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Purified Low-Molecular-Weight Protein Kinase from Murine Sarcoma Virus Particles Catalyzes Tyrosine Phosphorylation Endogenously but Phosphorylates Cellular Proteins at Serine

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The low-molecular-weight (LMW) protein kinase associated with high-titer murine sarcoma virions has been extensively purified by ammonium sulfate fractionation, Bio-Gel P-100 gel filtration, DEAE-cellulose and carboxymethyl cellulose chromatography. The purified enzyme migrates as a 16K polypeptide in polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The enzyme catalyzes phosphotransfer with ATP as a phosphate donor to various exogenously added proteins as acceptors; it requires Mg^{2+} and is independent of cyclic AMP. The enzyme preparation catalyzes a low level of phosphorylation in the absence of any exogenously added substrate and forms phosphotyrosine. However, in the presence of acceptor protein molecules including total soluble cytoplasmic proteins of murine sarcoma virus-transformed mouse cells, the phosphorylated end products contain predominantly phosphoserine. The virion-associated enzyme also shows a preference for phosphorylating certain polypeptides in the soluble cytoplasmic extracts of murine sarcoma virus-transformed cells.

A class of low-molecular-weight (LMW) protein kinase activity and phosphoprotein molecules both migrating at 14 to 16K are packaged in high-titer transforming virus stocks of a murine sarcoma virus-murine leukemia virus complex, m3MSV (IC-MuLV); neither the phosphoprotein nor the kinase is detectable in nontransforming IC-MuLV virions (20). These molecules bind to purified actin and also interact with microtubular proteins in vitro. In virion preparations containing a temperature-sensitive mutant of MSV (4), the actin-binding kinase activity was found to be thermolabile in its ability to phosphorylate exogenously added substrate phosvitin (21).

This report describes an extensive purification of the LMW kinase from MSV virions and an analysis of the phosphotransfer reaction catalyzed by this enzyme. The enzyme has been purified by gel filtration, ion-exchange chromatography, and ammonium sulfate fractionation from high-titer m3MSV virion particles harvested from serum-free culture fluid of a producer mouse 3T3 cell line, 3B11IC (1). The purified 16K enzyme is a cyclic AMP-independent protein kinase which requires Mg^{2+} to catalyze phosphotransfer from ATP as a preferred phosphate donor. The activity is stimulated by monovalent cation (Na^+ or K^+) concentrations of 75 to 100 mM, whereas >200 mM Na^+ or K^+ inhibits the phosphotransfer. The phosphotransfer catalyzed by the enzyme is inhibited by α-tosyl L-lysyl chloromethyl ketone (TLCK) and L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK). When no acceptor protein is added, the enzyme catalyzes a limited level of phosphotransfer, presumably an autophosphorylation reaction, leading to the almost exclusive formation of phosphotyrosine residues. In comparison, with a variety of acceptor proteins added exogenously, the enzyme catalyzes phosphotransfer reactions leading to the formation of predominantly (>95%) serine phosphates. When total mouse cellular cytoplasmic proteins are used as substrates, the enzyme shows a significant preference for five polypeptides migrating at 20, 25, 33, 36, and 46K. The results presented in this report, together with the previous studies on the LMW kinase of MSV, indicate that this virion-associated protein kinase shows certain similarities with the protein kinases coded by the transforming genes of certain other retroviruses. The ability of this virion kinase to preferentially seek out certain polypeptides in a pool of cytoplasmic proteins further suggests its possible participation in specific phosphorylation processes in MSV-transformed cells.

MATERIALS AND METHODS

Cells, culture conditions, and viruses. The NIH3T3 mouse lines infected with IC-MuLV (8) or producing m3MSV (IC-MuLV) sarcoma-leukemia

complex (1) have been previously described. Cells were maintained in Dulbecco modified Eagle medium supplemented with 10% calf serum at 37°C. Before the collection of virus, cells at approximately 70 to 80% confluency were rinsed twice with serum-free Dulbecco modified Eagle medium salts by incubating for 6 h for every rinse. Serum-free culture fluid from these monolayers was then collected three times at 12- to 16-h intervals.

Culture fluid was clarified free of cell debris by centrifugation at 10,000 × *g* for 20 min and stored on ice. Virus was first pelleted by centrifugation through a 20% glycerol cushion at 105,000 × *g* for 70 min and then banded isopycnically to 1.16 g/cm^3 in sucrose density gradients. The purified virus was further concentrated by pelleting through a second glycerol cushion.

Enzyme purification and chromatographic procedures. Approximately 10 mg of viral protein was disrupted in a total volume of 25 ml in 0.02 M Tris-hydrochloride (pH 8.0)–0.4 M KCl–5 mM $MgCl_2$–5 mM dithiothreitol –0.25% Triton X-100 by mixing on ice for 15 to 20 min. Undisrupted virus and other aggregates were clarified by centrifugation in a Spinco SW41 rotor at 35,000 rpm for 75 min at 4°C. The supernatant was adjusted to the desired ammonium sulfate concentration by adding a saturated solution of ammonium sulfate. Salting out was carried out at 4 to 6°C by gentle mixing for 1 h, and the precipitated material was removed by centrifuging at 30,000 rpm in a Spinco SW41 rotor for 30 min at 4°C. During stepwise ammonium sulfate fractionation, the supernatant was adjusted to the next desired concentration of ammonium sulfate. For preparative purification, the ammonium sulfate cut of 35 to 75% was dissolved in 2.0 ml of a buffer containing 0.02 M Tris-hydrochloride (pH 8.0)–0.3 M KCl–2.5 mM $MgCl_2$–1 mM dithiothreitol and applied to a 50- by 2.5-cm Bio-Gel P-100 column (Bio-Rad Laboratories, Richmond, Calif.) equilibrated with the same buffer. Fractions were collected, and a sample of each fraction was assayed for total protein kinase activity in the presence of phosvitin (see below). Appropriate fractions were pooled, and proteins were precipitated by adjusting ammonium sulfate concentration to 75%. The precipitated material was dissolved in 0.02 M Tris-hydrochloride (pH 8.0)–0.05 M KCl–1 mM dithiothreitol–1 mM $MgCl_2$ and applied to a 12.0-ml Whatman DE-52 column. After loading the sample and removing unbound material by washing with the above buffer, bound proteins were eluted with a 40-ml linear KCl gradient between 50 and 600 mM. Fractions were again assayed for total protein kinase activity. The KCl concentration in these assays was adjusted to between 75 and 110 mM, a range in which the kinase shows only a 20% variation in net activity due to the difference in monovalent cation concentration. Appropriate fractions were pooled, and the proteins were concentrated again by 75% ammonium sulfate precipitation and suspended in 0.01 M morpholineethanesulfonic acid (pH 6.5)–25 mM KCl–1 mM dithiothreitol–1 mM $MgCl_2$. This was applied to a carboxymethyl cellulose column equilibrated with the same buffer. Unbound proteins were first washed off in loading buffer, and bound proteins were then eluted with a linear KCl gradient of 25 to 500 mM. Fractions were assayed as before. The final enzyme preparation was stored on ice.

Protein kinase assays. The phosphotransfer reactions were usually performed at room temperature (20 to 22°C) in a total volume of 50 μl containing 0.02 M Tris-hydrochloride (pH 8.0)–5 mM $MgCl_2$–75 mM KCl and 200 μg of phosvitin or casein per ml. The substrates were omitted when assaying endogenous or autophosphorylation. [γ-^{32}P]ATP (New England Nuclear Corp., Boston, Mass.) was used at a final concentration of 1 μM and a specific activity of ~25 Ci/mmol. After a 20-min incubation, the reaction was quenched by the addition of 1.5 ml 10% trichloroacetic acid at 4°C. Acid-precipitable ^{32}P radioactivity was filtered on Whatman GF/C filter pads and counted in a toluene-based fluid by liquid scintillation spectrometry. The background of these assays without added enzymes ranged between 800 and 1,200 cpm.

Preparation of cytoplasmic extracts and phosphorylation assays. Cells were grown to approximately 80% confluency in 10% calf serum-supplemented Dulbecco modified Eagle medium. They were rinsed twice with Dulbecco modified Eagle medium salts and then incubated for 6 to 8 h with Dulbecco modified Eagle medium salts to remove most of the serum. Cells were then scraped off the monolayer, washed in phosphate-buffered saline at 4°C, and then swollen with a hypotonic buffer (15 mM sodium phosphate [pH 7.2]–10 mM NaCl–0.1 mM $CaCl_2$–0.5 mM $MgCl_2$–0.05% aprotinin) at a density of ~10^7 cells per ml for 10 to 15 min on ice. Cells were then disrupted in a Dounce homogenizer, and nuclei were centrifuged at 10,000 × *g* for 15 min at 4°C. Crude cytoplasmic supernatant was then subjected to high-speed clarification to remove most subcellular organelles by centrifuging at 32,000 rpm for 2.5 h in a Spinco SW50.1 rotor at 4°C. The clear final supernatant was then dialyzed against two 2-liter changes of a buffer containing 0.01 M sodium phosphate (pH 7.2)–50 mM KCl–0.05% aprotinin. At the end of dialysis insoluble material, if any, was removed by centrifugation at 12,000 × *g* for 15 min.

Appropriate samples of the crude cytoplasmic extract containing a known amount of protein were adjusted to 0.01 M sodium phosphate (pH 7.2)–75 mM KCl–4 mM $MgCl_2$–0.3 mM $CaCl_2$–0.005% aprotinin, and [γ-^{32}P]ATP was added to a final concentration of 1 μM (~25 Ci/mmol). Other components (for example, monovalent and divalent cations, protease inhibitors, etc.) were added as desired. After 20 min of incubation at room temperature, an equal volume of 2× electrophoresis sample buffer (13) was added; the samples were dissociated for 10 min at 75°C and then applied to 20-cm-long 12.5% resolving gels with 3.8% stacking gels. For the studies on phosphorylation of cytoplasmic proteins by viral enzyme, the cytoplasmic extract was inactivated by heating at 60°C for 10 min and then cooled down to room temperature before the addition of enzyme and [γ-^{32}P]ATP. Reactions were then carried out as usual.

Samples were electrophoresed at 100 V for 18 h, and gels were first stained with Coomassie blue to visualize and approximately quantitate the protein bands. Destained gels were dried and autoradiographed on Kodak XR-1 films with Du Pont Cronex

Lightning Plus screens.

Detection of phosphoamino acids. The end products of various reaction mixtures were adjusted to 0.1% sodium dodecyl sulfate (SDS) and 2 mM EDTA to stop further enzyme activities. Samples were then incubated with the desired concentrations of acid or alkali or without any additions at appropriate temperatures for the lengths of time specified. The samples were then adjusted to 10% with cold trichloroacetic acid, and the precipitated radioactivity was measured by liquid scintillation spectrometry.

Estimation of free phosphates released by various treatments was done by making the incubation mixture 1% in sulfuric acid and then adding an excess of ammonium molybdate. The ammonium phosphomolybdate was extracted in an isobutanol-benzene mixture (1:1, vol/vol), and the radioactivity in the organic phase was measured by liquid scintillation counting.

For complete hydrolysis, samples were adjusted to 6 N HCl and transferred to sealed capillary tubes. These were heated at 100°C for 2.5 h and then lyophilized. Samples were subjected to paper electrophoresis in 0.5% pyridine–5% acetic acid buffer (23) on Whatman 3MM chromatography paper. After the paper was dried, authentic serine and threonine phosphates (Sigma Chemical Co., St. Louis, Mo.) and tyrosine phosphate (a gift from Tony Hunter) were visualized by ninhydrin staining. The radioactivity associated with each was monitored by autoradiography on Kodak XR-1 film with Du Pont Cronex Lightning Plus intensifying screens. Blocks of each lane corresponding to free phosphate, serine plus threonine phosphates, tyrosine phosphate, and the origin plus the partially hydrolyzed products were cut out. The paper patches were treated with NCS tissue solubilizer (New England Nuclear Corp.) at 60°C for 2 h and then the radioactivity was measured by liquid scintillation counting.

RESULTS

Purification of the LMW kinase from m3MSV (IC-MuLV). The m3MSV (IC-MuLV) virus was concentrated by sedimentation and density banding from serum-free culture fluid of the producer mouse cell line, 3B11IC (1). Concentrated virus suspensions ($\sim 10^{12}$ particles per ml) were disrupted at 4°C with the nonionic detergent Triton X-100 in the presence of high concentrations of KCl as described above. In an attempt to obtain a partial purification and concentration of the LMW kinase from other virion proteins, a series of ammonium sulfate fractionations were performed. The fractions were analyzed in parallel for endogenous and substrate-dependent protein kinase activities by standard protein kinase assays (see above) as well as for polypeptide compositions by SDS-polyacrylamide gel electrophoresis (PAGE) (13). Initial studies showed that greater than 90% of the total LMW protein kinase activity could be recovered between 40 and 65% ammonium sulfate saturation. For preparative purification purposes, then, the material which salted out at 35% ammonium sulfate saturation was discarded, and the 35 to 75% fraction was subsequently processed through a Bio-Gel P-100 gel filtration as described above. Column fractions containing protein kinase activities migrating faster than the viral p30 protein were pooled (Fig. 1A). This pool exhibited only low levels of kinase activity in the absence of any added substrate and showed a 30- to 40-fold increase in the specific activity for substrate-dependent phosphotransfer. The enzyme in the pool was concentrated again by the precipitation with 75% ammonium sulfate saturation. The enzyme was suspended in 20 mM Tris-hydrochloride (pH 8.0)–50 mM KCl–1 mM $MgCl_2$–1 mM dithiothreitol and subjected to DEAE-cellulose chromatography as described above. The majority of the substrate-dependent activity eluted at about 0.2 M KCl (Fig. 1B) near the trailing end of the major protein peak. This activity was pooled, concentrated by 75% ammonium sulfate precipitation, and then applied to a carboxymethyl cellulose column. The main peak of enzyme activity (Fig. 1C) was pooled and stored on ice. To examine the purities of the enzyme preparations during the procedure, representative samples were analyzed in a 12.5% SDS-PAGE (Fig. 2). The final purified material yielded one major polypeptide band at 16.5K (lane 7, Fig. 2). When the specific activities of the enzyme preparations were compared to the polypeptide compositions of these pools, there was a direct correlation between the extent of substrate-dependent phosphorylation and the enrichment of the 16K band, indicating that this 16K protein represents the protein kinase activity.

Biochemical characterizations of the purified MSV kinase. The protein phosphorylating activity of the enzyme was resistant to nucleases but sensitive to treatments with proteases or heating at >60°C for 5 to 10 min. In the presence of an excess of substrate, phosvitin, the phosphotransfer reaction was linear for about 40 min at room temperature (20 to 22°C) and also showed a linear dependence on enzyme concentration (Fig. 3A and B). The enzyme required Mg^{2+} with an optimum at about 7 mM (Fig. 3C). Monovalent cations (Na^+ and K^+) showed similar effects; maximal incorporation was observed at a final concentration of 90 mM (Fig. 3D), whereas the addition of more than 200 mM NaCl or KCl inhibited the phosphotransfer. The enzyme efficiently used ATP as a phosphate donor, and maximal incorporation was reached at a concentration of about 8 μM ATP in the assay mixtures (Fig. 3E). Under standard assay conditions using [γ-^{32}P]ATP at a specific activity of ~25 Ci/mmol, only the effects on substrate phosphorylation could be monitored since the

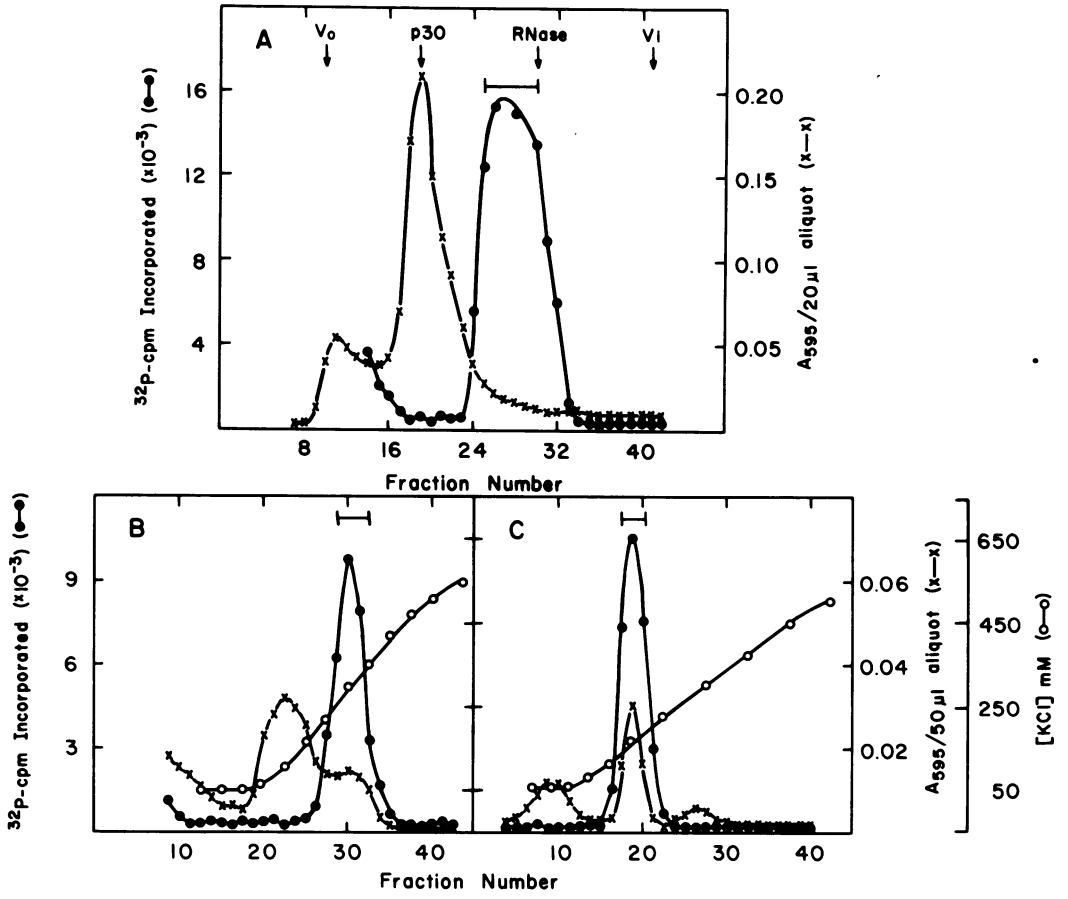

FIG. 1. *Purification of MSV kinase from m3MSV (IC-MuLV) virions by gel filtration and ion-exchange chromatography. A, Detergent (Triton X-100)-disrupted virions were subjected to ammonium sulfate fractionation, and the 35 to 75% cut was applied to a Bio-Gel P-100 column as described in the text. A sample (10 µl) of each fraction was assayed for protein kinase activity by using phosvitin substrate* (●). *The protein concentration in a 10-µl sample of each fraction was measured by the Coomassie blue G-250 assay* (×) *(5). Arrows, from left to right, indicate the positions of excluded volume (Vo), viral p30 protein (30K), pancreatic ribonuclease (14K), and included volume (Vi). B, The material in the fractions indicated by the horizontal bar in A was pooled, concentrated by 75% ammonium sulfate precipitation, and applied to a Whatman DE-52 column. Fractions were assayed for protein kinase activity* (●) *by using 10-µl samples. The amount of protein was estimated in 20-µl samples by Coomassie blue G-250 assay* (×). *The conductivity of every third fraction was measured, and the salt concentration (mM KCl) was obtained* (○). *C, The material under the horizontal bar in B was pooled, concentrated by 75% ammonium sulfate precipitation, and applied to a carboxymethyl cellulose column. Protein kinase activity was assayed in a 10-µl sample of each fraction* (●). *The amount of protein* (×) *was measured as before in a 40-µl sample of each fraction. The conductivity of every third tube was measured to obtain the salt concentration* (○).

amount of radioactivity incorporated in the absence of added substrates (autophosphorylation) was not significantly above the assay background (ca. 1,400 cpm compared with a background of 800 to 1,200 cpm).

In Table 1 some other biochemical properties of the enzyme are summarized. The enzyme activity was independent of cyclic AMP and cyclic GMP in concentration ranges from 0.5 to 2.5 µM. At a final concentration of 0.8 µM, ATP was a preferred phosphate donor over GTP by a factor of about 2. The Mg^{2+} requirement of the enzyme could not be substituted by Mn^{2+} or Ca^{2+}. When added in conjunction with 5 mM Mg^{2+}, low concentrations (≤1 mM) of Mn^{2+} or Ca^{2+} had no detectable effects, whereas concentrations of more than 5 mM of either ion caused a partial inhibition. Zn^{2+} caused a strong inhibition of the activity at a concentration as low as 0.1 mM. The enzyme was active over a broad

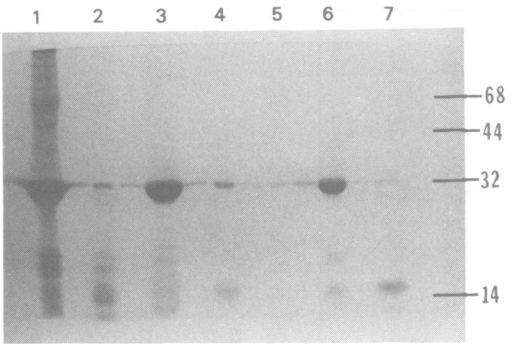

FIG. 2. *PAGE of m3MSV (IC-MuLV) viral proteins during the purification of MSV kinase. Appropriate fractions of viral protein were analyzed on a 12.5% polyacrylamide gel containing 1% SDS in a discontinuous Tris-buffered system as described by Laemmli (13). The gel was stained with 0.25% Coomassie blue R and destained. Lanes: 1, disrupted total virion; 2, material under the bar in Fig. 1A; 3, the 35 to 75% ammonium sulfate fraction of virion proteins before P-100 gel filtration; 4, protein eluting from DE52 column at the position indicated by the bar in Fig. 1B; 5, material which remained in the soluble fraction after 75% ammonium sulfate precipitation of material pooled from DE52 column (Fig. 1B); 6, material from fraction 10 from the carboxymethyl cellulose column (Fig. 1C); 7, peak enzyme activity pooled from carboxymethyl cellulose column as indicated by the horizontal bar in Fig. 1C. Arrows from top to bottom indicate the positions of standard molecular mass markers: bovine serum albumin (68K), ovalbumin (44K), carbonic anhydrase (32K), pancreatic ribonuclease (14K), and the bromophenol blue dye front. The stainable material migrating at ~32K is an artifact of staining the gel since it is visible in control lanes where only sample buffer was loaded.*

range of pH between 7.0 and 8.5. The enzyme catalyzed phosphotransfer, although at a reduced rate, when the incubation temperature was greater than 40°C. The optimum temperature for phosphorylation was between 33 and 35°C. Several polypeptide substrates were tested as acceptor proteins. Of the three efficient substrates, phosvitin was the best (100%); in comparison, α-casein was about 75% as efficient, and total calf thymus histone was only 40% as active; whether one specific type among the histones is strongly preferred as an acceptor is not clear at the present time.

The chloroketone family of serine protease inhibitors has been reported to inhibit the protein kinase activity of the avian SRC gene product (18); one of these, TLCK, also causes a partial reversion of the phenotype of avian sarcoma virus-transformed cells in culture (22). The two common inhibitors, namely, TLCK and TPCK, were tested for their effects on the endogenous phosphorylation and exogenous substrate-dependent phosphorylation catalyzed by purified LMW kinase preparations (Fig. 4). Neither of the two inhibitors showed any significant effect on the limited extent of autophosphorylation catalyzed by the LMW kinase. The phosphorylation of exogenously added phosvitin was efficiently inhibited by both the inhibitors. In addition, when a soluble cytoplasmic extract which had been heat inactivated (60°C, 10 min) to destroy the endogenous cytoplasmic kinases was used as the substrate, again ^{32}P incorporation catalyzed by the LMW kinase was strongly inhibited. In a control experiment where an active cytoplasmic extract of 3B11IC cells was incubated with [γ-^{32}P]ATP without the addition of LMW kinase, TLCK inhibited the incorporation by the endogenous cytoplasmic kinases; the final extent of inhibition in this case was, however, only 60%. This suggests that the LMW kinase can catalyze a series of TLCK-sensitive phosphorylation reactions which represent a subset of the phosphorylation reactions which can occur in MSV-transformed cell cytoplasm. The data indicate that TLCK and TPCK do not inhibit autophosphorylation of the virion LMW kinase, but inhibit phosphotransfer into acceptor proteins including candidate viral or cellular proteins (or both) present in cytoplasmic extracts of MSV-transformed mouse cells. The relevance of this sensitivity of MSV kinase to TLCK and TPCK and the possible involvement of the virion kinase in phosphorylation reactions associated with MSV-mediated cell transformation are unclear.

Analysis of phosphotransfers catalyzed by the LMW kinase. In the standard assays described above, the LMW kinase catalyzed only very low levels of autophosphorylation and showed a high degree of preference for the serine-rich protein phosvitin as the substrate. This suggested the possibility that a major end product of the kinase reaction might be phosphorylated serine residues in the substrate molecules. Recent examination of other retroviral transforming gene products has, however, revealed that the protein kinase activities associated with these polypeptides catalyze the formation of phosphotyrosine bonds (11, 23). Both serine and threonine phosphates are more labile to alkali treatment than are the tyrosine phosphate bonds; lysyl- and histidinylphosphate bonds are highly sensitive even to mild acid treatments (9, 10). To assess the nature of phosphoamino acids formed by the LMW kinase, the products of autophosphorylation reactions, performed with about 10-fold-higher specific activity of [γ-^{32}P]ATP (~250 Ci/mmol), were tested for sensitivities to various alkali and acid treatments.

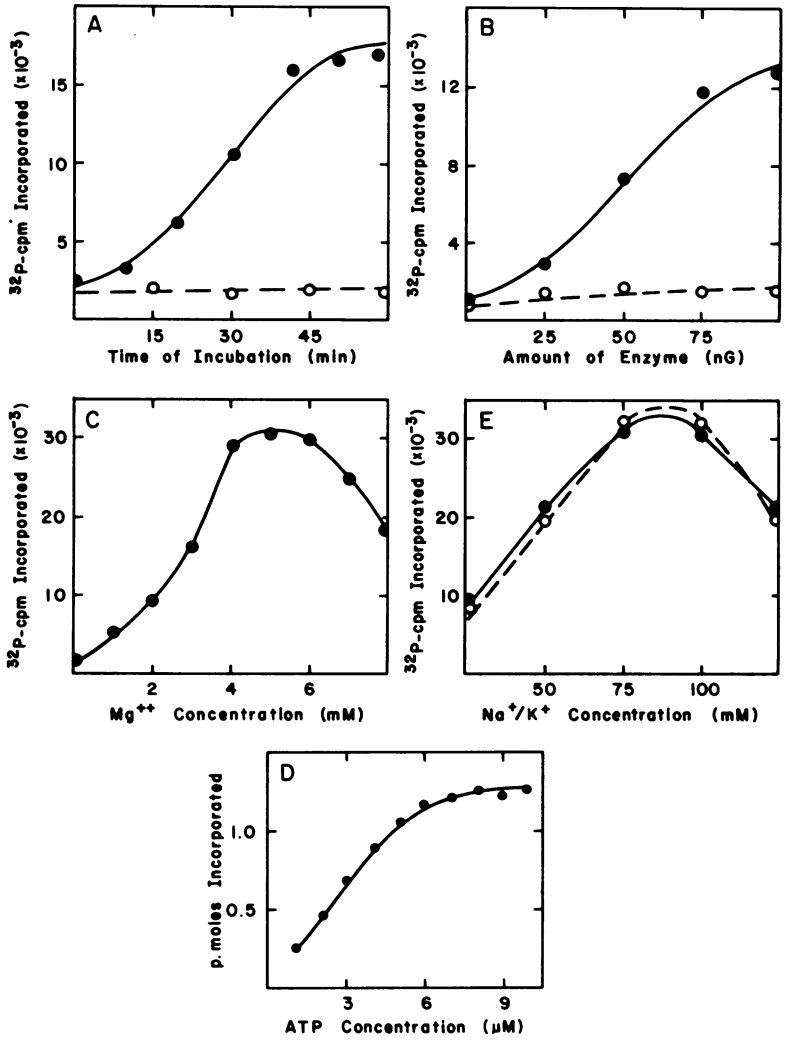

FIG. 3. *Biochemical characterization of the purified protein kinase from m3MSV (IC-MuLV) virions. The purified enzyme obtained after carboxymethyl cellulose chromatography (Fig. 1C) was used for these studies. A, Approximately 75 ng of enzyme was incubated with 10 μg of phosvitin under standard reaction conditions for the indicated lengths of time. Trichloroacetic acid-precipitable radioactivity was measured. Symbols: ●, purified enzyme plus phosvitin; ○, heat-inactivated (60°C, 15 min) enzyme plus phosvitin. B, Various amounts of enzyme were incubated with 10 μg of phosvitin under standard reaction conditions for 40 min at 22°C. Trichloroacetic acid-precipitable radioactivity was measured. Symbols: ●, enzyme plus phosvitin; ○, heat-inactivated (60°C, 15 min) enzyme plus phosvitin. C, Approximately 125 ng of enzyme and 10 μg of phosvitin were incubated under standard reaction conditions, except the amount of Mg^{2+} was varied as indicated. Trichloroacetic acid-precipitable radioactivity was measured after 40 min of incubation at 22°C. D, Approximately 125 ng of enzyme and 10 μg of phosvitin were incubated under standard reaction conditions with Mg^{2+}, except the concentrations of sodium chloride (●) or of potassium chloride (○) were varied. E, Approximately 150 ng of enzyme and 10 μg of phosvitin were incubated under standard conditions of Mg^{2+} and KCl at various concentrations of ATP. To vary the ATP concentration, a constant amount of [γ-^{32}P]ATP (~3,000 Ci/mmol) was used, and the final concentration was obtained by adding unlabeled ATP. Incorporation is expressed as picomoles of phosphate transferred into trichloroacetic acid-precipitable form.*

The use of higher-specific activity [γ-^{32}P]ATP was necessary to obtain adequate ^{32}P incorporation into protein (ca. 6,000 cpm per 100 ng of enzyme) for further analysis. The data were then compared to the stability of trichloroacetic acid-precipitable ^{32}P incorporated in reactions carried out in the presence of phosvitin or α-casein. In these substrate-dependent reactions, less than

TABLE 1. *Effects of variation in reaction conditions on the LMW kinase activity*[a]

Additions	^{32}P incorporated (pmol/mg of protein)	% of control activity
Enzyme (~150 ng)	91	
Plus phosvitin	216	100
Plus α-casein	165	76
Plus histones	83	39
Plus 2.5 μM cAMP	215	99
Plus 1.5 μM cAMP	219	101
Plus 0.5 μM cAMP	213	99
Plus 2.5 μM cGMP	218	101
Plus 1.0 μM cGMP	209	97
Minus Mg^{2+}	14	6
Minus Mg^{2+}, plus 5 mM Mn^{2+}	13	6
Plus Mg^{2+}, plus 1 mM Mn^{2+}	210	97
Plus Mg^{2+}, plus 5 mM Mn^{2+}	105	49
Minus Mg^{2+}, plus 2 mM Ca^{2+}	12	5
Plus Mg^{2+}, plus 1 mM Ca^{2+}	206	95
Plus Mg^{2+}, plus 5 mM Ca^{2+}	47	22
Plus Mg^{2+}, plus 0.1 mM Zn^{2+}	15	7

[a] Standard reaction mixtures contained ~150 ng of enzyme, 20 mM Tris-hydrochloride (pH 8.0), 5 mM $MgCl_2$, 75 mM KCl, and 1 μM [γ-^{32}P]ATP (~25 Ci/mmol) in a total volume of 50 μl. Substrates were added to a final concentration of 200 to 500 μg/ml. The effects of ions and cyclic nucleotides were tested with phosvitin as the substrate. Acid-precipitable counts in triplicate incubations were measured by liquid scintillation counting (at approximately 80% efficiency); a background of 0.8×10^3 cpm was subtracted from each value. The incorporation obtained with phosvitin substrate under standard assay conditions was normalized to 100%.

1% of the incorporated radioactivity could be accounted for by the endogenous phosphorylation of the enzyme (Table 1). As such, the analysis of acid and alkali stability of the total reaction products primarily reflects the nature of phosphomonoesters formed in the substrate molecules. In all cases all of the incorporated trichloroacetic acid-insoluble radioactivity was sensitive to treatments with bacterial phosphomonoesterases indicating the formation of phosphoamino acids. The results of acid and alkali stability experiments are presented in Table 2. Stability to treatments with either 0.1 M HCl at 37°C for 60 min or 1.0 M KOH at 55°C for 2 h indicates the absence of nitrogen-linked phosphate in the products of autophosphorylation reactions. In contrast, when phosvitin (which does not contain any tyrosine) was used as a substrate, less than 2% of the total incorporated radioactivity was alkali stable. Further, treatment with 6.0 M HCl for 100 min at 110°C converted a significantly larger fraction (28%) of the radioactivity incorporated in autophosphorylation reactions than the fraction (<10%) of radioactivity incorporated in the presence of substrates into free phosphate; this was measured by extraction of the reaction products in isobutanol-benzene in the presence of sulfuric acid and ammonium molybdate (15). Thus, it appears that the LMW kinase catalyzes the formation of serine or threonine phosphates (or both) in nonspecific substrate molecules.

To directly examine the phosphoamino acids formed, the products of autophosphorylation and substrate phosphorylation were concentrated by trichloroacetic acid precipitation and then hydrolyzed with 6.0 M HCl for 90 min at 100°C. The hydrolysates were analyzed on Whatman 3MM chromatography paper in 0.5% pyridine–5% acetic acid buffer (pH 3.5) for 2,900 V-h. The areas containing phosphotyrosine, phosphoserine, and phosphothreonine were marked by ninhydrin staining of authentic markers (Fig. 5). The reactions were performed using 0.3 μM [γ-^{32}P]ATP at a specific activity of 100 Ci/mmol. In the absence of any substrate (lane 3), the enzyme led to the formation of

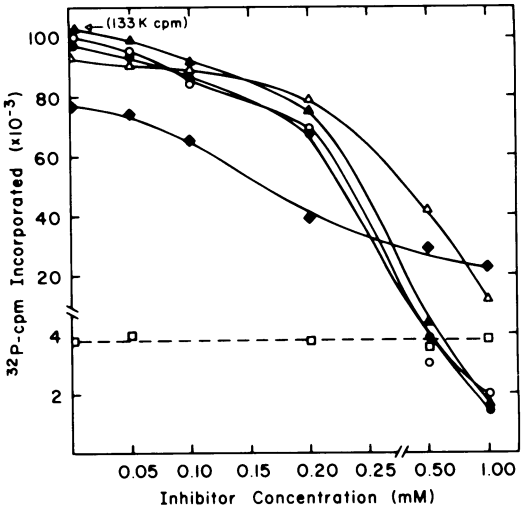

FIG. 4. *Effects of TLCK and TPCK on phosphotransfers catalyzed by purified MSV kinase. Incubations were carried out under standard conditions in the presence of various amounts of inhibitors for 30 min at 22°C. Trichloroacetic acid-precipitable ^{32}P counts per minute was measured. Symbols:* □, *enzyme alone (100 ng) incubated under standard conditions, except that ATP was added at a specific activity of ~150 Ci/mmol, with increasing concentrations of TLCK;* ●, *enzyme (100 ng) plus phosvitin (10 μg) incubated under standard conditions with increasing concentrations of TLCK;* ○, *enzyme (100 ng) plus phosvitin (10 μg) incubated under standard conditions with increasing concentrations of TPCK;* ▲, *heat-inactivated (60°C, 10 min) 3B11IC cytoplasmic extract incubated with 100 ng of purified kinase with increasing concentrations of TLCK;* △, *same as* ▲ *above, except with increasing concentrations of TPCK;* ◆, *3B11IC cytoplasmic extract incubated under standard conditions with increasing concentrations of TLCK.*

TABLE 2. *Acid and alkali sensitivities of the phosphomonoester bonds in the proteins phosphorylated by the LMW kinase*[a]

Treatment	% of incorporated radioactivity		
	Enzyme alone (auto-phospho-rylation)	Enzyme plus phosvitin	Enzyme plus α-ca-sein
None	100	100	100
0.1 M HCl (37°C, 1 h)	94	102	100
0.1 M KOH (37°C, 1 h)	96	98	94
1.2 M HCl (55°C, 2 h)	89	82	93
1.2 M KOH (55°C, 2 h)	96	<2	<2
Free phosphate (6 M HCl, 100°C, 100 min)	28	9	8

[a] Reaction mixtures contained, in 70 μl, about 150 ng of the enzyme, 2.0 μg of substrate (phosvitin or α-casein), 20 mM Tris-hydrochloride (pH 8.0), 5 mM $MgCl_2$, 75 mM KCl, and 1.0 μM [γ-^{32}P]ATP (~25 Ci/mmol). The average amount of trichloroacetic acid-precipitable incorporation in triplicate incubations was 122,000 and 81,000 cpm when the substrates used were phosvitin and α-casein, respectively. For autophosphorylation reactions, the substrate was omitted, and ATP was used to a final concentration of 0.3 μM at a specific activity of ~250 Ci/mmol to obtain adequate trichloroacetic acid-precipitable radioactivity; the average incorporation was 11,200 cpm. Control untreated and various acid- or alkali-treated materials were then precipitated with 10% trichloroacetic acid, and the acid-precipitable radioactivity was measured. The average trichloroacetic acid-precipitable radioactivity obtained in each incubation is considered as 100% to calculate radioactivities remaining trichloroacetic acid precipitable after the indicated treatments. To measure the amount of free phosphate released from the phosphorylated proteins, the trichloroacetic acid-precipitated proteins obtained after appropriate treatments were hydrolyzed with 6 M HCl at 100°C for 100 min. Free phosphate was converted to ammonium phosphomolybdate (15), and the radioactive phosphomolybdate was extracted in isobutanol-benzene and counted in a liquid scintillation counter.

phosphotyrosine. In the presence of phosvitin or casein (lanes 1 and 2), the majority of phosphate was transferred to phosphoserine. A low level of phosphotyrosine seen in the latter two might have come from phosphorylated enzyme molecules or from substrate molecules phosphorylated at tyrosine by the enzyme or from both. In either case, the major product of the phosphotransfer catalyzed by the LMW kinase is phosphoserine in exogenously added substrate molecules. For further quantitation, the phosphoamino acid spots were cut out and counted by liquid scintillation spectrometry. The ^{32}P radioactivity in phosphotyrosine was the highest (328 cpm) in the absence of substrates and much reduced (~90 cpm) when substrates were present. Phosphoserine contained about 2,900 and 2,300 cpm in reactions with phosvitin and casein, respectively. No detectable radioactivity was present in phosphoserine when no substrates were added. Under the reaction conditions ^{32}P radioactivity was not detectable in threonine phosphate. In all our hydrolysates, significant levels of free $^{32}P_i$ were detected; this could have arisen by the acid hydrolysis of free [γ-^{32}P]ATP contaminating the trichloroacetic acid-precipitated pellets which were obtained by using approximately 10 μg of bovine serum albumin carrier.

Taken together, the results of alkali stabilities and high voltage paper electrophoretic analyses of the phosphomonoesters formed in various phosphotransfer reactions further indicate that the LMW kinase of MSV forms phosphotyrosine in the apparent autophosphorylation reaction. As described below (Fig. 6, lane 1), the only

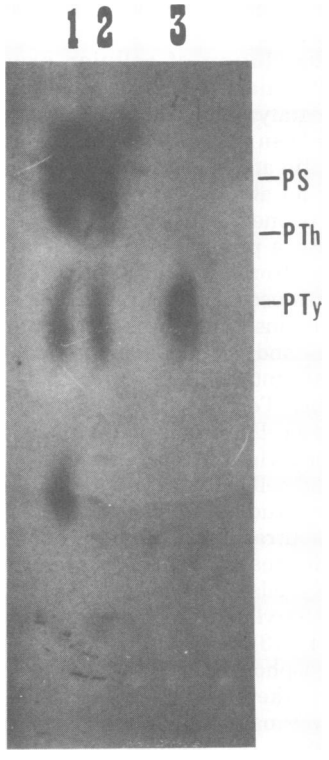

FIG. 5. *The identification of phosphoamino acids formed in reactions catalyzed by the LMW kinase. Standard reaction mixtures containing ~150 ng of purified enzyme were incubated with either 10 μg of phosvitin (lane 1), 10 μg of casein (lane 2), or in the absence of any substrate (lane 3). The phosphorylated protein products were concentrated by precipitation with trichloroacetic acid by using 20 μg of bovine serum albumin carrier. The proteins were then hydrolyzed with 6.0 N HCl at 100°C for 90 min and subjected to paper electrophoresis for 2,900 V-h. The positions of authentic markers from top to bottom are: phosphoserine (PS), phosphothreonine (PTh), and phosphotyrosine (PTy). The area containing free phosphates was cut out from the paper strip before autoradiography.*

protein which is detectably phosphorylated in an autophosphorylation reaction is the 16.5K peptide, the presumptive enzyme. The presence of a highly active protein kinase activity which phosphorylates tyrosine in the 16K substrate molecules cannot be ruled out. Whether the enzyme also catalyzes low levels of phosphotyrosine formation in reactions with exogenously added substrates like phosvitin and casein is not clear. The separation and recovery of phosphorylated polypeptides at the end of the reaction by gel electrophoresis were complicated because of the extent of ^{32}P incorporation and the fact that commercially obtained phosvitin and casein preparations, after phosphorylation reactions, yielded very diffuse bands in standard SDS-PAGE systems.

Phosphorylation of cellular proteins by the LMW kinase of MSV. Phosphorylation reactions catalyzed by the LMW kinase of MSV have so far been studied with artificial substrates like phosvitin and casein. The enzyme was then tested for its ability to phosphorylate cellular proteins. As one possible source of candidate in vivo targets, a preparation of soluble cytoplasmic proteins from the m3MSV producer culture, 3B11IC, was used. Such a crude preparation of cellular proteins would be likely to contain many of the viral and cellular gene products in their approximate intracellular proportions in transformed cells. To relate the phosphorylation of transformed cell proteins by the LMW kinase to the phosphorylation reactions which occur in transformed cell cytoplasm, three types of incubation were studied. First, cytoplasmic proteins from the nontransformed parent of the 3B11IC line were incubated in parallel with those from the 3B11IC cells in the presence of [γ-^{32}P]ATP. The phosphorylated products were analyzed by SDS-PAGE (13) on a long (20-cm) gel to detect unique phosphorylation products which might serve as markers for specific phosphorylation events in normal versus transformed cell cytoplasm. Second, transformed cell cytoplasm was preincubated at an elevated temperature (60°C for 10 min) to inactivate the endogenous kinases, and this heat-treated cytoplasmic protein preparation was used as a substrate mixture for the purified LMW kinase. The major phosphorylated products generated in such reactions by the LMW kinase were compared with those generated by the endogenous kinases in the cytoplasmic preparations of normal and transformed cells. Third, the phosphoamino acids in the products of the endogenous cytoplasmic incubations were compared to those generated by reconstruction of transformed cell cytoplasmic proteins with LMW kinase.

In Fig. 6A, a typical SDS-PAGE analysis of the products of phosphorylation reactions is presented. The products of the endogenous kinase reaction in the MSV-transformed cell line, 3B11IC, were not detectably altered by the addition of cAMP or low concentrations of Ca^{2+}. The bulk of the kinase reactions were dependent on Mg^{2+}. In the absence of added Mg^{2+}, a single band comigrating with the autophosphorylated LMW kinase enzyme was phosphorylated in 3B11IC cytoplasm (lane 2). It is tempting to speculate that this might represent the intracellular LMW kinase; these molecules could be autophosphorylated even without Mg^{2+} added exogenously. A band at about 46K was also seen to be weakly phosphorylated under these conditions; the possible relationship of these to the 16K molecules is not clear. One major artifact of this system would arise from simultaneous dephosphorylation and phosphorylation of cytoplasmic proteins during the incubation which would then be observed as phosphorylated products. To detect such products, a 15-min chase with 1,000-fold excess of unlabeled ATP was performed after the standard incubation. The majority of the bands did not appear to be phosphorylated by such simultaneous phosphatase-kinase activities since, in that case, these bands would have lost their associated radioactivity (lane 5). The 16K band appeared to lose its radioactivity during such a chase. This could be due to a reversible phosphorylation mentioned above, or it might represent an active turnover of phosphate residues from the autophosphorylated enzyme molecules, if indeed these represented an intracellular form of the virion LMW kinase.

A comparison of polypeptide bands phosphorylated in vitro in cytoplasmic extracts of normal and MSV-transformed mouse cells show four major phosphorylation products which are unique to the transformed cells (Fig. 6A, lane 4 compared with lanes 7 and 8). These polypeptides migrate at 46, 33, 20, and 16K; in addition, 25, 35, and 38K polypeptides also appear to be phosphorylated significantly more than in untransformed cells. One or more of these potentially represent new virally coded molecules expressed in cells and are phosphorylated by normal cellular kinases; alternatively, these could be cellular polypeptides (or viral polypeptides) phosphorylated by new protein kinase activities expressed in transformed cells.

After the identification of phosphorylation products which are preferentially generated in transformed cell extract, reconstruction experiments were performed to evaluate the possible participation of the virion LMW kinase in such phosphorylation reactions. The proteins phosphorylated by the virion LMW kinase with heat-

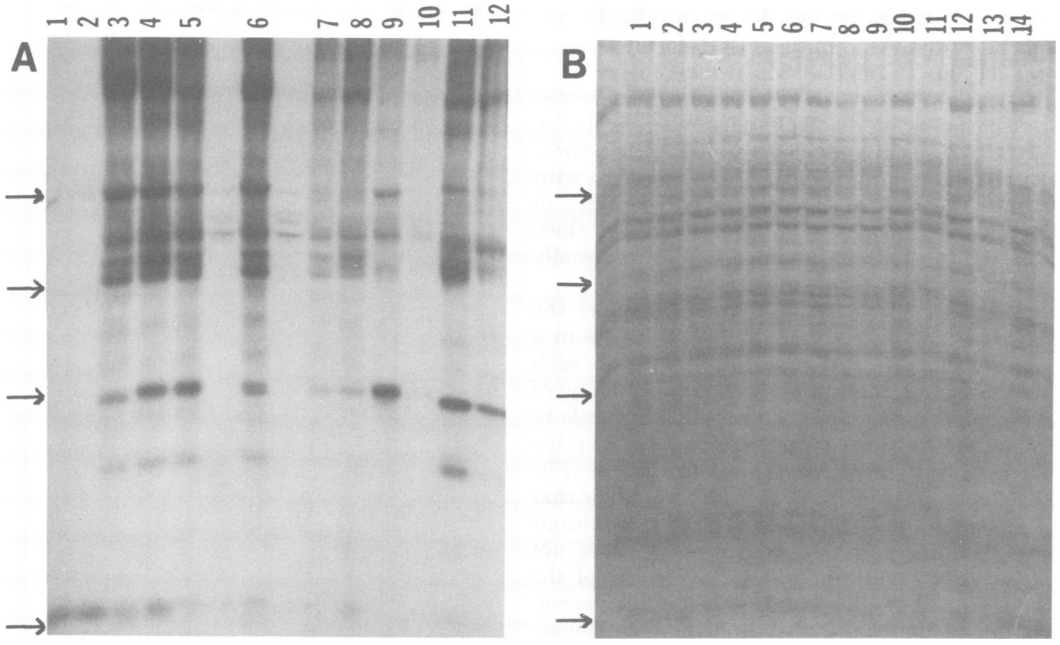

FIG. 6. *SDS-PAGE of 3B11IC cytoplasmic extracts incubated for phosphorylation studies. A, Incubations were made as follows: products were subjected to 20-cm 12.5% resolving gels which were then fixed, dried, and autoradiographed with Kodak XR-1 film using Du Pont Cronex Lightning Plus screens. The molecular mass markers indicated by the arrows from top to bottom are ovalbumin (44K), carbonic anhydrase (32K), α-chymotrypsinogen (26K), and ribonuclease (14K). The bottom 1.5 cm of the resolving gel is not shown. Lanes: 1, purified MSV kinase alone incubated with 10×-higher-specific activity [γ-^{32}P]ATP; 2, 3B11IC cytoplasmic extract incubated at high specific activity of [γ-^{32}P]ATP without Mg^{2+} or Ca^{2+}; 3, 3B11IC cytoplasmic extract incubated with Mg^{2+} but without Ca^{2+}; 4, 3B11IC cytoplasmic extract incubated with complete mixture described in the text; 5, incubation as in lane 4, except chased with a 100-fold excess of cold ATP added after the incubation and further incubated for 15 min; 6, 3B11IC cytoplasmic extract incubated in complete reaction mixture with added 1.5 μM cyclic AMP; 7, IC-MuLV-infected NIH cytoplasmic extracts incubated in complete reaction mixture; 8, IC-MuLV-infected NIH cytoplasmic extract incubated in standard reaction mixture with added 1.5 μM cyclic AMP; 9, crude m3MSV (IC-MuLV) virion lysate incubated under standard conditions; 10, 3B11IC cytoplasm heated at 60°C for 10 min and then incubated under standard conditions; 11, same as in lane 10, except 50 ng of purified MSV kinase was added; 12, heat-treated (60°C, 10 min) m3MSV (IC-MuLV)-infected NIH cytoplasmic extract incubated with 50 ng of purified MSV kinase. B, Coomassie blue staining of cytoplasmic extracts incubated under various conditions. Standard molecular mass markers are, from top to bottom, ovalbumin (44K), carbonic anhydrase (32K), α-chymotrypsinogen (26K), and ribonuclease (14K). Lanes 1 through 9 are 3B11IC cytoplasmic extracts incubated as follows: 1, without any additions; 2, with complete reaction mixture; 3, with Mg^{2+} omitted from the complete mixture; 4, with complete mixture and 50 ng of purified enzyme added; 5, with ATP omitted from the complete mixture; 6, with Ca^{2+} omitted from the complete mixture; 7, with complete mixture plus 1.5 μM cAMP; 8, with complete mixture plus 0.15 mM TLCK; 9, with complete mixture plus 1.0 mM TPCK. Lanes 10 through 14 are IC-MuLV helper-infected NIH cytoplasmic extracts incubated as follows: 10, without additions; 11, with complete mixture; 12, with complete mixture plus 1.5 μM cAMP; 13, with complete mixture plus 0.15 mM TLCK; 14, with complete mixture plus 1.0 mM TPCK.*

inactivated 3B11IC cell cytoplasm are resolved in lane 11 (Fig. 6A). The 33, 25, and 20K polypeptides show preferential phosphorylation by the LMW kinase; in addition, the 46 and 38K polypeptides as well as a new ~58K polypeptide also appear to be efficient acceptors. In contrast, various other polypeptides, including a major 62K and a 35K phosphorylated species (lanes 3 through 6), are not significantly labeled by the LMW kinase. The 16K peptide is also not phosphorylated under these conditions. When a comparable reconstruction experiment is performed with a commercially available protein kinase (data not shown), a random and complex array of phosphorylated polypeptides are seen with little or no distinct display of bands. Thus, the heat inactivation step does not appear to destroy the acceptor activity of the majority of cytoplasmic polypeptides. The virion LMW kinase, then, does exhibit a certain level of substrate prefer-

ence in this reconstructed system. When cytoplasmic proteins from the untransformed parental cell line (NIH/ICX-MuLV) were used (lane 12), the 46, 33, and 20K species were not detected as phosphorylated polypeptides, whereas the 58, 38, and 25K polypeptides were efficiently phosphorylated. The results are consistent with either the lack of the former polypeptides in nontransformed cells or their increased thermolability or their presence in the maximally phosphorylated forms in normal cells.

Although in vitro phosphorylation under the reaction conditions described here results in a reproducible distinct pattern of bands for each of the two cell lines, Coomassie blue staining of the polypeptides present in the extract fails to show any difference in the major polypeptide compositions of such cytoplasmic extracts (Fig. 6B, lanes 1 through 14). It is also apparent that incubation under various reaction conditions used in the phosphorylation assays does not cause any significant processing of any of the stained polypeptide bands. It should, however, be noted that most of the polypeptide species detected by phosphorylation assays do not represent any of the major polypeptide species visualized by staining. As such it is not clear whether these polypeptides undergo processing during the incubation. However, varying the length of incubation has not led to a progressive appearance of lower-molecular-weight phosphorylated peptides, thereby arguing against proteolytic processing to be responsible for the observed differences in phosphorylated peptides in the cytoplasmic extracts of normal and transformed cells.

The total phosphorylation products formed in the endogenous cytoplasmic incubations as well as in reconstruction incubations were analyzed for their acid and alkali stabilities as described above for the data in Table 2. In each case >95% of incorporated radioactivity was alkali labile, indicating that the majority of the products contained serine or threonine phosphates. To specifically analyze the phosphoamino acids in certain phosphorylated species obtained by these in vitro incubations, specific bands were cut out from the SDS-PAGE gel and eluted with 1% β-mercaptoethanol in 50 mM ammonium bicarbonate. The eluted radioactivity was concentrated by lyophilization and hydrolyzed in 5.5 N HCl at 100°C for 2 h. The products were analyzed by electrophoresing for 2,800 V-h on Whatman 3MM chromatography paper in 0.5% pyridine–5% acetic acid buffer (pH 3.5). In Fig. 7, lanes 1 and 2 represent the products from the 16K phosphorylated band generated by autophosphorylation of the LMW kinase (lane 1 of Fig. 6A) and the 16K band generated in the endogenous phosphorylation of 3B11IC cytoplasm (lane 4 of Fig. 6A), respectively. In each case the major phosphoamino acid comigrated with phosphotyrosine. The phosphorylated 33K cytoplasmic protein, on the other hand, gave predominantly phosphoserine with a small amount (<8%) of phosphotyrosine. Since the 33K polypeptide yielded a diffuse band in the gel, it is not clear whether a single protein species was eluted for acid hydrolysis. The data of this experiment further suggest that the 16K cytoplasmic protein resembles the 16K virion LMW kinase in containing phosphotyrosine, whereas

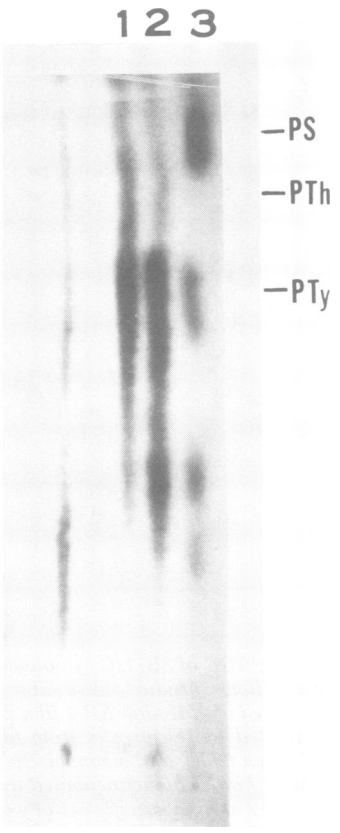

FIG. 7. *Identification of phosphoamino acids in phosphorylated cellular cytoplasmic proteins. Three SDS-PAGE-purified polypeptide species were eluted from gel slices as described in the text. The eluted polypeptides were hydrolyzed and electrophoresed as usual for 2,800 V-h. The positions of authentic markers phosphoserine (PS), phosphothreonine (PTh), and phosphotyrosine (PTy) are shown. The area containing free phosphate was removed from the paper strip before autoradiography. Lanes: 1, phosphorylated 16K protein generated by autophosphorylation of the LMW kinase; 2, phosphorylated 16K protein from the incubation of 3B11IC cytoplasm; 3, phosphorylated 33K protein in 3B11IC cytoplasm catalyzed by LMW kinase.*

one of the major cytoplasmic substrates phosphorylated by the LMW kinase contains predominantly phosphoserine.

DISCUSSION

The mouse sarcoma viral LMW kinase which binds to purified components of cellular cytoskeleton (20) has been purified from virion particles containing high titers of the m3 strain of MSV. The phosphotransfers catalyzed in vitro by this enzyme with candidate cellular protein substrates as well as artificial acceptor proteins have been analyzed with respect to the nature of phosphoamino acids formed, the sensitivity of the reactions to specific inhibitors, and certain other biochemical parameters of the reaction. In analogy with the protein kinase activities associated with the larger polypeptide products of other retroviral transforming genes like the $pp60^{src}$ prescribed by the SRC gene of avian sarcoma virus (6, 11, 14, 19) and the p120 protein of the Abelson murine leukemia virus (23, 24), the 16K LMW protein kinase of MSV is a cyclic AMP-independent, Mg^{2+}-requiring enzyme which prefers ATP as a phosphate donor and forms phosphotyrosine bonds. The phosphorylation reaction catalyzed by the enzyme is also sensitive to TLCK, a property that has been described for the immunocomplex kinase of avian sarcoma virus (18).

Total soluble proteins from cell lysates have been used to search for candidate preferred molecules phosphorylated by the virion kinase. A few of the cellular cytoplasmic proteins, ~58, 46, 38, 33, 25, and 20K, which are present in MSV-transformed mouse cells appear to be preferentially phosphorylated by the enzyme. Thus, although the enzyme can efficiently phosphorylate a variety of substrates in purified systems, it shows preference for certain species of cellular proteins in a mixture where the different substrate molecules are present in relative amounts approximating their intracellular concentrations. In the absence of substrates, the enzyme catalyzes an apparent autophosphorylation reaction in vitro when it forms predominantly phosphotyrosine residues. The addition of a serine protease inhibitor, TLCK, which has been shown to cause a partial phenotypic reversion of avian sarcoma virus-transformed cells (22), also inhibits the phosphotransfer catalyzed by the MSV kinase. The effects of TLCK or TPCK on the cellular phenotypes and the changes in intracellular protein phosphorylations after TLCK treatment of MSV-transformed cells are not known at the present time. The results of the in vitro inhibition of phosphotransfer are similar to those obtained in the absence of substrates, i.e., the apparent autophosphorylation is unaffected, and phosphotyrosine bonds are formed. A related inhibitor, TPCK, produced comparable effects with a lower efficiency especially when cellular substrates were used. A polypeptide protease inhibitor, soybean trypsin inhibitor, failed to cause any detectable affects on the MSV kinase activity (data not shown).

In the cells transformed by avian sarcoma virus, several size classes of related polypeptides have been identified as the products of avian sarcoma virus SRC gene (2, 12). Extensive studies on the genomes of various MSV isolates in relation to the nontransforming murine leukemia viral genome has suggested that the SRC gene of MSV could prescribe a polypeptide of about 45K (7). In vitro translation of the MSV genome and intracellular MSV-specific messages have also been shown to yield multiple molecular species of various size classes (16, 17). At this point, there is no formal evidence to indicate that the LMW kinase is a product of MSV, except for one indication from a temperature-sensitive mutant of MSV where the analogous LMW kinase is thermolabile (21). However, it is conceivable that the LMW kinase activity of MSV described here might represent one of the small biologically active products involved in transformation of cells by MSV. The larger products of various transforming genes might then represent multidomain polypeptides consisting of the "kinase domain" fused to other polypeptide sequences of other structural or catalytic functions (or both). The p120 protein molecules of Abelson murine leukemia virus (24) indeed represent fusion products between determinants specified by the GAG region and those specified by the transforming sequences in the viral genome. The availability of purified LMW kinase of MSV will now allow raising specific antisera to detect other species of polypeptides related to or closely associated with this small peptide and provide means for performing structure-function studies on these molecules in relation to cell transformation.

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