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Purification of Casein Kinase II From Lung Tissue and Kinetic Evaluations With Polyamines and Myc-Oncoprotein*

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Abstract: The overall goal of this study was to seek molecular linkages between polyamines, casein kinase II, and the myc oncoprotein by using a cell-free, highly purified enzyme kinetic analysis. Casein kinase II (CKII) was purified from sheep lung tissue by using an original 3-column/3-day set of procedures (DEAE cellulose, Sepharose 6L-4B, and poly-L-lysine agarose affinity chromatographies). CKII was purified approximately 550-fold with a recovery of near 40% and a final specific activity of about 250,000 pmol of phosphorylated protein substrate (casein)/min/mg of enzyme protein. Kinetic evaluations with the purified CKII using Mg-GTP as a phosphate donor, either casein or myc oncoprotein as substrate, and various polyamines (polylysine, polyornithine,

spermidine, or spermine) as stimulators showed: 1) phosphorylation of casein increased 2-4 fold with various polyamines; 2) without polyamines, phosphorylation of myc oncoprotein was about 10-20% when compared to casein; 3) phosphorylation of myc oncoprotein increased 10- to 30-fold in the presence of various polyamines. The phosphorylated form of myc oncoprotein is the only biologically active form which transactivates specific genes related to cell proliferation.

Key Words: Casein kinase II, polyamines, myc, cell proliferation abbreviations: CKII-casein kinase II; Pmyc-phosphorylated myc oncoprotein; Pmax-phosphorylated max protein.

Introduction

Casein kinase II (CKII) is a ubiquitous enzyme which is found in most cells and tissues of both prokaryotes and eukaryotes (1-3). It is in low activity in differentiated cells and in high activity in rapidly proliferating cells. Current laboratory data support theories that CKII may play a critical role in controlling cell proliferation by direct involvement in both the signal transduction and cell cycle control systems (4-5).

CKII phosphorylates more than 50 different proteins including regulatory enzymes, structural proteins, oncoproteins, and cancer suppressor genes's proteins (5-7). It uses either ATP or GTP, and magnesium or manganese as phosphate donor substrates. Several of the substrate proteins of CKII, such as myc oncoprotein and p53 cancer suppressor protein, are directly involved in the control of both cell proliferation and the cell cycle (8-10).

In addition, CKII is activated by various natural and

synthetic polyamines (11-14). The natural polyamines, spermidine and spermine, increase in all mammalian cells which undergo rapid cell proliferation (15-16). Various drugs have been developed which inhibit polyamine synthesis or polyamine function, thus inhibiting cell proliferation (17-19). Combinations of these two types of polyamine inhibitors are currently being evaluated in laboratory and clinical cancer studies (16-19).

Therefore, molecular linkages may exist, especially in rapidly growing cells, between polyamines, CKII, and certain CKII protein substrates such as the protein product of the myc oncogene. The phosphorylated myc oncoprotein is involved in events in the cell cycle, cell proliferation and differentiation, and apoptosis (20-23). The current report describes an original, very rapid and efficient purification of CKII from normal sheep lung and the phosphorylation of the myc protein as a substrate in the absence or presence of various polyamines.

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Materials and Methods

Most of the general chemicals and biologicals were purchased from commercial sources. Purification was performed using fresh sheep lung tissue. The tissue was minced and homogenized (1gm/3ml) in ice-cold buffer containing 0.1M Tris-HCl (pH 7.5), 2mM EDTA, and 2mM mercaptoethanol (Buffer A). All purification procedures were carried out at 3-5°C. The homogenate was centrifuged at 40,000Xg for 2 hours. The supernatant (cytosol) was used for the subsequent purification of CKII.

120ml of the cytosol was applied to a 2.5cm X 26cm DEAE cellulose column which was pre-equilibrated with a buffer containing 10mM TRIS-HCL (pH 7.5), 2mM EDTA, and 2mM mercaptoethanol (Buffer B). The column was washed continuously with Buffer B (2-3 column volumes) until the elutant was free of hemoglobin. The flow rate was then adjusted to 100ml/hr and proteins were eluted using 6ml batches of Buffer B containing NaCl from 0.1 to 1.0M (discontinuous gradient elution). All fractions were assayed for CKII activity as described below.

The CKII activity was assayed by measuring radioactive phosphate from gamma ^{32}P -GTP that was incorporated into acid precipitable proteins after the addition of protein substrates (24-25). Casein was used as substrate during the purification procedures, while casein or myc oncoprotein was used during the kinetic evaluations. The assay cocktail contained 20mM TRIS-HCl buffer (pH7.5), 10mM magnesium acetate, 80 μM EDTA, 80 μM mercaptoethanol, 0.2 μM of gamma ^{32}P -GTP (5000Ci/mmol) in 10 μM of nonradioactive GTP. Each assay contained approximately 200,000cpm, 10 μg of casein, 10 μg of myc protein, and/or 10 μg of poly-L-lysine. All assays were performed in 1.5ml microfuge tubes for 10min at 37°C in a water bath. The final assay volume was 250 μl . The reaction was stopped by the addition of 1ml of ice-cold 10% TCA and placing the microfuge tube into ice water. Acid precipitable materials were collected by centrifuging the microfuge tube at 12,000Xg for 15min at 4°C. The precipitate was washed twice with 1ml of the cold 10% TCA. The washed precipitate was dissolved in 0.5ml of 1N NaOH at room temperature. It was then added into 5ml of a scintillation cocktail containing 1% PPO, 0.025% POPOP, and 20% naphthalene in 1,4 dioxane and counted in the liquid scintillation counter.

Fractions containing CKII activity were pooled and concentrated 5-10-fold using a nucleopore concentrator with Diaflo ultrafilter YM 100 membranes. The

concentrated CKII activity was mounted onto a 1.5cm x 110cm glass column containing Sepharose CL-6B which was equilibrated with Buffer B. The column was washed with Buffer B at a flow rate of 5ml/hr. Fractions were collected and assayed for CKII activity as previously described. Fractions containing CKII activity were pooled and concentrated 5- to 10-fold as previously described.

The concentrated CKII activity was then mounted onto 1.2cm x 5cm glass column containing poly-L-lysine agarose equilibrated with Buffer B. The column was washed with several volumes of Buffer B. Proteins were then eluted using 5ml batches of Buffer B containing NaCl from 0.1 to 1.0M. Five ml fractions were collected and assayed for CKII activity. Fractions containing CKII activity were pooled, concentrated, and ultrafiltration dialyzed to remove NaCl. The sheep lung CKII as purified by this 3-column/3-day procedure was used for all subsequent kinetic studies

Results

CKII activity was released from the DEAE column with 0.15M NaCl in Buffer B (Figure 1). Elution from the Sepharose 6L-4B column occurred near the void volume using Buffer B, which indicated that CKII had an aggregated molecular weight near several million daltons at this stage in the purification scheme (Figure 2). CKII bound very tightly to the poly-L-lysine affinity agarose column and eluted using 0.9M NaCl in Buffer B (Figure 3). After concentration and ultrafiltration dialysis, this original 3-column/3-day set of procedures yielded a CKII enzyme preparation which was purified approximately 550-fold, gave a recovery of near 40%, and had a final specific activity of about 250,000 pmole of phosphorylated casein per min per mg of enzyme protein (Table 1). In addition, the purified lung CKII used MgATP as phosphate donor equally as well as it did MgGTP (data not given).

The kinetic evaluations of the purified CKII with casein, myc oncoprotein, and various polyamines are given in Table 2. Without added protein substrate, minimal phosphorylation (350+19) always occurred. Addition of polylysine in the absence of added protein substrate also allowed minimal (500+38) enzymatic activity. These observations are probably a result of enzyme self phosphorylation (14). With casein added as protein substrate, enzyme activity was high (2356+109) and increased 2-4 fold in the presence of polylysine, polyornithine, spermidine, and spermine (8021-310, 7346+356, 3901+196, and 4033+209), respectively. With myc oncoprotein added as protein substrate,

Purification (fold)	Volume Steps	PURIFICATION OF LUNG CASEIN KINASE II				Recovery /mg	Purification (%)
		Protein (ml)	Enzyme (mg)	Units Units* (x1000)	Enzyme (mg)		
cytosol	120	480	704	1,468	100	0	
DEAE Cellulose Column	70	74.6	1615	21,765	229	14.6	
Sepharose Column	40	24.8	1127	45,448	160	30.9	
Polylysine Affinity Column	20	0.36	289	802,778	41	546.8	

Table 1.

*Unuts-dpm ^{32}P /min assay

Final Specific Activity-near 2500,000 pmole of phosphorylated substrate protein (casein) per min per mg enzyme protein.

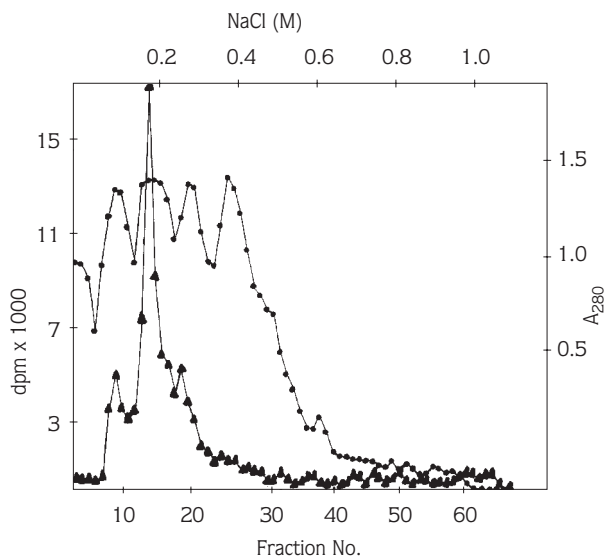


Figure 1. Elution Pattern for CKII from the DEAE-Cellulose Column: triangles-CKII activity; circles-optical absorbancy at 280nm.

enzymatic activity was low (621+55); however, it increased 5- to 15-fold in the presence of polylysine, polyornithine, spermidine, and spermine (8879+426, 7911+374, 3067+178, and 3711+224), respectively. And if one considers that the only-polylysine (no substrate added) assay represents CKII self phosphorylation (3, 6, 14), then the true non-stimulated CKII phosphorylation of casein and myc oncoprotein are approximately 2000 and 300, respectively. Using this assumption, polylysine stimulated phosphorylation of casein and myc

oncoprotein are about 7700 and 8500, respectively. Therefore, polylysine stimulation of myc oncoprotein phosphorylation could be as high 30 fold under these assay conditions.

Discussion

Homogenization of lung tissue in 0.1M Tris-HCl and mounting of the cytosol onto a DEAE cellulose column equilibrated with 10mM Tris-HCl prevents many protein kinases from binding, but the disaggregated form of CKII binds very efficiently. This procedure accomplished several critical objectives: it partially separates CKII from its naturally bound substrates, and it allows the enzyme to disaggregate into its minimal 4 subunit state (2 alpha and 2 beta peptides, totalling 140,000 daltons). In a NaCl free/low-ionic-strength buffer, CKII exists in a range of aggregated forms of several million daltons. These aggregated forms bind to and detach from DEAE with varying degrees of affinity. Under these conditions many other protein kinases do not bind, including the cyclic nucleotide dependent protein kinases. Therefore, this procedure allows for a more efficient binding and release of CKII on DEAE cellulose, removal of substrates and inhibitors which are endogenously bound to CKII, and a more accurate assay of 'native' CKII activity after elution from the DEAE column (11-17, 24-26).

In addition, the binding of CKII to a polylysine affinity resin in NaCl-free media and subsequent release at 0.9M NaCl allowed for a 20-fold increase in purification, but

KINETIC STUDIES WITH PURIFIED LUNG CASEIN KINASE II

Table 2.

	Enzyme Activity*
Using casein as substrate	
- CKII only (no substrate added)	350±019
- CKII+casein	2356±109
- CKII+polylysine (no substrate added)	500±038
- CKII+casein+polylysine	8021±310
- CKII+casein+polyornithine	7346±356
- CKII+casein+spermidine	3901±196
- CKII+casein+spermine	4033±209
Using myc protein as substrate	
- CKII only (no substrate added)	335±021
- CKII+myc	621±055
- CKII+polylysine (no substrate added)	423±047
- CKII+polylysine (no substrate added)	423±047
- CKII+myc+polylysine	8897±426
- CKII+myc+polyornithine	7911±374
- CKII+myc+spermidine	3067±178
- CKII+myc+spermine	3711±224

* Enzyme Activity is given as dpm 32P±standard error/10 min assay of 4-6 determinations. casein-10µg; myc protein-10µg; polylysine-10µg; polyornithine-10µg; spermidine-50µg; spermine-50µg.

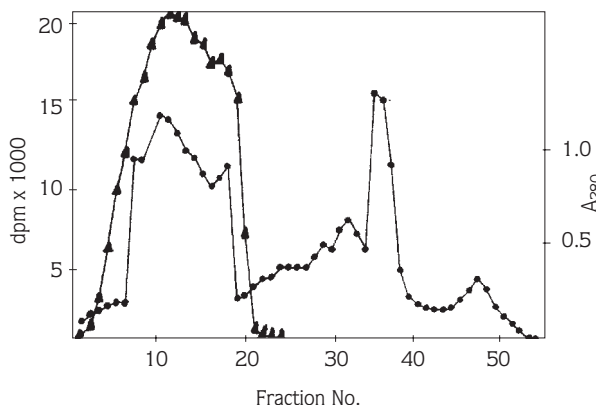


Figure 2. Elution Pattern for CKII from the Sepharose 6L-4B Column; triangles-CKII activity; circles-optical absorbancy at 280nm.

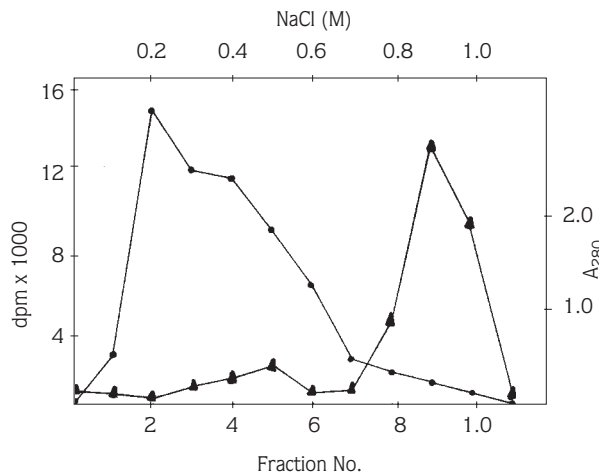


Figure 3. Elution Pattern for CKII from the Poly-L-lysine Agarose Affinity Column; triangles-CKII activity; circles-optical absorbancy at 280nm.

caused a 70% loss in total CKII activity from the previous purification step. Such a procedure has never been reported for CKII and suggests that various polymer forms of CKII may have different binding affinities to polyamines. This original 3-day/3-column set of purification procedures is the fastest and most efficient ever reported for CKII.

All types of fast growing normal and cancer tissues have extremely high levels of ornithine decarboxylase (ODC), polyamines, CKII activity, and myc oncoprotein (7,

9-10, 16, 22-23). Linkages between these molecular systems are being evaluated by many labs. Our current enzyme kinetics report evaluates a highly purified CKII, using myc oncoprotein as substrate, in the absence and presence of various polyamines. The data suggests that myc oncoprotein is a very poor substrate unless polyamines are present.

The myc oncoprotein can be phosphorylated on 4 or

5 serine/threonine residues by different protein kinases. Non-phosphorylated myc oncoprotein is not biologically active. Phosphorylation of myc oncoprotein (Pmyc) is necessary to allow a heterodimerization to a phosphorylated max protein (Pmax), the latter of which is usually present in abundance in most mammalian cells. The Pmyc-Pmax system is described below (27-31).

- 1) max-max homodimers have no activity
- 2) Pmax-Pmax homodimers bind to the DNA binding domain of CAGTG and inhibit gene transcription
- 3) myc-myc homodimers do not exist
- 4) Pmyc-Pmyc homodimers occur but have little activity
- 5) Pmyc-Pmax heterodimers bind tightly to the DNA binding domain of CACGTG and stimulate gene transcription.

In most fast growing cancers, amplification of the myc oncogene occurs either by chromosomal nondisjunction (allows for multiple copies of myc oncogene), or by mutation in a complex control region of the gene immediately before exon 1 (allows for increased transcription of the gene). Both types of amplification mechanisms result in increased quantities of a normal myc oncoprotein, not a mutated protein. However, the myc oncoprotein (and the max protein) are not biologically active until after several protein kinase

phosphorylations. Pmyc-Pmax is the only form which transactivates genes. It is currently not understood which protein kinase phosphorylates which serine/threonine residue, nor which phosphorylation allows biological activity. Because some protein kinases are cytoplasmic rather than nuclear, and because proteins usually gain nuclear entry only after being phosphorylated, certain phosphorylations on the myc oncoprotein may be related to nuclear entry and others to heterodimerization or DNA binding. Many oncoproteins and cancer suppressor proteins require phosphorylation to become biologically active molecules. However, further studies are necessary to clarify the effects that CKII phosphorylations have on myc oncoprotein functioning.

In summary, we have developed a new 3-step/3-day set of procedures to purify CKII from lung tissue. We used this highly purified enzyme to kinetically determine that myc oncoprotein phosphorylation by CKII is very low in the absence of polyamines but is increased 10- to 30-fold in the presence of polyamines. Only the phosphorylated form of myc oncoprotein (as a Pmyc-Pmax heterodimer) can transactivate genes such as the ODC gene, the rate-limiting enzyme for polyamine synthesis. Such potential linkages are critical in our attempts to understand the molecular mechanisms of cell proliferation (32-38).

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