, 8, 16, 17, 19, 22; P. Roy-Burman, B. K. Pal, M. B. Kaplan, M. Wright, and M. B. Gardner, Proc. 6th Int. Symp. Comp. Leuk. Res., in press) by guanidine-agarose chromatography, two of the peaks were found to contain superimposed $^{3}H$ and $^{32}P$ radioactivities (Fig. 1A). One of these two peaks eluted at the void volume of the column and was expected to contain $^{32}P$ label in the high-molecular-weight RNA and phospholipids of the virus. The other peak corresponded to the polypeptide p12. Treatment of the disrupted WM-275 virus with RNase prior to guanidine-agarose chromatography suggested that p12 might be a phosphoprotein, whereas the $^{32}P$ associated with the void volume and p10 was primarily due to RNA contamination (Fig. 1B). The remaining $^{32}P$ counts eluting at the void volume could be due mostly to phospholipids, which would not be affected by RNase treatment. Similar analysis of MuLV-R (Fig. 2), Ki-MSV, or MEV (Fig. 3) and WM-292 (data not shown) after RNase treatment showed that the p12 of each of these viruses was associated with both $^{3}H$ and $^{32}P$ radioactivities. Additional correspondence of $^{3}H$ and $^{32}P$ counts was observed with p10 of Ki-MSV (Fig. 3) and gp74 and a minor component, p21, of MEV (Fig. 3). Comigration of these radioactivities was also noted when the pooled peak fractions were analyzed by SDS-polyacrylamide gel electrophoresis; this is illus-


A
B
p30
gp71
p15
p12
p10
32P CPM x 10-3
3H CPM x 10-2
FRACTION NUMBER
40
60
80
100
120
140

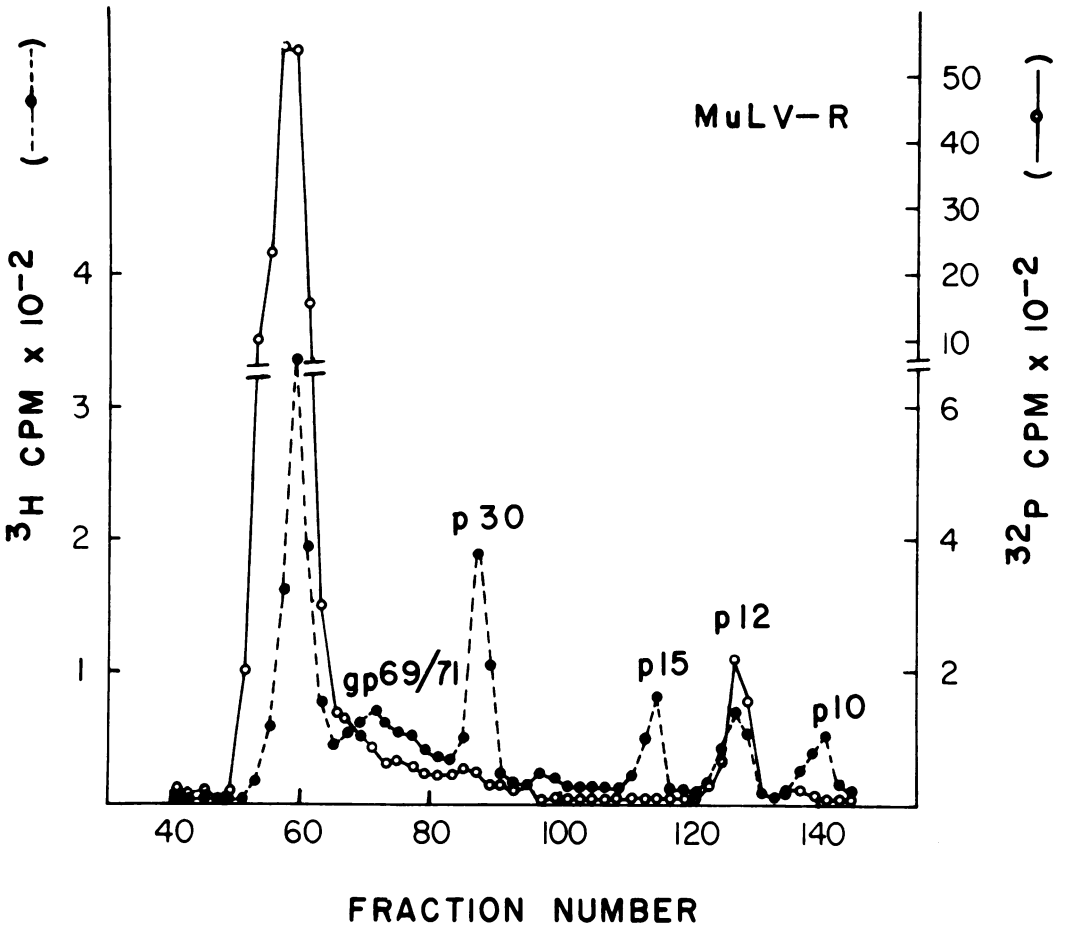



FIG. 2. *Separation of the polypeptides of MuLV-R virions, labeled with [$^3$H]amino acids and [$^{32}$P]phosphate, by guanidine-agarose chromatography.*

trated in Fig. 4 for p12 of MuLV-R and in Fig. 5 for p12 and p10 of Ki-MSV. The p10 of Ki-MSV was found to be further separated in SDS-gels into two components of which only the major component contained the $^{32}$P label (Fig. 5).

It should be noted that, whereas the polypeptide of MuLV-R primarily phosphorylated in vivo was p12, all of the major structural polypeptides of this virus were phosphorylated in vitro by the virion-associated protein kinase (26).

**Identification of the major structural phosphoproteins of feline oncornaviruses.** Guanidine-agarose chromatography of RNase-treated FeLV-GA labeled with [$^3$H]amino acids and [$^{32}$P]phosphate showed that, like WM-275, WM-292, and MuLV-R, this virus also contained both $^3$H and $^{32}$P labels in a single major polypeptide (p12) (Fig. 6). The comigration of both radioactivities was also observed in SDS-gel electrophoresis of this isolated peak. In contrast, RD-114 virus appeared to contain both labels in the polypeptide p16 (Fig. 6), and also the labels comigrated in SDS-gels (Fig. 4).

**Properties of the phosphate-polypeptide bonds.** The $^{32}$P label of the p12 of WM-275 was insensitive to RNase treatment (Fig. 1). In a separate experiment, the same virus was grown in the presence of (per milliliter): 2 $\mu$Ci of $^{14}$C-labeled amino acids and 50 $\mu$Ci each of [5,6-$^3$H]uridine (42 Ci/mmol), [5-$^3$H]cytidine (26 Ci/mmol), and [8-$^3$H]adenosine (32 Ci/mmol). This $^{14}$C- and $^3$H-labeled virus was subjected to guanidine-agarose chromatography after RNase treatment. Six components were detected by $^{14}$C counting, but there was no detectable $^3$H radioactivity in any one of the

FIG. 1. *Separation of the polypeptides of WM-275 virions, labeled with [$^3$H]amino acids and [$^{32}$P]phosphate, by guanidine-agarose chromatography. (A) Without RNase treatment; (B) with RNase treatment.*

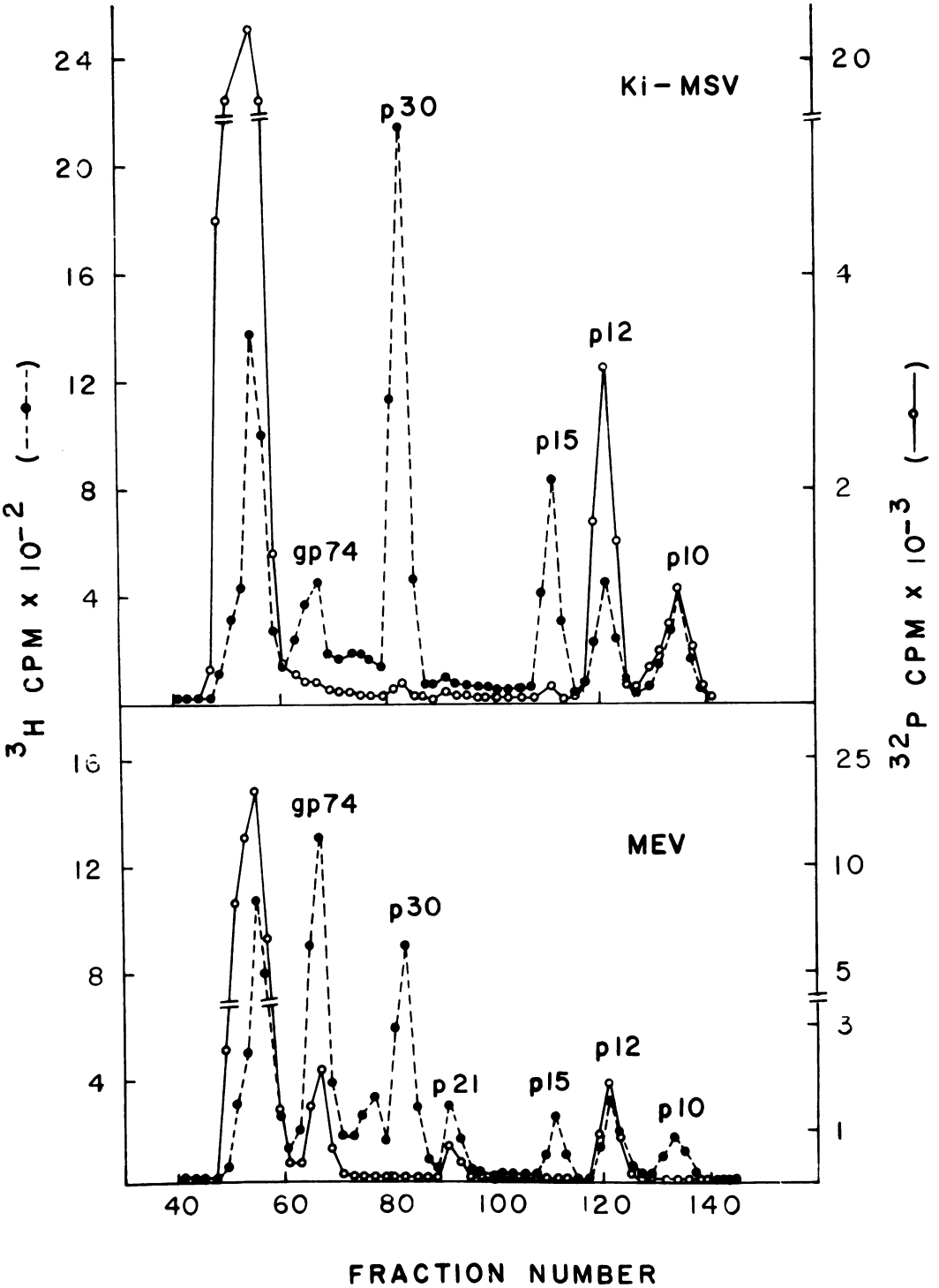



FIG. 3. *Separation of the polypeptides of Ki-MSV and MEV virions, labeled with [$^3$H]amino acids and [$^{32}$P]phosphate, by guanidine-agarose chromatography.*

peaks except a small amount in the void volume. This suggested that the $^{32}P$ in the polypeptide was not due to a covalent association of nucleotides with the polypeptide. Some properties of the phosphate-polypeptide bonds are shown in Table 1. The phosphate linkage was sensitive to treatment with alkali, but was stable in acid, as would be expected for a phosphoester of serine or threonine (20, 26). Acylphosphate sensitive to hydroxylamine at pH 5.5 was not detected. Also, $^{32}P$-labeled p16 of RD-114 was incubated in 10% trichloroacetic acid for 15 min at 50 C and extracted with chloroform-methanol (2:1). Almost all of the radioactivity was found to remain in the aqueous phase, thus eliminating the possibility of [$^{32}P$]phospholipids contamination in the polypeptides.

**Identification of the major phosphoamino acid.** The p12 of MEV and p16 of RD-114 phosphorylated in vivo were hydrolyzed in 6 N HCl at 110 C for 5 h and subjected to paper electrophoresis in the presence of phosphoserine and phosphothreonine markers. The major phosphoamino acid in p12 of MEV was found to be phosphoserine (Fig. 7), and that in p16 of RD-114 was phosphothreonine (Fig. 8).

## DISCUSSION

Our results demonstrate that oncornaviruses which contain a virion-associated protein kinase (9, 26) also possess phosphoproteins as their

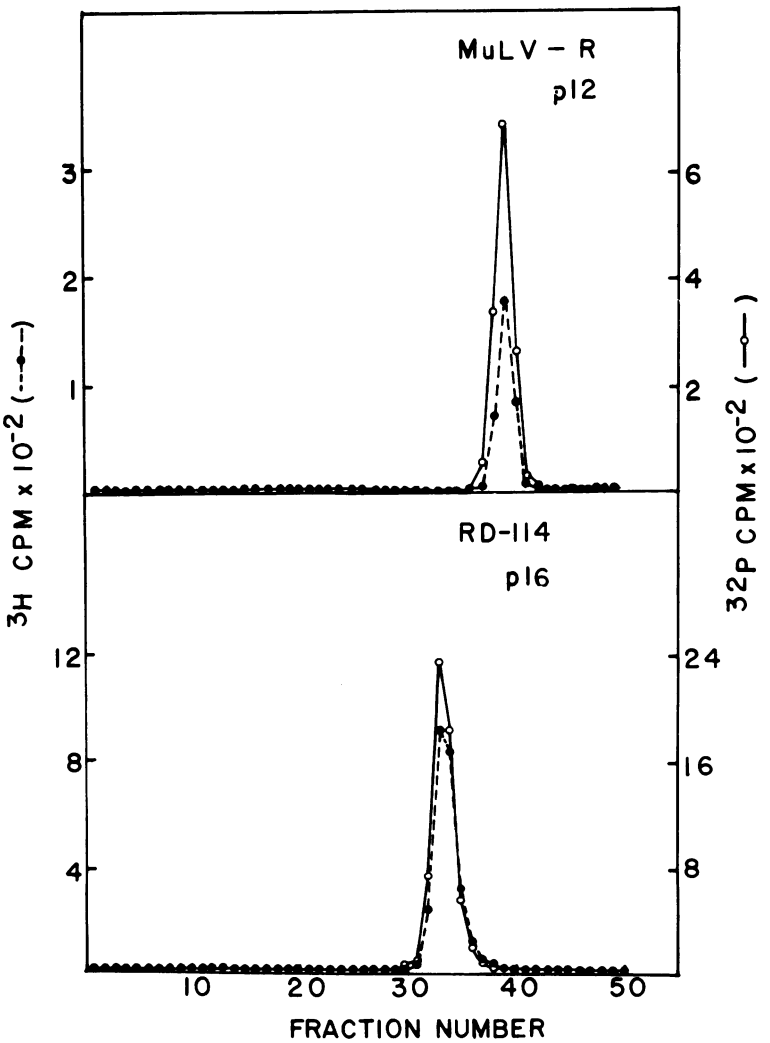



FIG. 4. *Polyacrylamide-gel electrophoresis of isolated MuLV-R p12 and RD-114 p16 phosphoproteins labeled with both [$^{3}H$]amino acids and [$^{32}P$]phosphate.*

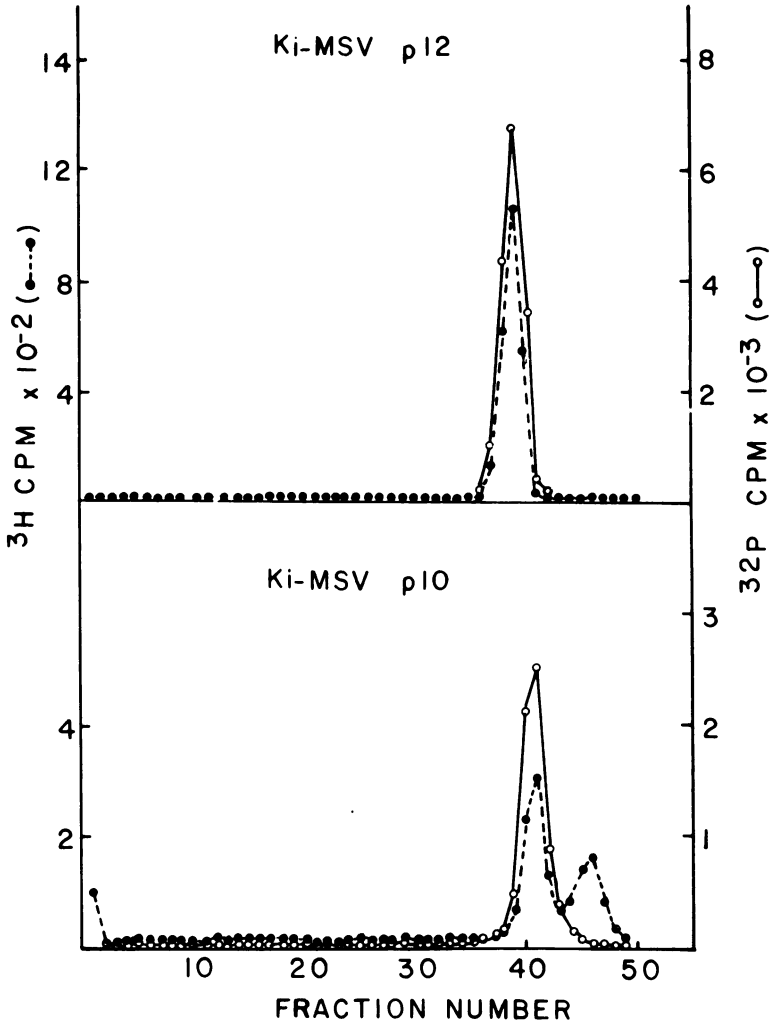



FIG. 5. *Polyacrylamide-gel electrophoresis of the isolated Ki-MSV phosphoproteins labeled with both [$^3H$]amino acids and [$^{32}P$]phosphate.*

structural components. One major phosphoprotein common to strains of laboratory and wild mouse oncornaviruses and a strain of feline leukemia virus is the polypeptide p12. In addition to this phosphoprotein, Ki-MSV contains a second phosphoprotein that is a major component of the polypeptides eluting as p10. The MEV, which gave rise to Ki-MSV after in vivo passage through rats (10, 11, 25), does not contain p10 in a detectable phosphorylated form. Instead, it contains another major phosphoprotein that comigrates with the virion glycoprotein gp74. At this time it is not known whether this phosphoprotein is identical to the major virion glycoprotein or whether it represents a viral or cellular phosphorylated polypeptide similar in size to that of gp74. A minor phosphoprotein, p21, was also detected in MEV. It is not known whether there exists a precursor-product relationship between these larger phosphoproteins of MEV and the smaller phosphoprotein, p10, of Ki-MSV. Also, other sarcoma viruses must be studied to determine the generality of occurrence of p10 in a phosphorylated state in these viruses. The possibility of the occurrence of other phosphorylated polypeptides that might be masked due to coelution with the phospholipids of these viruses in the void volume of the column can not be eliminated. It is known that aggregates of small- and large-molecular-weight species of viral glycoproteins and proteins appear in the void volume (16, 17, 19).

An interesting observation is that the major

phosphoprotein of RD-114 virus is not the p12 polypeptide. It is the p16 polypeptide. This difference in the nature of the structural phosphoprotein between RD-114 virus and FeLV-GA may be added to the list of criteria by which the two distinctly different major groups of oncornaviruses of the domestic cat have been distinguished (7, and the references cited therein). Our preliminary results suggest that a phosphorylated protein similar to RD-114 phosphoprotein is present in a baboon oncornavirus (29). In another independent study utilizing different techniques, it has been found that the major phosphoprotein of several strains of avian sarcoma virus is the p19 polypeptide (Michael M. C. Lai, personal communication). Thus,

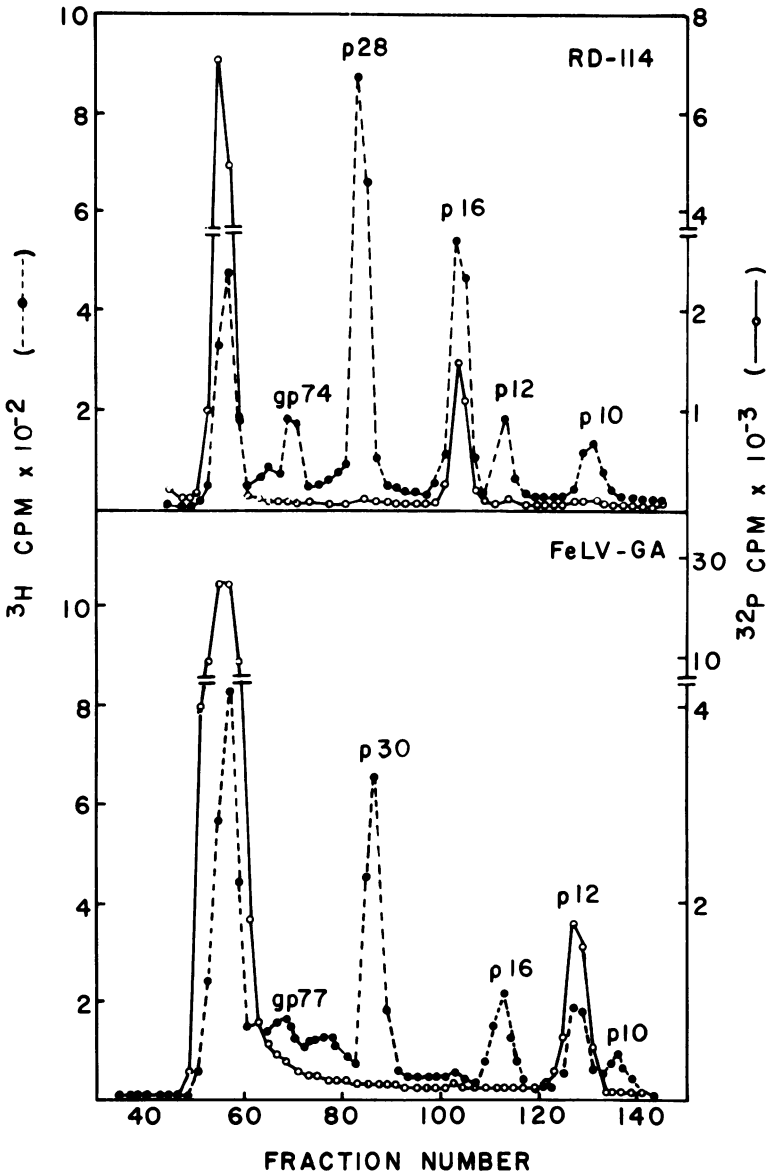



FIG. 6. *Separation of the polypeptides of FeLV-GA and RD-114 virions, labeled with [$^3$H]amino acids and [$^{32}$P]phosphate, by guanidine-agarose chromatography. The same column was used for fractionation of RD-114 and FeLV-GA polypeptides. For plotting, the elution positions of the void volumes were deliberately superimposed. The apparent differences in the elution position of equivalent molecular weight polypeptides were due to the variation in fraction volumes in the two runs. The indicated molecular weights were calculated from the elution position of internal marker proteins and from SDS-gel electrophoretic mobilities.*

TABLE 1. *Properties of an in vivo $^{32}P$-labeled phosphoprotein (p12 of MEV)*[a]

| Treatment | Acid-insoluble $^{32}P$ (%) |
|---|---|
| Control | 100 |
| 10% Trichloroacetic acid, 15 min at 50 C | 100 |
| 1 N NaOH, 15 min at 50 C | 45 |
| 1 N NaOH, 15 min at 100 C | 2 |
| 1 M $NH_2OH$, pH 5.5, 60 min at 37 C | 102 |

[a] Reaction mixtures (0.2 ml) were cooled at 0 to 4 C after treatment at specified conditions; 100 μg (0.1 ml) of bovine serum albumin was added, and the proteins were precipitated by addition of 0.2 ml of cold 50% trichloroacetic acid. The acid-insoluble materials were collected on membrane filters (Millipore Corp.), washed, dried, and counted for radioactivity as described previously (21). The control value represents 890 counts/min.

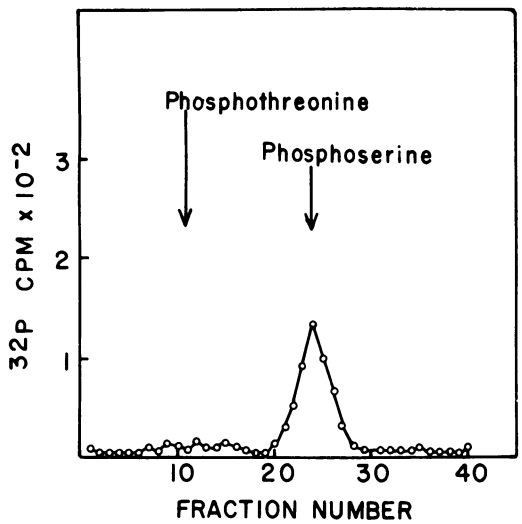



FIG. 7. *Identification of the phosphate acceptor amino acid residues of the p12 polypeptide of MEV by paper electrophoresis of the acid hydrolysates.*

there appears to be a pattern in the characteristics of the structural phosphoproteins of avian and lower mammalian oncornaviruses. A major phosphoprotein of avian oncornaviruses is p19, which is deficient in mammalian oncornaviruses (16). In the case of mouse and feline oncornaviruses, a major phosphoprotein that is common to the viruses tested is p12. RD-114 virus seems to be an exception in its phosphoprotein, and its similarity to endogenous primate viral phosphoprotein remains to be further investigated to aid in understanding a possible evolutionary relationship between these viruses (3).

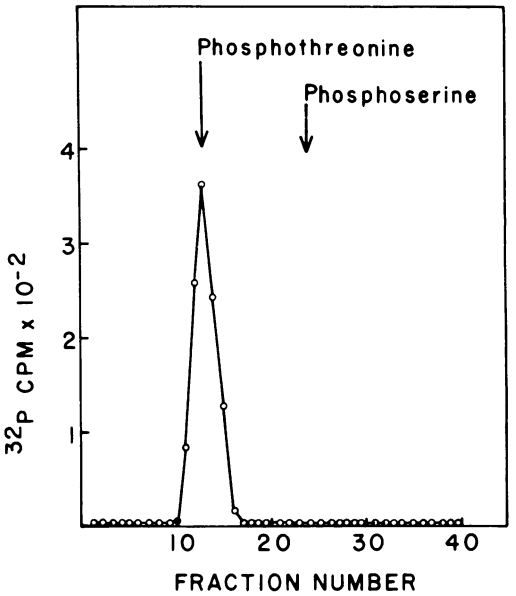



FIG. 8. *Identification of the phosphate acceptor amino acid residues of the p16 polypeptide of RD-114 by paper electrophoresis of the acid hydrolysates.*

At present we do not have any evidence for biological functions of the phosphorylated structural polypeptides of oncornaviruses, and we do not know whether all or some of the structural polypeptides like p12 or p16 are phosphorylated. However, it is realized that these phosphoproteins must have specific and important biological roles during the sequential steps of virus uncoating, replication, and assembly. Phosphorylation of core proteins by the virion-bound protein kinase may be involved in the regulation of transcription of the viral genome by the virion-associated reverse transcriptase. Such a regulatory process involving virion-associated transcriptase has been proposed in the case of rhabdoviruses in which the nucleocapsid proteins are phosphorylated by the virion protein kinase (24). Direct approaches to substantiate these speculations are limited. However, the combination of techniques employed in the present study to characterize the phosphoproteins of oncornaviruses will be readily applicable to study these proteins from temperature-sensitive, replication-defective, and transformation-defective mutants to obtain insight for the biological functions of phosphorylation and of oncornavirus phosphoproteins.

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