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Biochemistry

Tyrosyl protein kinases in normal rat liver: Identification and partial characterization

(peptide substrate/phosphotyrosine/subcellular fractionation)

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ABSTRACT **Rat livers were fractionated and subcellular components were assayed for tyrosyl protein kinase activity. About 60% of the kinase activity in the cytoplasm sedimented with the microsomal fraction, whereas 40% remained in the supernatant. Purification of cytosolic and microsomal kinases by ion-exchange and gel filtration chromatography resolved a major species whose molecular mass was 75 kilodaltons (referred to as TPK 75) and a minor one whose molecular mass was >160 kilodaltons. Partially purified TPK 75 phosphorylated a protein of the same molecular mass on tyrosine residues. The activity associated with TPK 75 was not stimulated by growth factors and was sensitive to thiol reagents.**

Tyrosyl protein kinase activities have been demonstrated to be associated with or intrinsic to the transforming gene products of a number of sarcoma-inducing retroviruses and have been postulated to be responsible for the transforming ability of these agents (for review, see ref. 1). Cellular receptors for growth factors also have been shown to possess tyrosyl kinase activities (2–5). The relationship between the tyrosyl kinase activities associated with growth factor receptors and those associated with viral-transforming proteins is not clear. However, it is apparent that analyses of these kinases will contribute to the understanding of how these enzymes are involved in the control of normal cell metabolism and how viral tyrosyl kinases effect transformation of normal cells to cancer cells.

Phosphotyrosine has previously been detected in proteins of normal cells, although it constitutes <0.1% of total cellular phosphoamino acids (6). Little is known about the cellular enzymes that are responsible for this particular postsynthetic modification of proteins. To date, investigations of tyrosyl protein kinases in normal cells have been limited to approaches that utilized antibodies directed against viral enzymes (7–9). However, the recent use of *in vitro* peptide substrates containing tyrosine, but lacking serine and threonine, has made it possible to assay a variety of tyrosyl protein kinases (10–13). We have made use of the *in vitro* phosphorylation of angiotensin peptides (13) to study the abundance of tyrosyl kinases in rat liver subcellular components. The most abundant of these kinases was partially purified and characterized.

MATERIALS AND METHODS

Materials. Enzyme-grade sucrose and ultra-pure sucrose were purchased from Schwarz/Mann. Aquacide IIA was from Calbiochem. Epidermal growth factor (EGF) was from Collaborative Research (Waltham, MA). [Val^5]Angiotensin II was synthesized in our laboratory. [γ-^{32}P]ATP (3,000 Ci/mmol; 1 Ci = 3.7×10^{10} Bq) was from Amersham and was diluted with unlabeled ATP to obtain the appropriate specific activity. Trasylol was from Mobay Chemical (New York). DEAE-Sephacel and Sephacryl S-200 were from Pharmacia. All other reagents were from Sigma.

Subcellular Fractionation. All operations were carried out at 4°C. Pathogen-free female rats (Charles River Breeding Laboratories) of Sprague–Dawley strain, weighing 150–250 g, were starved 14–16 hr before sacrifice. Livers were homogenized in 5 vol of cold homogenization buffer (0.25 M sucrose/25 mM Tris·HCl, pH 7.4/0.1 mM EDTA/5 mM $MgCl_2$/5 mM KCl/Trasylol at 100 units/ml/1 mM phenylmethylsulfonyl fluoride) with six strokes in a Teflon/glass Potter–Elvehjem homogenizer [0.012 inch (0.03 cm) clearance, Kontes Glass] driven at 1,000–1,200 rpm. The crude homogenate then was fractionated to yield purified cellular organelles (see Table 1) as described (14).

For fractionation of kinases in S_{100} and P_{100} fractions (see Table 2 and below), liver homogenate was centrifuged at 12,000 $\times$ *g* for 15 min to yield a postmitochondrial supernatant (PMS). The PMS fraction was filtered through three layers of cheesecloth and was centrifuged at 100,000 $\times$ *g* in a Beckman 70 Ti rotor for 1 hr. This step yielded a supernatant (S_{100}) and a pellet (P_{100}) that were further fractionated.

For fractionation of microsomes into smooth endoplasmic reticulum (SER) and rough endoplasmic reticulum (RER) fractions, livers were homogenized in ion-free 0.25 M sucrose and the homogenate was fractionated as described (15). The ribosomal pellet thus obtained was resuspended in 0.25 M sucrose/TEM buffer (25 mM Tris·HCl, pH 7.4/0.1 mM EDTA/5 mM $MgCl_2$/2 mM dithiothreitol) at a protein concentration of 5 mg/ml. The SER and RER fractions were collected, pelleted, and also resuspended in 0.25 M sucrose/TEM buffer at a final protein concentration of 5 mg/ml.

Kinase Assays. Tyrosyl protein kinase activity was determined by using angiotensin peptides as substrates as described (13). For measuring activities in subcellular fractions, assays were carried out in a total volume of 25 μl containing [Val^5]angiotensin II at 2.0 mg/ml, 40 μM [γ-^{32}P]ATP (22,000 cpm/pmol), 10 mM $MnCl_2$, 25 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 6.5), 12 mM *p*-nitrophenyl phosphate, 10 mM 2-mercaptoethanol, and 20–30 μg of protein. Reaction mixtures were incubated at 30°C for 5 min and reactions were stopped by further incubation of the samples at 90°C for 3 min. Five-microliter aliquots of the reaction products were separated by high-voltage paper electrophoresis at pH 3.5 and incorporation of radioactivity into phosphopeptides was determined as de-

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Abbreviations: EGF, epidermal growth factor; PCMB, *p*-chloromercuribenzoic acid; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; TLCK, *N*-α-tosyl-L-lysine chloromethyl ketone; SER, smooth endoplasmic reticulum; RER, rough endoplasmic reticulum; kDa, kilodalton(s); PMS, postmitochondrial supernatant.

Table 1. Distribution of tyrosyl protein kinase activity in rat liver subcellular fractions

Fraction	Protein, mg	Kinase activity, units/g of liver*	Marker enzyme activity, μmol/min per mg: 5′-Nucleotidase	Succinate-cytochrome c reductase	Glucose-6-phosphatase
Homogenate	2,900	77.2	0.084	0.018	0.029
Nuclei	33.6	0.851 (0.8)	0.044	0.017	0.032
Plasma membrane	8.10	2.02 (1.9)	0.607	ND	0.023
Mitochondria	171	2.27 (2.2)	0.207	0.292	0.068
Microsomes (P_{100})	150	64.0 (61.4)	0.237	0.031	0.143
Supernatant (S_{100})	940	35.2 (33.8)	0.013	ND	0.018

Rat livers (17 g) were homogenized and fractionated as described (14). ND, not determined.
*Numbers in parentheses are % of total kinase activity recovered.

scribed (13). One unit of enzyme activity is defined as 1 pmol of ^{32}P incorporated into peptide per min per reaction at 30°C.

When column fractions were assayed, kinase reactions were carried out as above for 30 min without *p*-nitrophenyl phosphate in the mixtures. Reactions were stopped by heating and samples were centrifuged briefly in an Eppendorf Microfuge to remove denatured proteins. Fifteen-microliter aliquots of the supernatants were acidified with an equal volume of 60% acetic acid. The mixtures were spotted on Whatman P81 paper squares (2 × 2 cm), which were washed with 30% acetic acid as described (16). During the purification of kinases from the microsomal salt-wash fraction, we found it necessary to perform kinase assays with and without peptide substrate to eliminate artifactual peaks. This was because of the presence of acid-soluble species in the microsomal salt-wash that became phosphorylated and adsorbed to the phosphocellulose paper.

Other Techniques. Protein concentration was determined by using bovine serum albumin as standard (17). RNA was determined as described by Fleck and Munro (18). Marker enzymes for cellular subfractionation were determined as described (19–21).

RESULTS

Tyrosyl Protein Kinases Were Found Predominantly in High-Speed Pellet and Supernatant of Rat Liver Homogenate. To determine the subcellular location of tyrosyl kinases, we prepared a homogenate from 17 g of rat liver, which then was fractionated by centrifugation into nuclei, plasma membranes, mitochondria, microsomes, and a high-speed supernatant. Each of these subcellular fractions was verified by assaying for marker enzymes characteristic of plasma membrane (5′-nucleotidase), mitochondria (succinate-cytochrome *c* reductase), and microsomes (glucose-6-phosphatase) (Table 1). These fractions were assayed for tyrosyl protein kinase activity by using [Val^5]angiotensin II as a substrate. The conditions of the assay have been optimized previously for the measurement of tyrosine-specific kinase activity and include the use of *p*-nitrophenyl phosphate as a phosphatase inhibitor (13). The sensitivity of the assay allows the precise detection of 0.001 unit of activity. In rat liver, we found that about 60% of the recovered kinase activity sedimented with the microsomal fraction and 30–35% was found in the 100,000 × *g* supernatant (Table 1). Approximately 2% or less of the total activity was associated with nuclei, plasma membranes, or mitochondria. The total activity that was recovered in the subcellular fractions was about 135% of that found in the homogenate. The lesser amount of activity in the homogenate may be due to the presence of inhibitory components.

We also attempted to devise procedures for further fractionation of the major tyrosyl kinases of rat liver and results are shown in Table 2. Tyrosyl kinases in the high-speed supernatant could be precipitated completely by adjusting the pH to 5.0. This procedure removes about 80% of the proteins in the high-speed supernatant and resulted in a significant enhancement in the specific activity. When the microsomal pellet was extracted with buffer that contained high concentrations of salt and EDTA, only about half of the kinase activity was released into the wash supernatant.

Tyrosyl Kinase Activities Are Associated with Free Ribosomes and Smooth and Rough Microsomes. To determine more precisely the association of tyrosyl kinase activity with cellular organelles, we further fractionated the microsomal fraction into its major individual components: free ribosomes and endoplasmic reticulum. Fractionation of the PMS on a discontinuous sucrose density gradient yielded a free ribosomal pellet, a dense interface of RER, a less dense interface containing SER, and a supernatant. All three particulate fractions were found to contain tyrosyl kinase activity (Table 3). The smooth microsomes contained about twice as much activity as was found in rough microsomes or free ribosomes. The supernatant was not characterized because of the difficulty in quantitatively recovering that fraction from the gradient. All of the kinase ac-

Table 2. Fractionation of tyrosyl kinases in high-speed supernatant and pellet

Fraction	Protein, mg/g of liver	Kinase activity, units/g of liver	Specific activity, units/mg
PMS	67.6	147	2.17
S_{100}	51.2	45.9	0.90
pH 5 fraction	6.65	41.7	6.27
P_{100}	10.5	59.8	5.70
Microsome wash	3.20	29.2	9.08
Washed microsomes	6.02	27.8	4.64

Rat livers (17 g) were homogenized and fractionated as described in the text. The S_{100} fraction was diluted with 1.5 vol of cold distilled water and was acidified to pH 5.0 by dropwise addition of 10% acetic acid over 5 min. The sample was stirred on ice for 75 min and was centrifuged at 8,000 × *g* for 5 min. The supernatant was discarded and the pellet was extracted twice with 15 ml of buffer A (10% glycerol/25 mM Tris·HCl, pH 7.4/0.1 mM EDTA/10 mM 2-mercaptoethanol) containing 0.05 M NaCl. Insoluble material was removed by centrifugation at 10,000 × *g* for 10 min. Supernatants were combined (pH 5 fraction) and were assayed for kinase activity. Results are expressed in units per g liver. The P_{100} fraction was resuspended with a Dounce homogenizer in buffer B (20% glycerol/25 mM Tris·HCl, pH 7.4/20 mM EDTA/1.0 M NaCl/10 mM 2-mercaptoethanol) to 5 mg of protein per ml. The suspension was incubated on ice for 1 hr and was centrifuged at 200,000 × *g* in a Beckman 70 Ti rotor for 1 hr. The supernatant (microsome wash) was removed and the pellet (washed microsomes) was resuspended in buffer B.

Table 3. Association of kinase activity with ribosomes, SER, and RER

Fraction	Protein, mg	RNA/protein	Activity, units
Free ribosomes	45.1	0.233	323
SER	85.0	0.066	523
RER	72.0	0.317	200

PMS was prepared from 15 g of rat liver and was fractionated on a discontinuous sucrose gradient (15). Fractions were collected and assayed for kinase activity.

tivity associated with free ribosomes could be extracted with buffer that contained 0.5 M KCl, whereas about 50% of the activity associated with SER and RER fractions was extractable with buffer B (data not shown).

Chromatographic Behavior of Cytosolic and Microsomal Tyrosyl Protein Kinases. The pH 5 fraction prepared from cytosol was freed of nucleic acids by chromatography on DEAE-Sephacel and then was purified further by phosphocellulose chromatography. Almost 80% of the protein applied to phosphocellulose was not retained on the column (not shown), whereas the majority of kinase activity eluted as a single peak between 0.26 and 0.32 M NaCl (Fig. 1A). Fractions that contained activity were pooled and further purified on a Sephacryl S-200 column. In this step, two peaks of activity were observed (Fig. 1B). The minor peak (peak I) eluted close to the void volume of the column, whereas the major peak (peak II) had a mobility corresponding to a molecular mass of about 75 kilodaltons (75 kDa) (referred to as TPK 75). Stored in 50% glycerol at −20°C, TPK 75 was stable for at least 5 months.

The microsomal salt-wash fraction was purified similarly. Again, all of the activity eluted from phosphocellulose as a single peak between 0.26 and 0.32 M NaCl (Fig. 1C). Further chromatography of that peak on a Sephacryl S-200 column resolved the activity into two peaks (I and II) with molecular masses of greater than 160 and 75 kDa (Fig. 1D).

In the chromatographic procedures described here, bovine serum albumin was added to all fractions because the enzymatic activities were extremely labile at low concentrations of protein when stored at 4°C. Therefore, it was not possible at this point to assess reliably the extent of purification.

A 75-kDa Phosphoprotein Was Found in Both the Cytosol and the Microsomal Salt-Wash Fraction. Both the cytosol and microsome salt-wash fraction contained kinases that behaved identically during ion-exchange chromatography on phosphocellulose and during gel filtration on Sephacryl S-200. To ascertain whether or not these kinases were identical, we incubated peak fractions with [γ-^{32}P]ATP and $MnCl_2$ and analyzed the reaction products by gel electrophoresis. After chromatography on phosphocellulose, both the cytosolic and microsomal fractions contained a large number of proteins with molecular masses ranging from 50 to >200 kDa that were phosphorylated during the *in vitro* incubation (Fig. 2A, lanes 1 and 4). The majority of these proteins eluted in peak I of the Sephacryl column (Fig. 2A, lanes 2 and 5). A 75-kDa phosphoprotein was the

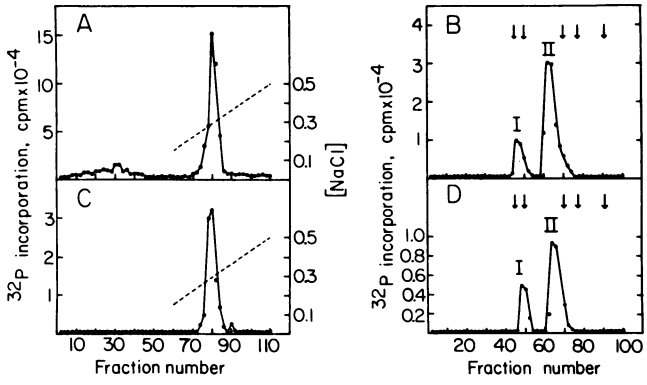

FIG. 1. Chromatography of rat liver tyrosyl kinases on phosphocellulose and Sephacryl S-200 columns. (A) Phosphocellulose chromatography of pH 5 fraction prepared from high-speed supernatant. The pH 5 fraction was prepared from 40 g of liver as described in the legend to Table 2. The sample (300 mg of protein) was chromatographed on a column (14 × 1.5 cm) of DEAE-Sephacel equilibrated in buffer A containing 0.05 M NaCl. The column was washed with 2 bed vol each of buffer A containing 0.05 M NaCl and buffer A containing 0.3 M NaCl. The flow-through (80 ml) and 0.3 M salt-wash (30 ml) fractions were pooled and applied to a column (10 × 1.5 cm) of phosphocellulose equilibrated in buffer A with 0.15 M NaCl. From this point on during the purification, bovine serum albumin was added to all column fractions to a final concentration of 0.1%. The column was washed with the same buffer and then washed with a linear gradient of 0.15 M (80 ml) to 0.50 M NaCl (80 ml) in buffer A. The flow rate was 20 ml/hr and 3-ml fractions were collected. (B) Sephacryl S-200 chromatography of fractions 76–84 (A). Fractions were pooled and concentrated by dialysis against Aquacide IIA. The concentrated sample was applied to a Sephacryl S-200 column (90 × 2.8 cm) equilibrated in buffer C (10% glycerol/25 mM Tris·HCl, pH 7.4/0.1 M NaCl/10 mM 2-mercaptoethanol). The column was washed with the same buffer at a rate of 20 ml/hr and 4-ml fractions were collected. Fractions that contained kinase activity were pooled and dialyzed against 50% glycerol/25 mM Tris·HCl, pH 7.4/0.1 M NaCl/10 mM 2-mercaptoethanol. (C) Phosphocellulose chromatography of microsomal salt-wash fraction. Microsomal fraction was prepared and extracted with buffer B as described in the legend to Table 2. The salt-wash fraction was dialyzed overnight against 4 liters of buffer A containing 0.05 M NaCl. The dialyzed fraction, containing 50 mg of protein, was chromatographed on DEAE-Sephacel and phosphocellulose as described in A. (D) Sephacryl S-200 chromatography of fractions 76–84 (C). Fractions were pooled, concentrated, and applied to a Sephacryl S-200 column as in B. Twelve-microliter aliquots of column fractions were used in kinase assays. Results are shown as radioactivity incorporated into peptide substrates (●). Arrows in B and D represent elution patterns of dextran blue, aldolase, bovine serum albumin, ovalbumin, and chymotrypsinogen, respectively.

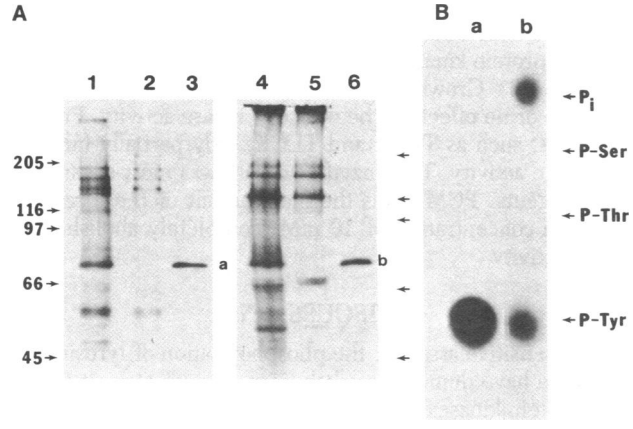

FIG. 2. Autoradiogram of a $NaDodSO_4$/polyacrylamide gel of *in vitro* phosphorylation products of rat liver kinase fractions. (A) Partially purified fractions of tyrosyl kinases were incubated in 25-μl reaction mixtures containing 25 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 6.5), 5 mM $MnCl_2$, 0.1 μM [γ-^{32}P]ATP (7 × 10^6 cpm/pmol), and 10 mM 2-mercaptoethanol and the reaction products were separated on a 10% gel (22). Lanes 1–3 are from the pH 5 fraction and lanes 4–6 are from the microsomal salt-wash fraction. Lanes 1 and 4 are fractions eluted with 0.26 to 0.32 M NaCl from phosphocellulose column (Fig. 1 A and C). Lanes 2 and 5 are products from peak I of Sephacryl S-200 columns (Fig. 1 B and D). Lanes 3 and 6 are products from peak II of Sephacryl S-200 columns (Fig. 1 B and D). Arrows indicate the positions of protein standards whose molecular masses are given in kDa. (B) Phosphoamino acid analyses of bands labeled a and b in A. P-Ser, phosphoserine; P-Thr, phosphothreonine; P-Tyr, phosphotyrosine.

Table 4. Effects of various reagents on tyrosyl protein kinase activity associated with TPK 75

Effector	Concentration	% activity
cAMP	100 μM	106
cGMP	100 μM	110
Heat-stable kinase inhibitor	0.8 mg/ml	103
EGF	0.3 μM	107
EGF	3.0 μM	117
Insulin	1.0 μM	102
Insulin	10 μM	113
N-Ethylmaleimide	10 mM	9.6
Iodoacetamide	10 mM	88
TPCK	10 mM	75
TLCK	10 mM	41
PCMB	10 mM	0

Purified TPK 75 (0.15 unit) was incubated at 0°C for 10 min with one of the above reagents and the reaction was started by the addition of ATP. Reaction products were separated by paper electrophoresis. Results are expressed as % of control values determined in reactions that did not contain any of the above reagents. TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; TLCK, *N*-α-tosyl-L-lysine chloromethyl ketone; PCMB, *p*-chloromercuribenzoic acid.

major phosphorylation product of peak II of both the cytosol and the microsome salt-wash (Fig. 2*A*, lanes 3 and 6). Several other very minor species became apparent upon prolonged exposure of the autoradiogram.

The 75-kDa phosphoproteins observed in both the cytosol and the microsome wash fraction were found to contain >95% phosphotyrosine when they were extracted from the gels and analyzed for phosphoamino acid content (Fig. 2*B*, lanes a and b). Upon long exposure, a trace of phosphoserine also was observed in each sample.

Effects of Growth Factors and Various Reagents on the Tyrosyl Kinase Activity Associated with TPK 75. We also examined the effects of a number of growth factors and reagents on the tyrosyl kinase activity associated with cytosolic TPK 75 and the results are shown in Table 4. The kinase activity was not affected by the addition of effectors of cyclic nucleotide-dependent protein kinases, such as cAMP, cGMP, or a heat-stable inhibitor (23). Growth factors, such as EGF and insulin, also had little or no effect on the cytosolic kinase activity. Protease inhibitors, such as TPCK and TLCK, only partially inhibited the kinase activity. The enzyme activity was most sensitive to thiol reagents. PCMB was the most potent of these reagents and, at a concentration of 10 mM, completely abolished the kinase activity.

DISCUSSION

Using a sensitive assay for the phosphorylation of tyrosine residues, we have demonstrated the presence of at least two tyrosyl protein kinases in the cytoplasm of normal rat liver cells. These kinases appear to be unique in that they are present in normal cells and are not stimulated by growth factors. Also, results of preliminary experiments indicate that these kinases are not immunologically related to the transforming gene products of Rous and Fujinami sarcoma viruses (unpublished data). The identification of these kinases represents another application of the angiotensin peptides as substrates for tyrosyl kinases (13). The results obtained do not exclude the possibility that other tyrosyl kinases that do not phosphorylate these particular peptide substrates exist in rat liver. No attempt has been made to characterize tyrosyl kinases associated with the plasma membrane fraction. Although they represent only a small fraction of the total activity of liver cells, the plasma membrane kinases have a relatively high specific activity. These kinases may be identical to growth factor receptor kinases previously identified in liver cell membranes (5).

The most abundant tyrosyl kinase in rat liver cytoplasm has a molecular mass of 75 kDa and was found in cytosol and the microsomal salt-wash fraction. The observation that the purified 75-kDa enzyme phosphorylates a 75-kDa protein on tyrosine residues suggests that the enzyme may possess autophosphorylating activity. A high molecular mass (>160 kDa) kinase also was found in both cytosol and the microsomal salt-wash fraction. Because this high molecular mass material was very heterogeneous, we did not attempt to analyze its reaction products in an *in vitro* phosphorylation reaction. There is as yet no evidence to indicate whether the high molecular mass enzyme is an aggregated form of TPK 75 or whether it is an entirely different enzyme. Furthermore, it is not clear how these two kinases relate to those that were not extractable from microsomal membranes. Resolution of these issues will have to await further purification and characterization of the different enzymes. To summarize, rat liver cytoplasm may contain as many as three tyrosyl protein kinases: TPK 75, a high molecular mass enzyme, and an enzyme that associates tightly with microsomes.

The available data do not allow us to conclude whether or not the association of tyrosyl kinases with ribosomes and endoplasmic reticulum is biologically significant. However, such an association is rather unique and suggests that tyrosyl kinases may play a role in regulating the activity of the protein synthetic machinery. Considerable evidence has been presented for the significance of phosphorylation of serine and threonine residues of ribosomal proteins and initiation factors in protein synthesis and cellular metabolic activities (24, 25). Although there has not been any report of tyrosine-specific phosphorylation in these components, the question of the involvement of phosphatases has not been dealt with adequately. In view of the findings presented in this report, these issues merit reevaluation.

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