JOURNAL OF VIROLOGY, Jan. 1974, p. 234-236
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Vol. 13, No. 1
Printed in U.S.A.

Protein Kinase Stimulated by Cyclic GMP in Uninfected and Simian Virus 40-Infected Monkey Kidney Cells

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Received for publication 23 August 1973

Uninfected African green monkey kidney cells contain a cyclic GMP-stimulated protein kinase. The level or specific activity of the enzyme was not significantly increased after infection of the cells with simian virus 40. This enzyme probably catalyzes the phosphorylation of the structural proteins of the virus.

We have recently shown that all the structural polypeptides of simian virus 40 (SV40) grown in African green monkey kidney (AGMK) cells are phosphorylated (5). Furthermore, purified SV40, which does not contain a virion-associated protein kinase, could be phosphorylated further by exogeneous protein kinases of different origins, including a protein kinase from uninfected AGMK cells (6). Although the biological significance of phosphorylation of SV40 is not known, it is possible that intracellular phosphorylation of infecting parental virus and of progeny viral proteins by cellular protein kinase(s) may play an important role in the replication of SV40 (5, 6). It was therefore of interest to purify and characterize AGMK cell protein kinase and to determine if the enzyme activity is increased after virus infection.

The distribution of protein kinase in the cell nuclear and cytoplasmic fractions prepared by the method of Berkowitz et al. (1) was determined by assaying the fractions (20 μg of protein) for protein kinase activity in a 0.2-ml standard reaction mixture containing 10 μmol of Tris-hydrochloride, pH 7.5, 4 μmol of $MgCl_2$, 2 μmol of dithiothreitol (DTT), 50 μg of calf thymus histone (Sigma Chemical Co., St. Louis, Mo.) and 2 nmol of γ-[^{32}P]ATP (New England Nuclear Corp., Boston, Mass.) with a specific activity of 88 counts per min per pmol. The mixture was incubated at 37 C for 30 min, and trichloroacetic acid-insoluble radioactivity was determined as described previously (5).

About 84% of the total cellular protein kinase activity was localized in the cell cytoplasm, and about 73% of this activity was recovered in the supernatant fluid after centrifugation at 100,000 × *g* for 90 min. Protein kinase was purified from secondary cultures of uninfected AGMK cells (5) by the method described in Fig. 1 and 2. Protein kinases were eluted from the DEAE-cellulose column with 0.2 M KCl (kinase I) (95% of total protein kinase activity) and 0.25 M KCl (kinase II) (Fig. 2). The yield and extent of purification of protein kinase I and II are shown in Table 1. Further purification of protein kinase I was attempted by chromatography on a

Cells scraped off the glass with rubber policeman, washed twice with 1 mM DTT in 50 mM Tris-hydrochloride, pH 7.5, and disrupted by ultrasonic vibration

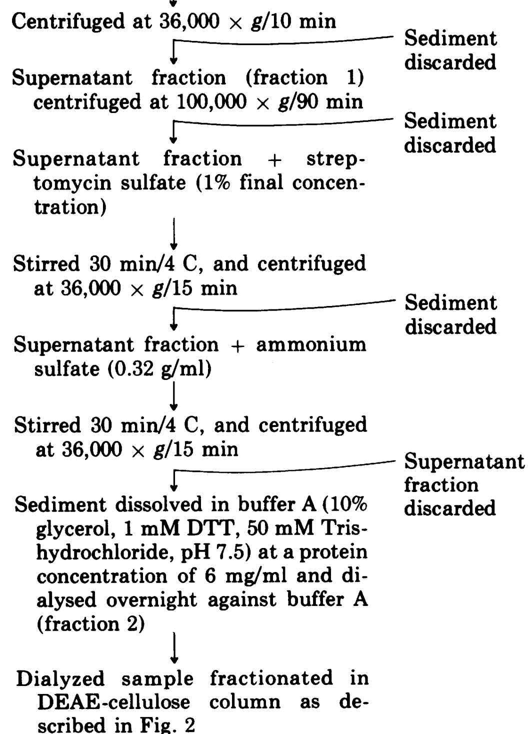

FIG. 1. *Purification scheme for protein kinase from secondary cultures of uninfected AGMK cells.*

phosphocellulose column which had previously been equilibrated with buffer A. About 85% of the total kinase activity did not adsorb to the column, and the remainder of the activity was eluted with 0.1 M KCl in buffer A. No further protein kinase activity was eluted from the column with higher concentrations of KCl, up to 0.5 M. The specific activity of the enzyme fraction eluted from the phosphocellulose column was not increased, probably because of inactivation.

The properties of kinase I were further investigated. Maximum enzyme activity was elicited when the reaction mixture was buffered with 50 mM Tris-hydrochloride or potassium phosphate at pH 7 to 7.5. The enzyme activity was dependent on the presence of Mg^{2+} ions but was inhibited by Ca^{2+} and Co^{2+} ions (Fig. 3a). NaCl, up to 0.2 M, had no effect on the enzyme activity, but higher concentrations of the salt were inhibitory. Results of the effect of cyclic nucleotides on kinase I activity are shown in Fig. 3b. Cyclic GMP (10^{-5} M), but not cyclic AMP, stimulated the enzyme activity from two- to threefold as compared with that in the absence of cyclic nucleotide. The protein kinase activity in the disrupted cells and ammonium sulfate precipitate (Table 1) was also stimulated by about threefold in the presence of cyclic GMP but not cyclic AMP. The presence of 2 to 50 mM DTT caused a twofold stimulation of the enzyme activity. Whole histones were the best substrate for kinase I, whereas arginine-rich histones, protamine, and SV40

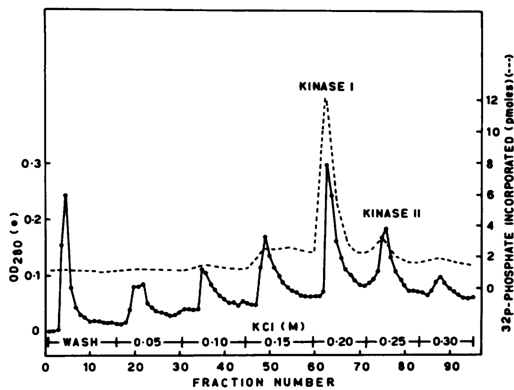

FIG. 2. *Chromatography of AGMK protein kinase on DEAE-cellulose. Fraction 2 (Fig. 1) containing 15 mg of protein was applied onto a DEAE-cellulose (DE-23, Reeves Angel, N.J.) column (1 by 11 cm) which had previously been equilibrated with buffer A. The column was washed sequentially with buffer A containing the indicated concentration of KCl. All the operations were carried out at 4 C. Fractions of 1.8 ml were collected and 50-μliter samples were assayed for protein kinase activity as described in the text.*

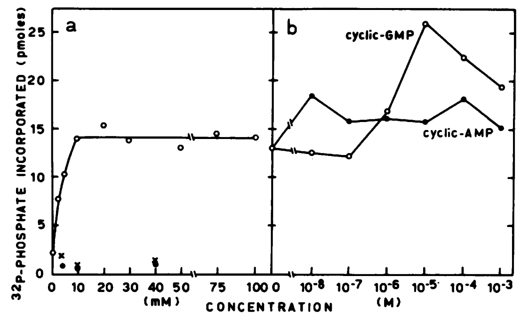

FIG. 3. *Effects of divalent cations and cyclic 3',5'-nucleotides on protein kinase I activity. The reaction mixture contained, in 0.2 ml: 10 μg of protein kinase I; 10 μmol of Tris-hydrochloride, pH 7.5; 2 μmol of DTT (omitted in sample containing Co^{2+}); 50 μg of histones; 5 nmol of γ-[^{32}P]ATP (190 counts per min per pmol); and the indicated concentration of divalent cations (●, $CaCl_2$; ×, $CoCl_2$; O, $MgCl_2$) (a), or cyclic nucleotide (b). Incubation was at 37 C for 30 min. In addition, the reaction mixture (b) contained 4 μmol of $MgCl_2$.*

TABLE 1. *Purification of protein kinases from uninfected AGMK cells*[a]

Sample	Total protein (mg)	Yield (%)	OD_{280}/OD_{260}[b]	Specific activity[c] (pmol/mg of sample)	Purification
Cell sonicate (fraction, 1, Fig. 1)	34.7	100		466	×1.0
Ammonium sulfate precipitate (fraction 2, Fig. 1)	15.8	45.5		710	×1.5
Protein kinase I	0.8	2.4	1.25	2953	×6.3
Protein kinase II	0.6	1.7	1.22	387	×0.9

[a] Purification procedures described in detail in Fig. 1 and 2.

[b] Optical density at 280 nm/optical density at 260 nm.

[c] Samples (20 μg) were incubated in a standard reaction mixture containing 50 μg of protamine and 5 nmol of γ-[^{32}P]ATP (400 counts per min per pmol) at 37 C for 30 min. The specific activity was corrected for incorporation observed in the absence of protamine.

empty capsids (5, 6) were also good phosphate acceptors (Table 2). The rate of phosphorylation of histones at 37 C was linear with time for up to 60 min and with enzyme concentration up to 60 μg/ml.

To determine if kinase I contains protein phosphatase activity, 50 μg of histones previously phosphorylated by beef heart kinase in the presence of γ-[^{32}P]ATP (6). was incubated with kinase I (15 μg) in a standard reaction mixture lacking γ-[^{32}P]ATP but containing 5 nmol of ATP, for 30 min at 37 C. About 15% of the ^{32}P radioactivity originally present in the histone was rendered trichloroacetic acid-soluble after incubation with kinase I.

All the structural polypeptides of SV40 grown in AGMK cells are phosphorylated (5). Since the virus does not contain a virion-associated protein kinase (5), phosphorylation of viral proteins must be mediated by a protein kinase(s) present in the infected cell. The level of protein kinase activity in the soluble cell fraction after virus infection did not differ significantly from that in uninfected cells (Table 3). Similarly, protein kinase activity in the sediment obtained after centrifugation of disrupted infected cells at 100,000 × g was only slightly increased. Identical patterns were obtained when the soluble extracts of whole uninfected and SV40-infected cells were analyzed by DEAE-cellulose chromatography as described above. The protein kinase of infected cells responded to the presence of cyclic nucleotides in a manner similar to that of uninfected cells.

Protein kinases from animal tissues and cells in vivo are stimulated by cyclic AMP and/or cyclic GMP or other cyclic nucleotides (2, 4), whereas those from cultured mammalian cells are stimulated only by cyclic AMP (3, 7, 8); stimulation by cyclic GMP was not investigated in the latter cell types. However, the AGMK protein kinase described in this communication is stimulated by cyclic GMP and not cyclic AMP.

TABLE 2. *Substrate specificity of protein kinase I*[a]

Substrate	[^{32}P]-phosphate incorporated (pmol)
Lysine-rich histones	3.3
Arginine-rich histones	12.5
Whole histones	25.6
Protamine	17.7
SV40 empty capsids	8.0
Phosvitin	3.8
Casein	4.0

[a] The protein substrate (50 μg) was incubated in a standard reaction mixture containing 15 μg of protein kinase I and 5 nmol of γ-[^{32}P]ATP (400 counts per min per pmol) for 30 min at 37 C.

TABLE 3. *Comparison of protein kinase activity in uninfected and SV40-infected AGMK cells*[a]

Cells	[^{32}P]phosphate (pmol) incorporated per mg of cell protein at indicated time (h) postinfection					
	12	24	33	48	55	72
Uninfected	1,168	1,281	2,077	1,853	2,103	1,881
Infected	1,993	2,349	2,680	2,444	2,196	2,313

[a] Secondary monolayer cultures of AGMK cells were infected with 100 PFU of SV40 per cell as described previously (5). At intervals postinfection the cells were suspended in buffer A, disrupted by sonication, and centrifuged at 100,000 × g for 90 min. A sample of the supernatant fluid (containing 20 μg of protein) was incubated in a standard reaction mixture containing 50 μg of protamine and 13 nmol of γ-[^{32}P]ATP (66 counts per min per pmol) for 30 min at 37 C.

We thank M. McFalls for technical assistance.

This study was supported by Public Health Service research grants CA-10594 and CA-10815 from the National Cancer Institute, by RR 05540 from the Division of Research Resources, and by the Commonwealth of Pennsylvania.

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