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Effects of Polyamines, Polyamine Synthesis Inhibitors, and Polyamine Analogs on Casein Kinase II Using Myc Oncoprotein as Substrate

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ABSTRACT. Polyamines, casein kinase II (CKII), and the *myc* oncogene are directly involved in the regulation of molecular events in cell proliferation, differentiation, and apoptosis. Each is increased in rapidly growing cancer cells. In our current study, we showed that the K_m values for purified CKII were similar for casein and Myc oncoprotein under a variety of assay conditions, and that specific natural and synthetic polyamines stimulated CKII phosphorylation of Myc oncoprotein 2- to 20-fold via increases in V_{max}. When polyamine synthesis inhibitors and analogs were studied with this purified enzyme system, two polyamine analogs (N^1,N^{12}-bis-(ethyl)-spermine [BESpm] and 1,19-bis-(ethylamino)-5,10,15, triazononadecane [BE4X4]), which did not affect basal enzyme activity, did prevent (or inhibit) polyamine-stimulated CKII activity by approximately 70 and 85 percent, respectively. Because the Myc oncoprotein transactivates several genes for key proteins involved in the regulation of cellular proliferation, including the ornithine decarboxylase gene (rate-limiting enzyme of polyamine synthesis), we suggest that there may be linkages between polyamines, CKII, and Myc in the control of cellular proliferation. We also suggest that the anticancer drugs BESpm and BE4X4 may inhibit cancer cell proliferation partially through interference with the above-suggested CKII linkages. BIOCHEM PHARMACOL **58**;2:251–254, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. polyamines; casein kinase II; Myc; cell proliferation

CKII[‖] is found in the cytoplasm and nucleus of most prokaryotic and eukaryotic cells [1–3] and is increased in rapidly proliferating cells, including cancer cells and tissues [4, 5]. Many laboratories consider that CKII plays a key role in cell cycle control, apoptosis, and cellular differentiation and proliferation [6, 7].

In recent years, more than 50 proteins which are phosphorylated by CKII have been identified, including: DNA topoisomerases I and II, several nuclear protein transcription factors (Myc, Jun, Myb, and Max oncoproteins), translation elongation factors (eIF3, eIF4B, and eIF5), cytoskeletal proteins (β-tubulin, microtubule-associated proteins, and myosin light chain), and membrane receptor system proteins (protein kinase C, insulin, and platelet-derived growth factor receptor [8–14]. However, research setting out to establish many of these proteins as "physiological" substrates, as compared to proteins which only contain the required phosphorylation sequence: RRREEET/SEEE [8, 13–15], has not yet been reported.

Polyamines are small organic molecules which increase several-fold in cells during rapid cell proliferation [16]. Even though the specific molecular mechanisms of polyamines are not yet understood, inhibition of polyamine synthesis, cellular uptake, and/or intracellular functioning inhibit cell proliferation [17, 18]. Currently, several polyamine synthesis inhibitors and polyamine analogs are being tested with several types of cancer [19–22]. In addition, because both natural and synthetic polyamines have been reported to stimulate CKII [5, 12, 23–28], we are currently studying the effects of several of these antipolyamine drugs on CKII *in vitro* and *in vivo*.

In the current study, we kinetically evaluated the effects of polyamines and antipolyamine drugs on CKII from purified sheep lung, using both casein and Myc oncoprotein as protein substrates and GTP and ATP as phosphate donors. Attempts were made to compare the effects of polyamine synthesis inhibitors and polyamine analogs on CKII activity under a variety of assay conditions.

MATERIALS AND METHODS

Most of the chemicals and biologicals were purchased from commercial sources. The antipolyamine drugs (polyamine

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‖ *Abbreviations:* CKII, casein kinase II; DFMO,2-(difluoromethyl)-DL-ornithine; BEPUT, *N*,*N*-diethyl-1,4-dibutane diamine; methyl GAG, methylglyoxal bis (guanylhydrozone); BESpm, N^1,N^{12}-bis-(ethyl)-spermine; and BE4X4, 1,19-bis-(ethylamino)-5,10,15, triazononadecane.

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synthesis inhibitors and polyamine analogs) were either purchased or kindly supplied by the Drug Synthesis and Chemistry Branch, National Cancer Institute, NIH.

Assay procedures and the purification of CKII from sheep lung tissue have been published previously [28, 29]. Briefly, the enzyme was routinely assayed in 20 mM Tris–HCl buffer at pH 7.5, 10 mM magnesium acetate, 80 μM EDTA, 80 μM mercaptoethanol, 0.2 μM of gamma ^{32}P-GTP or ^{32}P-ATP (5,000 Ci/mmol) in 0.1 mM of non-radioactive GTP or ATP (200,000 cpm/assay) respectively, 10 μg of casein or Myc oncoprotein, and varying concentrations of polyamines, antipolyamine drugs, and enzyme (final volume of 250 μL).

The purification procedures involved homogenization of fresh sheep lung tissue in 0.1 M Tris–HCl buffer at pH 7.5 containing 2 mM EDTA and 2 mM mercaptoethanol, and subsequent purification with 0.01 M Tris–HCl (+EDTA and mercaptoethanol) using diethylaminoethylamine cellulose, Sepharose CL-6B, and polyamine affinity column chromatographies (CKII tightly binds to polyamine affinity resin and only releases at 0.8 M NaCl). The procedures required 3 days and produced a CKII which was purified approximately 600-fold, with a final specific activity near 250,000 pmol of phosphorylated casein per min per mg enzyme protein at 35° [29].

RESULTS

All CKII kinetic studies were carried out in duplicate, with 2–4 repeats, and plotted as standard Lineweaver–Burk plots to obtain K_m, K_a, and V_{max} values ± standard deviations. In all kinetic assays, when the first substrate was varied, the second was not varied and was always maintained at saturating conditions. These standard procedures were always followed when performing kinetic evaluation of bisubstrate enzyme systems. The specifics for each varied first substrate within each study are given with the results table of that study.

Table 1 shows that in the absence of polyamines the K_m values for GTP with Myc or casein, for ATP with Myc or casein, for Myc with GTP or ATP, and for casein with GTP or ATP ranged from 0.28 to 6.74 μM. None of these K_m values changed when polyamines were added to the assays. Table 1 also shows that in the absence of polyamines the V_{max} for GTP with Myc or casein, for ATP with Myc and casein, for Myc with GTP or ATP, and for casein with GTP or ATP ranged from 5.8 to 12.5 pmol of ^{32}P from GTP or ATP per min per mg of enzyme protein. However, all V_{max} values increased from 4- to 12-fold when polyamines were added to the various combinations of Myc or casein as protein substrates and GTP or ATP as phosphate donors.

A variety of natural and synthetic polyamines were studied with CKII using Myc and GTP (Table 2). Putrescine, spermidine, spermine, polylysines, and polyornithine stimulated CKII from 2- to 20-fold. The K_a values ranged from 0.018 to 0.97 mM for polylysine (100,000 Da) to putrescine, respectively. The monoamines (lysine, ornithine, arginine) and polyarginine did not stimulate CKII. Similar results were observed with Myc + ATP, casein + GTP, and casein + ATP (data not shown).

TABLE 1. Enzyme kinetics of lung CKII

	−polyamine	+polyamine
K_m GTP (with Myc)	4.83 ± 0.99	5.20 ± 0.71
V_{max} GTP (with Myc)	5.8 ± 1.21	68.3 ± 8.12
K_m GTP (with casein)	6.74 ± 1.51	5.93 ± 0.84
V_{max} GTP (with casein)	10.9 ± 1.86	60.7 ± 7.97
K_m ATP (with Myc)	8.71 ± 1.72	6.97 ± 0.93
V_{max} ATP (with Myc)	8.0 ± 1.75	71.4 ± 9.79
K_m ATP (with casein)	3.32 ± 0.69	2.91 ± 0.37
V_{max} ATP (with casein)	12.5 ± 3.55	58.6 ± 8.03
K_m Myc (with GTP)	0.28 ± 0.07	0.33 ± 0.05
V_{max} Myc (with GTP)	7.0 ± 1.59	80.2 ± 9.79
K_m casein (with GTP)	1.01 ± 0.34	1.05 ± 0.17
V_{max} casein (with GTP)	10.6 ± 2.31	63.6 ± 8.93
K_m Myc (with ATP)	0.37 ± 0.09	0.39 ± 0.05
V_{max} Myc (with ATP)	8.9 ± 2.16	76.4 ± 9.11
K_m casein (with ATP)	0.78 ± 0.19	0.71 ± 0.10
V_{max} casein (with ATP)	12.2 ± 2.73	60.3 ± 8.81

K_m = μM; V_{max} = pmol of ^{32}P incorporated per min per mg enzyme protein at 37°; polyamine was 0.1 μM of polylysine (100,000 Da).

Several antipolyamine drugs were kinetically evaluated with purified lung CKII using Myc + GTP and Myc + ATP (Table 3). None of the drugs affected the enzymatic activity when polyamines were absent. In the presence of added polylysine, the polyamine synthesis inhibitors DFMO and BEPUT did not alter the polyamine stimulation of CKII and methylGAG prevented (or inhibited) polyamine stimulation about 10–15%, while the polyamine analogs BESpm and BE4X4 prevented (or inhibited) polyamine stimulation by approximately 70 and 85 percent, respectively. When CKII was studied with casein as substrate protein, either ATP or GTP as phosphate donor, and spermidine or spermine as stimulator, the results were similar (our unpublished observations).

Several other types of inhibitors (not antipolyamine system drugs) were studied with CKII using Myc + GTP (Table 4). Both in the absence and presence of polyamines,

TABLE 2. Polyamine activators of lung CKII

	K_a	V_{max}
No polyamines added	—	4.9 ± 2.1
+putrescine	0.971 ± 0.21	7.3 ± 1.8
+spermidine	0.523 ± 0.13	17.5 ± 3.1
+spermine	0.371 ± 0.80	21.1 ± 3.6
+lysine	—	5.1 ± 2.2
+polylysine (100,000 Da)	0.018 ± 0.01	87.1 ± 7.4
+polylysine (10,000 Da)	0.027 ± 0.01	83.5 ± 8.3
+polylysine (1,000 Da)	0.035 ± 0.02	79.9 ± 9.1
+ornithine	—	4.2 ± 1.9
+polyornithine (20,000 Da)	0.031 ± 0.01	81.6 ± 9.4
+arginine	—	3.9 ± 2.2
+polyarginine (20,000 Da)	—	4.7 ± 2.3

K_a = mM; V_{max} = pmol ^{32}P incorporated per min per mg enzyme protein at 37°; Myc and GTP were protein substrate and phosphate donor, respectively.

TABLE 3. Polyamine inhibitors of lung CKII

Inhibitors	V_{max} (−polylysine)	V_{max} (+polylysine)
Myc + GTP		
None	6.08 ± 1.9	80.2 ± 9.5
DFMO	5.73 ± 2.2	77.9 ± 9.9
BEPUT	6.11 ± 2.1	79.3 ± 9.0
MethylGAG	5.32 ± 1.8	61.6 ± 8.4
BESpm	5.53 ± 1.9	18.9 ± 3.5
BE4X4	5.29 ± 1.7	10.7 ± 2.9
Myc + ATP		
None	7.98 ± 2.7	67.0 ± 8.2
DFMO	8.53 ± 2.6	69.1 ± 9.4
BEPUT	7.68 ± 2.3	66.5 ± 7.9
MethylGAG	7.92 ± 3.1	51.1 ± 6.7
BESpm	7.35 ± 2.7	18.6 ± 2.9
BE4X4	7.23 ± 2.2	13.4 ± 2.7

The calculated K_i values for methylGAG, BESpm, and BE4X4 using Myc + GTP and Myc + ATP were 25.8, 6.2, 4.7, and 38.7, 9.1, 6.5 μM, respectively. V_{max} = pmol ^{32}P incorporated per min per mg enzyme protein at 37°.

dextran sulfate and heparin inhibited CKII activity from 60 to 90%. Neither dextran nor $MgSO_4$ had any effect. Similar effects have been previously reported with other casein kinases using casein as substrate, ATP as phosphate donor, and both natural and synthetic polyamines [4–8, 23–25, 29].

DISCUSSION

We have shown here that Myc oncoprotein is an excellent protein substrate for non-stimulated or polyamine-stimulated CKII. The K_m values for Myc, with GTP or ATP, were near those for casein under similar assay conditions. However, in the current study we did not evaluate changes in the biological activity of Myc before and after phosphorylation.

In recent years, more than 50 proteins have been reported to be phosphorylated by CKII [1–5]. Many of these proteins/enzymes are critical for rapid cell proliferation, the Myc oncoprotein being one these proteins [29, 30]. The Myc oncoprotein is a nuclear transcription control factor which has been observed to be amplified severalfold in many types of cancers [31–34]. The myc gene/protein system is controlled at the level of transcription, translation, and posttranslation [32–40]. The posttranslation control apparently involves phosphorylation of 8–10 serine/threonine amino acid residues by several different protein kinases (including CKII, glycogen synthetase, cdc kinase, and mitogen-activated protein kinase [32, 35–37]). Only the phosphorylated forms of the Myc protein can dimerize with a phosphorylated max protein (also phosphorylated by CKII) to form a biologically active transcription initiation factor complex. The active phosphorylated heterodimer PMyc:PMax protein complex binds to DNA and transactivates several genes involved in rapid cell proliferation, cell cycle control, and apoptosis [36–44]. One of these is the gene for ornithine decarboxylase [39, 40]. ODC is the rate-limiting enzyme for polyamine synthesis. Therefore, there may be possible linkages between polyamines, CKII, Myc oncoprotein, and rapid cell proliferation. Further studies are necessary to elucidate such linkages.

TABLE 4. Inhibitors of lung CKII

Inhibitors	V_{max} (−polyamine)	V_{max} (+polyamine)
No inhibitors added	4.64 ± 2.3	86.3 ± 9.4
+dextran sulfate	3.03 ± 1.8	7.0 ± 2.1
+heparin	2.72 ± 1.6	6.1 ± 2.9
+dextran	4.26 ± 1.9	85.5 ± 9.2
+$MgSO_4$	5.01 ± 2.3	81.9 ± 9.8

Myc and GTP were substrate protein and phosphate donor, respectively. K_i values for dextran sulfate and heparin were 2.1 and 1.3 μM, respectively. V_{max} = pmol of ^{32}P incorporated per min per mg enzyme protein at 37°.

Using Myc or casein as protein substrate and ATP or GTP as phosphate donor, CKII activity was stimulated severalfold when certain natural or synthetic polyamines were added to the assay system. This suggests that one potential molecular target of polyamine action may be CKII. No stimulation was observed with monoamines, whereas the stimulation was quite specific for three or four carbon/one amino units (polyarginine did not stimulate, but polylysine and polyornithine did). In addition, two polyamine analogs, BESpm and BE4X4, prevented (or inhibited) polyamine stimulation of CKII, but did not affect basal (non-polyamine-stimulated) enzyme activity. The polyamine synthesis inhibitors showed no effect on basal or polyamine-stimulated CKII activity. Therefore, it is possible that one of the molecular mechanisms for the inhibition of cell proliferation by these two polyamine derivatives may involve the enzyme, CKII.

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